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ENVIRONMENT AND HEALTH

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Editorial: Environment and Health

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Editorial on the Research Topic

Environment and Health

INTRODUCTION

Ten thousand years of protracted and increasing interaction between humans and their environment has influenced, beside the quality of ecosystems, also our quality of life, healthy life span, and health inequalities. In 2016 WHO reported that globally some 12.6 million deaths each year can be ascribed to unhealthy environments. Moreover, 24% of global deaths (and 28% of deaths among children under five) come from modifiable environmental factors (Prüss-Ustün et al., 2016). Human epidemiological studies and experiments in laboratory animals proved that exposure to some pollutants can increase susceptibility to diseases such as diabetes, heart disease, and reproductive cancers. Exposures to toxic agents can also cause immunosuppression, which increases vulnerability to infections, such as COVID-19 (Birnbaum and Heindel, 2020). Environmental factors which represent specific threats for humans and ecosystems are directly associated to exposure to hazardous substances in air, water, soils, and food. Marine sediments and seawater represent an additional major source of contaminants to the environment through multiple pathways. Risks and consequences are also amplified by climate changes and linear and non-linear combinations are reflected in multi-hazard effects. Consequences of living and working in a poor-quality environment could be further magnified in groups of people with poorer health and socio-economic status. In this view, the strategic framework of Agenda 2030 launched by UN refers to the Sustainable Development Goals as critical sectors where all Countries are urged to promote actions to protect the planet (https://www.who.int/topics/millennium_development_goals/en/). This comprehensive vision recognizes that building economic growth requires address a range of social needs including education, social protection and job opportunities, while tackling climate change and environmental defense. The highly integrated 17 Sustainable Development Goals represent a solid framework that ensuring healthy lives and promoting well-being at all ages is essential to sustainable development. Major progress was made in improving the health of millions of people and relevant efforts were also oriented in increasing life expectancy and reducing some of the common negative impacts. At the same time, worldwide consumption and production depend on the use of the natural environment and resources in a way that continues to have destructive impacts on the planet. Economic and social progress over the last century has been accompanied by environmental degradation that is endangering the ecological systems on which the human future depends. Thus, the development of multidisciplinary approaches for understanding mechanisms and dynamics of interference between environment and health is a crucial commitment. The holistic "One Health" approach (e.g., Gibbs and Paul, 2014; CE29.6.2017-COM, 2017 COM 339 Final, 2017) combining efforts of WHO, Food and Agriculture Organization, and World Organization for Animal Health,

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promotes multi-sectorial responses to food safety hazards, risks from zoonoses, and other public health threats. Thus, the human-animal-ecosystem-human interfaces represent primary fields of investigation for valuable improvements of the well-being of human population, ecosystem and environment.

This Research Topic comprises 14 studies including original research articles, method developments, reviews, and perspectives covering several aspects of the complex interaction between environment and health, with critical reviews on key environmental sectors as outdoor and indoor air quality, food, and marine environments. Also, specific proposals of modern approaches to monitor environment and health outcomes as well as restoration and recovery of polluted environments represent a crucial part of this topic. The collection contributes to create and implement effective conceptual links and "bridges" between distinct scientific communities, working in a multidisciplinary way. Most of the articles concern air and sea, as well as some insights dedicated to modern strategies to restore and recover polluted environment, also considering the impacts on human health and the economy.

AIR QUALITY

Poor air quality leads to premature death, cancer, and long-term damage to respiratory and cardiovascular systems. WHO estimates that environmental air pollution caused some 4.2 million deaths in 2016, while household air pollution from cooking with polluting fuels and technologies caused an estimated 3.8 million deaths in the same period (https://www.who.int/health-topics/air-pollution#tab=tab_1). Progress has been made to reduce unhealthy air emissions, an important step in creating a healthier environment. In particular, the Declaration of the Sixth Ministerial Conference on Environment and Health (held in Ostrava, Czech Republic, on June 15, 2017) includes a commitment to "prevent and eliminate adverse effects on the environment and health, costs and inequalities relating to waste management and contaminated sites, progressing toward the elimination of the disposal and trafficking of uncontrolled and illegal waste, and the proper management of waste and contaminated sites in the context of the transition toward a circular economy" (Sixth Ministerial Conference on Environment and Health. Available at: http://www.euro.who.int/en/media-centre/events/events/2017/06/sixth-ministerial-conference-on-environment-and-health). In this context, Manisalidis et al. (2020) documented, through a comprehensive knowledge-based analysis, modes and dynamics of air pollution and related impact on human health (at different systems level) and suggests specific science to policy actions as valuable trajectories to sustainable solutions. Perrino et al. (2020) measured continuous trace gases and erosol (including NOx, SO_2, O_3, NMHC) organic and inorganic pollutants on particulate matter (PM_{10} and $PM_{2.5}$) over the period 2016–2018 from two highly polluted sites in southern Italy and offer original insights and understanding on the role of anthropogenic emissions and particularly on mechanisms of ozone formation, also considering the WHO limits. Kim et al. (2020) studied risk factors correlated to outdoor air pollutant exposure (PM_{10}, $PM_{2.5}$, and NO_2), specifically in relation to cognitive impairment in elderly and gender-differentiated population of South Korea. The preliminary results suggest higher adverse effects of outdoor air pollution on cognitive function in women, indicating specific actions for prevention and intervention.

Stapleton et al. (2020) reported on a pilot study in 21 homes selected from the NIH funded cohort residing in Iowa of current and former smokers, with and without a history of respiratory exacerbations. The aim of the research was at assessing whether indoor air particulate collected from their homes would affect *in vitro* bacterial growth, biofilm formation, and primary human airway surface liquid antimicrobial activity, identifying mechanisms in the development of respiratory tract infections, including bacterial growth, biofilm formation, and innate immunity. Herting et al. (2020) assessed a systematic review on the available literature on the evidence from Magnetic Resonance Imaging studies on how early-life exposure responds to outdoor air pollution on neuro-development. Correlations between pollutants and physiological brain features suggested that outdoor air pollution may significantly affect structure and function of brain. Further air pollution-neuroimaging studies are urgently needed in a developmental neuroscience perspective. Viegas et al. (2020) presented a comprehensive study on the effects of mineral sulfurous thermal waters on lung epithelial-immune crosstalk through the action of its main component, H_2S as valuable inhalational treatment of respiratory diseases.

Marine Environment and Human Health

The marine environment is essential for human health through the provision and quality of air, food and water and offering health-enhancing economic and recreational opportunities. However, human activities such as transport, industrial processes, agricultural and waste management exerts an increasing pressure on marine environment. Many knowledge gaps still significantly limit planning of policies orienteered to a sustainable use of marine resources and environmental and human health protection. The European Marine Board (2013) reported a global estimate of 250 million cases of gastroenteritis from bathing in contaminated water, and 50,000–100,000 annual deaths caused by infectious hepatitis. The global burden of human disease caused by sewage pollution of coastal waters has been estimated at four million casualties, annually. The knowledge about the behavior and ecotoxicity of pollutants (including those of emerging concern) in the marine environment is particularly relevant given the complex interactions among different matrices (sediments, seawater, atmosphere, and biota), and represents a field of cutting-edge multidisciplinary science. In this context, Ausili et al. (2020) presented a comprehensive view on the current status of monitoring and recovery actions of highly industrialized coastal-marine areas in Italy, and provide quantitative indicators to inform policies for restoring huge territory affected by pollution. D'Agostino et al. (2020) reported on the environmental status of contamination by persistent and emerging contaminants (polycyclic aromatic hydrocarbons, Pesticides, polybrominated diphenyl ethers, etc.) from a

highly contaminated site in southern Italy, and offer new approaches to estimate cancer and non-cancer human health risk due to dermal absorption from contaminated seawater and/or ingestion of contaminated fish. Mekni et al. (2020) examined a mixture of legacy (polybrominated diphenyl ethers) and emerging contaminants, such as halogenated (norbornenes (HNs)) and organophosphate (OPFRs) flame retardants, in sediments and samples of eel (*Anguilla anguilla*) from the Tunisian Bizerte Lagoon. Chemical behavior at the environmental interfaces has been explored and health risk associated to the consumption of eel has been estimated, offering original methodological approaches to evaluate impacts by new classes of pollutants in the marine environment. Simmons et al. (2020) reported on a bead-based salivary IgG antibody multiplex immunoassay to determine and quantify infections from environmental exposures to six waterborne pathogens. Results refer to a wide spectrum of 2,091 study participants at Boquerón Beach, Puerto Rico during the summer of 2009 and show how simultaneous infections could affect human health, in synergistic and/or antagonistic interactions. Combined to water quality studies, environmental microbial pathogenesis provides valuable risk assessment tools in estimating exposure potential and facilitating the development of disease surveillance and screening tools.

Strategies for Solutions

The scientific community is urged to provide valuable knowledge about the dynamics of pollutants in the environment but also to offer solutions at short to mid-term to improve environmental quality and ecosystem and human health. In this view, Drago et al. (2020) reported preliminary results from a new birth cohort in highly contaminates sites in southern Italy. The approach offered an unprecedented opportunity to monitor effects of integrated environmental effects on the health of mother-son couples in the short- to long-term period. Di Bella et al. (2020) presented original results about a suite of organic and inorganic contaminants detected in food (terrestrial and seafood) from highly contaminated sites in southern Italy offering original approaches to assessment of associated health risk for different age profiles. Moxley et al. (2020) presented detailed results on the biodegradation efficiency of halogenated compounds in contaminated soils using groups of meso- to thermophilic microorganisms under the extreme conditions of arid and semi-arid areas. Francocci et al. (2020) presented a comprehensive view and original applications of the circular bioeconomy applied to contaminated sites as valuable holistic solution for restoration/recovery of large polluted sites.

CONCLUSIONS

This Research Topic should inspire future science trajectories focused on multi- and interdisciplinary investigations to meet environment restoration and healthier societies. The complexity and significant heterogeneity of the contributions reinforce the urgency to broaden and integrate science approaches and visions on the relationship between environment and health. An issue of increasing priority and urgency.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Influence of Abiotic Factors Temperature and Water Content on Bacterial 2-Chlorophenol Biodegradation in Soils

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Halogenated compounds are environmental pollutants toxic to humans and wildlife. Certain microorganisms degrade these halogenated compounds. However, little is known about the potential of microorganisms in bioremediation under extreme conditions, specifically in arid and semi-arid soils frequently exposed to high temperatures and desiccation periods. Arid and semi-arid environments and deserts make up vast areas of Earth's landmass. To investigate the degradation of 2-chlorophenol (2-CP) in soils as a function of temperature and water availability, three bacterial species were tested, two soil mesophiles of the genus *Rhodococcus, R. opacus and R. erythropolis,* and a soil thermophilic isolate, *Parageobacillus thermoglucosidasius*. Degradation trials in soil samples with these species were performed over a range of water activity from 1 to 0.4. At their optimum growth temperature, *R. opacus* showed maximum 2-CP degradation at water activity 0.9 sharply decreasing when lowering water activity. Nevertheless, the *Parageobacillus* isolate (optimum growth temperature 60°C) showed maximum 2-CP degradation rates at water activity 0.5 which represented highly desiccating conditions. *Parageobacillus* degradation of 2-CP was very low at water activity above 0.9. Thus, biodegradation of 2-CP in soils is possible even under arid conditions although different microbial species might be involved in this task depending on the interactions of abiotic factors and the diversity of microbial communities in soils. These results contribute to understand the potential biodegradation of specific halogenated compounds in the environment which is of great relevance to comprehend the fate of halogenated pollutants (i.e., 2-CP) in deserts, arid and semi-arid soils.

Keywords: 2-chlorophenol, temperature, water availability, desiccation, soil, biodegradation *Rhodococcus*, *Parageobacillus*, bioremediation

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INTRODUCTION

Halogenated compounds, such as chlorophenols, represent important environmental pollutants. Chlorophenols are released into the environment through anthropogenic activities, for instance by their use in pesticides, herbicides, their presence in industrial wastes (Arora and Bae, 2014) and also as a result of organic matter combustion and biological chlorination (Ahling and Lindskog, 1982). Chlorophenols can adhere onto particulate organic matter persisting in the environments for

extended periods of time and leading to accumulation in the organisms through different trophic levels. Understanding the fate of chlorophenols in nature is complex and it is certainly the result of an interplay of biotic and abiotic processes.

The biological degradation of chlorophenols has been studied (Arora and Bae, 2014) and several microorganisms have been reported to be able to mineralize chlorophenols (Arora and Bae, 2014). The extent to what these microorganisms could perform chlorophenol degradation in the natural environment remains to be fully understood. In addition, the influence and effect of multiple abiotic factors needs to be added to our comprehension of chlorophenol degradation to be able to provide a knowledgeable management of bioremediation procedures and models on the environmental fate of chlorophenol.

Soils are highly heterogeneous environments (Or et al., 2007) holding a huge microbial diversity (Curtis et al., 2002). As a consequence, following the fate of chlorophenols in these environments is a complex task. Environmental factors, such as temperature and water content, are important contributors to soil heterogeneity and changes in microbial activity (Manzoni et al., 2012) which must directly influence soil biodegradation of chlorophenols. Nevertheless, the influence of these factors and their interplay with specific chlorophenol-degrading microorganisms remains to be understood.

Meteorological factors can influence the functioning and mechanisms occurring at upper soil layers. For instance, high temperature and sun exposure can result in elevated temperature measurements at upper soil layers (Portillo et al., 2012), which implies the activation of soil thermophilic bacteria (Portillo et al., 2012), and, consequently, cause a switch on the functionally dominant bacteria and metabolic processes (Portillo et al., 2012; Santana and Gonzalez, 2015). This scenario can have important consequences both at local and global scales (Santana and Gonzalez, 2015). In addition, high temperature events imply increased evaporation leading to soils with low water availability, desiccation and aridity. Temperature and water content are intimately related at upper soil layers and could be implicated in important consequences on the behavior of soil microbial communities (Manzoni et al., 2012; Biederman et al., 2016), including their potential for biodehalogenation. There is a current gap in our knowledge on the influence of these factors (temperature and water availability) on the microbial potential to decompose chlorophenols.

The extension of landmass assigned to deserts, arid and semi-arid ecosystems represents a major area of terrestrial Earth surface located mostly at medium and low latitudes (Portillo et al., 2012; Santana and Gonzalez, 2015). Many of the arid-classified sites are potential targets for halogenated compound disposal in the field because these lands frequently belong to under-developed countries or present relatively low inhabiting human population. In this scenario, understanding the fate of chlorophenols in dry terrestrial environments is an important concern to envision if these pollutants are to be decomposed or will long persist in these environments.

This study will focus on 2-chlorophenol (2-CP) as a case study of biodehalogenation to better understand the fate of this pollutant in soils. We directed special attention to the effect of temperature and desiccating conditions on the fate of 2-CP in the environment. The aim of this study is to assess the influence of high temperature and low water content on the differential removal of 2-CP by distinct soil bacteria as a first approach to understand the potential bioremediation of halogenated pollutants, above all, in arid and dry soils.

MATERIALS AND METHODS

Bacterial Strains and Soil Samples

Two mesophilic (optimum growth temperature 28°C), 2-CP-degrading strains of the genus *Rhodococcus*, *R. opacus* IG (DSM 43205) (Klatte et al., 1994) and *R. erythropolis* DSM 43066 (Goodfellow and Anderson, 1977), were used in this study as soil isolates previously evaluated for their ability to degrade 2-CP (Bondar et al., 1999; Goswami et al., 2002; Arora and Bae, 2014). These strains were grown in medium 535 (Klatte et al., 1994) as recommended by the DSMZ (German Collection of Microorganisms and Cell Cultures) which composition (per liter) included: pancreatic digest of casein (17.0 g), enzymatic digest of soy bean (3.0 g), sodium chloride (5.0 g), dipotasium hydrogen phosphate (2.5 g), and glucose (2.5 g). Medium 535 was adjusted to pH 7.3. In addition, a soil thermophilic isolate (optimum growth temperature 60°C), *P. thermoglucosidasius* strain 23.6 (previous *Geobacillus*) (Aliyu et al., 2016) was a representative of the ubiquitous thermophilic bacterial community detected in soils (Marchant et al., 2002; Portillo et al., 2012) and grown in Nutrient Broth which contains (per liter): beef extract (5.0 g), peptone (10.0 g), and sodium chloride (5.0 g). Nutrient Broth was adjusted to pH 7.2 at 60°C. Strain 23.6 was confirmed to decompose 2-CP in liquid medium.

Soil samples were collected at Coria del Rio (Sevilla, Spain) at the experimental agricultural area "La Hampa" (IRNAS-CSIC) which soil characteristics have been previously reported (Cardoso de Barros, 1996).

Biodegradation of 2-Chlorophenol

Most studies have performed estimates of dehalogenation in aqueous solution. Unlike them, herein, we present a more realistic analysis by quantifying decomposition of a halogenated compound (i.e., 2-CP) in soils considering its high physical and chemical heterogeneity. The procedure allows to evaluate decomposition of 2-CP (and similarly other halogenated pollutants) at different water availabilities (i.e., water activity). To evaluate the decomposition of 2-CP over time by the studied strains under realistic soil conditions, 400 mg of autoclaved soil (3 consecutive days each at 121°C for 20 min) were supplemented with 2-CP (50 μl, 0.5 g/l) and 70 μl of a culture at its exponential phase of growth. Controls lack the addition of cells which were replaced by the same volume of sterile distilled water. The soil plus cell mixtures were desiccated in a vacuum concentrator (Micro-Cenvac, N-Biotek, Gyeonggi-do, South Korea) to obtain the desired water activity (a_w). Once the desired a_w was obtained, the samples (in triplicates) were placed in an incubator to the

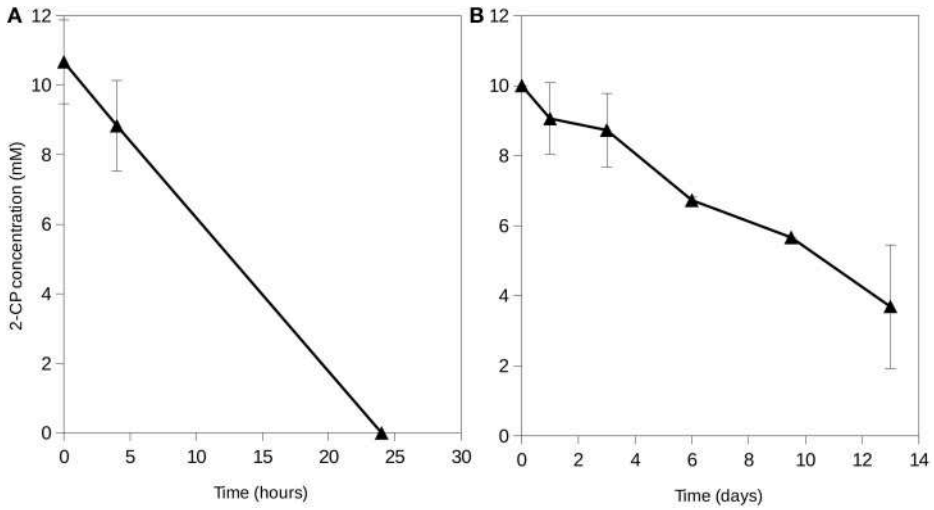

FIGURE 1 | Examples of the decay curves of 2-CP decomposition over time by *Rhodococcus opacus* (**A**; at a_W 0.93) and *Parageobacillus thermoglucosidasius* (**B**; at a_W 0.96) in soil samples.

required growth temperature (either 20 or 60°C for mesophiles or thermophiles, respectively). Over time, samples were collected at 0, 4, 8, 24, and 72 h incubation to determine the concentration of 2-CP in the soil mixtures.

Water activity represents the water available for microorganisms and it is defined as the partial vapor pressure of water in a sample divided by the partial vapor pressure of pure water, and it ranges from 0 (no water) to 1 (water). The a_W was determined using a water activity probe (Rotronic AG, Bassersdorf, Switzerland). The soil sample to be determined was placed in the temperature controlled probe chamber to perform the measurement following the manufacturers' recommendations. As a reference, the minimum a_W that allows the growth of a microorganism (the fungus *Xeromyces bisporus*) is >0.63 (Stevenson et al., 2015).

Quantification of 2-CP was carried out by HPLC (Agilent Infinity 1260; Agilent, Santa Clara, California, USA) using a mobile phase consisting on Acetonitrile:Water (70:30), and an injection volume of 10 µl through a column Agilent Zorbax Eclipse Plus C18 (Agilent, USA) at 35°C. Soil aliquots collected over time were supplemented with 1 ml acetonitrile to dissolve 2-CP, sonicated at 40°C for 15 min and vortex at room temperature for additional 15 min. The suspension was centrifuged at 8,000 × g for 4 min. The supernatant was collected and filtered through a 0.2 µm-pore-diameter filter. The extracted solution was injected in the described HPLC system and 2-CP quantified by peak integration after absorbance measurement at 273 nm (Goswami et al., 2002). 2-CP quantifications over time were used to estimate the decomposition rate (per hour) through the linear portion of the decay curve (**Figure 1**). These decomposition rates were estimated by linear regression (Sokal and Rohlf, 1995). All experiments were carried out in independent triplicates and the averages and standard deviations are presented.

RESULTS AND DISCUSSION

Understanding the functioning of soil ecosystems under a variety of environmental conditions is a requirement for a proper management of soils. Soils can be used as a source for economical benefit and food products as well as a potential site for the biodecomposition of human activity pollutants. Soils, and specifically, semi-arid and arid soils, are potential targets for dumping halogenated pollutants. Their environmental risks are high and it is of most importance to understand when and how the biodecomposition of 2-CP (as well as other halogenated pollutants) occurs in these soils.

Environmental conditions, such as high temperature and dry periods, affect soil upper layers. Besides, these upper layers are the most influenced by pollutants. However, the range of environmental conditions allowing bacteria to biodegrade halogenated compounds remains to be studied. Herein, we approach the biodegradation of 2-CP in a soil by different soil bacteria under a variety of temperature and water availability conditions commonly observed in natural soils.

The analysis of the capability of *Rhodococcus* species to decompose 2-CP in natural soils revealed that these soil mesophiles are only able to carry out efficient biodegradation of 2-CP under soggy and temperate conditions (**Figure 2**). Nevertheless, soil upper layers frequently reach high temperatures (e.g., up to 75°C during summer in Southern Spain and over 90°C at desserts) (McCalley and Sparks, 2009; Portillo et al., 2012) and consequently, their water availability is highly reduced, reaching highly desiccating values. Under these frequently encountered soil conditions, it is unknown if bacteria can carry out the process of decomposition of halogenated pollutants in soils and which bacteria and under which conditions this could be processed. Common 2-CP decomposing soil bacteria, such as *R. opacus* and *R. erythropolis*,

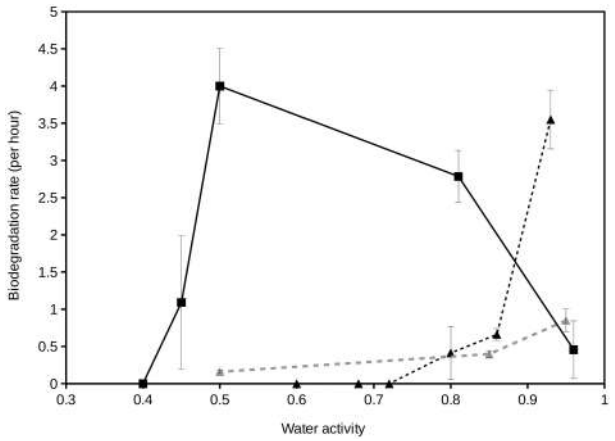

FIGURE 2 | Biodegradation of 2-CP in soil as a function of water activity for three bacterial species: a thermophilic *Parageobacillus thermoglucosidasius* (black continuous line and squares), and two mesophiles, *Rhodococcus opacus* (black discontinuous line and triangles) and *R. erythropolis* (gray discontinuous line and triangles). Note that the optimum 2-CP decomposition rate for the thermophilic species, *P. thermoglucosidasius*, was observed at water activity 0.5 (corresponding to a quite dry soil). For *Rhodococcus*, the optimum 2-CP decomposition rate occurred under soggy conditions, at water activity >0.9. Error bars represent one standard deviation.

are unable to carry out efficient decomposition of 2-CP in dry soils and at high soil temperatures (**Figure 2**).

Nevertheless, our results have shown that common soil thermophilic bacteria (i.e., *Parageobacillus*) (Portillo et al., 2012; Santana and Gonzalez, 2015; Aliyu et al., 2016) are capable of efficiently biodecompose 2-CP under high temperature and dry conditions commonly observed at upper soil layers (**Figure 2**). This suggests that thermophilic bacteria could replace mesophiles during hot soil periods. In fact, soils can get frequently hot at medium and low latitudes which is coincident with those land areas on Earth most exposed to high temperatures (Gonzalez et al., 2015; Santana and Gonzalez, 2015). These results confirm that under arid conditions, some soil bacteria can continue the biodegradation process of halogenated pollutants (i.e., 2-CP) and so these polluted soils can be bioremediated even under extreme temperature and water scarcity conditions.

Previous reports have indicated that thermophilic bacteria are ubiquous in soils (Marchant et al., 2002; Portillo et al., 2012). This presence of thermophilic bacteria in soils has explained the observation that maximum enzymatic hydrolytic activities in soils always occur at high temperatures (Gonzalez et al., 2015) and this has been related to soils with reduced organic matter content (Santana and Gonzalez, 2015). Those results represented previous evidence on the great potential of soil thermophiles in soils functioning and, definitively, in soil health (Marchant et al., 2002; Portillo et al., 2012; Santana and Gonzalez, 2015; Wong et al., 2015), even on their potential for dechlorination (Larsen et al., 1991) and the decomposition of recalcitrant hydrocarbons (Wong et al., 2015) in soils. Consequently, soil microbial communities present a great dynamism so that different species can be temporarily replaced depending on the environmental conditions that soils experience. That dynamism and high diversity of microbial soil communities represent the basis to maintain healthy and well-functioning soil ecosystems. These are important aspects to be considered when analyzing local and global consequences of natural microbial communities in response to the variable environmental conditions existing in our planet. Consequently, the above represent critical factors to understand soil bioremediation processes as a function of environmental parameters and under a current climate change scenario.

AUTHOR CONTRIBUTIONS

EM executed the experiment in collaboration with EP-F and EG. JG proposed the project and, in collaboration with EP-F and EG, designed and worked out the new methodology. JG prepared the manuscript and provided funding.

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REVIEW
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Biological Effects of Thermal Water-Associated Hydrogen Sulfide on Human Airways and Associated Immune Cells: Implications for Respiratory Diseases

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Natural mineral (thermal) waters have been used for centuries as treatment for various diseases. However, the scientific background of such therapeutic action is mostly empiric and based on knowledge acquired over time. Among the various types of natural mineral waters, sulfurous thermal waters (STWs) are the most common type in the center of Portugal. STWs are characterized by high pH, poor mineralization, and the presence of several ions and salts, such as bicarbonate, sodium, fluoride, silica, and carbonate. Furthermore, these waters are indicated as a good option for the treatment of various illnesses, namely respiratory diseases (e.g., allergic rhinitis, asthma, and chronic obstructive pulmonary disease). From the sulfide species present in these waters, hydrogen sulfide (H_2S) stands out due to its abundance. In healthy conditions, H_2S-related enzymes (e.g., cystathionine β-synthase and cystathionine γ-lyase) are expressed in human lungs, where they have mucolytic, antioxidant, anti-inflammatory, and antibacterial roles, thus contributing to airway epithelium homeostasis. These roles occur mainly through S-sulfhydration, a post-translational modification through which H_2S is able to change the activity of several targets, such as ion channels, second messengers, proteins, among others. However, in respiratory diseases the metabolism of H_2S is altered, which seems to contribute somehow to the respiratory deterioration. Moreover, H_2S has been regarded as a good biomarker of airway dysfunction and severity, and can be measured in serum, sputum, and exhaled air. Hence, in this review we will recapitulate the effects of STWs on lung epithelial-immune crosstalk through the action of its main component, H_2S.

Keywords: sulfurous thermal waters, hydrogen sulfide, S-sulfhydration, allergic rhinitis, asthma, chronic obstructive pulmonary disease

INTRODUCTION

Natural mineral waters from thermal springs (thermal waters) are used in Europe since ancient Greece for hygiene and later for the treatment of several diseases (e.g., respiratory, skin, and musculoskeletal diseases). Nowadays these waters are also used beyond their conventional purposes, namely with preventive, anti-stress, and aesthetic functions. The classification of thermal waters (35–40°C) is based upon their physicochemical features, which allows their subdivision into sulfurous, salso-bromo-iodic, bicarbonate, and bicarbonate-sulfate waters (1, 2). In fact, a beneficial link between sulfurous thermal water (STWs) and clinical improvement of several illnesses has been suggested (3–6). Such beneficial effects may be due to analgesic, antioxidant (7), antibacterial (8), and anti-inflammatory (9) properties of STWs. Thus, the main advantages of the therapeutic use of STWs lies in the fact that these provide a non-aggressive treatment, without considerable side effects, and which also has preventive properties (6, 8, 10, 11). Nevertheless, knowledge associated with the clinical properties of STWs in the context of respiratory diseases is mainly empiric, acquired over a period of centuries, since few well-designed clinical studies exist. Furthermore, although there are some very interesting *in vitro* studies on the effects of STWs on cells of the immune system, such studies are scant. Hence, the question of how exactly these waters modulate the observed clinical amelioration is poorly understood.

In this non-systematic review, we will analyze recent and past data obtained from a number of studies that have contributed toward the elucidation of the mechanisms of action of STWs on the lung epithelia-immune interface. To do that, we have performed a compilation of PubMed publications combining the following search terms "sulfurous thermal waters," and "hydrogen sulfide" with the terms "allergic rhinitis," "asthma," "chronic obstructive pulmonary disease" (COPD) "lung," and "lung endothelial cells" with Boolean operator "AND" and "OR." Various combinations were used, in order to focus on specific search questions of the various analyzed topics. No limitations were made in duration of the study or the demographic data of subjects. Literature published in the last 30 years was included. The inclusion criteria in this review were studies conducted with STW, H_2S-enriched waters, or H_2S for airway diseases, allergic, chronic, rhinosinusitis, COPD, or biological targets and effects of H_2S.

The following outcome parameters examined were included in this review: mucocilliary clearance time, nasal respiratory flow and resistance, adverse effects, and immunoglobulin values.

Overall, in the different searches performed, 7,345 studies were obtained. Of these, in total, 7,114 studies were excluded by reading the title or the abstract since they were not relevant or focused on cellular features or disease-related aspects that were not related to the topic of this review. Of the remaining 231 studies, 59 were excluded from analysis because of redundant information of lower quality than that in included studies or because the work focused on issues that were not fully relevant.

Abbreviations: STWs, Sulfurous thermal waters; H_2S, Hydrogen sulfide; IL, Interleukin; COPD, Chronic obstructive pulmonary disease; MAPK, Mitogen-activated protein kinase; NF-κb, Nuclear factor-kappa B; AR, Allergic rhinitis.

THERMAL WATERS: COMPOSITION AND BIOCHEMICAL PROPERTIES

Depending on their geographical localization, STWs present different physicochemical characteristics. These differences are due to their diverse chemical composition and to the presence of varying amounts of ions and salts, resulting in different therapeutic indications (2, 12).

Portugal is a country with abundant natural mineral (thermal) waters from north to south, as well as in the Portuguese islands, and the frequent visits to bath spas are quite common by the Portuguese population. Among the different types of thermal waters, sulfurous ones are the most common in the north and center of Portugal, with Termas das Caldas da Felgueira (13), Termas de São Pedro do Sul (14), Ferreira et al. (15), and Termas de Unhais da Serra (16) being some of the more representative ones in terms of sulfur-predominant waters. These thermal waters are alkaline (pH = 8.4–8.9), poorly mineralized, and are indicated for the treatment of respiratory, circulatory, digestive, rheumatic and musculoskeletal, as well as metabolic-endocrine diseases (12).

Concerning respiratory diseases, the therapeutic exposure to STWs is performed mostly through inhalation (6, 11, 17), and recently, significant clinical efficacy (e.g., nasal resistance and nasal flow improvement, and reduction of mucocilliary clearance time) was demonstrated when adult and elderly patients underwent hydrogen sulfide (H_2S)-enriched nasal water inhalations (18). Inhalator exposure efficacy depends upon various aspects which may affect particle deposition in the airways. These include adopted nebulizer, particle size, airway caliber, and patient's breathing pattern. For instance, the nebulizer must be able to produce particles with a diameter $<3\,\mu m$ in order to reach the bronchioli (11). Side effects are another aspect that must always be taken into consideration, whatever the implemented therapy. Even though STWs are generally well-accepted as a safe therapeutic tool due to their low number of side effects (10), these can still occur. In a systematic review and meta-analysis, Keller et al. (19), analyzed all side effects occurring in the pooled total patient population that took part in 13 clinical studies. Focusing on sulfurous waters, after 90 days of STWs treatment, only 19 out of 370 patients presented some adverse events. From those, 13 experienced mild nasal irritations and a sensation of burning, 5 suffered from very limited epistaxis, and one from dermatological hypersensitivity. Moreover, it is of note that even when subjects presented those effects, most of them were local and reversible (19). However, despite common use STWs to achieve a state of well-being and disease amelioration, the cellular and molecular bases underlying these beneficial effects remain unclear.

It was recently found that STWs can induce the production of moderate amounts of neutrophil-attracting chemokines, and low levels of tumor necrosis factor α and interleukin (IL)-6 (9). Even though pro-inflammatory mediators are frequently linked

to detrimental situations, moderate inflammatory stress may be regarded as positive, according to hormesis theory (20). Thus, mild stress can stimulate body systems repair, in order to prevent further and more severe damage, provided that this state does not involve the accumulation of irreversible changes (20). In addition, with time, inflammation may change the composition of nasal, sinusal, and lung bacterial flora, which may be associated with the development of resistant strains of bacteria (21). This means that inadequately controlled inflammation may lead to bacterial superinfection. Thus, for subjects with inflammatory respiratory disease, which is stable or in its initial phases, STWs may be a good complement to drug therapy since they may contribute toward prevention of recurrent infections caused by various bacteria (11) and viruses (22), and subsequently avoid the progression to a chronic state. Additionally, thermal waters might be also a useful tool in post-operative recovery in cases of chronic rhinosinusitis with or without polyposis, as observed by Staffieri et al. (23). These authors detected a significant reduction of the numbers of inflammatory cells (particularly eosinophils and mast cells) in the nasal mucosa of patients who were treated with sulfurous-arsenical-ferruginous thermal water inhalation after 6 months of having undergone endoscopic sinus surgery (23). Hence, by playing a preventive role in the progression of inflammatory states, STWs may have a prophylactic effect against further inflammation or subsequent superinfection. Furthermore, as a prophylactic agent, STWs may also reduce or even avoid additional hospital costs and degradation of patients' quality of life which are associated with events, such as frequent infection-induced exacerbations or prolonged hospitalizations. Thus, either as prophylactic or therapeutic tools, STWs may not only improve patients' general health and disease-related clinical parameters, but may also show other benefits from a social and financial point of view (e.g., reduction of drug-related costs, decreased hospitalization and disease-specific healthcare costs, national health care decongestion, and a decrease in school and work absenteeism), although some of these benefits were shown in rheumatological diseases and still need to be addressed in cost-effectiveness studies of STWs treatments for respiratory diseases (see **Table 1**).

TABLE 1 | Major beneficial effects induced by sulfurous thermal waters.

		References
Prophylactic	• Preventive role in the progression of inflammatory states or subsequent superinfection	(11, 22, 23)
Therapeutic	• Nasal resistance and flow amelioration	(18, 19)
	• Reduction of mucocilliary clearance time	(19)
	• Reduction of inflammatory influx	(23, 24)
	• Reduction of drug intake	(18, 19)
Socio-economic	• Reduction of school and work absenteeism	• Possible in respiratory diseases, but still needs to be addressed in cost-effectiveness studies
	• Decrease of hospitalization and other disease-related healthcare costs	

STW inhalation can have either a prophylactic or a therapeutic role, which may be associated with potential socio-economic benefits.

In thermal waters H_2S, hydrosulfide ion, and sulfide anion are the most common sulfide species present, with H_2S being the most abundant (7).

BIOLOGICAL PROPERTIES OF HYDROGEN SULFIDE

Due to its inflammable and corrosive nature, H_2S was thought to be a poisonous gas. However, in the last decades, it has been reported, along with nitric oxide and carbon monoxide, as a gaseous signaling molecule (25). Indeed, in contrast to nitric oxide, H_2S is relatively stable in body fluids, appearing as a promising therapeutic agent in several diseases. Nevertheless, it is important to take into account that H_2S can easily pass from water to air (26) and it is a gas which is soluble in water as well as in physiological liquids, and which is volatilized and broken down *in vivo* (namely in the lungs due to the abundant presence of oxygen) (27). Thus, H_2S frequently mentioned in studies may indicate a mix of H_2S and hydrosulfide and sulfide species, alongside with their effects (25, 28, 29).

H_2S is a colorless gas with potent reducing properties resulting from geothermal activities (sulfurous mineral water and volcanoes) and found in vegetable proteins (broccoli, garlic), and synthetic compounds (NaHS, GYY4137) (28, 30–34) (**Figure 1A**). In the human body, H_2S is produced by a variety of cells (e.g., epithelial, vascular, and smooth muscle cells), and is mainly synthesized from L-cysteine via cytoplasmic and mitochondrial cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes (30, 35). Endogenous H_2S is also generated by the combined action of cysteine aminotransferase, localized in the cytosol and 3-mercaptopyruvate sulfurtransferase, present in the mitochondria (28, 30, 34, 36). Apart from these enzymatic pathways, H_2S can also derive from indirect or secondary endogenous sources, particularly from glucose (via glycolysis), glutathione (GSH), inorganic and organic polysulfides, elemental sulfur, and even from bacterial sources present within the gastrointestinal and pulmonary tracts (29, 31, 33) (**Figure 1B**). After H_2S synthesis, it can either act on its biological targets or be stored as a bound sulfane sulfur pool, through oxidative formation of hydrodisulfides/persulfides, hydropolysulfides, and polysulfides) (33, 37) as well as an acid-labile sulfur pool, through metal-sulfur clusters, free hydrosulfide ion, and persulfides (29, 34) (**Figures 1C,D**). In the presence of oxygen, H_2S undergoes oxidation in the mitochondria by sulfide quinone reductase and also via methylation in the cytoplasm, through thiol-S-methyltransferase (30, 38) (**Figure 1E**). In addition, free H_2S can be scavenged by methemoglobin and molecules with metal or disulfide bonds (**Figure 1E**), and excreted together with biological fluids (urine and flatus), as well as exhaled in breath (**Figure 1F**) (30, 35).

Due to its high ability to diffuse across lipid membranes without the need of a transporter, H_2S can easily reach its molecular targets in a variety of cells, including those in

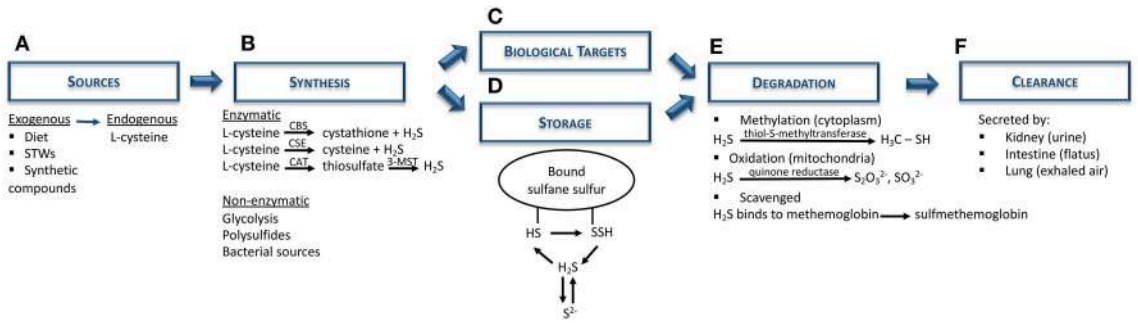

FIGURE 1 | Hydrogen sulfide metabolism: synthesis, storage, degradation, and clearance. **(A)** Both exogenous (diet; sulfurous thermal waters; synthetic compounds, e.g. NaHS) and endogenous (L-cysteine) are the main sources of H_2S biosynthesis. **(B)** H_2S is synthesized mainly via CBS and CSE enzymes, although it can also be produced through the combination of CAT and 3-MST. Nevertheless, non-enzymatic sources (e.g., polysulfides, bacterial sources) can also be H_2S sources. **(C)** Subsequently, H_2S can either act on its biological targets or **(D)** be stored as bound sulfane sulfur and acid-labile sulfur pools. **(E)** In order to maintain H_2S levels balanced, this gas undergoes further degradation via oxidation in mitochondria (quinone reductase) or, methylation in the cytoplasm (thiol-S-methyltransferase), or it can be scavenged by binding to hemoglobin. **(F)** Finally, H_2S is excreted by the kidney (urine), intestine (flatus) or lung (exhaled air). CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; H_2S, hydrogen sulfide; HS, hydrosulfide; 3-MST, 3-mercaptopyruvate sulfurtransferase; NaHS, sodium hydrosulfide; S^{2-}, acid-labile sulfur; SSH, hydrodisulfide; STWs, sulfurous thermal waters.

respiratory, cardiovascular, and neuronal systems (25, 28, 35, 39, 40). Its stability, storage, and release depend upon pH, temperature, and oxygen concentration of the environment. Thus, at physiological pH only a third of total sulfur amount is in the H_2S form. At acidic pH, however, H_2S is the only form present. In contrast, at an alkaline pH only the bisulfide form exists (28). Oxygen also influences H_2S stability since the presence of oxygen induces its conversion to sulfur, which can be further oxidized to hyposulfite, sulfites, and sulfates. In other words, under aerobic conditions H_2S is consumed and consequently its effective concentration decreases (29, 41). These findings corroborate the hypothesis that H_2S may act as a cellular oxygen sensor (42). Indeed, the decrease in H_2S oxidation under hypoxic conditions has been associated with the production of significant amounts of H_2S by airway epithelial cells (AECs) (43), akin to production via reduction of preexisting sulfites in mitochondria (44). Fu et al. suggested that this occurs as a result of CSE enzyme translocation to mitochondria, thereby allowing H_2S synthesis even after a stress-inducing stimulus, such as hypoxia (45). However, a significant increment of mitochondrial H_2S levels and a decrease of its clearance can, therefore, lead to harmful effects, including vasoconstriction and pro-apoptotic effects (46).

The measurement of H_2S has turned out to be of major importance to ascertain its putative role in a number of diseases. Although various methods have been used for measuring H_2S levels in blood and plasma/serum, such as the methylene blue method, sulfite-sensitive ion selective electrodes, and others, these methods have provided discrepant results, with H_2S levels in plasma ranging between 1 and 100 μM. This high variability has been attributed to the limitation and lack of sensitivity of the techniques (29, 47), to its capacity to diffuse through cellular membranes, and to the extremely short H_2S half-time *in vivo* (48). Hence, to overcome this obstacle it is mandatory to perform precise biological measurements of H_2S.

Regarding the airways, different approaches are used to quantify H_2S levels. On the one hand, Saito et al. have suggested that sputum H_2S may be a better biomarker than serum H_2S in lung-related diseases, since its serum levels may reflect other diseases of peripheral organs (49). Moreover, induced sputum was shown to be an effective sample to assess and identify asthma subtypes (50). However, Saito et al. found a negative correlation between H_2S levels in sputum and serum in COPD patients with acute exacerbations (51). This led the authors to propose that an increase in H_2S levels in sputum may reflect sequestration of H_2S from the circulation into the lungs, with the sputum-to-serum H_2S ratio being a good predictor of exacerbations (51). On the other hand, the measurement of exhaled H_2S is a non-invasive technique that is not affected by oral conditions (52) and which has been shown to positively correlate with the lung function, particularly forced expiratory volume in 1s (53). Nevertheless, unlike exhaled nitric oxide, H_2S levels in exhaled air are not used as an H_2S metabolism detector (54). Therefore, either exhaled or sputum H_2S could become promising and accurate indicators of airway diseases and airway disease phenotypes.

Biological Targets of Hydrogen Sulfide

Most of the biological activities of H_2S are exerted through protein S-sulfhydration, a post-translational modification process whereas an H_2S-derived sulfur group (sulfhydryl) is added to the thiol groups of reactive cysteine residues, originating hydropersulfide (35, 55) (**Figure 2**).

This formed group enhances reactivity of the protein and may be associated with an increment of its biological activity (55). The degree of protein S-sulfhydration can be influenced by cell pH as well as by the distance between the target amino acids and the active core of the protein (35). Unlike S-sulfhydration, S-nitrosylation caused by nitric oxide results in a reduction of cysteine reactivity. As an example, through S-sulfhydration the endothelial nitric oxide synthase (eNOS) activity increases, as a consequence of eNOS dimerization. In contrast, S-nitrosylation

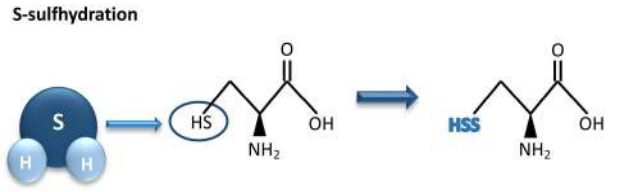

FIGURE 2 | Hydrogen sulfide-mediated S-sulfhydration reaction. H_2S adds a sulfhydril group to thiol groups of reactive cysteine residues, resulting in sulfhydration of its biological targets. H_2S, hydrogen sulfide.

of eNOS induces the formation of eNOS monomers, leading to a reduction of its activity (56).

Thus, H_2S is regarded as a gaseous signaling molecule that targets a number of important biological processes and pathways, and in this sense contributes to the maintenance of body homeostasis (35, 57, 58). The biological targets include ion channels, second messengers, signaling molecules, and transcriptions factors. Nevertheless, some of the observed H_2S effects are contradictory and probably result from different cell types and concentrations used.

Ion Channels

H_2S is able to interact with many ion channels and, as a consequence, alter their activity by inducing or inactivating their action. It has been reported that by sulfhydrating ATP-activated potassium channels (K^+_{ATP}) in smooth muscle cells (SMCs) (35, 59, 60) and small to medium calcium-dependent potassium channels in vascular endothelial cells (61), H_2S can induce the opening of these channels, thereby allowing K^+ ion influx, which leads to vasorelaxation.

In contrast, this molecule has the ability to inhibit big conductance calcium-activated potassium channels (35, 57, 61). Moreover, H_2S also exerts a regulatory effect on intracellular calcium levels by stimulating or inhibiting the T/L-type calcium channels and in turn may up- or down-regulate several calcium-dependent signaling pathways and enzymes, depending upon cell type (35, 61, 62). Furthermore, through the reduction of inositol-1,4,5-triphosphate receptor present in airway SMCs, H_2S can also affect calcium efflux in cells (62) (**Figure 3A**).

Second Messengers

Second messengers are important intracellular signaling molecules involved in several cellular pathways and their availability can be affected by H_2S in a direct or indirect way.

Bucci et al. have observed that H_2S has the ability to suppress the degradation of cyclic nucleotides by inhibiting the activity of phosphodiesterase, an enzyme that is responsible for their conversion into AMP and GMP (63). Thus, cAMP and cGMP availability increases, making these molecules available for intracellular signal transduction pathways that they are involved in.

The interaction of H_2S with calcium ion channels directly affects calcium availability in cells. Therefore, the interaction of this gaseous signaling molecule with calcium ion channels and intracellular calcium pools, can lead to a rise in intracellular calcium by inducing its influx and release, respectively (35). This subsequently induces endothelial proliferation. In parallel, the enhancement of calcium levels can also prompt the translocation of CSE from cytosol to mitochondria, thereby stimulating H_2S synthesis inside this organelle, and subsequent mitochondrial ATP production (45). On the other hand, the decrease in intracellular calcium can also be induced by H_2S via inhibition of calcium channels, and in turn this suppression seems to be involved in the induction of airway smooth muscle relaxation (64). Moreover, L-type calcium channel inhibition by H_2S induces membrane permeability and, subsequently, decreases elastase release (65) (**Figure 3B**).

Recently, it was reported that, under pathological conditions, the S-sulfhydration of the alpha subunit of ATP synthase supports its activation. In this manner, mitochondrial bioenergetics is maintained (66).

Signaling Molecules

Signaling molecules act as signal transmitters allowing the cross-talk between cells. H_2S has also been reported as capable of modulating the activity of several signaling molecules involved in biological processes, including phosphorylation, oxidation and degradation of proteins.

Thus, H_2S has been shown to regulate the activity of the mitogen-activated protein kinase (MAPK). In primary human lung endothelial cells, exogenous H_2S was able to prevent MAPK activation (67). H_2S has also been reported to increase the levels of other kinases, including PI3K/Akt, and protein kinase C, some of which can inhibit the production of pro-inflammatory cytokines (48, 57, 67–69) (**Figure 3C**).

Transcription Factors

Transcription factors are proteins responsible for regulating genetic information transcription and can also be a target for H_2S action. Thus, by undergoing S-sulfhydration transcription factors can induce up- or downregulation of gene expression.

The inactivation of nuclear factor-kappa B (NF-κB) through S-sulfhydration, blocks its translocation to the nucleus, which leads to the suppression of pro-inflammatory mediators and production of adhesion molecules (40). Nonetheless, depending upon the specific inflammatory stage, an opposite effect can be observed (70). Thus, in an initial phase of the inflammatory response, pro-inflammatory cytokine tumor necrosis factor α (TNF-α) can induce CSE transcription, thereby enhancing H_2S synthesis. Hence, the newly generated H_2S sulfhydrates the p65 subunit of NF-kB, promoting its binding to the coactivator ribosomal protein S3, which results in an increment of anti-inflammatory cytokine transforming growth factor-β secretion as well as the stimulation of anti-apoptotic transcriptional activity (57, 70). However, it should be highlighted that in both situations the inflammatory response is diminished, and consequently, oxidative stress decreases.

Moreover, as a consequence of S-sulfhydration of Kelch-like ECH-associated protein 1 (a negative regulator of factor erythroid-related factor 2 activity), the nuclear factor erythroid-related factor 2 is then translocated to the nucleus. This

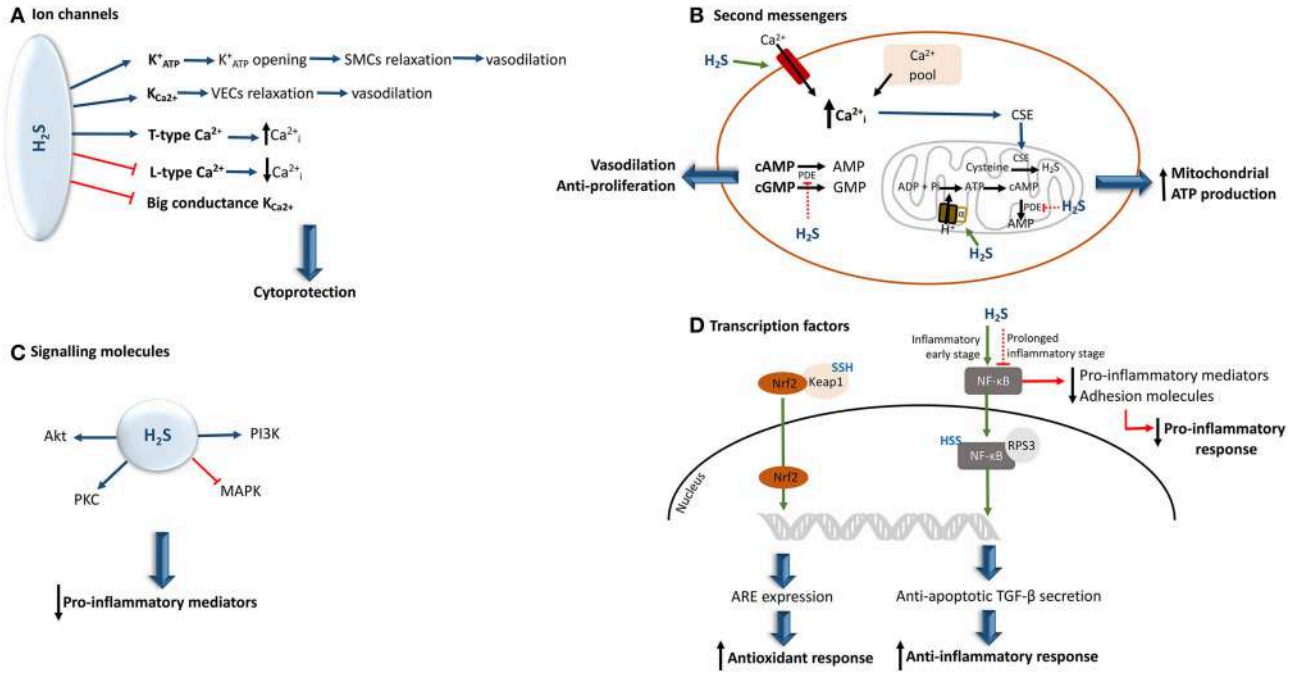

FIGURE 3 | Hydrogen sulfide interaction and effects upon the activity of its biological targets. **(A)** In a physiological concentrations, H_2S can either activate or inhibit different ion channels, providing a cytoprotective effect to the cell. **(B)** By inducing an increment of intracellular calcium, H_2S induces the translocation of CSE to the mitochondria, resulting in the generation of H_2S. In parallel, the H_2S-mediated S-sulfhydration of the mitochondrial alpha subunit of ATP synthase, allows the augmentation of cAMP levels. This results in the increase of mitochondrial ATP production. Moreover, H_2S can also inhibit PDE activity, allowing the increase of cAMP and cGMP net levels, thereby conferring vasodilatory and anti-proliferative properties **(C)** If, on the one hand, H_2S seems to be able to prevent MAPK activation, on the other hand it increases the levels of other kinases, including PI3K/Akt, and protein kinase C, some of which can inhibit the production of pro-inflammatory cytokines. **(D)** The H_2S-induced S-sulfhydration of Keap 1 induces the translocation of Nrf2 to the nucleus leading to the expression of ARE, which results in the augmentation of antioxidant responses. Moreover, H_2S can induce NF-κB activation or inhibition. This depends upon the stage of underlying inflammation. ARE, antioxidant response elements; Ca_i^{2+}, intracellular calcium; CSE, cystathionine γ-lyase; K^+_{ATP}, ATP-activated potassium channels; Keap 1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor-kappa B; Nrf2, nuclear factor erythroid-related factor 2; PI3K, phosphoinositide 3-kinase; PDE, phosphodiesterase; PKB, protein kinase B; PKC, protein kinase C; SMCs, smooth muscle cells; TGF-β, transforming growth factor-β; SSH, S-sulfhydration; VECs, vascular endothelial cells; the green arrow indicates that the pathway is stimulated and the red arrow indicates that the pathway is inhibited.

results in the expression of antioxidant response elements (68, 71) (**Figure 3D**).

Consequently, S-sulfhydration appears to play an important role in protecting cellular damage, since it has vital implications in anti-inflammatory as well as in antioxidant defenses.

Biological Effects of Hydrogen Sulfide in the Lung and Associated Immune System Cells

The respiratory mucosal epithelium is the first internal line of defense by acting as a major physical barrier between internal and external environments. The airway surface liquid (comprising mucus and periciliary liquid layers) covers this epithelial surface made up of goblet cells, ciliated cells, basal cells, and club–Clara cells (72–74). It is produced by the epithelial cells, which are also in contact with respiratory gases, thereby constituting the air-liquid interface (75). A connective tissue fibroblast-rich layer is located underneath the epithelial surface, and is involved in the maintenance of tissue homeostasis and wound healing (76). This cell layer also contains a variety of cell types, including cells of the innate and adaptive immune system (76). In this layer is located another lung barrier formed by endothelial cells, which separate the bloodstream and vessel walls. The endothelium also has other functions (e.g., blood and oxygen supply, nutrient delivery, and immune cell trafficking) (75, 76).

In physiological conditions, H_2S-related enzymes are expressed in the human lungs, namely in SMCs and primary fibroblasts (60, 77). It is now acknowledged that H_2S is required for the development of lung vasculature and alveolarisation (78), and in other lung functions, including airway tone and pulmonary circulation (30). Furthermore, H_2S appears to be involved in various processes namely airway mucolytic activity, oxidative stress, inflammatory state, cell proliferation, and apoptosis (10, 30). These will be briefly reviewed below (see also **Table 2**).

Mucolytic Effect

Mucins are secreted by goblet cells, submucosal glands in upper airways (75, 87), and alveolar cells in lower airways (74). By secreting mucins, the mucus traps and absorbs potential harmful pathogens and irritants which are subsequently removed from the respiratory tract by the process of ciliary beating which

TABLE 2 | General biological effects of hydrogen sulfide at physiological levels in the lungs.

Biological effects		Model of study	Disease	Thermal water or H_2S donor/control	Application	References
Mucolytic	Less viscous mucus	Human patients	Chronic inflammatory processes	Sulfur-chloride-bicarbonate-alkaline/-	12-days 1x daily humid-hot inhalation for 10 min	(10)
	Reduction of mucociliary transport time	Human patients	Chronic sinonasal disease	Sulfurous-arsenical-ferruginous/ISCS	12-days 1x daily warm vapor followed by nasal aerosol	(8)
			Chronic rhinosinusitis	Sulfurous/Physiological solution	12-day 1x daily warm vapor inhalations and nasal irrigations	(6)
			Chronic sinonasal inflammation	Radioactive water/-	14-days 1x daily warm vapor inhalations followed by nasal aerosol for 10 min	(24)
	Increment of mucociliary clearance	*In vitro* pig tracheae and cultured H441 cell line	Hypoxia	Na_2S/Hyperoxia (100% O_2)	100 μM Na_2S applied to the apical compartment	(43)
Antioxidant	Increment of GSH availability and SOD levels	*In vivo* female BALB/C mice sensitized with ovalbumin (OVA)	Inflammatory lung diseases	NaHS/iNOS inhibitor	Treatment 30 min before each OVA challenge during 6 days	(79)
Anti-inflammatory	Suppression of leukocyte adherence and migration	*In vivo* male C57BL6/J mice 8 weeks of age	Myocardial ischemia-reperfusion injury	Na_2S/-	NA_2S administered into the left ventricular at the time of reperfusion at different doses	(80)
		In vivo rats	Acute inflammation	NaHS and Na_2S/-		(81)
	Inhibition of macrophage inflammation	*In vitro* THP-1 Monocytes and RAW Macrophages cell lines	Macrophage inflammation	NaHS/Hexyl acrylate	Pre-treatment with NaHS for 30 min	(82)
	Inhibition of myeloperoxidase activity	*In vitro* inflamed colon cells from male Wistar rats and isolated neutrophils from human blood	Colitis and healthy state	Na2S/-	Interaction of sulfide and MPO assessed using several spectroscopic techniques	(83)
Antiviral/antibacterial	Protective role in controlling viral assembly/release	*In vivo* 10- to 12-week old BALB/c mice or C57BL/6J mice genetically deficient in the CSE enzyme	RSV infection	GYY4137/-	GYY4137 administration performed i.n. at different doses and timing of RSV infection	(22)
		In vitro A549 cell line and SAE cells	RSV infection	GYY4137/-	GYY4137 administrated either prior to infection, but not throughout the duration of infection, or at different times p.i. after the viral inoculum was removed	(84)
	Inhibition of bacterial biofilm production	Human patients	RURT infection	Sulfurous/physiological solution	12-day 1x daily warm vapor inhalations	(11)
Analgesic	Induction of endogenous opioid system activation	*In vivo* male Wistar rats	Visceral pain	Na_2S/vehicle	100 μmol/kg Na_2S administered 5 min before CRD	(85)
Anti-proliferative	Inhibition of SMCs proliferation	*In vitro* airway SMCs from donor lungs	–	NaHS or GYY4137/-	100 μM H_2S donors exposure for 2 or 3 days	(32)

(Continued)

TABLE 2 | Continued

Biological effects		Model of study	Disease	Thermal water or H_2S donor/control	Application	References
Anticancer	Increase in the production of metabolic acid lactase of cancer cells Impairment of pH regulatory system of cancer cells	*In vitro* MCF10A, MCF7, WI38, and HepG2 cell lines	Cancer	NaHS or GYY4137/ZYJ1122	400 μM H_2S donors exposure for 5 days	(86)

A549 cell line, human alveolar type II-like epitelial cell line; CRD, colorectal distension; GYY4137, morpholin-4-ium 4 methoxyphenyl [morpholino] phosphinodithioate, a novel water-soluble, slow releasing H_2S compound; GSH, glutathione; H_2S, hydrogen sulfide; H441, human lung epitelial cell monolayers; HepG2, human hepatocelular carcinoma; ISCS, isotonic sodium chloride solutioN; i.n., infected intranasally; MCF10A, human breast adenocarcinoma; MCF7, human breast epithelial; MPO, myeloperoxidase; Na_2S, sulfur salt; NaHS, sodium hydrosulfide; p.i., post-infection; RURT, recurrent upper respiratory tract infections; RSV, Respiratory syncytial virus; SMCs, smooth muscle cells; SOD, superoxide dismutase; SAE cells, small alveolar epitelial cells; WI38, normal human lung fibroblast; ZYJ1122, structural analog of GYY4137, which does not release H_2S.

Exposure to H_2S at physiological levels appears to have beneficial effects in various pulmonary processes, namely airway mucolytic activity, oxidative stress, inflammatory state, viral and bacterial infection, pain relief, cell proliferation, and carcinogenic.

mediates appropriate mucociliary clearance (73). An effective removal of mucus protects the respiratory epithelium, acting as a vital role in airways homeostasis (88). Both endogenous and exogenous H_2S show positive effects upon the respiratory tract by modulating the mucolytic activity. This appears to result from the interactions between H_2S and the disulfide bonds of mucins, resulting in breakage of the latter, which allows the mucus to become less viscous (10). The production of endogenous H_2S induces the opening of K^+_{ATP} and the activation of the cAMP pathway (30). Additionally, exogenous H_2S inhibits Na^+/K^+-ATPase and calcium-sensitive potassium channels in human bronchiolar epithelia, thereby triggering electrolyte absorption (33, 89).

This leads to an increase in mucociliary clearance, and therefore the elimination of foreign microorganisms can be more effective (43). Krause et al. also proposed that inhibition of transepithelial sodium absorption (via inhibition of Na^+/K^+-ATPase) is favored under acute hypoxia, in order to avoid H_2S degradation, as well as exogenous H_2S exposure (43). Additionally, an amelioration of mucociliary function was observed with inhalation of exogenous H_2S, as confirmed by a substantial reduction of mean mucociliary transport time in patients with chronic rhinosinusitis (8, 24).

Antioxidant Effect

In a well-controlled environment, reactive oxygen species (ROS) are responsible for cell signaling activation, enhancement of pro-inflammatory cytokines acting as mediators of immunity, and in addition, ROS are also able to protect the cells/tissues. Simultaneously, a balance is achieved with a tight regulation performed by antioxidants (e.g., reduced glutathione, GSH) which directly scavenge those species that are formed, thereby inhibiting any excessive production, by removing or repairing cellular damage or modifications induced by these reactive species.

In the lungs, exposure to H_2S promotes a boost of antioxidant effects, such as (i) increase in GSH availability, and superoxide dismutase levels (28, 79, 90), and (ii) generation of ATP, replacing oxygen in mitochondrial respiration (91). The reported H_2S-induced mitochondrial protection related to this antioxidant effect is known to result from mitochondrial cytochrome *c* oxidase inhibition and also from its capacity to modulate cellular respiration, thereby preventing the generation of ROS (48). Consequently, there is an increase in scavenging capacity (30, 34, 92), as well as an induction of endogenous antioxidant defenses (39). Hence, H_2S antioxidant features seem to involve both an indirect action and an induction of endogenous antioxidant defenses. For instance, by stimulating cysteine and cysteine transporter activity, H_2S induces an augmentation of substrate levels that are necessary for GSH production (29). Unlike large size antioxidants, H_2S can easily cross both plasma and mitochondrial membranes. This allows H_2S to more promptly reach its biological targets, and therefore it is considered to be more effective at diminishing cellular oxidative stresses, and at increasing antioxidant defenses (80). Braga et al. demonstrated that antioxidant effects of STWs, an exogenous H_2S source, provide protection against oxidative DNA damage (93).

Anti-inflammatory Effect

As the first line in contact with foreign species, AECs can act either as a modulator (during homeostasis) or as a sensor (post-injury lung homeostasis) of innate and adaptive immune systems (74). In healthy states, macrophages are the main immune cell type present in the airways, accounting for about 60–70% of all cells. Neutrophils represent 30–40% of total cells, while eosinophils are rare, not exceeding 2% (94). Beneath the epithelial layer, dendritic cells, macrophages, and mast cells can be found in the lamina propria. Dendritic cells and macrophages, in particular, can detect pathogens that have crossed the lung epithelium, phagocytose and destroy by microbicidal mechanisms, thereby contributing toward avoidance of significant infections.

Under a normal state the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) occurs through specific membrane or cytosolic receptors present on epithelial cells, and results in the activation of several signaling pathways (e.g., via MAPK, NF-κB, among others) (95–97). Therefore, pro-inflammatory cytokines, chemokines, antimicrobial proteins, and antiviral substances are produced and released by AECs, which allows them to have a crucial role in the recruitment and activation of innate (e.g., macrophages, eosinophils, dendritic cells) and adaptive (e.g., T and B cells) immune cells (96, 97). The presence of these cells allows several responses (phagocytosis, dendritic cell maturation, chemotaxis, inflammasome activation, and others) to be triggered (97). Subsequently, cells of the adaptive immune system (e.g., T and B cells) are activated. Thus, when homeostasis is affected, the usual function of AECs is reduced and pro-inflammatory activities increase significantly.

The anti-inflammatory properties of H_2S can in part be explained by its potent reducing, antioxidant, and scavenging features. Nevertheless, there are controversial results concerning H_2S properties, since it appears to exert both pro- and anti-inflammatory effects. Some authors have shown an *in vitro* promotion of granulocyte survival via H_2S-induced inhibition of caspase-3-cleavage and p38 phosphorylation (98, 99) and in an oxidative stress environment, activated neutrophils seem to be able to convert H_2S into sulfite, which is associated with inflammation (100). Moreover, there are studies suggesting that this gaseous transmitter is involved in GSH depletion and ROS formation (101), and consequently induction of mitochondrial cell death pathways (102). Meanwhile, others have suggested the participation of H_2S in some key anti-inflammatory pathways, such as: (i) suppression of leukocyte adherence and migration, mediated by K^+_{ATP} activation in endothelial cells and leukocytes (81), (ii) inhibition of oxidized low-density lipoprotein-induced macrophage inflammation via NF-κB suppression (82), leading to a reduction of several pro-inflammatory cytokines (e.g., IL-1β, IL-6, and IL-8) (103–105), and (iii) reduction of neutrophil toxic effects by inhibiting myeloperoxidase activity (83). However, these discrepancies may be related to differences in H_2S concentration. In fact, beneficial effects of H_2S generally prevail at lower concentrations, whereas deleterious effects are observed at higher levels and at fast-releasing rates, which is comparable to effects seen with carbon monoxide and cyanide (28, 34, 48). In addition, inhalation of H_2S at high acute levels or chronic lower-level exposure can also induce lung inflammation and toxicity (48).

Nonetheless, it has been known for some time that STWs can exert a direct anti-inflammatory effect in the lung. Exposure to these natural mineral waters may induce increased release of IL-10 levels by *in vitro* exposed primary human monocytes (9). Furthermore, an increase in the levels of IL-10 in saliva were also described in patients who had been treated with STWs (9). Moreover, STWs can simultaneously up-regulate immunoglobulin (Ig) A (an anti-inflammatory immunoglobulin) levels, as well as down-regulate the levels of IgE (a pro-inflammatory immunoglobulin) and cytokines secreted by eosinophils (90). This suggests that by stimulating anti-inflammatory defenses, STWs decrease the formation of ROS, and therefore modulate the pro-inflammatory state.

Antiviral and Antibacterial Effects

In several pulmonary diseases (asthma, COPD, among others) recurrent respiratory tract infections may occur due to a fragile protective mechanism system, which may enhance a pro-inflammatory state (106). As these diseases progress, viral and bacterial infections may become more frequent, and increasingly more difficult to treat. Consequently, the patients' health state worsens and their quality of life decreases.

Using AECs, Chen et al. shown a link between CSE inhibition and a significant increase in viral replication and chemokine secretion, which are reduced when these cells are exposed to a slow-releasing H_2S donor (107). Serum H_2S levels are significantly decreased in COPD subjects with very symptomatic exacerbations induced by bacteria and viruses (Type I; Anthonisen criteria), compared to control subjects. However, when COPD exacerbations are less symptomatic (Type II or III; Anthonisen criteria), serum H_2S levels are higher than those in control subjects (107). Moreover, when COPD patients who exacerbated are treated with antibiotics, serum H_2S levels are lower than in those patients who did not require antibiotic treatment. Taken together, these results indicate that endogenous H_2S synthesis is induced in order to counter the infections-mediated exacerbations (107). Currently, the most frequent therapies used to treat lung viral and bacterial infections are antiviral and antibiotics. However, over time, antibiotic-resistant bacteria may be increasingly observed.

To overcome these problems, some studies focused on the clinical efficacy of therapeutic S-based compounds (e.g., H_2S donors, STWs) (22, 84). Previous data showed that these compounds exhibit a protective role as an antiviral agent since they were able to control viral assembly/release (84). In turn, an improvement in viral infection-induced airway hyperresponsiveness can be prompt and may be associated with a decrease in the expression of pro-inflammatory mediators as well as in inflammatory cell influx into the lung (22). Furthermore, a study where patients with chronic sinonasal disease inhaled STWs showed a significant nasal flow improvement in these patients, which was associated with lowering the numbers of nasal bacteria (8). Also, a study in children with frequent

upper respiratory infections showed an important reduction in frequency, duration, severity, and social impact of the infectious episodes with STWs treatment (11). This antibacterial effect may be due to H_2S toxicity since only sulfur-bacteria and some microorganisms survive in the presence of S-based compounds, such as those present in STWs (108). This anti-pathogenic role also seems to be linked to the inhibition of bacterial biofilm production, as a result of blockage of the synthesis of microbial adhesins, thereby interfering with bacterial adherence to epithelia and reducing pro-inflammatory potential (6). Also, Varrichio et al. observed a significant reduction of bacteria in children with respiratory infections treated with salso-sulfide thermal water (17). In this context, Benedetti et al. proposed NF-kB pathway as a target of H_2S donors to reduce bacteria-induced inflammation (40).

Thus, S-based compounds can be a good complementary approach to conventional drug therapy, thereby increasing clinical efficacy of these treatments.

Analgesic Effect

Whether and how exactly H_2S can modulate an anti-nociception effect remains to be clarified. In patients with perennial allergic rhinitis (AR), a previous study involving inhalation of heated water showed that heat therapy may contribute to relief of perinasal pain and can somehow prevent nasal congestion (109). However, in this study, the heated water was not described in terms of H_2S content. Nevertheless, hyperthermia acts as a stimulus of neuroendocrine responses, with an increase in opioid (β-endorphin and Met-encephalin) and adrenocorticotropic hormone levels (110, 111). As mentioned previously, the main compound present in STWs is H_2S, and since this therapy is usually performed through inhalation at a temperature between 36 and 38°C (13, 14, 16), STWs therapy can somehow mediate an analgesic effect in respiratory diseases, mainly in the upper airways, during treatment. This can be explained in part, by the high temperatures of STWs but also by the role of H_2S in the activation of K^+_{ATP}. In fact, some studies have suggested that the H_2S-mediated analgesic effect is based upon the opening of neuronal K^+_{ATP}, which in turn is related with endogenous opioid system activation (85, 112).

Anti-proliferative Role

In the lung, cAMP and cGMP are responsible for mediating endothelium-dependent dilatation, since they are involved in lung vascular homeostasis. In fact, a reduction in their levels may lead to pulmonary hypertension, a disease characterized by high blood pressure that affects the lungs' arteries which become narrowed, blocked or destroyed (113). Since H_2S inhibits the activity of phosphodiesterase, cGMP net levels increase (63). Therefore, H_2S might eventually act as a good auxiliary agent in the treatment of pulmonary hypertension by preventing the proliferation of vascular SMCs and consequently promoting vasodilation of lung blood vessels.

Perry et al. proposed that both endogenous and exogenous H_2S might be able to control airway SMCs proliferation and cytokine release, namely IL-8, by suppressing these cell types. This occurs through inhibition of extracellular signal-regulated kinase 1/2 phosphorylation and extracellular signal-regulated kinase-1/2 p38 MAPK, with CBS, and not CSE, the main enzyme involved in the endogenous H_2S production (32). Although H_2S inhibits the proliferation of SMCs, it promotes the growth of endothelial cells (60, 114).

The inhibitory effect upon cell growth induced by exogenous H_2S was also observed by a reduction in lymphocyte subset proliferation, with a subsequent decrease in T cell-derived IL-2 production (115). Similar results were obtained in an *in vitro* study using STWs (116). This anti-proliferative effect was also shown by Baskar et al. when exogenous H_2S exposure caused human lung fibroblast cell death by inducing an increase in DNA lesions in these cells (102). In parallel, cell cycle regulators (p53, p21, and Ku proteins) were activated, being responsible for cell death via apoptosis (102). Therefore, the anti-proliferative effect prevents further degradation of DNA and allows the removal of injured cells by a controlled cell death process. In accordance with this, H_2S has been shown to exert a direct suppression effect on human lung fibroblast migration, proliferation, and transdifferentiation into myofibroblasts (117).

Anticancer Effect

During the last decade, some data have suggested various anticancer effects related to H_2S action (118–121). It was proposed that H_2S-mediated anticancer role is based upon the combined capacity of H_2S to increase the production of metabolic acid lactase and to impair pH regulatory system of cancer cells (86).

However, in contrast, and despite the anticancer efficacy in various types of cancer cells (e.g., gastric carcinoma, renal tumor), other studies have shown that H_2S may induce tumor cell proliferation, and migration, as well as angiogenesis, via inhibiting apoptosis (122–124). Jia et al. observed hepatoma cell suppression when CBS/H_2S pathway was inhibited (125). Moreover, Szczesny et al. have also found that besides normal lung epithelial cells, epithelial lung adenocarcinoma cells also have the ability to synthesize H_2S, in order to maintain their bioenergetics function and to increase its mitochondrial DNA repair processes. In this sense, cancer cell viability was preserved (126).

Hence, in a cancer situation the type of treatment must be re-assessed since H_2S seems to be advantageous for cancer cell proliferation. Nevertheless, the H_2S-induced inhibitory effects on cancer cell proliferation depend not only on H_2S dose and length of exposure, but also on tumor types. In this context, a continuous and prolonged exposure to low H_2S concentrations significantly affects cancer cell survival activity (127). In turn, with continuous, high-level exposure to H_2S, the cell survival rate is severely affected in both cancer and non-cancer cells (128). Oláh et al. have also indicated varying H_2S levels as an explanation for these inconsistent findings regarding anticancer effect of both H_2S inhibitors and donors (129). Nevertheless, these authors suggested that if on the one hand optimal endogenous H_2S concentration may induce proliferation of cancer cells, on the other hand both endogenous H_2S inhibition (decrease of its levels to below optimal concentration) and exogenous delivery (increment of its levels to above optimal

concentration) may suppress cancer cell proliferation (129). Clearly, H_2S-mediated effects have to be further studied, namely with various cancer cells lines *in vitro* as well as in animal models of various cancers, before any firm conclusions can be drawn.

It should also be taken into account that H_2S may have a biphasic biological effect, since at low-to-moderate concentrations H_2S has a physiological role, whereas at higher concentrations, it exerts a more pathological role, as summarized in **Table 3**.

Effects of Hydrogen Sulfide in Respiratory Diseases

The human nose and lung are continually exposed to indoor and outdoor agents (e.g., allergens, tobacco smoke, and pathogens). As a result, the airway mucosal epithelium, as an internal line of defense needs to play numerous roles in order to eliminate these foreign agents, thereby allowing normal airway function. On the other hand, after chronic injury and inflammation, the dysregulation of airway epithelial cell function may lead to the pathogenesis of lung diseases, such as AR, asthma, and COPD. These airways diseases are characterized by frequent or persistent respiratory symptoms and intermittent or persistent airflow limitation (130, 131). Moreover, besides the involvement of inflammatory cells and oxidative stress in the pathogenesis of AR, asthma, and COPD, there are various studies suggesting that these patients present alterations in H_2S metabolism (132–137).

Allergic Rhinitis

AR is a chronic inflammatory disease that affects the nasal airways, which become inflamed and engorged after exposure to an allergen to which patients are sensitized. In the nasal mucosa of AR patients, the relevant allergens bind to membrane-bound IgE on mast cells. Cross-linking of various IgE molecules by the allergen induces mast cell activation with rapid release of various pro-inflammatory mediators which are involved in development of acute symptoms. Eosinophils and lymphocytes are also involved, being crucial to the development of chronic symptoms (138, 139). In fact, this inflammatory process disturbs some features of the nasal mucosa, since inflammation is associated with swelling of sinusoidal capacitance vessels, reduction of nasal airway passages size, and an increase in mucus production (140). In addition, nasal airway remodeling may also take place in AR, although whether this really occurs remains controversial. In fact, some studies claim that remodeling is involved, since structural changes (e.g., nasal tissue glandular hypertrophy, collagen or extracellular matrix deposition) were observed, as compared with healthy controls (141, 142). However, in contrast other studies have not found such an association (143, 144).

Park et al. have shown that H_2S can be found in human nasal mucosa as well as in the plasma of healthy subjects. This may be partially explained by the presence of CBS and CSE in human nasal epithelium (134). While CBS is mainly distributed in the superficial epithelium and submucosal glands, CSE is exclusively localized in vascular endothelium and surrounding smooth muscles (133, 134). In the nasal and sinus mucosa, the amount of H_2S was shown to be increased in mild and moderate/severe persistent AR (134). In this context, there is an enhancement of human H_2S-synthesized enzymes, mRNA and protein levels, which consequently leads to a significant increment of H_2S levels in human airway (134). This may indicate a compensatory mechanism to attempt to revert the pro-inflammatory state.

Although there are few data specifically regarding STWs-treatment of AR, an amelioration in AR patients suffering from allergen-specific non-seasonal rhinitis when treated with a S-based compound water was observed, and this was associated with a reduction in total IgE and an increase in IgA serum levels (145). In accordance with this, it was suggested that STWs may exert an immunomodulatory activity by inducing an increase in IgA levels in nasal mucus (90). Another study also showed significant amelioration when AR patients were treated with STWs, namely in terms of a significant decrease in nasal flow resistance and nasal mucociliary transport time in 84% of subjects (146). Likewise, a significant reduction of IgE and an increase in IgA levels as well as an improvement of subjective symptomatology assessment scale were also observed (146). Data from these studies suggest a compensatory mechanism in order to reduce the presence of pro-inflammatory mediators and an improvement of the inflammatory state. However, clearly more thorough studies on the immunomodulatory and anti-inflammatory effects of STWs treatments in AR patients (as well as in patients with chronic rhinosinusitis) are needed.

Due to common immunopathophysiology, there is a close relationship between AR and asthma ("single airways concept"). In fact, AR is regarded as a risk factor for the development of asthma (147). Thus, although few studies have been carried out with STWs treatment in patients with asthma, we shall now analyse this context.

Asthma

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. Asthma is thus regarded as a long-term inflammatory disorder that results in excessive smooth muscle contraction, hyper-responsiveness, and variable airflow obstruction and bronchospasm. The development and progression of asthma are associated with airway inflammation (inflammatory cells and cytokines), mitochondrial dysfunction, and oxidative stress (148). Excessive airway mucus production is a feature of asthma, particularly in more severe stages, and mucus may even block the airways, which may lead to death by suffocation (149). The difficulty in controlling asthma is partially explained by its clinical and cellular phenotype heterogeneity. Thus, asthma patients may be grouped into different clusters which are associated with preferential features. In this context, one partial but possible subdivision of patients may be into those who preferentially present an eosinophilic bronchial infiltrate and those that have a preferentially neutrophilic profile (150, 151). In addition, it is fundamental to remember that AECs and SMCs are also some of the major cell types involved in asthma pathology, since they are sources of excessive mucus production and the mediators of cell contraction, respectively. This is supported by the underlying episodes of bronchial smooth muscle contraction in asthma patients (152). Likewise, both AECs and SMCs are responsible for facilitating the amplification

TABLE 3 | Dual biological effects of hydrogen sulfide: beneficial vs. deleterious.

	Beneficial effects (low H_2S levels)	**References**	**Deleterious effects (high H_2S levels)**	**References**
Antioxidant/oxidant	• Increase in GSH availability • Scavenging of ROS • Reducing agent	(79, 90) (48) (39, 92)	• Increase in ROS • DNA damage	(101) (100)
Anti-inflammatory/inflammatory	• Suppression of leukocyte adherence and migration • Inhibition of pro-inflammatory genes/cytokines • Increase in anti-inflammatory cytokines	(80, 81) (90)	• Promotion of granulocyte survival • Conversion of H_2S into sulfite • GSH depletion and consequent ROS formation	(98, 99) (100) (101)
Bioenergetic	• Mitochondrial ATP synthesis • Mitochondrial cytochrome *c* oxidase inhibition	(91) (48)	• Induction of mitochondrial cell death pathways • Reduction of ATP levels • Inhibition of mitochondrial respiration	(102)
Modulation of cancer	• Procarcinogenic	(122–124, 126)	• Anticarcinogenic	(34, 118–120)

H_2S, hydrogen sulfide; GSH, glutathione; ROS, reactive oxygen species; K^+_{ATP}, ATP-activated potassium channels.
Exposure to low levels of is associated with beneficial effects. In contrast, exposure to higher levels and fast-releasing rate of exposure to H_2S is associated with deleterious effects.

of lung inflammation, by recruiting T cells, which contributes to an uncontrolled pro-inflammatory environment. All of these actions are associated with histological changes in the airways (e.g., increased bronchial wall thickness, mucous metaplasia, and smooth muscle hyperplasia and hypertrophy) which may lead to the impairment of pulmonary function (153).

In human studies, a significant decrease in H_2S serum levels was shown in patients with stable asthma, in comparison with healthy subjects. Such a decrease was even more pronounced in asthmatic patients with severe acute exacerbations. It should be pointed out that a similar type of reduction in H_2S serum levels was also observed in smokers compared to non-smokers. Moreover, such a decrease was negatively correlated with the percentage of sputum neutrophils in patients with acute asthma (137). Furthermore, both serum H_2S levels and lung function parameters were found to be decreased in asthmatic children, suggesting a positive correlation between these two parameters (136). In contrast, Saito et al. found elevated H_2S levels in the sputum of asthmatic subjects, but without significant differences between mild and severe stages (49). These authors also observed a positive correlation between sputum H_2S levels and sputum neutrophil counts. Nevertheless, serum H_2S levels presented a negative correlation with sputum macrophage (49) and eosinophil counts (53).

Previous data have also shown that H_2S donors induce vascular smooth muscle relaxation, while they suppress the proliferation of airway SMCs and IL-8 release in humans (32). Since airway SMCs express high amounts of H_2S-related enzymes, it appears that the lack of H_2S may be involved in the progression and worsening of asthmatic airway obstruction.

However, exposure to environmental H_2S levels in excessive concentrations may have deleterious effects in asthma patients. In an epidemiological study carried out in northeast Nebraska (USA), a positive correlation was observed between the frequency of hospital visits by pediatric and adult asthma patients, due to exacerbations, and high (>30 ppb) outdoor total reduced sulfur and/or general H_2S levels on the previous day (154). However, it should be noted that actual individual levels of exposure to these sulfur species were not recorded but only inferred. In contrast, Bates et al. did not observe a significant increase in the risk of development of symptoms in asthmatic subjects when exposed to H_2S from geothermal activity in New Zealand (155). This suggests that, in certain settings, or in prolonged ambient exposure inhalation of high levels of environmental sulfur species may trigger asthma exacerbations, although this must be studied with more robust study designs and compared with intermittent and controlled exposure which is the situation observed in Spas.

Chronic Obstructive Pulmonary Disease

COPD is an airways disease caused by significant exposure to noxious particles or gases. The chronic, persistent, airflow limitation associated with COPD is caused by a mixture of small airways disease (e.g., obstructive bronchiolitis) and parenchymal destruction (emphysema) (131). Thus, COPD is characterized by an irreversible persistent decline of airflow associated with an enhanced chronic inflammatory response and emphysematous changes (156). It is well-known that COPD non-smokers and smokers share epithelial susceptibilities features, with direct or indirect cigarette smoking exposure being the main trigger for the development of this disease. In addition, most COPD patients have other chronic co-morbidities, with a proportion of them also having features of asthma–potential Asthma-COPD overlap/ACO (157, 158).

COPD is well-characterized by severe lung structural and functional changes, namely basal, goblet and mucous cell hyperplasia, as well as airway fibrosis (159, 160). Furthermore, a chronic influx of inflammatory cells (T lymphocytes, neutrophils, and alveolar macrophages) in bronchial wall and lumen is also involved in the pathophysiology of the disease (161). As a result of the pro-inflammatory actions, particularly those induced by neutrophil-derived enzymes, the lung parenchyma is destroyed in many cases, leading to emphysema (106, 161, 162).

In a small study performed by Sun et al. using human peripheral lung tissue samples from six patients with COPD, as well as from eleven healthy non-smokers and seven smokers with normal lung function, despite H_2S levels being quite similar among the groups, a significant decrease in CSE protein levels and an increase in its mRNA levels was observed in COPD patients as well as in smokers (135). However, in contrast, CBS mRNA levels were reduced in COPD patients as well as in smokers, suggesting that H_2S metabolism may be altered in the lung tissue of COPD patients, just as it is in smokers (135). Moreover, the same group showed a negative correlation between exhaled levels of H_2S and sputum eosinophilia, in a larger group of 77 COPD patients, suggesting that increased levels of exhaled H2S may predict a non-eosinophilic phenotype in these patients (163). Nevertheless, temporal stability of such phenotypes must be confirmed, since it may vary over time. In fact, Chen et al. also showed that serum H_2S levels in COPD subjects vary longitudinally, with higher levels being more frequently seen in a stable state than in acute exacerbations of COPD (132). Both in healthy subjects and in COPD patients with acute exacerbations, H_2S levels were more significantly reduced in smokers than in non-smokers. Also, in this study, regarding serum H_2S levels, a negative correlation with sputum neutrophils, and a positive association with sputum lymphocytes and macrophages was observed in all patients with COPD. Finally, it seems that H_2S availability is also related to the stage of airway obstruction in COPD since its levels are significantly decreased in the more advanced stage III than in the milder stage I (132). Thus, the authors justify the enhancement of serum H_2S levels in stable COPD patients as a compensatory mechanism. Nevertheless, increased levels of H_2S were observed in COPD subjects with an exacerbation, in comparison with patients with stable COPD, healthy smokers, and non-smokers (51). Despite certain contradictions founded in the previous studies, all indicated significant changes of H_2S and H_2S-synthesized enzymes levels in COPD subjects. These H_2S metabolism alterations seems to contribute, at least in part, to exacerbations and worsening of this respiratory disease state, affecting general lung function.

A significant reduction of oxidative stress and an amelioration of symptoms in subjects suffering from moderate to severe COPD were observed after a 12-day inhalation with STWs and 1 month after the end of the treatment (92). Similar beneficial effects have been observed in patients with chronic rhinosinusitis (6). Further studies focusing on mechanisms underlying such STWs-driven improvement in COPD patients are warranted.

In summary, in spite of generally effective drug-based treatments for most cases of AR, bronchial asthma, and COPD, some patients still show a sub-optimal response to such treatments. Furthermore, over time, long-term high-dose therapy may be associated with the development of some adverse effects. Thus, additional thermal spa complementary therapeutic tool mainly for subjects whose symptoms are not adequately controlled with the usual drug-based therapeutic approach seems to be a good option. With supplementary STWs treatment it may be possible to regain symptom control and eventually reduce baseline drug therapy. Another aspect which must be borne in mind is that with STWs treatment at Spas, additional psychological components may also contribute toward clinical improvement. These components include leisure time, opportunity for relaxation, being aware of regular clinical monitoring, and various cultural aspects, all of which may play a part in Spa treatment-associated final results (164).

CONCLUSIONS AND FUTURE PERSPECTIVES

Mainly due the presence of H_2S, STWs might be an advantageous and promising option as an add-on non-pharmacological complementary therapy for respiratory diseases, such as AR, chronic rhinitis/rhinosinusitis, bronchial asthma and COPD, since these natural mineral waters have been associated with significant, quick onset, and relatively long-lasting improvement of clinical parameters in patients with these diseases. Furthermore, H_2S-rich STWs have various effects upon inflammatory and immunological parameters that may contribute toward their clinical efficacy. It may thus be possible to use STWs in a preventive way in terms of disease progression and exacerbations, although this needs to be better ascertained. Moreover, if correctly applied, no significant side effects have been reported with STWs. Nevertheless, in spite of the potential benefits of STWs in respiratory diseases, further elucidation of the mechanisms underlying such benefits warrants further studies. To that end, *in vitro* models appear to be a promising choice for studying the effects of STWs on human respiratory mucosa. Recently, Epithelix Sàrl (Switzerland) and MatTek Corporation (USA) have developed an *in vitro* 3D human lung models with a morphologically and functionally differentiated structure as well as an ability to be maintained in a homeostatic state for a long period of time and to be a ready-to-use model (165, 166). In addition, this 3D cell model can also be used to perform safety testing of occupational and environmental chemicals, pharmaceutical development, drug delivery, and inflammatory responses (167–172). Overall, since these *in vitro* 3D models seem to reproduce human biological responses, they are an interesting choice in the study of various respiratory diseases, such as AR, asthma, and COPD, namely in terms of their response to STWs exposure, and this is one of the current interests of our research, which is in accord with current ethical concerns regarding the use of animals in research investigations, i.e., the 3R (Replace, Reduce, Refine) and animal welfare.

CONTRIBUTION OF THIS REVIEW TO THE FIELD

This review is novel andignificantly contributes toward having a clearer and more organized view of the role of sulfurous thermal/natural mineral waters (STWs), in inhalational treatment of respiratory diseases, such as rhinitis, asthma or chronic obstructive pulmonary disease. Furthermore, this review also recapitulates the main effects of STWs on lung epithelial-immune crosstalk through the action of its main component, H2S, thereby establishing a relationship between clinical efficacy

of these treatments and the underlying immunopathological and immunotherapeutic mechanisms.

AUTHOR CONTRIBUTIONS

JV and AE carried out literature searches and wrote the manuscript. EC checked the biochemical aspects. FA checked the immunological aspects. MV and LT-B checked the clinical aspects. All authors made contributions to the review. EC, FA, MV, and LT-B checked the final version of the manuscript.

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Visitors to a Tropical Marine Beach Show Evidence of Immunoconversions to Multiple Waterborne Pathogens

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Determining infections from environmental exposures, particularly from waterborne pathogens is a challenging proposition. The study design must be rigorous and account for numerous factors including study population selection, sample collection, storage, and processing, as well as data processing and analysis. These challenges are magnified when it is suspected that individuals may potentially be infected by multiple pathogens at the same time. Previous work demonstrated the effectiveness of a salivary antibody multiplex immunoassay in detecting the prevalence of immunoglobulin G (IgG) antibodies to multiple waterborne pathogens and helped identify asymptomatic norovirus infections in visitors to Boquerón Beach, Puerto Rico. In this study, we applied the immunoassay to three serially collected samples from study participants within the same population to assess immunoconversions (incident infections) to six waterborne pathogens: *Helicobacter pylori, Campylobacter jejuni, Toxoplasma gondii,* hepatitis A virus, and noroviruses GI. I and GII.4. Further, we examined the impact of sampling on the detection of immunoconversions by comparing the traditional immunoconversion definition based on two samples to criteria developed to capture trends in three sequential samples collected from study participants. The expansion to three samples makes it possible to capture the IgG antibody responses within the survey population to more accurately assess the frequency of immunoconversions to target pathogens. Based on the criteria developed, results showed that when only two samples from each participant were used in the analysis, 25.9% of the beachgoers immunoconverted to at least one pathogen; however, the addition of the third sample reduced immunoconversions to 6.5%. Of these incident infections, the highest levels were to noroviruses followed by *T. gondii.* Moreover, many individuals displayed evidence of immunoconversions to multiple pathogens. This study suggests that detection of simultaneous infections is possible, with far reaching consequences for the population. The results may lead to further studies to understand the complex interactions that

occur within the body as the immune system attempts to ward off these infections. Such an approach is critical to our understanding of medically important synergistic or antagonistic interactions and may provide valuable and critical information to public health officials, water treatment personnel, and environmental managers.

Keywords: immunoconversions, incident infections, exposure, multiplex immunoassay, salivary antibodies, antibody response, Luminex, Boquerón Beach

INTRODUCTION

Understanding the complex relationship between water quality, human exposure to waterborne pathogens, and subsequent infections is an ongoing public health challenge. Previous studies have shown considerable risk of illness related to polluted recreational and potable water (1–4). One of the primary risks associated with such exposures is the manifestation of acute gastrointestinal illness which may be caused by a variety of viruses, bacteria, and protozoa. In lieu of classic approaches to studying exposure (e.g., water sampling, risk modeling, epidemiological surveys, enzyme-linked immunosorbent assays), we developed a bead-based salivary IgG antibody multiplex immunoassay using the Luminex^{TM} xMAP platform. Many traditional immunological studies involve examining antibodies in a monoplex, one pathogen at a time. Monoplex immunoassays, in general, do not truly capture the dynamic nature of the immune system and obscure our ability to observe simultaneous antibody responses to multiple pathogens. The multiplex developed and applied in this study assesses IgG antibodies in saliva to examine exposure to multiple pathogens simultaneously (5–7).

Saliva is a biofluid with emerging applications in research and clinical settings. We previously demonstrated that salivary IgG antibodies offer a viable alternative to the traditional use of blood, serum, and plasma for assessing immunoprevalence to waterborne pathogens (5, 8, 9). Researchers have noted the many benefits of using saliva including ease of collection, transport, and storage, applicability to remote collection and point-of-care settings, non-invasiveness, and the use of sampling mechanisms which are easily tolerable by children. The use of assays based on saliva are thus expected to have positive impacts on infant and child health (10–12).

We previously determined the prevalence of salivary antibodies against six waterborne pathogens: *Helicobacter pylori, Campylobacter jejuni, Toxoplasma gondii,* hepatitis A virus, and norovirus genogroups G.I.I and GII.4 in beachgoers and swimmers at Boquerón Beach, Puerto Rico using the multiplex immunoassay (5–7, 13, 14). We also linked epidemiological factors to the norovirus exposures and were able to identify asymptomatic infections (i.e., detectable infections unlinked to symptomology) associated with swimming at a fecally contaminated beach (15). As a follow-up, we aim to determine immunoconversions and evidence of multiple infections to the six target pathogens using samples collected from visitors to the beach which has been shown to be impacted by sewage from publicly-owned treatment works.

Immunoconversion relates to the development of detectable antibodies in the blood and saliva, usually within days after exposure to infectious agents. Soon after an initial exposure and during the acute phase of an infection, IgM antibodies along with other immune system components dominate the response. Later IgG antibodies that retain memory of the exposure are produced and these immunoglobulins rapidly expand in the event of a secondary infection as part of what has been described as the anamnestic response (16).

Previous immunoconversion studies relied on two serum samples (one pre-exposure and another post-exposure) obtained via invasive procedures. These studies measured a 4-fold increase in antibody response from the first to second sample to determine whether there was a change in antibody levels indicative of an active infection. In our study, we collected an initial sample (S1) at the beach before swimming, and the remaining samples (S2 and S3) were self-collected by participants 10–14 and 30–40 days later, respectively. After collecting and processing the samples, we compared immunoconversion rates given traditional criteria based on two samples to the rates using criteria based on all three-samples. Further, we explored the data to assess whether there was evidence of simultaneous co-infections (immunoconversions to two or more pathogens). Co-infections have been shown to aggravate and exacerbate the symptoms of AGI-related diarrhea (17, 18).

MATERIALS AND METHODS

Reagents

5.6 micron polystyrene microsphere (bead) sets were obtained from Luminex Corp. (Austin, TX, USA) at a concentration of 12.5×10^6 beads/ ml each. Biotinylated goat anti-human IgG (λ) secondary detection antibody was obtained from KPL (Gaithersburg, MD, USA). Antigens were purchased as described and coupled to the beads in accordance with the optimized multiplex immunoassay (5, 8). Characterized sera was obtained from SeraCare (Milford, MA, USA) to validate the assays.

Antigen Coupling and Confirmation Using Animal-Derived Antibodies

Beads were activated and coupled, as previously described (5, 8) and serial dilutions of primary capture antibodies specific to each antigen were used to confirm that the beads were coupled properly thus ensuring that the dynamic range of the assay could be defined (5, 8). Briefly, coupled bead stocks were diluted in PBS-1% BSA to a final concentration of 100 beads/μl of each unique bead set. 5×10^3 beads from each bead set was

added to individual wells of a pre-wet 96-well filter plate. An equal volume 2-fold serial dilutions of anti-species IgG primary antibody was added to the beads, mixed gently, covered, and allowed to incubate in the dark, at room temperature for 30 min at 500 rpm on a VWR™ microplate shaker (Radnor, PA, USA).

After incubation, supernatant was vacuumed out, wells were washed twice with 100 μl of PBS pH 7.4 containing 0.05% Tween 20 (PBS-T) (Sigma, St. Louis, MO, USA) and vacuumed again to remove excess buffer. Beads were resuspended in PBS-1% BSA buffer and exposed to 0.8 μg of biotinylated anti-species IgG secondary detection antibody. The filter plates were covered and allowed to incubate in the dark at room temperature for 30 min on a plate shaker. After a 30-min incubation in the dark on a plate shaker to protect the beads from bleaching, the wells were washed twice as above. Then the samples were incubated for 30 min with 1.2 μg of streptavidin-R-phycoerythrin, vacuumed, washed X2, and resuspended in 100 μl of PBS-1% BSA. The plates were then analyzed on a Luminex 100 analyzer (Luminex Corporation, Austin, TX, USA).

Saliva Collection, Processing, and Analysis

Saliva samples were collected from 2,091 study participants at Boquerón Beach, Puerto Rico during the summer of 2009 as part of the National Epidemiologic and Environmental Assessment of Recreational Water Study (NEEAR) conducted by the United States Environmental Protection Agency (19). A key criterion for selecting the beach was ensuring that local residents were the primary visitors; hence, while tourists do recreate at the beach, visitors are largely locals with over 70% indicating six or more visits to the beach annually. Informed consent was obtained from subjects in accordance with Institutional Review Board approval (IRB # 08-1844, University of North Carolina, Chapel Hill, NC, USA). Study participants were instructed to rub the Oracol™ saliva collection device (Malvern Medical Developments, Worcester, U.K) against the gingival crevices of the oral mucosa (between the gums and teeth) to absorb saliva. Infants younger than 1 year old were excluded from the study because of the potential for contamination by maternal antibodies and high rates of non-waterborne infections. Individuals who reported dental or other illnesses at the time of the initial collection were also excluded.

The baseline samples (S1) saliva samples were collected on the beach by trained study staff members while the second and third samples were self-collected by the participants at home and within a day or two post-collection, the samples were shipped overnight on ice to USEPA in Cincinnati for storage and analysis. Upon receipt, the swabs were stored at −80°C until ready for processing which typically occurred in <1 week. The Oracol™ saliva collection devices were thawed to room temperature and centrifuged twice (first at 491 × g, 10°C for 5 min to recover the saliva off the collection sponge and then at 1,363 × g, 10°C for an additional 5 min to pellet debris from the saliva) and transferred to 1.5 ml microcentrifuge tubes. Finally, the samples were centrifuged at 1,500 × g for 3 min and the supernatant transferred to a fresh 1.5 ml microcentrifuge tube and stored at −80°C.

A 1:4 dilution of the saliva samples in phosphate buffered saline containing 1% bovine serum albumin (PBS-1% BSA) was added to prewet and vacuumed 96-well filter plates (Millipore, Billerica, MA, USA). 5×10^3 beads from each bead set and an equal volume of diluted saliva were loaded onto each well resulting in a final dilution of 1:8 in a total volume of 100 μl per well. The loaded filter plates were processed, as previously described (5, 8), reporter fluorescence was measured using a Luminex 100 analyzer and expressed as MFI (Median Fluorescence Intensity) of at least 100 beads per bead set.

Assay Controls, Cross-Reactivity, and Signal to Noise Ratio (SNR)

Assay controls are described in greater detail in previous publications. Briefly, an uncoupled unique bead set was added to the assay to evaluate non-specific binding and sample to sample variability (5, 8). These control beads were treated identically to antigen conjugated beads and blocked with BSA. However, they were not coupled to any antigen during the coupling step. To control for non-specific binding and/or contamination of the saliva by serum from gum disease or other sources, samples with reactivity to uncoupled control beads at ≥500 MFI were discarded.

Tests for cross-reactivity were performed in monoplex, duplex, and multiplex for each antigen. Assay sensitivity was validated with characterized human plasma samples as previously described (8). A signal to noise ratio (SNR) was calculated by dividing the MFI of the specific antigen signals by the MFI of the uncoupled control beads for each sample (8, 20).

Defining Immunoconversions and Data Analyses

Only study participants who gave all three samples were considered and two definitions, or immunoconversion criteria (IC) were evaluated. Criteria A is based on the commonly used approach for determining seroconversions for clinical samples collected in a controlled environment: a 4-fold MFI increase from S1 to S2 ($S2 \geq 4 \times S1$) (14, 21, 22). The second criterion (Criteria B) was developed by extending the traditional definition to ensure that the S2 sample MFI is immunopositive [greater than the cut-off point (9)]; and the S3 sample MFI is at least three times that of the S1 sample ($S2 \geq 4 \times S1$; $S2 \geq$ cutoff; $S3 \geq 3 \times S1$). To reduce potential false positives, we measured antibody responses in the S3 sample. IgG levels are expected to remain relatively high and not drop to zero during the 30–40 day period after initial exposure for all the organisms in this assay. The addition of the S3 sample may guard against considerable variation in volume and composition as well as possible errors in labeling since S2 and S3 were self-collected. All data analyses were performed using Microsoft Excel 2016, JMP 14, and MATLAB Release 2018b.

RESULTS

Study Population

Study participants provided 5,533 serially collected samples which were processed to prepare the immunoassay results for statistical analyses (**Supplementary Material**). Before assessing

immunoconversions, we removed samples with control bead MFI ≥500 (as previously noted) and computed a mean MFI of samples with duplicate IDs. The remaining samples included saliva collected from 2,078 individuals at the beach (S1) and then self-collected by 1,694 individuals at S2 and 1,666 individuals at S3. Scatterplots of the MFI results suggest that IgG antibodies to the noroviruses were more prevalent (the most frequently detected of the targeted pathogens) in the study population followed by hepatitis A virus (HAV) and *H. pylori* (**Figure 1**). In contrast, most of the *T. gondii* and *C. jejuni* MFI results were near the level of the control beads. Of these samples, MFI results were available from 1,399 individuals who provided saliva during all three collection periods. This cohort was used to estimate the number of immunoconversions by measuring IgG antibody levels against the six antigens in the Luminex multiplex immunoassay and computing immunoconversions based on the criteria developed.

Immunopositivity and Immunoconversions

A cut-off value of 505 [criteria = $10^{\text{mean(h)}+3\text{SD(h)}}$, where $h = \log$ (control) and SD = standard deviation] was previously developed as a part of the Boquerón immunoprevalence study (9); hence, samples with MFI ≥505 for the study pathogens are considered immunopositive. A heatmap for the target pathogens (**Figure 2**) provides a visualization of the immunopositive samples (in red) at each collection time point (**Figure 2**, upper panel). Results indicate that over 60% (68, 61, and 62% in S1, S2, and S3, respectively) of the individuals were immunopositive to at least one pathogen during each sample period. As expected, the cohort of beachgoers was primarily exposed to noroviruses (NoV GI.1: 45.34% and NoV GII.4: 30.05%) followed by HAV (15.68%; reported as a mean over the sample periods) and the highest levels were found in the baseline sample (S1), indicating that these participants were exposed prior to providing the initial sample on the beach.

MFI trends reflect antibody response patterns and afford the ability to estimate immunoconversions in the study population. When comparing the two immunoconversion definitions, there were 363 (25.9%) and 91 (6.5%) of the 1,399 individuals who immunoconverted to at least one pathogen, given Criteria A and Criteria B, respectively. For Criteria A, the immunoconversions amongst the specific pathogens ranged from 97 (6.93%) for NoV GII.4 to 166 (11.87%) for *T. gondii*. Conversely, there were much fewer immunoconversions based on Criteria B with most being to noroviruses (NoV GI.1: 39(2.79%); NoV GII.4: 36(2.57%) followed by *T. gondii* (22(1.57%) (**Table 1**). While most of the immunoconversions were to one pathogen, many individuals immunoconverted to multiple simultaneously (Criteria A: 46.56 %; Criteria B: 24.18 %).

Results from the less stringent criteria (Criteria A) reflected multiple simultaneous immunoconversions to all the pathogens in the study. Although several of the samples were found to meet the 4-fold increase guideline between S1 and S2, many of these samples never exceeded the immunopositivity cut-off level (MFI ≥505). Therefore, these samples were considered immunonegative. Accordingly, we shifted away from Criteria A and used the more stringent definition (Criteria B) which not only assures that the second sample is immunopositive, but by including S3, also affords the ability to capture antibody patterns that mimic the anamnestic response and could help reduce the number of false positives.

Using Criteria B to further examine immunoconversions, we note that 69 individuals (75.82%) immunoconverted to one pathogen and 22 individuals immunoconverted to multiple (24.18%), resulting in 120 total immunoconversions. **Figure 3** provides IgG antibody response curves for the immunoconversions captured during the study period. In the plots, the dashed red line represents the immunopositivity cut-off value (MFI = 505) and helps to highlight the number of ways individuals immunoconverted to the target pathogens based on Criteria B. All immunoconversions met the core criteria (S2 ≥ 4 × S1; S2 ≥ cutoff; S3 ≥ 3×S1), yet a small number of them (n = 9) presented with immunopositive baseline samples indicating previous exposures, mostly to noroviruses. In addition, rather than declining from S2 to S3, some of the MFIs continued to increase suggesting the possibility of new exposures at S2. Of the immunoconversions to multiple pathogens, the majority (n = 18) were to only two pathogens. **Figure 4** provides a breakdown of the co-immunoconversions observed by specific pathogen pairs. Nearly half of these co-immunoconversions were to the noroviruses (44.4%) and over a fifth were to *H. pylori* and Nov GI.1 (22.2%) and *H. pylori* and hepatitis A (22.2%).

DISCUSSION

Detecting exposure and incident infections to environmental pathogens has long been a complex environmental and public health challenge. Classic approaches involve epidemiological studies and surveys capturing demographic and symptomology information, collection of water, soil, and/or air samples to detect organisms in media and complex modeling to estimate potential impacts on human populations. As an augmentation of this practice, this effort extends the use of salivary antibodies as biomarkers of exposure. Rather than estimating the risk of exposure, our approach relies on the body's immunological response to determine whether exposure and subsequent infection to specific pathogens has occurred. Capitalizing on the availability of antibodies in saliva samples, we developed a multiplex immunoassay to measure IgG antibodies against waterborne pathogens. The optimized assay was used to estimate immunoprevalence to targeted pathogens given saliva samples collected from beachgoers at Boquerón Beach, Puerto Rico (9). Additionally, we identified a breakthrough connection between asymptomatic norovirus infections and swimming at the beach (impacted by sewage from publicly-owned treatment works) finding that infected parties did not necessarily present with gastrointestinal symptoms (15). The assay was also applied in a prospective community study of waterborne infections to characterize associations between water-related exposures, *Cryptosporidium* and norovirus infections and gastrointestinal symptoms (6).

In this work, we applied the multiplex immunoassay to investigate antibody responses and detect evidence of immunoconversions to six target pathogens in visitors at

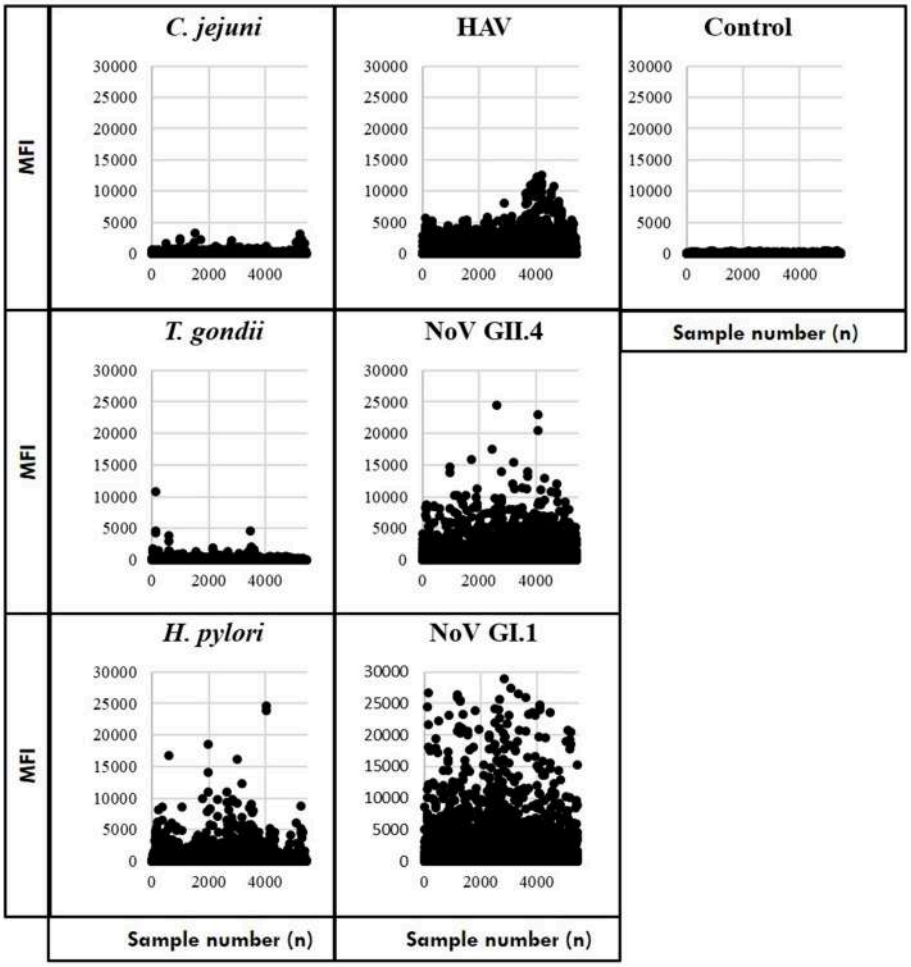


FIGURE 1 | Median fluorescence intensity (MFI) for the target pathogens and assay controls.

Boquerón beach, Puerto Rico. Our effort was a blind examination of the study population focused on IgG antibodies characterized by patterns in median fluorescence intensity and aimed at providing an immunological response screening approach for all pathogens in the multiplex.

After applying the immunoassay and processing the MFI results, we found that 1,399 visitors provided samples during all three collection periods and on average, 892 (64%) of them displayed evidence of detectable antibodies to at least one pathogen (9). This is consistent with previous studies of acute gastrointestinal illness (AGI) performed in multiplex (23–26). These observations demonstrate the potential for multiplex assays to identify multiple infections and may be applied either cross-sectionally or longitudinally to provide insight into the epidemiology of these conditions, as well as understanding the role of potential risk factors.

When only a single pre- and post-exposure sample is available, calculating immunoconversion is typically based on a 4-fold increase in antibody titer between the initial and follow-up sample (14, 21, 22). This may be suitable when the samples are collected in controlled clinical settings; however, collection inconsistencies arise when samples are collected in a field setting where antibody responses in individuals may be more variable due to factors related to self-collection (e.g., sample contamination, spitting). While many individuals demonstrated typical immunoconversions, with a 4-fold increase in antibody levels that was sustained 6 weeks later (S2), we noticed some variations in the data. Examples of this variability included cases in which: (1) we found a 4-fold increase in antibody levels from S1 to S2 but the MFI during both periods was below the cut-off point (MFI = 505); hence, neither sample was immunopositive, or (2) cases where the baseline sample (S1) level was already above the immunopositivity threshold, suggesting previous exposure, and the individual did not experience a 4-fold increase MFI for the targeted pathogen at S2. Using the traditional approach (Criteria A), we found 363 (25.9%) immunoconverted individuals compared to 91 (6.5%), resulting in an almost 4-fold decrease when using the more stringent definition (Criteria B).

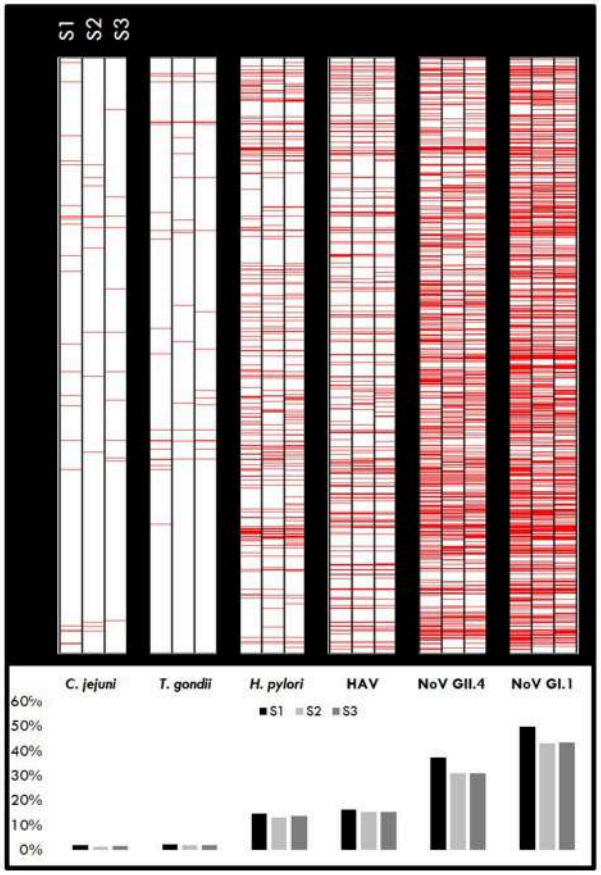


FIGURE 2 | Immunopositivity heatmaps showing MFI response by pathogen for individuals who gave all three samples ($n = 1{,}399$). Upper panel: Red lines represent positive samples ($\geq$ cut-off) for S1, S2, and S3 samples for each pathogen. The darker areas indicate a higher number of positive samples. Lower panel: Percentage of samples positive for each pathogen.

In our study, some of the saliva samples were self-collected and varied considerably in quality, volume, and composition. We used the follow up sample (S3) to more fully characterize the immunological response of individuals who showed the initial increase in antibody levels and account for the variability associated with the saliva samples. The addition of the third sample afforded the ability to examine patterns reflective of an anamnestic response more tightly linking the immunoconversion criteria (Criteria B) to expected immunological patterns. The more stringent immunoconversion criteria (Criteria B) may present the best option to analyze the data because, in most cases, IgG responses are expected to remain elevated for months to years, in some cases, following initial exposure and infection (27–32). As a result, the third sample may protect against potential false positives resulting from sample to sample variability in antibody concentrations due to collection technique or volume of sample collected. Further, immunoconversions to pathogens with relatively long incubation periods (for example, HAV−19–49 days) may not be detected using the two-sample approach with S2 being collected about 10 days after the initial sample.

TABLE 1 | Immunoconversions to target pathogens: number (*n*) and percentage (%) of individuals that immunoconverted.

To Single and Multiple Pathogens: *n* (%)		
IC	**Criteria A**	**Criteria B**
Single ($N = 1$)	194 (53.44)	69 (75.82)
Multiple ($N \geq 2$)	169 (46.56)	22 (24.18)
To Specific Pathogens: n (%)		
Pathogen	**Criteria A**	**Criteria B**
Campylobacter jejuni	107 (7.65)	1 (0.07)
Helicobacter pylori	127 (9.08)	2 (0.14)
Toxoplasma gondii	166 (11.87)	22 (1.57)
Hepatitis A virus	99 (7.08)	20 (1.43)
Norovirus GI.1	121 (8.65)	39 (2.79)
Norovirus GII.4	97 (6.93)	36 (2.57)

As noted in Wade et al. (15), the number of participants who reported symptoms of AGI was much lower than expected. The low number of symptomatic individuals may be attributed to the fact that many of these individuals had been exposed to those pathogens previously and therefore were immunologically protected and accordingly, did not exhibit symptoms (e.g., diarrhea, vomiting). We observed evidence of protective immunity during our analysis of immunoprevalence where participants were found to have very high antibody levels in the baseline (S1) samples which were indicative of prior exposures (9) most without complaints of the symptoms of gastrointestinal illness (15). This phenomenon of high underlying immunity in a population is not uncommon and as a recent example, was observed during an outbreak of hepatitis E virus in Chad (33).

There are several limitations to our study. Firstly, although antibody binding appears to be specific within the context of these antigens, we cannot eliminate the possibility of cross-reactivity. The issue of cross-reactivity (binding to similar or overlapping ligands) or multi specificity (binding of distinctly different ligands or different conformations of the same antibody (34–37) continue to plague immunological studies. Questions pertaining to non-specific binding and cross-reactivity were addressed by using PVX buffer vs. PBSA (8, 9, 38). Our analysis showed that PVX buffer was superior to PBSA in reducing non-specific binding in plasma and serum samples but had very little effect on saliva samples (9).

Another limitation of the study is that antibody levels in saliva samples are typically lower than levels observed in sera or plasma (36). Some individuals with low specific serum IgG will have even lower saliva IgG concentrations and therefore, these individuals may not be considered when reporting immunoprevalence or immunoconversions. The implication of these findings is that immunoprevalence and immunoconversions may be underestimated by oral fluid IgG detection because the assay may not have sufficient sensitivity when the antibody titer range is low.

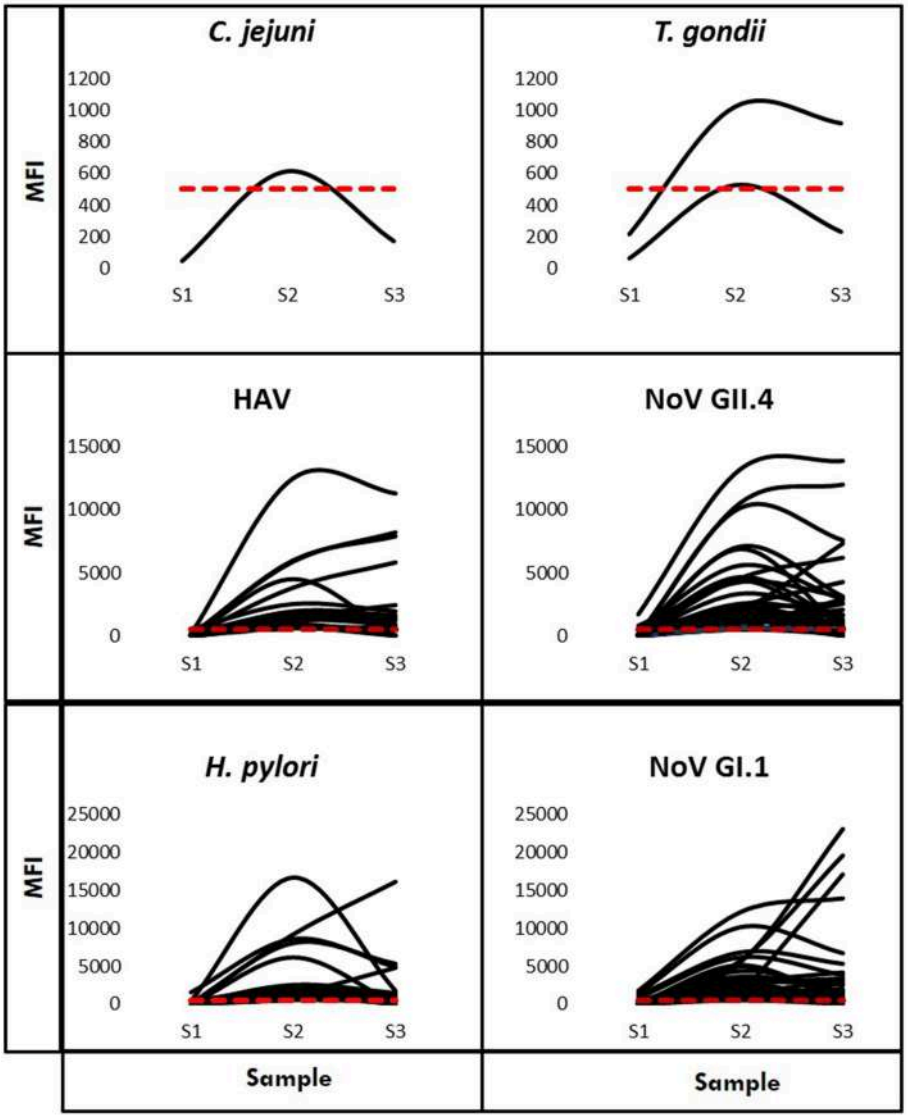
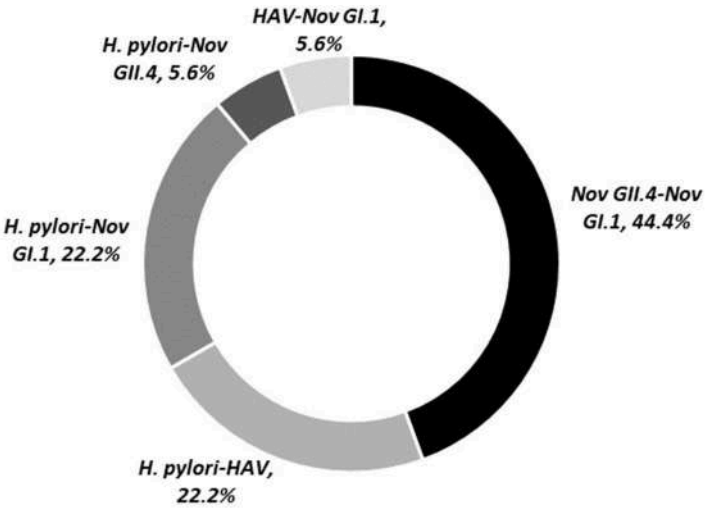


FIGURE 3 | IgG antibody response curves for the six multiplexed pathogens (Criteria B). Characteristic curves showing antibody responses measured in MFI from the baseline (S1) to the final (S3) sample for individuals who immunoconverted. The red dashed line represents the cut-off point (MFI = 505).

Although the immunoglobulin concentration in crevicular saliva is lower than that found in serum, we used the Oracol sampler because it collects crevicular fluid enriched with serum antibodies. In this study, we focused on systemic rather than on mucosal immune responses particularly in the IgG isotypes. It was previously shown that IgA antibody measurement in saliva was unreliable and influenced by several factors including donor's age, secretory flow rate and acute or chronic stresses (39). Furthermore, it has been shown that the proportion of specific to total IgG is similar in saliva and serum (40).

Finally, the ability to separate specific environmental exposures (i.e., at the beach) from other exposures, including those leading to underlying immunity in the population is an ongoing public health challenge. Many pathogens are not solely waterborne, and humans are constantly exposed to potentially harmful agents through what they eat, breathe, touch, etc. Accordingly, it is important to link the findings from exposure studies to occurrence data gathered through water quality studies, soil sampling, air monitoring, etc. to help identify potential sources of exposure for monitoring and management.

Despite these limitations, this multiplex salivary antibody immunoassay has been very useful in measuring immunoprevalence to the six pathogens in the assay as well as identifying asymptomatic norovirus infections in swimmers at Boquerón Beach (9, 15). In September 2017, Hurricane Maria severely devastated the island of Puerto Rico. The hurricane caused significant damage to the island's health, electrical, water, and sewage infrastructure. A comparative study of the post-hurricane immunoprevalence and incident infections would be highly desirable. Such a study would improve



	C. jejuni	T. gondii	H. pylori	HAV	NoV GII.4	NoV GI.1
C. jejuni	X	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)
T. gondii		X	0(0.00)	0(0.00)	0(0.00)	0(0.00)
H. pylori			X	4(22.22)	1(5.56)	4(22.22)
HAV				X	0(0.00)	1(5.56)
NoV GII.4					X	8(44.44)
NoV GI.1						X

FIGURE 4 | Co-Immunoconversions to specific pathogen pairs calculated using Criteria B. Number of individuals (*n*) who immunoconverted to both pathogens simultaneously.

our understanding of the impact of natural disasters in the pathogenesis of infections and disease as well as inform risk assessment and management in the aftermath of these events. This assay is being deployed in large epidemiological studies of fresh water rivers, lakes and wells and marine beaches to study the impact of water quality on human health. The results being obtained from these studies will help to improve our understanding of the transmission of environmental pathogens.

The salivary antibody multiplex immunoassay presented here can measure the presence of human salivary antibodies to multiple antigens simultaneously to determine immunoprevalence and immunoconversions in individuals. Furthermore, this assay can identify simultaneous infections and co-immunoconversions in individuals exposed to environmental pathogens. Recreational water sources are typically contaminated with multiple pathogens and hazardous chemicals, each of which can assault the health of exposed individuals simultaneously.

Very little information is available about how these simultaneous infections affect human health and whether they result in synergistic or antagonistic interactions. Griffiths et al. studied the impact of co-infections in humans and concluded that they have a deleterious effect on health exacerbating infection outcomes in humans (41). Moreover, Bhavani et al. observed synergistic effects between rotavirus and co-infecting pathogens on diarrheal disease, noting that the pathogenic potential of each organism appeared to be enhanced during co-infection (42).

We have applied this immunoassay in epidemiological studies at several locations across the United States and were able to estimate immunoprevalence to environmental pathogens, identify asymptomatic norovirus infections, and demonstrate associations between water-related exposures, AGI symptoms and *Cryptosporidium* and norovirus infections. When used in conjunction with water quality studies, this assay can potentially enhance our knowledge and understanding of environmental microbial pathogenesis and assist risk assessment modelers in estimating exposure potential thus facilitating the development of disease surveillance and screening tools thereby leading to better health outcomes and a cleaner, more sustainable environment for the general population.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

Approval was obtained from the University of North Carolina, Chapel Hill, NC, USA (IRB # 08-1844), for the collection of saliva samples from beachgoers at Boquerón Beach, Puerto Rico, as part of the USEPA NEEAR Water Study. Study subjects provided informed consent and were instructed on the use of the Oracol™ saliva collection device. Infants younger than 1 year were not included. Informed consent was obtained from parents of minors.

AUTHOR CONTRIBUTIONS

SA designed the study with KS and TE. TW, KO, AD, and ES provided the NEEAR saliva samples. SA, KS, SG, CC, and MR conducted the laboratory experiments and processed the raw assay data. TE performed the data analysis. KS, TE, and SA wrote the original manuscript and KS, TE, SA, CC, SG, MR, KO, ES, TW, AG, and AD participated in the review and discussion of the study data and the criteria development.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2019.00231/full#supplementary-material

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Outdoor Air Pollution and Brain Structure and Function From Across Childhood to Young Adulthood: A Methodological Review of Brain MRI Studies

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Outdoor air pollution has been recognized as a novel environmental neurotoxin. Studies have begun to use brain Magnetic Resonance Imaging (MRI) to investigate how air pollution may adversely impact developing brains. A systematic review was conducted to evaluate and synthesize the reported evidence from MRI studies on how early-life exposure to outdoor air pollution affects neurodevelopment. Using PubMed and Web of Knowledge, we conducted a systematic search, followed by structural review of original articles with individual-level exposure data and that met other inclusion criteria. Six studies were identified, each sampled from 3 cohorts of children in Spain, The Netherlands, and the United States. All studies included a one-time assessment of brain MRI when children were 6–12 years old. Air pollutants from traffic and/or regional sources, including polycyclic aromatic hydrocarbons (PAHs), nitrogen dioxide, elemental carbon, particulate matter (<2.5 or $<10\,\mu m$), and copper, were estimated prenatally ($n = 1$), during childhood ($n = 3$), or both ($n = 2$), using personal monitoring and urinary biomarkers ($n = 1$), air sampling at schools ($n = 4$), or a land-use regression (LUR) modeling based on residences ($n = 2$). Associations between exposure and brain were noted, including: smaller white matter surface area ($n = 1$) and microstructure ($n = 1$); region-specific patterns of cortical thinness ($n = 1$) and smaller volumes and/or less density within the caudate ($n = 3$); altered resting-state functional connectivity ($n = 2$) and brain activity to sensory stimuli ($n = 1$). Preliminary findings suggest that outdoor air pollutants may impact MRI brain structure and function, but limitations highlight that the design of future air pollution-neuroimaging studies needs to incorporate a developmental neurosciences perspective, considering the exposure timing, age of study population, and the most appropriate neurodevelopmental milestones.

Keywords: air pollution, development, brain, neuroimaging, magnetic resonance imaging (MRI)

INTRODUCTION

There is increasing evidence that air pollutants may disrupt brain and nervous system development in vulnerable populations (1). Given that the brain's structure and function continues to mature well into the third decade of life (2–14), the neurotoxic effects of outdoor air pollution may be present throughout the entire dynamic neurodevelopmental process, as a result of exposure from childhood, through adolescence, and into young adulthood (15–17). Extant data on neurodevelopmental effects of air pollution suggest that both prenatal and postnatal exposure is associated with deficits in intelligence quotient (IQ) (18–22), as well as a broad range of cognitive domains (21, 23, 24), subclinical mental health problems (25, 26) as well as risk for Autism Spectrum Disorders (27–29). Together, these findings suggest outdoor air pollution exposure may impact cognitive development and emotional behaviors, yet questions remain regarding the mechanisms influencing the structural and functional brain alterations that may underlie these associations observed in children and adolescents. The aim of this study was to systematically review the current air pollution epidemiologic literature that used Magnetic Resonance Imaging (MRI) techniques to study brain structure and function in children and adolescents. While recent reports have highlighted this growing area of research (30, 31), the current review not only offers an updated state-of-the-science summary and synthesis from the developmental neuroscience perspectives, but also provides a thorough methodological critique of the literature that was largely missed in earlier reviews.

METHODS

We conducted an extensive search of the MEDLINE database through PubMed using a combination search of a list of "air pollution" terms and a list of "brain imaging" terms. A comprehensive strategy for a combination search was developed to identify articles published before September 6, 2018 and with keyword components including air pollution and functional and/or structural MRI, using both MESH terms and Title/Abstract keywords and excluding animal studies and studies published in non-English languages. The search algorithm was as follows: [("magnetic resonance image"[title/abstract] OR "magnetic resonance images"[title/abstract] OR "magnetic resonance imaging"[title/abstract] OR "MRI"[Title/Abstract] OR "white matter hyperintensity"[title/abstract] OR "white matter hyperintensities"[title/abstract] OR "neuroimage"[title/abstract] OR "neuroimages"[title/abstract] OR "neuroimaging"[title/abstract] OR "neuroinflammation"[title/abstract] OR "systemic inflammation"[title/abstract] OR "white matter volume"[title/abstract] OR "white matter volumes"[title/abstract] OR "brain structure"[title/abstract] OR "brain volume"[title/abstract] OR "brain volumes"[title/abstract] OR "neurotoxic"[Title/Abstract] OR "neurotoxicity"[Title/Abstract] OR "neurotoxicities"[Title/Abstract] OR "functional connectivity"[Title/Abstract] OR "Brain/pathology"[mesh] OR "Brain/physiopathology"[Majr] OR "Magnetic Resonance Imaging"[Mesh] OR "Cognition Disorders/pathology"[Mesh] OR "Cognition Disorders/chemically induced"[Majr] OR "White Matter/pathology"[Majr]) AND ("Air Pollution"[Title/Abstract] OR "Air Pollutant"[Title/Abstract] OR "Air Pollutants"[Title/Abstract] OR "Particulate Matter"[Title/Abstract] OR "Ozone"[Title/Abstract] OR "Nitrogen dioxide"[Title/Abstract] OR "Nitrogen oxides"[Title/Abstract] OR "Sulfur Dioxide"[Title/Abstract] OR "black carbon"[title/abstract] OR "elemental carbon"[title/abstract] OR "Vehicle Emission"[Title/Abstract] OR "Vehicle Emissions"[Title/Abstract] OR "diesel"[Title/Abstract] OR "diesel exhaust"[Title/Abstract] OR "diesel exhausts"[Title/Abstract] OR "vehicle exhaust"[Title/Abstract] OR "vehicle exhausts"[Title/Abstract] OR "vehicular exhaust"[Title/Abstract] OR "vehicular exhausts"[Title/Abstract] OR "road traffic"[Title/Abstract] OR "PM2.5"[Title/Abstract] OR "PM10"[Title/Abstract] OR "coarse particle"[Title/Abstract] OR "coarse particles"[Title/Abstract] OR "ultrafine particle"[Title/Abstract] OR "ultrafine particles"[Title/Abstract] OR "Polycyclic aromatic hydrocarbon"[Title/Abstract] OR "Polycyclic aromatic hydrocarbons"[Title/Abstract] OR "Air Pollution"[Mesh] OR "Particulate Matter"[Mesh] OR "Ozone"[Mesh] OR "Nitrogen dioxide"[Mesh] OR "Nitrogen oxides"[Mesh] OR "Sulfur Dioxide"[Mesh] OR "Vehicle Emissions"[Mesh] OR "Air Pollution/adverse effects"[Majr] OR "Polycyclic Aromatic Hydrocarbons/adverse effects"[Mesh] OR "Polycyclic Aromatic Hydrocarbons/poisoning"[Mesh] OR "Polycyclic Aromatic Hydrocarbons/toxicity"[Mesh] OR "Inhalation Exposure/adverse effects"[Mesh])]. For resulting articles, we obtained their abstracts, plus full texts if needed, to determine each publication's relevance to our review. The selection process to identify relevant papers for a full review consisted of two phases of screening, including a title and abstract screening, followed by a full-text screening. Two reviewers conducted the two phases independently (C.C. and D.Y.), and in case of doubt, a third reviewer (M.H.) was consulted. When selected for full-text screening, additional inclusionary criteria were met to be reviewed in detail. Inclusionary criteria for this review were as follows: (1) full-length original research article; (2) reporting individual-level data on exposures to outdoor air pollution, (3) at least one of the MRI outcome measures was examined (e.g., structural T1, diffusion-weighted T2, echo planar imaging T2, etc.); and (4) studying children, adolescents, or young adults (aged $\leq$30 years) at the time of MRI scans. Studying exposure effects during young adulthood is relevant to the scope of our review because the brain continues to develop into the third decade of life (4, 7–9, 11–14, 32, 33). Studies were excluded if they met the following criteria: (1) not an original paper; (2) indoor or occupational air pollution; (3) no MRI outcome of interest; (4) experimental studies; (5) human studies without individual-level exposure data; and (6) studies conducted in adults aged >30 years. For all the identified relevant original and review articles, we also looked into the title/abstract of listed bibliographies to identify additional articles that may not have been captured through our initial search of the MEDLINE

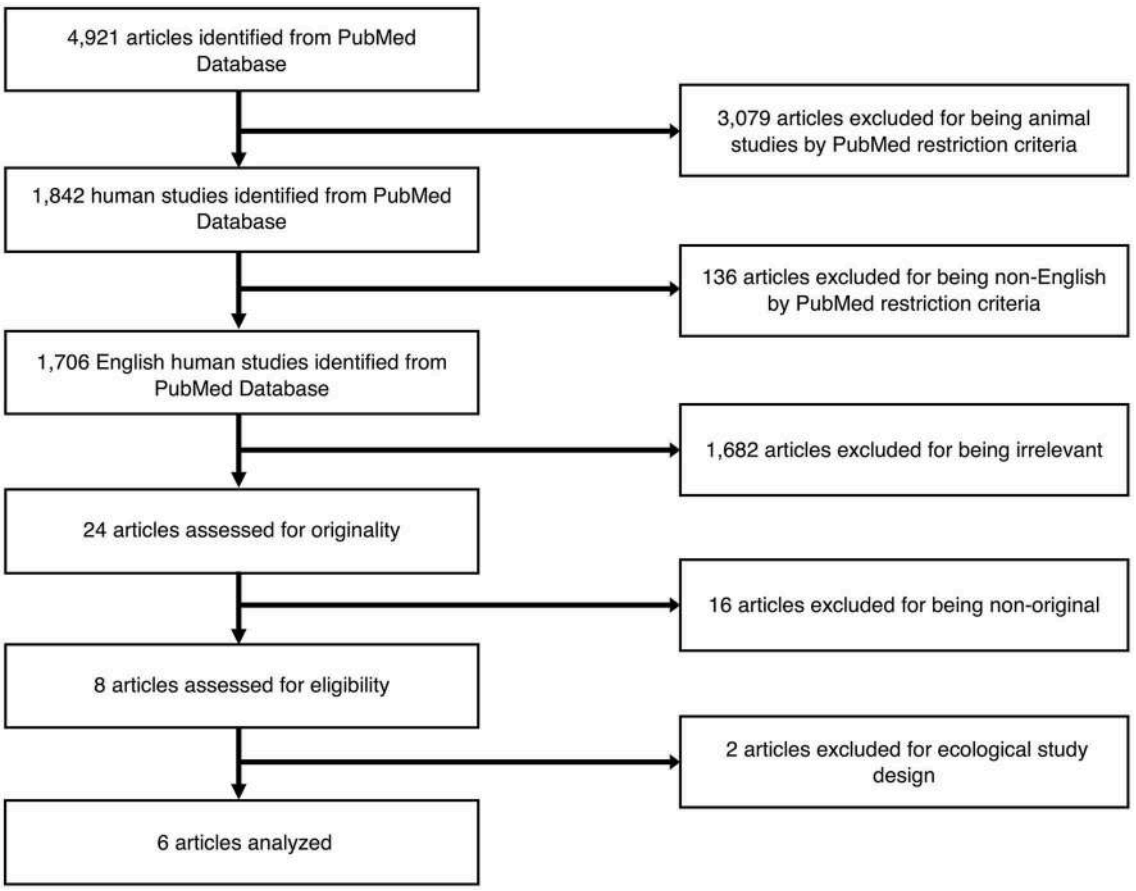

FIGURE 1 | Selection process of the articles to be reviewed.

database. The same extensive search process was repeated using the ISI Web of Knowledge dataset.

RESULTS

Figure 1 outlines the study selection process. Using the comprehensive search strategy, 4,921 unique articles were identified from PubMed, with 1,842 of them meeting the criteria of including humans. One hundred thirty-six articles were then removed for being published in a language other than English. Of the remaining 1,706 studies, 1,682 articles were removed following title and abstract screening, as the articles did not meet the following criteria outlined above (i.e., exclusion of individual-level data on exposures to outdoor air pollution or MRI outcome measures; participants were adults or elderly subjects at the time of MRI scans). The remaining 24 articles were selected for a full-text review, with 16 articles ineligible as they did not meet the criteria of being an original research article (i.e., review articles or commentaries). The remaining 6 articles were considered relevant original research articles and included in the final review. No additional studies were identified from our ISI Web of Knowledge database or through the manual search of listed bibliographies of relevant articles. Details of the 6 studies are presented in **Table 1**. All studies included only cross-sectional MRI data from pre-adolescents (<13 years old), with no studies conducted during mid-to-late adolescence (13–19 years) or young adulthood (20–30 years old). Participant samples were subsets from 3 larger children cohorts: (1) the Brain Development and Air Pollution Ultrafine Particles in School Children (BREATHE) project with traffic as the primary source of air pollution in Barcelona, Spain ($n = 4$); (2) the Generation R Study in Rotterdam, The Netherlands where air pollution is generated from traffic, industrial, and other point sources ($n = 1$); and (3) the Columbia Center for Children's Environmental Health (CCCEH) cohort with traffic as the primary outdoor source of intra-urban air pollution in New York City ($n = 1$). These studies employed various approaches to exposure assessment. The CCCEH cohort used personal air samples and urinary biomarkers to estimate prenatal and post-natal exposures to polycyclic aromatic hydrocarbons (PAHs), respectively. In the BREATHE project, investigators directly measured particulate matter with aerodynamic diameter of $<2.5\,\mu m$ ($PM_{2.5}$) and nitrogen dioxide (NO_2), in both the school courtyard (outdoor) and in the classrooms (indoor) during two 1-week campaigns for the warm and cold seasons,

TABLE 1 | Summary studies.

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
OUTDOOR AIR POLLUTION AND BRAIN IMAGING						
Peterson et al. (34)	40 right-handed children born to nonsmoking Dominican and African-American women 18–35 years old residing in Washington Heights, Harlem, or the South Bronx in NYC were recruited 1998–2006 through local prenatal care clinics and followed *in utero* to 7–9 years of age	Personal air monitors to measure 8 airborne PAHs (benz[a]anthracene, chrysene, benzo[b]fluroanthene, benzo[k]fluroanthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, disbenz[a,h]anthracene, and benzo[g,h,i]perylene) and determine maternal exposures over 48 h period during 3rd trimester, with mean ± standard deviation (SD) of 5.13 ± 6.20 ng/m^3. Spot urine samples collected from child at age 5 used to measure postnatal urinary PAH metabolites, with $N = 38$ and mean ± SD of 14999.4 ± 19795.8 ng/L. Source of pollutants: traffic emissions	3T GE, with 8 channel head-coil; T1 weighted: TR = 4.7 ms, TE = 1.3 ms, acceleration factor = 2, 256 × 256 matrix, 160 slices, 1 mm thickness; ANALYZE 8.0 and in-house software; structural MRI's, surface mapping. Correction using False Discovery Rate ($p < 0.05$).	PAH correlated inversely with morphological measures in frontal, superior, temporal, parietal, and rostrocaudal extent of the mesial surface mostly in the Left hemisphere; underlying white matter mainly driving effect. Pearson correlation coefficients and 95% confidence intervals: Prenatal PAH levels: Left lateral superior prefrontal cortex: −0.57 (−0.75, −0.304); Left superior temporal gyrus: −0.51 (−0.71, −0.23); Left precuneus: −0.50 (−0.71, −0.22); Left medial prefrontal cortex: −0.56 (−0.75, −0.30) Postnatal (age 5) PAH levels: Left dorsal-lateral prefrontal cortex: −0.47 (−0.69, −0.18); Left medial dorsal prefrontal cortex: −0.52 (−0.72, −0.25)	Child's age and sex; prenatal cotinine levels, measures of postnatal PAH exposure at age 5 years, handedness	Prenatal PAH relates to less left hemisphere white matter; while postnatal PAH contributes to less white matter in dorsal prefrontal regions
Pujol et al. (35)	263 children (mean age 9.7 ± 0.9 years old; range: 8–12.1 years) from the BREATHE project who were selected from 39 schools in Barcelona (schools selected based on modeled NO_2 values) and had been in the school for at least 18 months	Pollutant levels in school courtyards were sampled twice during 1-week periods (8 h/day) separated by 6 months in the warm (year 2012) and cold (year 2012/2013) periods. Elemental carbon (measured in $PM_{2.5}$) was measured with High-Volume samplers and NO_2 with passive dosimeters (96 h period), with mean ± SD of 0.92 ± 0.30 μg/m^3 and range of 0.42–1.92 μg/m^3. A single traffic-related pollutant indicator was computed using the weighted average of these two, using the following formula:	1.5T GE with 8 channel head-coil; T1 weighted: TR = 11.9 ms, TE = 4.2 ms, Flip = 15°, 256 × 256 matrix, 134 slices, 1.2 mm thickness; SPM (version 8) VBM and FREESURFER (version not reported). DTI: 25 directions with *b*-value = 1,000 s/mm^2, TR = 8,300 ms, TE = 94 ms, Flip = 90°, 128 × 128 matrix, 26 slices, 5 mm thickness with no gap, spin-echo single-shot EPI; FMRIB FDT, accounted for head motion.	No significant association was identified between air pollution and any anatomical, DTI or metabolic brain measurement. Traffic-related air pollution was significantly associated with: (1) weaker functional connectivity between regions belonging to the DMN (i.e., between the medial frontal cortex and the angular gyrus bilaterally); (2) stronger functional connectivity between the medial frontal cortex seed region and the frontal operculum at the lateral boundary of the DMN; (3) lower deactivations (rest > task map) during passive viewing and listening in the supplementary motor area and somatosensory cortex.	Age, sex, academic achievement, difficulties scores, obesity, parental education, home and school vulnerability index, distance from home to school and public/non-public school category	Traffic-related air pollution was associated with functional brain changes, but there was no relationship with brain anatomy, white matter microstructure, or membrane metabolism

(Continued)

TABLE 1 | Continued

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
		[(EC/group median) + (NO_2/group median)/2] Source of pollutants: traffic emissions	RS-FMRI (eyes closed): TR = 2,000 ms, TE = 50 ms, Flip = 90°, 64 × 64 matrix, 22 slices, 4 mm with 1.5 mm gap, 180 EPI volume; SPM8 preprocessing with 8 mm Gaussian filter; high-pass filter (∼0.008 Hz), white matter, CSF, and global signal removal; scrubbed for motion; Functional connectivity maps using 3.5 mm spheres of frontal lobe ($n = 4$) and caudate ($n = 2$) seeds; Correction with Monte Carlo and a family-wise error rate ($p < 0.05$) and Bonferroni per connectivity map ($n = 4$). Sensory FMRI task: TR = 2,000 ms, TE = 50 ms, Flip = 90°, 64 × 64 matrix, 22 slices, 4 mm with 1.5 mm gap, 120 EPI volume; block design (ABABABAB) of 30 s fixation and 30 s of visual-auditory input; Correction with Monte Carlo and a family-wise error rate ($p < 0.05$). Proton (1H) Spectroscopy: PROBE-SV and STEAM sequence, TR = 2000 ms, TE = 30 ms, 128 signal averages, voxel = 23 ×14 × 14 mm in the left frontal white matter; NAA FWHM 0.09 ppm and creatine RMS >8.	*T*-values (non-adjusted vs. adjusted by age and sex): RS-FMRI Medial frontal seed map: Left Lateral frontal cortex (+ correlation): 3.6 vs. 4.1; Left Parietal cortex (– correlation): 3.5 vs. 3.2; Right Parietal cortex (– correlation): 3.5 vs. 3.2 RS-FMRI Dorsal frontal seed map: Left Parietal cortex (+ correlation): 3.6 vs. 3.5; Right Lateral frontal cortex (– correlation): 3.7 vs. 3.5; Right Insula (– correlation): 4.3 vs. 4.3 RS-FMRI Posterior cingulate seed map: Left Lateral frontal cortex (+ correlation): 3.3 vs. 3.2 RS-FMRI Supplementary motor area seed map: Left Prefrontal cortex (+ correlation): 4.4 vs. 4.6; Right Prefrontal cortex (+ correlation): 3.5 vs. 3.6; Left Parietal cortex (+ correlation): 3.3 vs. 3.4; Right Parietal cortex (+ correlation): 4.1 vs. 3.9; Left Anterior cingulate cortex (– correlation): 3.6 vs. 3.7 Sensory FMRI task: Right Somatosensory cortex (+ correlation): 3.7 vs. 3.8; Left Premotor cortex (+ correlation): 3.7 vs. 3.6		

(Continued)

TABLE 1 | Continued

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
Pujol et al. (36) Brain and Behavior	263 children (mean age 9.7 ± 0.9 years old; range: 8–12.1 years) from the BREATHE project who were selected from 39 schools in Barcelona (schools selected based on modeled NO_2 values) and had been in the school for at least 18 months	Copper (measured in $PM_{2.5}$) levels in school courtyards were sampled with High-Volume samplers twice during 1-week periods (8 h/day) separated by 6 months in the warm (year 2012) and cold (year 2012/2013) periods and analyzed using ICP-MS, and then averaged to obtain the yearly exposure levels, with mean ± SD of 8.7 ± 3.0 ng/m^3 and range of 3.7–13.8 ng/m^3. Source of pollutants: traffic emissions, industrial sources, and railway	1.5T GE with 8 channel head-coil; T1 weighted: TR = 11.9 ms, TE = 4.2 ms, Flip = 15°, 256 × 256 matrix, 134 slices, 1.2 mm thickness; SPM (version 8) VBM (5 FWHM) and FREESURFER (version not reported). DTI: 25 directions with *b*-value = 1,000 s/mm^2, TR = 8,300 ms, TE = 94 ms, Flip = 90°, 128 × 128 matrix, 26 slices, 5 mm thickness with no gap, spin-echo single-shot EPI; FMRIB FDT, accounted for head motion. RS-FMRI (eyes closed): TR = 2,000 ms, TE = 50 ms, Flip = 90°, 64 × 64 matrix, 22 slices, 4 mm with 1.5 mm gap, 180 EPI volume; SPM8 preprocessing with 8 mm Gaussian filter; high-pass filter (~0.008 Hz), white matter, CSF, and global signal removal; scrubbed for motion; Functional connectivity maps using 3.5 mm spheres of frontal lobe (n = 4) and caudate (n = 2) seeds; Correction with Monte Carlo and a family-wise error rate (p < 0.05).	Higher copper levels were associated with: (1) higher gray matter concentration in the striatum, specifically in the caudate nucleus, with no effect on tissue volume; (2) higher FA in white matter close to the caudate nucleus and in the caudate nucleus; (3) changes in the complex architecture of neural tissue diffusion; (4) reciprocal reduction of functional connectivity between the caudate nucleus and the frontal lobe operculum (bilaterally). Beta coefficients and 95% confidence intervals: Gray matter density: Left caudate nucleus: β = 0.3 (0.1, 0.5) DTI Fractional Anisotrophy (FA): Left caudate nucleus: β = 0.1 (0.05, 0.2) RS-FMRI: Left frontal cortex to Left caudate: β = −0.1 (−0.2, −0.1)	Age, sex, academic achievement, academic difficulties score, obesity, parental education, home and school vulnerability index, public/nonpublic school category, socioeconomic status, elemental carbon, other toxic agents (Pb, Mn, Sb, and Fe)	Association between environmental copper exposure in children and alterations of caudate structure and function
Mortamais et al. (37)	242 children (median age 9.7 years; range: 8–12 years) from the BREATHE project who were selected from 35 of 39 schools in Barcelona (schools selected based on modeled NO_2 values) and on average at school for 6.5 years before study; removed 19 participants b/c parents said smoked at home	Pollutant levels in school courtyards were sampled twice during 1-week periods (8 h/day) separated by 6 months: Jan to June 2012 and Sept 2012 to Feb 2013. PAHs (measured in $PM_{2.5}$) was measured with High-Volume samplers. Outdoor and indoor PAHs: (benz[a]anthracene (BAAN), chrysene (CHRYS),	1.5T GE with 8 channel head-coil; T1 weighted: TR = 11.9 ms, TE = 4.2 ms, Flip = 15°, 256 × 256 matrix, 134 slices, 1.2 mm thickness; FREESURFER volumes (version not reported). No correction for multiple comparisons.	No associations with brain parenchymal fraction, putamen, or globus pallidus volumes. Higher outdoor school PAHs was associated with caudate volumes. Beta coefficients and 95% confidence intervals per IQR increase: Caudate nucleus, mm^3: Total PAHs: β = −132.9 (−245.0, −20.8); p = 0.020; BAP: β = −150.6 (−259.1, −42.1); p = 0.007	Age, sex, ICV, maternal education, home socioeconomic vulnerability index, residential exposure to NO_2 and $PM_{2.5}$, classroom noise	Exposure to PAHs associated with subclinical changes on the caudate

(Continued)

TABLE 1 | Continued

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
		benzo[b+j+k]fluroanthene (BFL), benzo[e]pyrene (BEP), benzo[a]pyrene (BAP), indeno[1,2,3-c,d]pyrene (IP), and benzo[g,h,i]perylene (BGP)) levels were obtained by averaging the two one-week measures, which were seasonalized, with BAP mean ± SD of 99 ± 62 pg/m^3 and range of 20–304 pg/m^3, and a PAHs mean ± SD of 1,458 ± 704 pg/m^3 and range of 597–3,235 pg/m^3. Weekly-averaged NO_2 concentrations with passive dosimeters were also obtained. Source of pollutants: traffic emissions		Boys (n = 123): Total PAHs: β = −19.2 (−211.0, 172.6); $p = 0.844$; BAP: β = −48.6 (−219.8, 122.5); $p = 0.577$ Girls (n = 119): Total PAHs: β = −192.3 (−364.1, −20.5); $p = 0.028$; BAP: β = −212.0 (−382.8, −41.2); $p = 0.015$ No significant interactions between sex and PAHs observed for brain volumes Null findings: Brain parenchymal fraction, %: Total PAHs: β = 0.3 (−0.2, 0.7); $p = 0.240$; BAP: β = 0.0 (−0.4, 0.5); $p = 0.929$. Putamen, mm^3: Total PAHs: β = 26.4 (−128.3, 181.2); $p = 0.738$; BAP: β = 13.0 (−139.2, 165.2); $p = 0.867$; Globus pallidus, mm^3: Total PAHs: β = 4.5 (−46.1, 55.2); $p = 0.861$; BAP: β = −16.9 (−64.9, 31.0); $p = 0.488$		
Guxens et al. (38)	783 children (6–10 years old) from the Generation R Study, a population-based birth cohort in Rotterdam, The Netherlands, who were born between April 2002 and Jan 2006. Oversampled based on certain maternal exposures during pregnancy (i.e., cannabis, nicotine, selective serotonin reuptake inhibitors, depressive symptoms, and plasma folate levels) and child behavior problems (i.e., ADHD, pervasive developmental problems, dysregulation problems, and aggressive problems)	Air pollution monitoring campaigns took place over three 2-week periods for NO_2 in 80 sites and PM_{10}, $PM_{2.5}$, and $PM_{absorbance}$ (a proxy for elemental carbon) in 40 sites in 2009 to 2010 across The Netherlands and Belgium. Coarse PM calculated as the difference between PM_{10} and $PM_{2.5}$. The 3 measurements were averaged, adjusting for temporal variation. LUR models were then used to estimate air pollution exposure levels at mothers' geocoded residential address during the entire fetal period. Background monitoring network sites ($n = 7$) were used to back-extrapolate to the fetal period, accounting	3T GE with 8 channel head coil; T1 weighted: TR = 10.3 ms, TE = 4.2 ms, Flip = 16°, 0.9 × 0.9 mm in-plane resolution, 186 slices, 0.9 mm thickness; FREESURFER (version 5.1) volumes and cortical thickness. Corrected using Monte-Carlo null-Z w/10,000 iterations ($p < 0.1$).	No associations were seen between air pollution exposure during fetal life and global brain volume measures at 6–10 years of age. Significant associations between air pollution exposure during fetal life and regional cortical thickness at 6–10 years of age. Fully adjusted beta coefficients and 95% confidence intervals representing the differences in thickness (mm) per each increase of 5 mg/m^3 of $PM_{2.5}$, 5 mg/m^3 of PM_{10}, and 10^{-5} m^{-1} of $PM_{absorbance}$: $PM_{2.5}$: Right precuneus region: β = −0.045 (−0.062, −0.028); $p < 0.001$; Right Pars opercularis region: β = −0.024 (−0.033, −0.014); $p < 0.001$; Pars orbitalis region, right: β = −0.028 (−0.043, −0.012); $p = 0.001$; Right rostral middle frontal region: β = −0.029 (−0.041, −0.018); $p < 0.001$; Right superior frontal region: β = −0.029 (−0.043, −0.016);	Parental educational levels, monthly household income, parental countries of birth, parental ages, maternal prenatal smoking, maternal prenatal alcohol use, maternal parity, family status, and maternal psychological distress, calculated pre-pregnancy BMI, maternal IQ when children were 6; child's gender, age at scanning, genetic ancestry	Children exposed to higher PM levels during fetal life had thinner cortex in several brain regions of both hemispheres

(Continued)

TABLE 1 | Continued

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
		for changes of home address during pregnancy. a range of 16.8–28.1 $\mu g/m^3$, the coarse (PM_{10}) median value was 11.8 $\mu g/m^3$ with a range of 9.2–17.8 $\mu g/m^3$, the proxy for elemental carbon ($PM_{absorbance}$) median value was 1.9×10^{-5} m^{-1} with a range of 1.2–3.6 10^{-5} m^{-1}. Source of pollutants: intra-ubran road traffic, traffic on highways, and industrial and other point sources		$p < 0.001$; Left cuneus region: $\beta = 0.022$ (−0.035, −0.009); $p = 0.002$ PM_{10}: Right Lateral orbitofrontal region: $\beta = -0.037$ (−0.059, −0.016); $p = 0.001$ $PM_{absorbance}$: Left fusiform region: $\beta = -0.105$ (−0.160, −0.049); $p < 0.001$ Null findings: NO_2: Total brain volume: $\beta = 124$ (−1118, 1,375); $p = 0.84$; Cortical gray matter volume: $\beta = -60$ (−853, 733); $p = 0.88$; Cortical white matter volume: $\beta = 199$ (−287, 685); $p = 0.42$; Subcortical gray matter volume: $\beta = 36$ (−17, 89); $p = 0.18$; Ventricular volume: $\beta = 4$ (−57, 64); $p = 0.90$ $PM_{2.5}$: Total brain volume: $\beta = -3079$ (−7790, 1,632); $p = 0.20$; Cortical gray matter volume: $\beta = -2598$ (−5583, 387); $p = 0.09$; Cortical white matter volume: $\beta = -268$ (−2096, 1,559); $p = 0.77$; Subcortical gray matter volume: $\beta = -60$ (−258, 138); $p = 0.55$; Ventricular volume: $\beta = -96$ (−323, 131); $p = 0.40$; PM_{10}: Total brain volume: $\beta = -4868$ (−10337, 822); $p = 0.09$; Cortical gray matter volume: $\beta = -3542$ (−7059, 8); $p = 0.05$; Cortical white matter volume: $\beta = -1129$ (−3215, 1127); $p = 0.34$; Subcortical gray matter volume: $\beta = -92$ (−325,148); $p = 0.46$; Ventricular volume: $\beta = -100$ (−372, 168); $p = 0.45$; $PM_{absorbance}$: Total brain volume: $\beta = -2861$ (−18745, 24,467); $p = 0.79$; Cortical gray matter volume: $\beta = -2683$ (−16377, 11,012); $p = 0.70$; Cortical white matter volume: $\beta = 5,807$ (−2566, 14,180); $p = 0.17$; Subcortical gray matter volume: $\beta = 418$ (−497, 1,334); $p = 0.36$; Ventricular volume: $\beta = -64$ (−1108, 979); $p = 0.90$		

(Continued)

TABLE 1 | Continued

References	Study design and MRI sample	Exposure	Outcome	Main findings	Confounders adjusted/controlled	Conclusions
Alemany et al. (39)	163 children (mean age 9.3 ± 0.82 years old; range: 8–12.1 years) from the BREATHE project who had genetic data available. Children were selected from 38 schools in Barcelona and 1 adjacent city, Sant Cugat del Vallés (schools selected based on modeled NO_2 values) and had been in the school for at least 18 months. 22.7% ($n = 37$) were APOE-e4 carriers	Pollutant levels in school courtyards were sampled twice during 1-week periods (8 h/day) separated by 6 months: Jan to June 2012 and Sept 2012 to Feb 2013. PAHs (measured in $PM_{2.5}$) was measured with High-Volume samplers. Outdoor and indoor PAHs: (benz[a]anthracene (BAAN), chrysene (CHRYS), benzo[b+j+k]fluroanthene (BFL), benzo[e]pyrene (BEP), benzo[a]pyrene (BAP), indeno[1,2,3-c,d]pyrene (IP), and benzo[g,h,i]perylene (BGP)) levels were obtained by averaging the two one-week measures, which were seasonalized, with PAHs mean ± SD of 1546.29 ± 775.08 pg/m^3. Elemental carbon (measured in $PM_{2.5}$) was measured with High-Volume samplers and NO_2 with passive dosimeters (96 h period), with NO_2 mean ± SD of 47.74 ± 12.95 $\mu g/m^3$. Source of pollutants: traffic emissions	1.5T GE with 8 channel head-coil; T1 weighted: TR = 11.9 ms, TE = 4.2 ms, Flip = 15°, 256 × 256 matrix, 134 slices, 1.2 mm thickness; FREESURFER (version 5.3). No correction for multiple comparisons.	Beta coefficients and 95% confidence intervals of basal ganglia volumes by annual average of air pollution exposure to PAHs, EC, and NO_2 (per IQR increase): Caudate nucleus, mm^3: Total PAHs: β = −120.1 (−211.2, −29); EC: β = −110 (−250.5, 30.6); NO_2: β = −265.1 (−474.3, −56) Putamen, mm^3: Total PAHs: β = 9.1 (−115.2, 133.5); EC: β = −15.2 (204.7, 235.1); NO_2: β = 93.4 (−364.3, 177.5) Globus pallidus, mm^3: Total PAHs: β = 1.1 (−37.2, 39.5); EC: β = −22.2 (−101.6, 57.1); NO_2: β = −35.6 (−133.9, 62.7) In e4 carriers and non-carriers: Caudate nucleus, mm^3: Total PAHs: e4 carrier β = −590.2 (−1032.3, −148.15) vs. non-carrier β = −137.1 (−322.0, 47.84); $p = 0.04$; EC: e4 carrier β = −375.0 (−776.81, 26.88) vs. non-carrier β = −49.8 (−236.6, 137.1); $p = 0.11$; NO_2: e4 carrier β = −737.9 (−1201.3, −274.5) vs. non-carrier β = −157.6 (−388.8, 73.6); $p = 0.03$ Putamen, mm^3: Total PAHs: e4 carrier β = −13.6 (−272.5, −245.3) vs. non-carrier β = 19.45 (−456.4, 495.3); $p = 0.90$; EC: e4 carrier β = −31.0 (−444.3, 382.25) vs. non-carrier β = 52.9 (−206.3, 312.1); $p = 0.55$; NO_2: e4 carrier β = −63.75 (−586.3, 458.8); non-carrier β = −82.4 (−405.4, 240.5); $p = 0.74$ Globus pallidus, mm^3: Total PAHs: e4 carrier β = −43.1 (−212.9, 126.75)vs. non-carrier β = −5.1 (−81.0, 70.88); $p = 0.40$; EC: e4 carrier β = −13.1 (−166.3, 140.2) vs. non-carrier β = −26.10 (−102.0, 49.8); $p = 0.97$; NO_2: e4 carrier β = −49.6 (−237.9, 138.6) vs. non-carrier β = −44.15 (−138.7, 50.35); $p = 0.79$	Age, sex, ICV, maternal education, maternal smoking, exposure to tobacco at home, home socioeconomic vulnerability index, residential exposure to NO_2 and $PM_{2.5}$.	Association between annual average air pollution exposure to PAHs and NO_2 and smaller caudate volumes was larger in children carrying the APOE-e4 allele compared to non-carriers

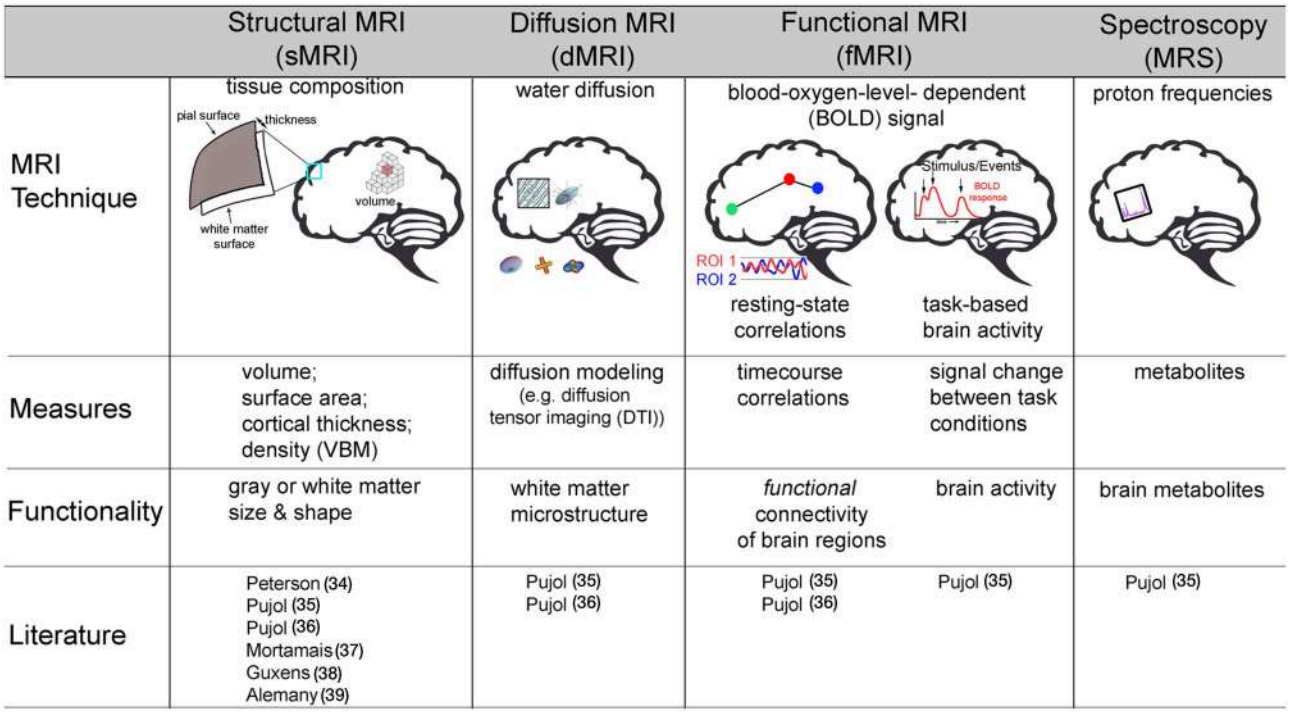

FIGURE 2 | Description of various non-invasive magnetic resonance imaging (MRI) techniques used to assess how outdoor air pollution exposure relates to brain structure and function in children. VBM, voxel based morphometry; DTI, diffusion tensor imaging; ROI, region of interest. Pujol (35); Pujol (36).

as well as residential NO_2 and $PM_{2.5}$ at the time of the study and the prenatal period. The Generation R study used residential location data during pregnancy and applied several validated land-use regression (LUR) models to estimate particulate matter with aerodynamic diameter of $<10\,\mu m$ (PM_{10}), $PM_{2.5}$, NO_2, and absorbance of fine particles as a proxy of elemental carbon (EC) during the entire fetal period, accounting for changes in home address during pregnancy. All included studies reviewed were commendable in using either a strategic participant sampling approach to reduce other pollutant exposures and/or adjusting for second-hand smoke (i.e., a source of indoor pollution) and socioeconomic factors. However, tactics for reducing additional confounds, including socioeconomic status, parental or child's education, and other social adversities in the residential or school neighborhood, were diverse (see **Table 1** for details). Below, we synthesize these details and the reported findings on the exposure-outcome associations, according to the primary exposure time window under study.

Prenatal Exposure

Both prenatal exposure studies utilized structural MRI, which is a technique that allows for the quantification of tissue types and morphological metrics of the brain, including gray matter (i.e., cell bodies) and white matter (i.e., myelination of axons) volume (size in mm^3), surface area (mm^2), density, and cortical thickness (i.e., mm, distance between gray/white matter boundary and pia matter) (**Figure 2**). Using structural MRI, Peterson et al. was the first study to examine the association between outdoor air pollution and brain structure in 40 children (7–9 years) selected from a larger African-American and Dominican birth cohort study in New York City from 1998 to 2006 (34). Of the 727 mother-newborn pairs in the CCCEH cohort, 40 children were identified to capture a full range of prenatal PAHs exposure measured by personal monitoring for 48 h in the 3rd trimester. The results suggested that white matter, comprised of myelination, or the insulation of axons (i.e., connections) between brain cells, was most affected. Higher levels of prenatal PAHs exposure were associated with reductions in white matter surface area and were largely located on the left side of the brain. After adjusting for prenatal exposure, urinary level of PAH metabolites measured at age 5 were also found to be associated with smaller white matter surface area in a specific region of both the right and left side of the frontal lobe, known as the dorsolateral prefrontal cortex. Correlations were assessed between PAHs (prenatal and postnatal) and various behavioral scores measured by The Wechsler Intelligence Scale for Children (IQ and processing speed) and Child Behavior Checklist (Anxiety-Depression, Attention, DSM-ADHD, DSM-Conduct, and Externalizing scores) at age 7 years. Of the fourteen correlations, only prenatal PAH and processing speed were found to be significant ($p < 0.05$, uncorrected). Using a Sobel test, white matter surface area partially mediated an association between higher prenatal PAHs exposure and slower processing speed. A key strength of this study was a strategic sampling approach of the children in order to minimize confounding factors of other ambient chemicals (e.g., second-hand smoke, insecticides). In addition, in MRI studies where the brain is segmented into multiple regions or into smaller units, up to 500,000 or

more statistical tests are commonplace. For this reason, multiple comparison corrections are an imperative step in reducing Type I error in MRI brain research (40, 41). Importantly, Peterson and colleagues' results were corrected accordingly; adding an important level of rigor to its methodology. However, this study was also not without limitations. In addition to the small sample size, the current study only included age and sex of the child, and did not account for family socioeconomic status or other social factors as covariates in the fully adjusted models. While the children were all selected based on a minority population, it is unclear if socioeconomic factors even within this strategically chosen sample may be confounding results. Nonetheless, this pioneering study provided evidence that prenatal and postnatal PAH exposure in particulate matter may impact a child's brain development.

In a large ($n = 783$) group of school-age children (ages 6–10 years) based on the Generation R study, investigators examined prenatal exposure and total brain volumes and thickness of gray matter in the cortex (38). Based on an air pollution monitoring campaign of NO_2 across 80 sites and PM across 40 sites in The Netherlands and Belgium in 2009–2010, LUR models were developed, and exposures to NO_2, coarse particles (PM_{10} minus $PM_{2.5}$), $PM_{2.5}$, and a proxy for EC were assigned to maternal residential locations. Investigators found no association between prenatal exposure and overall brain size (e.g., total whole brain, total gray, total white, total subcortical gray, and total ventricular volume). However, greater prenatal $PM_{2.5}$ exposure was associated with reduced cortical thickness in portions of the frontal lobe, as well as the parietal and occipital lobes. Higher levels of coarse particle and absorbance of fine particles (e.g., marker for EC) exposure during the fetal period were associated with thinner gray matter in separate frontal and occipital regions of the cortex, including the lateral orbitofrontal and fusiform regions, respectively. Causal mediation analyses were then employed using bootstrapping to determine whether cortical thickness is a mediator of the association between the pollutant and behavioral performance on a set of cognitive and emotional tasks. Adjusted associations were determined between air pollutants and behavioral performance based on the cognitive function of each identified regions. Of the 18 statistical tests performed, only $PM_{2.5}$ was found to significantly relate to inhibition errors on the response set task ($p = 0.02$). Of the 5 regions associated with $PM_{2.5}$, inhibition errors on this test was found to only relate to thickness in one region in the frontal lobe (i.e., rostral middle frontal; $p = 0.02$) and another region in the parietal lobe (i.e., precuneus; $p = 0.05$). Formal causal mediation analyses showed that thickness in these brain regions partially mediated the relationship between prenatal fine particle exposure and poor inhibitory control. The strengths of the current study were the large sample size and the number of social factors adjusted for in their final models, including family socioeconomic status (i.e., parental education, household income) as well as parental and other prenatal factors (i.e., parental age, maternal smoking, and other drug use, etc., maternal stress, maternal IQ). In addition, the authors performed a thorough adjustment for multiple comparisons for their MRI outcomes. A limitation to the study, however, is that the initial design of the Generation R study was to oversample on both maternal exposures (i.e., drug, alcohol, psychiatric medication) during pregnancy as well as child behavior problems. Thus, the generalizability of the current study may be limited by its potential sampling bias.

In the two prenatal studies, to date, the interpretations are also limited in that they did not account for the exposure measures temporally proximate to the MRI scans or other brain health outcomes. Specifically, these studies have not characterized, or accounted for, the exposure of the individual spanning the various years prior to assessing brain development. For example, Peterson and colleagues measured one 48-h period of exposure during the third trimester period and a single urine sample at age 5. Using two relatively abbreviated periods of exposure to assign prenatal and postnatal exposure is likely not accurate in reflecting both the amount and variability of exposure of a given individual prior to measuring the outcome. Similarly, Guxens and colleagues use of LUR of exposure were limited to the prenatal period, without exposure information for over 6–10 years leading up to the MRI visit. As such, it is difficult to rule out potential air pollution effects during the postnatal period in which the brain continues to develop. The following section on childhood exposure further highlights the potential importance of postnatal exposure on MRI related brain outcomes.

Childhood Exposure

Pujol et al. was the first to examine how childhood air pollution exposure relates to brain MRI-measured structure and function. Using various types of MRI neuroimaging, the investigators included 263 children (ages 8–12 years) recruited from the BREATHE project in Barcelona, Spain. High-volume active samplers for $PM_{2.5}$ and passive dosimeters for NO_2 were placed both inside classrooms and outside in the school courtyard during a 1–week (8 h/day) air monitoring campaign, which was conducted twice (one in warm season and the other in cold season) during the study period. An MRI assessment, including structural MRI, diffusion MRI (dMRI), resting-state functional MRI (rs-fMRI), Magnetic Resonance Spectroscopy (MRS), and task-based fMRI, was collected 1 year after measuring air pollution exposure at school. The investigators calculated traffic-related pollution scores using EC and NO_2 (35). Given that EC and NO_2 are highly correlated and have been found to be related to vehicle exhaust in Barcelona, the authors calculated a traffic related pollutant indicator using the weighted average of exposure to EC and NO_2. This traffic-related pollution index was associated with differences in brain function as measured by fMRI. FMRI measures the blood oxygen level-dependent (BOLD) signal (42) as an estimate of neural activity. The BOLD signal capitalizes on the tight coupling of blood flow and oxygenation when local neurons are activated. By mapping changes in the BOLD signal, fMRI can indirectly measure neuronal activity across time while individuals rest, known as resting-state fMRI (rs-fMRI) (43), or in relation to completing some sort of activity or task, often referred to as task fMRI (42) (**Figure 2**). Using both of these types of fMRI, Pujol and colleagues, found that higher traffic-related pollution was related to brain activity during rest as well as during a sensory fMRI task (35). Specifically, higher

traffic air pollution exposure was associated with less functional connectivity (e.g., BOLD signal correlations) between the frontal to parietal lobes (i.e., medial frontal to parietal cortex), between two frontal lobe regions (i.e., dorsal frontal cortex to lateral frontal and insula), and between motor and frontal regions (i.e., supplementary motor area to anterior cingulate) while subjects were at rest. Also while at rest, higher traffic-related exposure was also linked to stronger brain activity between motor areas and both the right and left side of the prefrontal and parietal lobes, as well as the distinct frontal lobe regions with the parietal lobe and cingulate cortex (i.e., medial frontal to lateral frontal, dorsal frontal to parietal, and posterior cingulate to lateral frontal cortex). An entire field of literature has focused on understanding common patterns of brain activity while individuals rest, which are known as functional connectivity networks (43, 44). Pujol and colleagues noted that altered patterns of functional activity with traffic pollution included some brain regions that are part of the resting state default mode network (DMN) whereas other regions are part of a functional connectivity networks known to be involved in more goal-directed behaviors, like the frontal-parietal (FP) network. Thus, the authors highlighted that traffic-related air pollution may lead to poor brain activity *within these specific networks* as well as impairments in brain activity *between these different networks* in children (35). The map of brain activity seen in the medial frontal lobe during rest was also found to relate to motor reaction time (collected from a cognitive battery performed with the children at school) (35). This study also found that greater air pollution from vehicle exhaust was linked to less deactivation in the somatosensory and premotor brain regions when individuals were presented with both visual and auditory stimuli in the MRI machine. Interestingly, no associations were found between the traffic-exposure score and other MRI outcomes collected, including brain volume or cortical thickness (sMRI), membrane metabolism (MRS), or white matter microstructure (via dMRI).

Using the data collected from the same cohort of children from the BREATE project, copper was measured in $PM_{2.5}$ and found to correlate with road traffic, industrial activity, and in some schools, train proximity. Higher average 1-year exposure to copper was associated with greater gray matter density, but not volume, of both the right and left caudate as well as increased gray matter thickness in the left supplementary motor area (36). In addition, this study also found copper to relate to white matter microstructure using the technique dMRI (**Figure 2**). DMRI is an MRI techniques that provides additional insight on water diffusion to assess microstructural properties of various tissues (32). More uniform restriction, quantified with dMRI using a term known as "fractional anisotropy" (FA), is thought to reflect more myelination in white matter regions, as well as axon organization, and/or axon size. Using this technique, higher copper exposure was related to higher values of FA in the caudate nucleus and its adjacent white matter tract (36). It is important to note that in the field of developmental neuroscience, higher values of FA in children are thought to reflect more myelination (e.g., insulation of the cell connections) and bigger axons, which typically occur with healthy, normative brain development (9, 32). Therefore, an association between higher copper exposure and "more mature" biomarkers of white matter development is rather counterintuitive. The authors postulate that this unexpected relationship may be due to a difference in the number of connections that may cross in the region; which is a known methodological limitation of this method (45). Unfortunately, the authors did not report other common diffusion metrics (e.g., axial or radial diffusivity) or estimate individual white matter tracts for all participants in the study; which are necessary to help clarify these unexpected findings between copper and white matter microstructure. Lastly, higher copper exposure in the year preceding neuroimaging collection correlated with reduced functional connectivity (e.g., negative correlations) for the caudate nucleus to portions of the frontal lobe (i.e., fronto-operculum) and the supplementary motor area to a portion of the parietal lobe, known as the supramarginal gyrus. However, again there were also increased connectivity between regions of the frontal lobe (i.e., medial prefrontal cortex to fronto-operculum) and regions of the frontal lobe (i.e., medial prefrontal cortex) to auditory cortex (36). Although a negative association was also found between copper exposure and reaction time variability on an attention network task in this MRI sample, the authors did not report whether additional analyses were performed to examine the relation among exposure to airborne copper, behavior, and brain-MRI measures.

More recently the BREATHE project has also examined how PAHs, EC, and NO_2 relate to basal ganglia volumes using structural MRI (37, 39). A negative association was found between outdoor benzo[a]pyrene (BAP) and both the right and left caudate nucleus volumes; a similar finding was found when using a total score of outdoor PAHs (37). Neither BAP nor total PAH levels related to the ratio of total gray and white matter to intracranial volume (i.e., overall brain size) (37). Expanding on these findings, 163 children with both MRI and genetic information were examined to determine if vulnerability to air pollution may vary by the e4 allele in the apolipoprotein E (APOE) gene – a primary genetic risk factor for Alzheimer's disease (39). Higher average annual NO_2 and PAH were again shown to relate to smaller caudate volumes from the BREATHE study, with a larger effect estimate in children carrying the APOE-e4 allele (39). In both studies, no associations were found between exposure levels (PAHs, EC, or NO_2) and other nearby regions, such as the putamen or globus pallidus volumes. Adjusting for residential-based NO_2 and $PM_{2.5}$ exposure (assigned via LUR) at the time of the study (37, 39) was not found to change the results.

Important in helping reduce the likelihood of spurious associations, the BREATHE project considered a number of confounding factors in their fully-adjusted models. For example, family socioeconomic status was accounted for by maternal education, whereas a vulnerability index (e.g., education, unemployment, and occupation by census tract) was assessed to account for potential neighborhood socioeconomic effects (35–37, 39); although the use of these variables seemed to vary by study reports. In addition, classroom noise was measured in one study; albeit the data were not shown and inclusion as a covariate during model testing was unclear (37). In terms of limitations, only two of the four studies from the BREATHE project corrected for multiple comparisons (see **Table 1** for

details). Although there is less concern about the proximity between exposure and outcomes, the BREATHE project also faces the challenge of accounting for potential effects of exposure at other periods of the child's development. Of the studies to date, only one of the recently published BREATHE studies accounted for prenatal exposure based on residential LUR estimates (37). In fact, regardless of examining the prenatal or postnatal period of exposure, no air pollution-MRI study to date has fully characterized the potential temporal exposures across the life span of the child and their various impacts on brain maturation. For example, each study focused on just a handful of exposures and quantified them over abbreviated periods of maturation. Together, these methodological challenges of understanding both the temporal dynamics of exposure as well as characterization of various air pollutants may result in inaccurate prediction of exposure, which suggests existing studies may suffer from information bias (e.g., misclassification of exposure). Moving forward a more thorough assessment of various exposures across development are necessary to strengthen the conclusions that can be drawn regarding the timing of exposure and potential impacts on neurodevelopment.

DISCUSSION

Our systematic review identified 6 studies on outdoor air pollution that reported associations between outdoor air pollution and white matter (e.g., surface area and microstructure), cortical and subcortical gray matter (e.g., cortical thickness, volumes), as well as brain function (e.g., resting state and task-based brain activity patterns). These findings also suggest that outdoor air pollution exposure may harm neurocognitive maturation through its impact on brain structure, with partial mediations being reported in Peterson et al. (34) and Guxens et al. (38). When considering the type and timing of exposure, various social confounders, as well as the different MRI outcomes under study, these findings are far from conclusive. For instance, the two prenatal exposure studies found opposite effects on the type of brain tissue impacted. Peterson et al. (34) found significant associations between PAH and white matter surface area (but not with gray matter), whereas in a larger sample Guxens et al. (38) found associations with particles (EC, PM_{10}, $PM_{2.5}$) and gray matter thickness, but not white matter measures. These two studies employed different methodological MRI preprocessing and analytic techniques, making it difficult to directly compare or amalgamate their imaging results. For example, cortical thickness, surface area, and gray matter volume capture distinct neurobiological phenotypes and these outcomes vary as a function of development across childhood and adolescence (46). In contrast, the reported associations with postnatal exposures were much more similar; albeit conducted by a single research group. In the BREATHE project, 1-year average exposures to traffic-related air pollutants (e.g., copper, PAHs, TRAP [EC+NO_2], and NO_2) was associated with smaller caudate volume (35, 37, 39), altered functional brain activity to sensory stimuli and altered patterns of intrinsic connectivity of large-scale functional networks (35, 36). However, these postnatal exposure studies to date are based solely on the BREATHE project; the generalizability and the reproducibility of their novel findings need to be examined in other studies of different population context. These and other considerations need to be addressed in future research.

Recommendations for Future Research

Incorporation of a Developmental Neuroscience Perspective

Brain development is hallmarked by an orchestra of events that span both the prenatal and postnatal period of development (**Figure 3**). Longitudinal studies of child and adolescent brain development using MRI provide strong evidence that brain development is dynamic and continual, and that individual differences exist in brain structure and function at the beginning of an MRI study and change over time (i.e., intercepts and slopes) (49). Postnatal neurodevelopmental trajectories vary by brain region, with sensory, motor, and language reaching peak volumes in early childhood and the prefrontal cortex, hippocampus, amygdala, and cerebral white matter volumes continuing to mature across adolescence and into the mid-to-late twenties (4, 7, 9, 10, 32, 50). Moreover, new cell growth and plasticity of cell connections are especially apparent in the hippocampus and amygdala across the lifespan, and are required for adult cognitive and emotional-based learning and memory processes.

Evidence from the reviewed studies suggests that exposure to ambient air pollution during both the prenatal (34, 38) and childhood periods (34–37, 39) are associated with MRI brain outcomes in children under age 12. Two studies found adverse effects of postnatal estimates in statistical models that adjusted for prenatal exposure (34, 37), providing evidence for the independent effects of postnatal exposure periods and highlighting the importance of studying the continuing impact of neurotoxic air pollutants on the extended developmental trajectories beyond school age. However, the studies that included postnatal periods of exposure focused primarily on the pre-adolescent period and no study examined how outdoor air pollution exposure may impact the dynamic periods of adolescent and young adult brain maturation despite known differences in adolescent and young adult brain structure and function as compared to children. Future research needs to be conducted in adolescents and young adults in order to better understand how air pollution may impact the unique neural systems throughout the entire developmental process of brain maturation. Since trajectories of growth vary by brain region, one might expect that early exposure (prenatal and/or early childhood) may impact early developing systems such as sensory, motor, and language systems, while executive functions and self-regulation of emotion and reward processes may remain vulnerable to neurotoxic insults during the teenage years and as individuals transition into young adulthood. Although the literature remains scant, the observation of air pollution exposure during childhood and its effect on caudate volumes may illustrate the importance of exposure time window. Specifically, prenatal studies did not detect this association (34, 38), whereas associations of average annual BAP exposure and bilateral caudate nucleus volumes was seen in

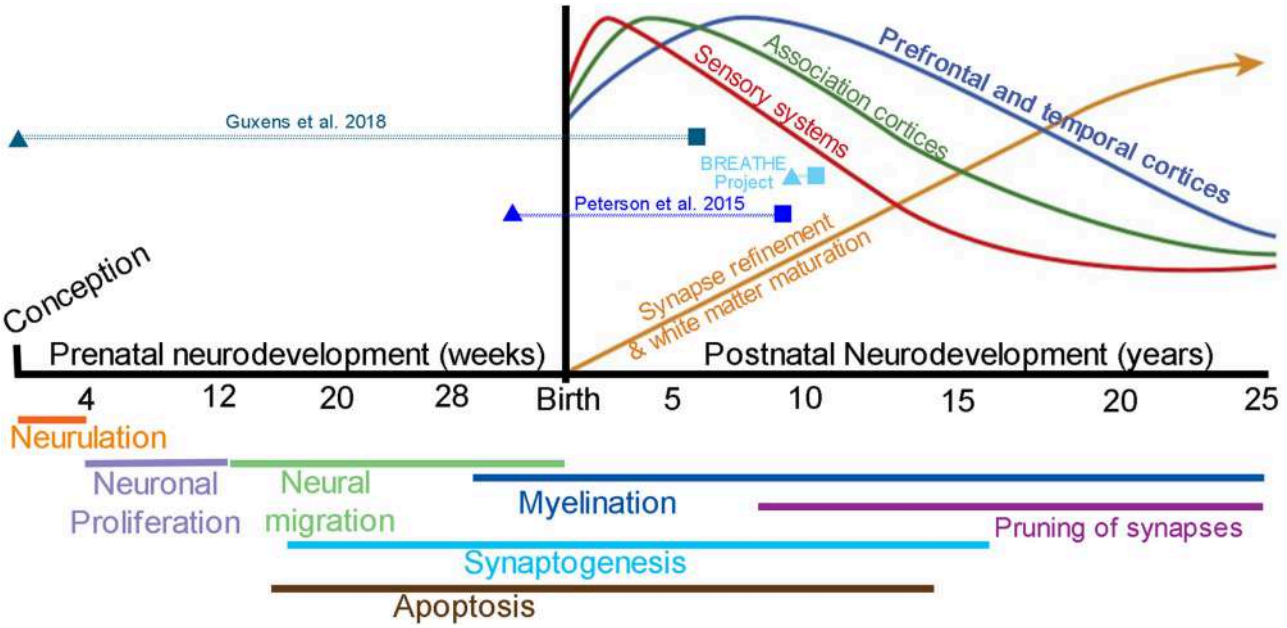

FIGURE 3 | Brain development from conception to young adulthood. Stages of brain development during the prenatal period (weeks) thru postnatal period of childhood and adolescence (years). Top: Postnatal quadratic trajectories of gray matter volume patterns seen by MRI, with sensory systems (red) peaking earlier in development, followed by association (green) and prefrontal and temporal (blue) cortices; a continual increase in white matter volume is seen well into the 3rd decade of life (yellow); Adapted from Nelson et al. (47) with permission from National Academies Press. The timing of outdoor air pollution (triangles) and the age of MRI assessment (squares) are presented for each of the included studies [note BREATHE project includes (35–37, 39)]. Bottom: Molecular and cellular processes that contribute to patterns of brain maturation as seen by MRI, which may be vulnerable to outdoor air pollution from fetal development to young adulthood; Adapted from Tau and Peterson (48) with permission from Springer Nature. Neurulation, folding of the neural plate into the neural tube; Neuronal proliferation, formation of two major brain cell types known as neurons and glial cells; Neural migration, movement of neurons to their final destination in the brain; Apoptosis, planned cell death; Synaptogenesis, creation of synapses allowing for communication via neurotransmitters between neurons; Myelination, formation of a myelin sheath to allow for faster cell conduction (e.g., myelination is often termed "white matter" via MRI); Pruning of synapses, removal of connections between cells (i.e., axon or dendrite) allowing for improvements in network capacity and efficiency.

children after adjusting for prenatal exposure (37). Moreover, growth of the caudate has been shown to continue from childhood to young adulthood (4, 51), and together, with the prefrontal cortex, portions of the caudate play a vital role in various executive functions, including cognitive control (52). Therefore, the design of future air pollution-neuroimaging studies needs to incorporate a developmental neurosciences perspective, considering the exposure timing, age of study population, and the most appropriate neurodevelopmental milestones, regardless of whether the study is focused on the neurotoxic effects in the period of fetal, childhood, adolescent, or young adult development. Studies interested in exposure later in neurodevelopment will also have the challenge of addressing how earlier periods of exposure may influence their results, as questions also remain if early neurotoxic effects persist or become exaggerated across the lifespan.

Longitudinal Design With Repeated Exposure Assessment and MRI Scans

Existing studies included only one-time assessment of structural morphometry and functional measures by brain MRI scans. Similarly, previous studies have been limited by not being able to fully characterize the exposure histories across different time windows relevant to the neurodevelopmental stages. For instance, previous studies on the prenatal exposure and developing brains rarely account for the recent exposures in close temporal proximity to MRI measures. Studies with more comprehensive exposure characterization over time as well as more than one MRI assessment can help determine whether ambient air pollution may change the "starting points" (i.e., intercepts) and/or may change the trajectory (i.e., slopes) of brain maturation as well as offer a more accurate understanding of the amount and timing of exposure that may be most detrimental. For example, longer durations of exposure to ambient air pollution may presumably result in more severe and/or permanent effects on brain structure and function. If true, rather than assessing a single time window of exposure such as the prenatal window and/or adjusting for more recent exposure during childhood, larger effects may be seen if we examine how annual and/or cumulative child or adolescent exposure is associated with trajectories of neurodevelopment. Therefore, longitudinal studies with more comprehensive exposure characterization and repeated MRI scans are needed to enhance the validity of the study findings to date, as well as fully characterize the specific neurodevelopmental trajectories affected by outdoor air pollution exposure during vulnerable windows across both the prenatal and postnatal periods.

Confounders and Covariates

Studies to date have used different approaches in addressing the presence of other potential confounders, including socioeconomic status, parental or child's education, social adversities in the residential or school neighborhood, and related spatial confounders. Further studies should consider following the lead of Guxens et al. (38) who implemented a directed acyclic graph approach based on existent literature. All studies to date have adjusted for age, sex, and intracranial volume (i.e., overall differences in head size when the dependent variable of interest was brain volume) (see **Table 1** for details), but other known confounders are likely to include the participant's family- and neighborhood-level socioeconomic background (e.g., family income, maternal education, neighborhood poverty) and various lifestyle factors (e.g., physical activities). This is especially important in places like the United States since exposures are higher among minorities (53), as well as for people with less than a high-school education compared to those with a high-school or college education, and among persons with income below the poverty line (54). Depending on sources, air pollution levels can be different in urban vs. rural environments, suggesting spatial covariates may also confound the exposure effect estimates if known to affect neurobehavioral development, such as traffic noise and greenspace. Similarly, neurodevelopment covariates to explore may include prenatal factors (e.g., maternal age, pre-term birth) and other physical health (e.g., body mass index (BMI), pubertal maturation, medications). Future studies need to clearly present the methods by which they measured and determined to include or exclude confounding factors in fully adjusted models. This will strengthen the results of future studies to mitigate concerns about differences in social class and race as well as other physical environmental factors that may otherwise lead to potential confounding bias.

Different Sources and Exposure Characteristics

Further research is needed to improve our understanding of the specificity and source(s) of exposure, as well as other characteristics most harmful to the developing brain. The reported primary pollutants included $PM_{2.5}$, PM_{10}, NO_2, or various $PM_{2.5}$ constituents (e.g., PAHs, copper, and EC). Most studies alluded to traffic as being the primary source of exposure, although not clearly documented in all studies. To reduce the potential confounding by other known environmental neurotoxins, Peterson et al. (34) recruited an informative sample with no or very low levels of prenatal exposures to second-hand smoke, lead, and chlorpyrifos. Others performed statistical adjustment in their data analyses to control for the potential influences by other co-pollutants or conducted sensitivity analyses regarding second-hand smoke exposure (36–39). One other study dealt with correlated pollutants by creating a composite score of exposure, and therefore did not adjust for multiple pollutants (35). Discrepant findings are likely to occur if future studies do not consider a multitude of sources and various exposures that children may encounter within and between various studies. Even when studies both assess the same criterion pollutant, such as $PM_{2.5}$, the chemical composition may vary between geographical locations (55). By assessing and accounting for various exposures in the same sample, stronger conclusions can be made to strengthen policy implications regarding which exact types of exposure are most harmful. For example, while all the studies investigated derivatives of particle pollution, these studies did not quantify various other air pollutants, such as airborne gases, carbon monoxide, sulfur oxides, or other volatile organic compounds. In addition, the exposure methods of the previous studies varied from personal monitoring, stationary monitoring, and LUR prediction modeling. Advantages and disadvantages of various types of exposure assessments for outdoor air pollution exposure, including LUR, urinary biomarkers, and personal monitoring sensors, have been reported in detail by recently focused reviews (56, 57). Detailed critiques of these approaches were beyond the scope of this review, but we note that the appropriate choice of exposure assessment methods depends on both the study design and research questions. For instance, studies that aim to retrospectively capture long-term exposure on neurodevelopmental processes in a well-characterized existing cohort may find that spatiotemporal models are useful to gather information about exposure over days, months, and years; especially given recent advances in establishing more fine grain spatiotemporal (i.e., 1×1 km grid, daily estimate) predictions for various particle and gaseous pollutants (58–61). For studies built on ongoing or newly launched cohorts, it will be desirable to collect information on individual-level behaviors of the child, such as the amount of time and patterns of activities for playing outdoors or time spent in other microenvironments (e.g., in vehicles; in classrooms), as such data may be used to determine the exposure sources, refine the specific characteristics of exposure, and reduce the exposure measurement error. On the other hand, although personal sampling allows for the most complete assessment of personal exposure across various microenvironments, technical limitations, such as participant compliance and burden have made this method more difficult for large scaled, population-based studies (57). For these same reasons, personal sampling is usually limited to a very brief period of time (i.e., 24 or 48 h), leading to a rather crude estimate of possible exposure to try and associate with neural processes of brain development that occur over months and years when captured with MRI. This latter point is especially of importance, as it remains to be elucidated if acute time-varying factors, such as exposure the day or week before, would impact various MRI outcomes. For example, reliability differences in MRI estimates within the same individuals (62) may suggest macrostructural changes detected by MRI may be better suited for detecting effects of chronic exposure, whereas functional metrics may be more sensitive to acute conditions. Future studies should consider these strengths and weaknesses of each technique depending on their specific question(s) in relation to how air pollution may impact the brain during development.

Assessing the Heterogeneity of Exposure Effect

The putative developmental neurotoxic effects of exposure to ambient air pollution may vary by demographic features and genetic factors (63). Of the included studies, two studies examined sex differences in the effect, with one suggesting larger effects in girls (34) and the other reporting no difference between the sexes (37). Given the sex differences seen in the

associations between air pollution exposure and cognition, with some studies reporting greater impairments in males compared to females (21, 22, 24, 64, 65), future studies should examine sex as a potential modifier and report summary statistics by sex. Similarly, outdoor air pollution exposure is associated with various adverse health effects in children (66), such as pre-term birth (67, 68), increased BMI (69), increased asthma risk (70), delayed lung function growth (70, 71), and vitamin-D deficiency (72, 73). These clinical attributes may modify the impact exposure has on brain structure and function, and future studies need to examine their potential contributions to the heterogeneity in the neurotoxicity of exposure to ambient air pollution. In addition to a confounder, SES may also be an effect modifier. While Peterson et al. (34) was based on the larger CCCEH cohort of minority pregnant women of low SES backgrounds; none of the MRI studies to date included a large and diverse enough sample to examine SES as a modifier. This is likely an important issue to address, as previous cognitive studies in adolescents and young adults have found that the adverse effects of exposure can be exponentially larger in low SES families (22). Thus, more research is needed to examine the possible social disparities in exposure effects on MRI brain outcomes. Lastly, only one study examined how genetics influenced the association between air pollution and brain structure (39). These findings of an interaction between genotype and exposure (39) corroborate animal studies (74), further highlighting the importance of genetic differences in how neurodevelopment may be affected by air pollution. Genes involved in oxidative stress and inflammatory pathways are logical candidates for future studies as well as functional polymorphisms that are involved in neuronal proliferation and differentiation (e.g., brain-derived neurotrophic factor) (75) and neurotransmitter transporters and receptors (e.g., 5-HTTLPR) (76). Large-scale studies with adequate statistical power will be needed to examine whether individuals may be differentially affected by early life exposure to outdoor air pollutants based on additional environmental and biological factors.

Advanced Multi-Modal Neuroimaging

Several MRI techniques were used based on 1.5-Tesla ($n = 4$) or 3-Tesla ($n = 2$) MRI with an 8-channel head-coil, including structural MRI (CCCEH, BREATHE, and Generation R), functional MRI (BREATHE), diffusion MRI (BREATHE), and magnetic resonance spectroscopy (MRS) (BREATHE). **Figure 2** illustrates key concepts about each of these non-invasive MRI techniques. Future studies have a number of advancements in hardware and sequence development in the field of neuroimaging at their disposal, such as 3T and 7T scanners combined with a 32- or 64-channel radiofrequency head-coil (instead of a 1.5 or 3T with an 8-channel head-coil), which improves the MRI signal-to-noise ratio. Similarly, sequences with higher spatial resolution for diffusion MRI and fMRI scans are readily available with voxel sizes around 1.5–2 mm^3 as compared to 3–5 mm^3 a few years ago. Like the BREATHE project, studies should aim to perform various advanced MRI modalities and examine multiple metrics per imaging modality within the same population to allow for a more complete characterization of brain structure and function.

Regional-specific findings with structural MRI

Despite previous studies linking vehicle related traffic to smaller head circumferences during gestation (77), MRI findings in children suggests air pollution exposure does not lead to global differences in whole brain volume or intracranial size (35–38). The contrasting findings between head circumferences and total brain size could be due to a number of reasons. First, head circumference measurements were taken by ultrasound and only examined the exposure and outcome variables during pregnancy. The included studies have examined both prenatal and postnatal windows of exposure, but all brain MRIs outcomes were measured during childhood (6–12 years). Furthermore, while head circumference and brain volume have been found to correlate at very young ages (<6 years), at older ages (7–42 years) head size is no longer an accurate proxy for brain volume (78). Thus, brain MRI may provide a much more precise measure of brain development, especially given the dynamic structural changes seen across childhood and adolescence. Recent studies also suggest that head size and intracranial volume may be strongly linked to genetics (79), which may explain why no associations exist between air pollution exposure and intracranial volume or whole brain metrics, even in the largest MRI sample studied to date (38).

Rather than global volume impairments, regional specificity in the existing studies suggest some neural processes may be more vulnerable to the neurotoxic effects of air pollution, including the prefrontal cortex, white matter, and caudate. Depending on data preprocessing, structural MRI can segment gray matter (primarily composed of cell bodies and synapses) and white matter (primarily myelination of axons) into volume estimates based on regions of interest (ROI) (reported in BREATHE, Generation R), assess density with voxel based morphometry (VBM) (as reported in BREATHE), cortical thickness (reported in BREATHE and Generation R), and/or surface area (as reported in CCCEH). These structural outcomes are not synonymous, and each morphometric (e.g., thickness, volume, surface area) has been found to follow a different developmental trajectory (7, 46). Therefore, a more granular targeted analysis in terms of both regional-specificity as well as examination of each morphometric outcome will help to elucidate which brain regions and neural processes may be most vulnerable to the neurotoxic effects of air pollutants.

Task FMRI and rs-FMRI

Of the reviewed studies, only a single fMRI task was utilized to measure BOLD response to sensory stimuli. We found no studies that utilized task-based fMRI to examine the neural substrates underlying the reported deficits in various cognitive (e.g., working memory or inhibitory control) (80) or emotional (e.g., anxiety or aggression) behaviors that have been linked with outdoor air pollution exposure in children and adolescents (21, 23–26). Air pollution MRI studies using working memory, attention, inhibitory control, and emotion fMRI tasks are warranted. In terms of resting state, only four seed regions were previously used to examine functional connectivity of regions included in the DMN and the fronto-parietal networks (35). However, it is unclear if exposure affects other neural networks, including the somatomotor, visual, cingulo-opercular, or dorsal

attention, which are also known to develop across childhood into young adulthood (81–84). Moreover, future studies combining resting state fMRI and graph theory analyses (44) will be able to more directly test if air pollution exposure results in maintaining strong *within-network* connectivity and poor *between-network* connectivity, ultimately reflecting altered maturation of resting state networks. Given that the BREATHE project was the only study to implement these techniques, fMRI remains an untapped resource for elucidating how outdoor air pollution affects brain activity across development.

Structural white matter connectivity

Multi-shell diffusion-weighted imaging may provide an unparalleled opportunity to further our understanding of how air pollution impacts white matter maturation. Sequences using High Angular Resolution Diffusion Imaging (HARDI) sequences (i.e., higher number of gradient directions) allows for more complex models of water dispersion to more accurately perform tractography to assess white matter fiber bundles (85). Similarly, multi-shell dMRI acquisition (e.g., including more than one b-value) can allow for *neurite orientation dispersion and density imaging* (NODDI) analyses (86). This allows for calculation of orientation dispersion (ODI) and neurite density (NDI) indices, of which increases in ODI may best reflect myelination (87), whereas reduced NDI may reflect geometrical complexity of neurite architecture in the human central nervous system (88). Utilization of these novel techniques in future studies may improve our ability to further elucidate our understanding of how outdoor air pollution impacts white matter. For example, previous associations between air pollution and white matter surface area (34) and FA (36) suggested that myelination (i.e., the lipid and protein sheath along axons that allows for faster signal conduction) and/or oligodendrocytes (i.e., myelin forming glial cells) may be affected. Moreover, by comparing the reported findings of copper and white matter FA values (36) with a well-known white matter tract atlas (89), these affected white matter regions can be further categorized into the superior corona radiation (SCR), anterior corona radiation (ACR), internal capsule (IC), or genu of the corpus callosum (gCC). Employing multi-shell and NODDI techniques may elucidate the degree by which outdoor air pollution affects the development of specific white matter fiber bundles and myelination along those fiber tracts. Thus, studies using these novel dMRI techniques have an unparalleled opportunity to further assess how air pollutants may impact the structural integrity and myelination of these white matter tracts as they develop across childhood, adolescence, and well into the third decade of life (32, 90).

Cerebral blood flow

It remains unknown if outdoor air pollution leads to alterations in neurovasculature. Despite known effects on cardiovascular health (91), including increased blood pressure and hypertension (92, 93) and neurovasculature inflammation (94), no study has examined if outdoor air pollution may alter cerebral perfusion. A common MRI technique that can complement structural MRI and fMRI is arterial spin labeling (ASL). ASL measures the delivery rate of blood flow to brain tissue, termed cerebral blood flow (CBF) (95). CBF is regulated by astrocytes (e.g., neuronal support cells), based on the demands of neuron activity (which utilizes oxygen and glucose), and is thus variable across the brain. Thus, measuring CBF can be a useful technique to clarify if fMRI results are confounded by changes in the hemodynamic response, but also may elucidate additional mechanisms—neurovasculature and astrocytes (given their vital role in regulating CBF)—by which outdoor air pollution may affect the developing brain.

A Translational Approach Toward Understanding Mechanisms

While the exact mechanisms remain unknown, it is thought ambient air pollution may impact the brain *in utero* or during postnatal development either via systemic and/or direct effects. During the prenatal period, possible mechanisms include nanoparticles (smaller than 240 nm) bypassing the placenta to impact the developing fetus (96) and/or maternal changes in the immune system (97, 98), as indicated by changes in cord blood immune system seen to occur with air pollution exposure (99). Postnatal exposure may also affect the brain by direct neurotoxicity and/or acting secondarily through systemic changes (1, 100). Fine and coarse PM can deposit into airways and lung tissue (101). Trace metals, endotoxins, and other smaller soluble compounds can then interact with tissues to produce reactive oxygen species or induce signaling cascades that increase inflammation. Following an increase in inflammation, inflammatory markers enter the circulatory system and disrupt the blood brain barrier, allowing pro-inflammatory cytokines, and other substances to enter the brain. In addition to systemic effects, it has been hypothesized that metals and other toxins, including ultrafine particles, may take a more direct route to the brain through the olfactory system (102, 103), although support for this hypothesis is mixed. Animal models continue to show that PM exposure leads to various changes within the brain, including neuroinflammation, oxidative stress, microglial activation, neurovascular damage, altered neurotransmitters, and up-regulation of genes encoding inflammatory cytokine pathways (100, 104–108). These experimental findings were supported by several neuropathological reports suggesting activation of neuroinflammatory markers and microglia, impairments in vasoconstriction, and lesions in the frontal lobe in the brain autopsies of children, young adults, and animals living in highly polluted cities (109, 110).

A limitation of human MRI is that it is unable to decipher cellular and molecular level changes that may occur as a result of outdoor air pollution. Thus, in studying the fundamental questions of how air pollution affects these brain systems, future research should aim to perform a translational approach that bi-directionally informs researchers performing both human and animal studies. For example, MRI studies will be vital to elucidate which neural networks are especially vulnerable to the neurotoxic effects of air pollutants in humans. These findings can then inform animal exposure studies to

elucidate the key cellular and molecular changes. In the same realm, existing animal studies already have shown additional key brain regions that are impacted by exposure, including lateral ventricle size and the hippocampus (65, 111, 112). These animal studies provide insight into the need to take a targeted approach to consider examining the volumes of these two structures, but also the function and connectivity of the hippocampus with larger networks. These are just a few examples, yet the possibility of using a translational research approach will ultimately aid us in understanding the mechanisms underlying how outdoor air pollution exposure impacts neurodevelopment.

CONCLUSIONS

In summary, prenatal, and postnatal exposure to chemicals and particulate matter from outdoor sources, including the chemical components indicative of exposures to traffic-related air pollution, may lead to alterations in specific neural networks. Longitudinal studies with multi-modal, advanced MRI measures are needed to determine how the timing of exposure from conception to young adulthood influences brain structure and function. Examining the potential heterogeneity by which outdoor air pollution impacts both regional specificity as well as large-scaled neural networks will greatly advance our understanding of air pollution neurotoxicology in populations and contribute to the critical knowledge base needed to inform both prevention strategies and public health policy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MH made substantial contributions to the conception of this review, analysis, interpretation of the data, and writing the manuscript. DY and CC were involved with the acquisition, analysis, and interpretation of the data as well as revising the manuscript for intellectual content. J-CC was involved in the conceptualization of the project as well as the interpretation of the data and revising of the manuscript. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of their respective contributions of the work.

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Gender Difference in the Effects of Outdoor Air Pollution on Cognitive Function Among Elderly in Korea

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Background/Aim: Given a fast-growing aging population in South Korea, the prevalence of cognitive impairment in elderly is increasing. Despite growing evidence of air pollution exposure as one of the risk factors for declining cognition, few studies have been conducted on gender difference in the relation of cognitive function associated with outdoor air pollution. The aim of this study is to investigate the effect modification of gender difference in the association between cognitive function and air pollutant exposure (PM_{10}, $PM_{2.5-10}$, and NO_2).

Methods: The study focused on elderly, and the resulting sample included 1,484 participants aged 55 and older with no neurologic diseases, recruited from the four regions in Korea (Seoul, Incheon, Pyeongchang, and Wonju). We used the Mini-Mental State Examination (MMSE) score (with the conventional cut-off point "23–24") to assess cognitive decline as the primary outcome of the study. Air pollution data used in this study were based on the 5-year average of predicted PM_{10} and NO_2 concentrations, as well as the 2015 average $PM_{2.5}$ concentration. Additionally, a survey questionnaire was utilized to obtain information about general health assessment. To explore gender differences in the effects of air pollution exposure on cognitive function, we used penalized logistic regression, negative binomial regression, and generalized linear mixed model analyses. Subgroup analyses were also performed by the geographic location of residence (metropolitan vs. non-metropolitan).

Results: We found that women than men had a higher risk for decreased cognitive function associated with increased exposure to PM_{10} and $PM_{2.5-10}$, respectively, even after adjustments for confounding factors (OR 1.01 [95%CI 1.00-1.03] in PM_{10}; OR 1.03 [95%CI 1.01–1.07] in $PM_{2.5-10}$). After stratification by metropolitan status, we also found that the adverse effect of NO_2 exposure on cognitive function was higher in women than men [OR 1.02 [95%CI 1.00–1.05] in metropolitan; OR 1.12 [95%CI 1.04–1.20] in non-metropolitan]. Notably, the magnitude of the effect sizes was greater among those in non-metropolitan regions than metropolitan ones.

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Conclusions: Although our findings suggest that the adverse effects of outdoor air pollution on cognitive function appeared to be higher in women than men, this should be tentatively reflected due to some limitations in our results. While additional research is warranted to confirm or dispute our results, our findings suggest an indication of the need for developing and implementing prevention or interventions with a focus on elderly women with increased risk for air pollution exposure.

Keywords: air pollution, gender difference, cognitive function, particulate matter, nitrogen oxide, mini-mental state examination

INTRODUCTION

The decline of cognitive function is one of the reasons for low independence and likely disability among older adult populations (1, 2). In South Korea, as one of the countries with a fast-growing aging population, the prevalence of cognitive deficits or decline among older adults (aged 65 years and older) had been increasing considerably (3). A nationwide survey conducted by Kim and colleagues (3) estimated the prevalence of mild cognitive impairment and dementia in elderly people aged 65 and older in South Korea and projected that the expected number of dementia cases would be doubled every two decades before the coming 2050 (3). The burden associated with increased cognitive decline among the elderly is likely to grow with rapidly growing aging populations.

Essential is to reduce risk factors for cognitive decline or impairment. For example, the known risk factors such as age, sex/gender, socioeconomic conditions, genetic component, health risk behaviors (i.e., smoking and drinking), high blood pressure, and disease morbidities were examined in prior studies (4–10). Among risk factors, comparatively less is known about environmental factors, including air pollution, which could have played a role in risk for cognitive decline or deficits (2, 11, 12). In South Korea, in recent years, the issue of ambient particulate air pollution has become more severe than before. This aspect could play a role in adversely affecting the health of the population. Evidence showed that high levels of exposure to $PM_{2.5}$, PM_{10}, and NO_2 were adversely associated with respiratory health (13–16), cardiovascular diseases (14, 17), and increased risk of psychiatric disorders (18).

It has been gradually suggested that there is a relationship between air pollution exposure and cognition in elderly (2, 19, 20). For instance, a prospective cohort study by Weuve et al. (19) examined whether long-term PM exposure may be related to cognitive decline among the US women aged 70–81 years in the Nurses' Health Study Cognitive Cohort and found that higher levels of $PM_{2.5-10}$ and PM_{10} exposure were related to faster decline in cognitive function, respectively. Meanwhile, Ailshire and Crimmins (20) explored the relation of cognitive function associated with high concentrations of $PM_{2.5}$ among older US adults using data from the Health and Retirement Study. They found that high levels of $PM_{2.5}$ exposure had an adverse effect on cognitive function, but no notable significant effect modification by individual-level characteristics, including demographics and socioeconomic factors was found.

In spite of growing indication of a positive link between air pollution exposure and cognitive decline, much less consideration has been directed to the extent of gender difference in the relation of cognitive function associated with air pollution exposure. Although a few studies explored effect modification by gender/sex as a supplementary purpose concerning examining the main effects of air pollution on cognitive function (11, 20), the results were varied. Specifically, previous research found that the effect of air pollution exposure on cognition was different between males and females (21, 22), while no such result was found for other research (23). Notably, there is a discrepancy over whether the effect is higher in males than females or vice versa (21–23). Therefore, this study aims to clarify this inconsistency by investigating gender differences in the association between exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2) and cognitive functioning in a cohort of Korean older adults. Detecting gender differences and identifying the mechanism can help develop gender-specific interventions or policies to reduce the risk of cognitive decline associated with outdoor air pollution among older adult populations.

MATERIALS AND METHODS

Study Subjects

Participants were included among the cohorts recruited from the four different regions of South Korea (i.e., Seoul, Incheon, Wonju, and Pyeongchang) between 2014 and 2017. Specifically, they were voluntary participants recruited through community advertisement from the Nowon-gu, Yangcheon-gu, Mapo-gu, Seodaemun-gu, Eunpyeong-gu, and Gangnam-gu of Seoul, the Namdong-gu and Ganghwa-gun of Incheon, and Wonju and Pyeongchang, respectively. Since it was voluntary recruitment conducted in small districts, our study has a limitation on generalizability. The inclusion criteria were: aged 55 years or above and no history of having dementia, Parkinson's disease, or stroke. We excluded patients with Alzheimer's disease and amnestic mild cognitive impairment, as well as missing data concerning the characteristics of participants except for smoking status since most of the women were likely to be non-smokers or social smokers. Although we kept them in the study, we did not adjust for smoking status in our regression analyses, which will follow in the statistical analysis section. The resulting sample was comprised of 1,484 participants (569 men and 915 women). We enrolled the participants from Seoul

and Incheon, separately, in Gachon University Gil Medical Center and Yonsei University Severance Hospital for a medical examination, and those from Wonju and Pyeongchang in the Lifetime Management Center at Yonsei University Wonju College of Medicine and Wonju Severance Christian Hospital for a medical checkup, respectively. The study was approved by the Institutional Review Boards of the Yonsei University Health System (IRB Approval No. 4-2014-0359). Trained nurses as research staff administered a survey questionnaire, constructed based on the prior studies for outcome assessment (12, 15, 24) as well as the Korea Centers for Disease Control and Prevention for epidemiological characteristics (25), to all subjects following the protocols and ethical standards of the human experimentation committee at Yonsei University Severance Hospital. The study participants were asked to answer the questions about demographics, anthropometric measurement, socioeconomic condition, health risk behaviors (smoking, drinking, etc.), physical activity, disease morbidities including family history, and residential characteristics.

Outcome Assessment

Mini-Mental State Examination (MMSE) as the most commonly used cognitive test (26, 27) was used to assess participants' cognitive function. Notably, MMSE, which detects individual cognitive impairment by evaluating mental status, is frequently used for dementia screening in the primary care setting (28, 29). MMSE with 30 points in total comprises questions on orientation, attention, calculation, language, and recall (29). The study used the conventional cut-off point for the MMSE ("23–24") as an indication for a decline in cognitive functioning, based on the criteria employed in the prior studies (12, 30). Therefore, the dependent variable in the study was "decreased cognitive function" as binary ("1" if MMSE score $\leq$ 23 corresponding to a decreased cognitive function, "0" if 24 $\leq$ MMSE score $\leq$ 30 corresponding to a normal cognitive function), and we also included MMSE score as a continuous variable in the models.

Prediction for Long-Term Concentrations of Particulate Matter and Nitrogen Dioxide

Based on the residential addresses of the participants, each was assigned the predicted exposure concentration based on a national point-wise exposure prediction approach using regulatory monitoring data for 2010. In spite of this prediction, however, we acknowledge some weaknesses in our data: (1) limitation about one chronic exposure incorporated; (2) the presence of a possible regression toward null effect subject to modeled data used without reflecting individual exposure appropriately. However, it should be noted that regression toward null effect is considered as a limitation in most of the studies using modeled air pollutants data. That is, results may be shown with no impact of air pollution due to using the average concentrations of air pollutants, although it may exist when using the actual values. A specific method of prediction was documented in another published article (31).

Specifically, Kim et al. (31) developed the national prediction model by incorporating the annual mean concentration values of log-transformed NO_2 and PM_{10} estimated at the 277 air quality monitoring sites between 2010 and 2016 after excluding 11 places that did not meet the inclusion criteria. In the process of developing a prediction model, 322 geographic variables that consist of proximity and buffer in eight categories, including traffic, physical geography, land use, demographic characteristics, etc. were used to get information about geographic characteristics that could be attributed to the spatial variability of air pollution. This universal kriging model comprises two elements: mean and variance. That is, the mean was characterized with the two and three predictors which were estimated by partial least squares, while the variance was assessed using the three parameters for covariances such as partial still, range, and nugget which could find the presence of spatial correlation and both spatial and non-spatial variability. Consequently, we calculated the predicted values of the annual average concentrations of NO_2 and PM_{10} at the residential addresses of the study participants over the past 5 years, using the model developed. The rationale for using the 5-year mean values of the air pollution levels was that the information about participants' residential addresses was solely available in those 5 years. For $PM_{2.5}$ data, however, we merely used the 2015 average concentration as a proxy for the 5-year values because information about $PM_{2.5}$ had become publicly available since 2015.

Concerning air pollution concentration levels in the four regions, Seoul had an average of 22.23 (μg/m3) for PM_{10-25}, 47.88 (μg/m3) for PM_{10}, and 33.80 (μg/m3) for NO_2. Incheon had an average of 21.61 (μg/m3) for PM_{10-25}, 47.90 (μg/m3) for PM_{10}, and 33.33 (μg/m3) for NO_2. Wonju had an average of 13.86 (μg/m3) for PM_{10-25}, 39.37 (μg/m3) for PM_{10}, and 10.88 (μg/m3) for NO_2. Pyeongchang had an average of 16.82 (μg/m3) for PM_{10-25}, 42.50 (μg/m3) for PM_{10}, and 15.35 (μg/m3) for NO_2.

This study included "exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2)" as continuous variables in the main models, while we also examined them with categorization as part of our sensitivity analysis in the statistical analysis section.

Potential Confounding Factors

Following the status of the participants, potential confounding factors were included. Age (in years) was included, recoded as a categorical variable (55–64 and 65+), given the fact that older age is a strong risk factor for cognitive decline (7). Other factors such as gender, marital status, education, income, smoking status, drinking, body mass index (BMI), systolic blood pressure, disease morbidities, and geographic location were included, since women, low educational and income levels, being unmarried, smoking and drinking, higher BMI, high blood pressure, and disease morbidities (hypertension, diabetes, hyperlipidemia, and depression) were indicated as risk factors for cognitive decline or impairment in the literature (4, 6, 7, 10, 32–34). Marital status indicating whether an individual is currently married was recoded and included as a dichotomous variable (yes or no). As indicators for socioeconomic condition, education ("less than middle school" and "middle school graduate or more") and income (X, Korean won) (X<1,000,000, 1,000,000 $\leq$ X< 2,000,000, 2,000,000 $\leq$ X< 4,000,000, and X $\geq$ 4,000,000) were included. Smoking status was assessed based on the questions of

whether a participant smoked more than five packs of cigarettes in each entire life and if he or she currently smokes (yes or no), which was categorized into "current smoker," "former smoker," and "never smoker." Alcohol drinking was assessed based on the question of whether a participant does not drink at all, which was recoded and included as a binary variable. We calculated BMI (kg/m^2) using individual weight and height and included it as a categorical variable [underweight (<18.5), normal weight (18.5–22.9), overweight (23.0–24.9), and obese (≥25.0)], based on Asia-Pacific BMI classifications (35, 36). Disease morbidities (hypertension, diabetes, and hyperlipidemia) were included, indicated by the question of whether or not an individual has ever been diagnosed by a physician, which was conducted through a trained nurse's interview. Blood pressure was measured twice, and average of systolic blood pressure was included in the models. Being depressed was included as a dichotomous variable, assessed by the Korean version of the Short Geriatric Depression Scale (SGDS-K) (37) (i.e., depressed=yes if $8 \leq$ SGDS-K ≤ 15; depressed = no if $0 \leq$ SGDS-K < 8). Moreover, we included the variable "geographic location" based on the residence area of the participants (Seoul, Incheon, Wonju, and Pyeongchang), categorized into "metropolitan" (Seoul and Incheon) and "non-metropolitan" (Wonju and Pyeongchang). In addition to those risk factors, we included the variables, physical activity ["often (4 days or above)," "a few times (1 to 3 days),"and "never (<1 day)"]and quality of daily life ("good quality," "neither poor nor good," "poor"), as related protective factors indicated by prior studies (38, 39), which were assessed based on the questions, "Recently, how many days did you walk at least 10 min?" and "How would you rate the quality of your daily life? (i.e., work, house chores, leisure activities, etc.), respectively.

Statistical Analysis

The dependent variable in this study was: "decreased cognitive function" as binary ("1" if MMSE score ≤ 23, "0" if $24 \leq$ MMSE score ≤ 30). We performed different regression analyses to detect and examine gender differences in the relationship between air pollution exposure and cognitive function in the participants. Because the dependent variable has extra zero observations after categorizing, in which general logistic regression models may not be adequate to use (40), we first conducted penalized logistic regression analyses to examine the odds of declining cognitive function associated with increased exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2). Notably, penalized logistic regression analysis can solve problems, including multicollinearity and overfitting, which commonly occur in the use of general logistic regression models (41). Next, we analyzed negative binomial regression models since we also examine the MMSE score as a count variable with over-dispersion in which the general poisson regression model does not fit adequately. Furthermore, we conducted generalized linear mixed model analyses to include a geographic location (metropolitan vs. non-metropolitan) as random effects concerning examining the effects of air pollution exposure on cognitive function in the participants. To detect and investigate the gender differences, we created and employed two different models: interaction models and stratified models. Specifically, we incorporated interaction terms in the regression analyses, based on the results from the Rao-Scott Chi-square test and a two-sample *t*-test for categorical variables and continuous variables, respectively. Moreover, we conducted regression analyses by stratifying gender.

In a sensitivity analysis, we additionally examined exposure to air pollutants (PM_{10}, PM_{10-25}, and NO_2) as interval variables using label values at the 25, 50, and 75 percentiles, respectively to see whether and how the estimated effect of air pollution on cognitive function would change at intervals. Specifically, we divided air pollutants such as PM_{10}, PM_{10-25}, and NO_2, respectively, into the four classes: (1) PM_{10}: Q1 ($PM_{10} \leq 43.65$), Q2 ($43.65 < PM_{10} \leq 47.26$), Q3 ($47.26 < PM_{10} \leq 48.67$), Q4 ($48.67 < PM_{10} \leq 55.40$); (2) PM_{10-25}: Q1 ($PM_{10} \leq 17.91$), Q2 ($17.91 < PM_{10-25} \leq 21.08$), Q3 ($21.08 < PM_{10-25} \leq 22.76$), Q4 ($22.76 < PM_{10-25} \leq 28.33$); (3) NO_2: Q1 ($NO_2 \leq 20.44$), Q2 ($20.44 < NO_2 \leq 32.34$), Q3 ($32.34 < NO_2 \leq 35.10$), Q4 ($35.10 < NO_2 \leq 44.38$). We also estimated the effect of air pollution exposure on cognition with the log-transformation as commonly used to deal with non-normal or skewed data (42). It is because our air pollutant data were indicated as non-normal from the result of the Shapiro-Wilks normality test ($p < 0.0001$). Thus, we wanted to see whether the effects on cognition would alter with the log-transformed air pollutants. Further, we investigated whether there exists any non-linear effect of air pollution exposure on cognition using zero-truncated negative binomial regression models, which can confirm our results. In addition to looking over and exploring air pollution in multiple ways, we examined MMSE score using a different cut point ("24–25") since after excluding patients with a previous diagnosis such as Alzheimer's disease and amnestic mild cognitive impairment, many participants were resulted in having MMSE scores ≥25 (87.61%). In this regard, we investigated the effect of outdoor air pollution on cognition by incorporating MMSE score values ≤25 as an indication of lower cognition.

The significance threshold was set at 0.05. All statistical analyses were conducted using SAS, version 9.4 (NC, USA).

RESULTS

Table 1 shows the characteristics of the Korean cohort and the difference by gender. The mean exposure levels of PM_{10}, $PM_{2.5-10}$, and NO_2 were significantly higher in women than in men (46.56, 20.66, and 30.11 $\mu g/m^3$ in women vs. 45.09, 19.24, and 25.83 $\mu g/m^3$ in men). Being >65 years old (74.17 vs. 63.83%), married (93.66 vs. 72.79%), with a high education (52.20 vs. 39.56%) and income (14.94 vs. 10.49%) were significantly more common in men than in women. Doing physical activity (94.10 vs. 91.04%) and having a metropolitan area of residence (85.90 vs. 64.50%) were significantly more prevalent in women than in men. Having a good quality of life (92.96 vs. 88.31%) and a BMI > 25.00 (41.12 vs. 37.70%) were significantly more common in men than in women. Smoking and drinking habits were significantly higher in men than in females: 71 vs. 2.85% for smoking status (both current and past); 71.88 vs. 36.50% for alcohol drinking. For disease morbidities, hypertension and hyperlipidemia were significantly more common in women than in men: 36.17 vs.

TABLE 1 | Characteristics of the study participants.

Characteristics	All (n = 1,484)	Men (n = 569)	Women (n = 915)	Statistical test[a/]	p-value
	Mean ± SD[b/]	Mean± SD[b/]	Mean ± SD[b/]		
PM_{10} ($\mu g/m^3$)	46.00 ± 3.93	45.09 ± 4.30	46.56 ± 3.57	$t = -7.15$	<0.0001
$PM_{2.5-10}$ ($\mu g/m^3$)	20.11 ± 3.71	19.24 ± 4.04	20.66 ± 3.38	$t = -7.26$	<0.0001
NO_2 ($\mu g/m^3$)	28.47 ± 10.19	25.83 ± 11.24	30.11 ± 9.11	$t = -8.03$	<0.0001
Systolic blood pressure (mmHg)	127.99 ± 13.49	128.56 ± 12.91	127.64 ± 13.84	$t = 1.74$	0.082
	N (%)	%	%		
Age				$\chi^2 = 17.17$	<0.0001
55–64	478 (32.21)	25.83	36.17		
65+	1,006 (67.79)	74.17	63.83		
Marital status				$\chi^2 = 98.37$	<0.0001
Yes	1,198 (80.78)	93.66	72.79		
No	285 (19.22)	6.34	27.21		
Education				$\chi^2 = 22.68$	<0.0001
Less than middle school	825 (55.59)	47.80	60.44		
Middle school graduate or more	659 (44.41)	52.20	39.56		
Income (X) (won)				$\chi^2 = 11.80$	0.008
X < 1,000,000	382 (25.74)	23.55	27.10		
1,000,000 ≤ X < 2,000,000	493 (33.22)	30.23	35.09		
2,000,000 ≤ X < 4,000,000	428 (28.84)	31.28	27.32		
X ≥ 4,000,000	181 (12.20)	14.94	10.49		
Physical activity				$\chi^2 = 6.15$	0.046
Often	1,040 (70.08)	70.13	70.06		
A few times	339 (22.84)	20.91	24.04		
Never	105 (7.08)	8.96	5.90		
Quality of daily life				$\chi^2 = 8.85$	0.012
Good	1,336 (90.09)	92.96	88.31		
A little	142 (9.57)	6.69	11.36		
Not at all	5 (0.34)	0.35	0.33		
Ever smoker				$\chi^2 = 798.01$	<0.0001
Current	86 (5.80)	14.06	0.66		
Former	344 (23.18)	56.94	2.19		
Never	4 (0.27)	0.53	0.11		
Not reported [c/]	1,050 (70.75)	28.47	97.04		
Drinking				$\chi^2 = 175.63$	<0.0001
Yes	743 (50.07)	71.88	36.50		
No	741 (49.93)	28.12	63.50		
BMI				$\chi^2 = 14.21$	0.002
< 18.50	25 (1.68)	1.76	1.64		
18.50–22.90	437 (29.45)	23.90	32.90		
23.00–24.90	443 (29.85)	33.22	27.76		
≥ 25.00	579 (39.02)	41.12	37.70		
Depressed [d/]				$\chi^2 = 2.72$	0.098
Yes	219 (14.76)	12.83	15.96		
No	1,265 (85.24)	87.17	84.04		
Hypertension				$\chi^2 = 15.99$	<0.0001
Yes	480 (32.35)	26.19	36.17		
No	1,004 (67.65)	73.81	63.83		
Hyperlipidemia				$\chi^2 = 23.42$	<0.0001
Yes	481 (32.41)	24.96	37.05		

(Continued)

TABLE 1 | Continued

Characteristics	All (n = 1,484)	Men (n = 569)	Women (n = 915)	Statistical test[a/]	p-value
No	1,003 (67.59)	75.04	62.95		
Diabetes				$\chi^2 = 7.05$	0.007
Yes	244 (16.44)	19.68	14.43		
No	1,240 (83.56)	80.32	85.57		
Geographic location of residence				$\chi^2 = 92.73$	<0.0001
Metropolitan	1,153 (77.70)	64.50	85.90		
Non-metropolitan	331 (22.30)	35.50	14.10		
Outcome	Mean ± SD [b/]	Mean± SD [b/]	Mean ± SD [b/]		
Mini–Mental State Examination Score	27.23 ± 2.56	27.25 ± 2.39	27.21 ± 2.65	$t = 0.31$	0.759
	N (%)	%	%		
Decreased cognitive function[e/]	126 (8.49)	5.62	10.27	$\chi^2 = 9.76$	0.001

There could be a round error in the percentages shown above.
[a/] Rao-Scott Chi-Square test and t-test to compare the difference between males and females in terms of categorical variables and continuous variables, respectively.
[b/] SD: standard deviation.
[c/] Missing information were usually excluded except for smoking status, because most of women were likely to be non-smokers or social smokers.
[d/] Created based on the Korean version of Short Geriatric Depression Scale (SGDS-K) (i.e., depressed=yes if 8 ≤ SGDS-K ≤15; depressed=no if 0 ≤ SGDS-K < 8).
[e/] Decreased cognitive function was defined as MMSE score≤23.

TABLE 2 | The odds of declining cognitive function associated with exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, NO_2) by geographic location of residence and effect modification by gender.

	All	Metropolitan	Non-metropolitan
	OR (95% CI)[a/]	OR (95% CI)[a/]	OR (95% CI)[a/]
PM_{10}[b/]	0.93 (0.84–1.04)	0.97 (0.85–1.10)	0.82 (0.65–1.02)
$PM_{2.5-10}$[b/]	1.02 (0.91–1.15)	1.07 (0.93–1.24)	0.83 (0.64–1.06)
NO_2[b/]	0.97 (0.92–1.01)	0.98 (0.93–1.04)	0.88 (0.78–0.99)
Female	2.38 (1.28–4.42)	2.21 (0.99–4.91)	5.45 (2.52–11.80)
(PM_{10} [b/])*(Female)	**1.01 (1.00–1.03)**	1.01 (0.99–1.03)	**1.04 (1.02–1.07)**
($PM_{2.5-10}$ [b/])*(Female)	**1.03 (1.01–1.07)**	**1.04 (1.00–1.07)**	**1.13 (1.06–1.22)**
(NO_2 [b/])*(Female)	1.01 (0.98–1.02)	**1.02 (1.00–1.05)**	**1.12 (1.04–1.20)**

[a/] OR: odds ratio; CI: confidence interval. The bold values mean statistically significant. Analyses were conducted with adjustment for confounding factors (age, gender, marital status, alcohol drinking, systolic blood pressure, BMI, education, income, physical activity, quality of daily life, disease morbidities, being depressed, metropolitan status).
[b/] Air pollutants scale was increased by 10 units since 1 unit change was too small.

26.19% for hypertension; 37.05 vs. 24.96% for hyperlipidemia. On the contrary, diabetes was significantly more prevalent in men than in females: 19.68 vs. 14.43%. Having a decreased cognitive function was significantly more common in females than in men: 10.27 vs. 5.62%. Only systolic blood pressure (128.56 vs. 127.64 mmHg), being depressed (12.83 vs. 15.96%), and MMSE score (27.25 vs. 27.21) did not significantly differ between men and women.

Tables 2, **3** present results from our penalized logistic regression analyses to estimate the odds of decreased cognitive function associated with exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2) by geographic location of residence and detect gender differences. Specifically, our interaction models (**Table 2**) find that women than men had a higher risk for decreased cognitive function associated with an increase in PM_{10} and $PM_{2.5-10}$, respectively, even after adjusting for related factors [OR 1.01 [95%CI 1.00–1.03], $p = 0.016$ (PM10); OR 1.03 [95%CI 1.01–1.07], $p = 0.023$ ($PM_{2.5-10}$)]. After stratification by metropolitan status, our results also find that the adverse effect of NO2 exposure on cognitive function was higher in women than men [OR 1.02 [95%CI 1.00–1.05], $p = 0.024$ (metropolitan); OR 1.12 [95%CI 1.04–1.20], $p = 0.001$ (non-metropolitan)]. Notably, the magnitude of the effect sizes was shown stronger among those in non-metropolitan regions than metropolitan ones, which indicates that the adverse effects of air pollution exposure were higher in non-metropolitan women compared with metropolitan women. Meanwhile, our stratified models (**Table 3**) find that among the metropolitan group, $PM_{2.5-10}$ exposure was significantly associated with risk for decreased cognitive function in women (OR 1.21 [95%CI 1.02–1.44], $p = 0.028$), which, however, no significant result was found for men.

Further, our generalized linear mixed models find that an increase in $PM_{2.5-10}$ exposure had a negative effect on the MMSE score, even after adjusting for related factors ($\beta = -0.11$, $p = 0.003$). Similarly, our negative binomial regression model finds that an increase in $PM_{2.5-10}$ exposure had an adverse, crude relationship with the MMSE score ($\beta = -0.007$, $p = 0.021$). The details about the results are provided in **Table 4**.

In sensitivity analyses, first, after using a different cut point ("24–25"), our stratified models find that among the metropolitan group, $PM_{2.5-10}$ was associated with risk of lower cognition in women (OR 1.26 [95%CI 1.11–1.42], $p = 0.0002$), which, however, no significant result was found for men. After examining exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2) as interval variables, there seemed to be a pattern showing a negative relationship between the higher quartile of outdoor air pollution and MMSE score in the metropolitan group, indicating that higher levels of air pollution exposure were associated with decreased cognitive function (for PM_{10},

TABLE 3 | The odds of declining cognitive function associated with exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, NO_2) by gender and geographic location of residence.

	Men			Women		
	All	Metropolitan	Non-metropolitan	All	Metropolitan	Non-metropolitan
	OR (95% CI)[a/]	OR (95% CI)[a/]	OR (95% CI)[a/]	OR (95% CI)[a/]	OR (95% CI)[a/]	OR (95% CI)[a/]
PM_{10}[b/]	1.01 (0.93–1.11)	0.77 (0.61–0.97)	1.10 (0.79–1.54)	1.04 (0.90–1.19)	1.09 (0.93–1.28)	0.83 (0.59–1.17)
$PM_{2.5-10}$[b/]	1.04 (0.95–1.15)	0.89 (0.70–1.12)	1.12 (0.77–1.61)	1.12 (0.96–1.30)	**1.21 (1.02–1.44)**	0.88 (0.59–1.29)
NO_2[b/]	1.01 (0.98–1.04)	0.92 (0.84–1.02)	1.02 (0.87–1.20)	1.00 (0.94–1.06)	1.02 (0.95–1.10)	0.90 (0.76–1.07)

[a/] OR, odds ratio; CI: confidence interval. The bold value means statistically significant. [b/] Air pollutants scale was increased by 10 units since 1 unit change was too small.

TABLE 4 | The estimated effects of exposure to air pollutants (PM_{10}, $PM_{2.5-10}$, NO_2) on MMSE score by geographic location of residence and effect modification.

	Generalized linear mixed model[a/]		Negative binomial model			
			Metropolitan		Non-metropolitan	
	β[b/]	*P*[b/]	β[b/]	*P*[b/]	β[b/]	*P*[b/]
PM_{10}	0.0140	0.670	−0.0004	0.903	0.0057	0.364
$PM_{2.5-10}$	**−0.1106**	**0.003**	−0.0046	0.194	0.0054	0.440
NO_2	0.0203	0.097	−0.0001	0.962	0.0027	0.367
Female	−0.3786	0.044	−0.0051	0.705	−0.0488	0.071
$(PM_{10})^*$ (Female)	−0.0062	0.127	−0.0002	0.561	−0.0010	0.115
$(PM_{2.5-10})^*$ (Female)	−0.0126	0.162	−0.0007	0.383	−0.0032	0.120
$(NO_2)^*$ (Female)	−0.0003	0.955	−0.0003	0.507	−0.0027	0.297

[a/] Incorporated geographic location of residence (metropolitan vs. non-metropolitan) as random effects.

[b/] β, coefficient; P, p-value.

After adjustment for confounding factors (age, gender, marital status, alcohol drinking, systolic blood pressure, BMI, education, income, physical activity, quality of daily life, disease morbidities, being depressed, metropolitan status).

OR 1.18 [95%CI 0.36–3.83] (Q2 vs. Q1), OR 1.25 [95%CI 0.38–4.07] (Q3 vs. Q1); for $PM_{2.5-10}$, OR 0.86 [95%CI 0.22–3.29] (Q3 vs. Q1), OR 0.94 [95%CI 0.25–3.59] (Q4 vs. Q1); for NO_2, OR 0.54 [95%CI 0.09–3.35] (Q2 vs. Q1); OR 0.73 [95%CI 0.12–4.52] (Q3 vs. Q1). With the log-transformation of air pollutants, our results from the generalized linear mixed models find that PM_{10} and $PM_{2.5-10}$ exposures, respectively, affected MMSE scores more adversely in women than men ($\beta = -0.07$, $p = 0.041$ ("PM^*_{10}female"); $\beta = -0.09$, $p = 0.05$ ("$PM^*_{2.5-10}$female"). Although our negative binomial models find no significant effect medication, our results show that among the metropolitan group, $PM_{2.5-10}$ was negatively associated with the MMSE score ($\beta = -0.161$, $p = 0.028$), similar to the results from the models without the log-transformation. Furthermore, our zero-truncated negative binomial models for exploring a non-linear effect of air pollution exposure on cognition find that $PM_{2.5-10}$ exposure had an adverse effect on cognitive function among the metropolitan group ($\beta = -0.007, p = 0.006$).

DISCUSSION

In this cross-sectional study of Korean older adults, we explored gender differences in the relation of cognitive functioning associated with particulate air pollution. Albeit not strong, we found that women than men had a higher risk for declining cognitive function associated with increased exposures to PM_{10} and $PM_{2.5-10}$, and after stratification by geographic location of residence, the adverse effect of NO_2 exposure on cognitive function was greater in women than men. Furthermore, our results from both negative binomial and generalized linear mixed models find that an increase in $PM_{2.5-10}$ had an adverse effect on declining cognitive function. Our main findings are both congruent and incongruent with prior studies (11, 21, 22). For example, a study of 105 healthy children who were highly exposed to $PM_{2.5}$ and ozone in Mexico City examined the role of other risk factors including Apolipoprotein 4, gender, and BMI on the risk of declining cognition and found that girls than boys may be more likely to develop cognitive deficits associated with air pollution exposure (22). In the meantime, Chen et al. (21) examined the impact of air pollution on individual cognitive performance using the data from the China Family Panel Studies and found a significant, adverse effect of air quality measured by the air pollution index on both the verbal and math test scores of the respondents. Especially, the adverse effects of air pollution on cognitive performance were higher in men compared with women. However, a study conducted with a sample from the National Survey of Health and Nutrition in Mexico did not find any significant modification by sex in the relationship between $PM_{2.5}$ concentrations and cognitive function (43).

A probable explanation may apply for our finding that women than men may have a higher risk for declining cognition associated with increased exposures to PM_{10} and $PM_{2.5-10}$. It may be due to the different neurological structure or system between men and women, which could have affected cognitive function associated with air pollution exposure differently in them. For example, prior research investigated the relationship between the structural brain organization and general intelligence in men and women and found that women than men have less gray matter and more white matter as related to intelligence (44). Moreover, Gallart-Palau et al. (45) examined the extent of gender-specific molecular differences in developing Alzheimer's disease with cerebrovascular disease and found that despite the presence of a similar amount of brain protein (i.e., myelin-associated glycoprotein) in men and women, women

than men were more likely to receive a stronger influence from the pathology of white matter.

It is well-documented that there exist the effects of sex/gender in cognition-related research (46–50). For example, a literature review by Nebel and colleagues (46) explored how sex and gender played a role in Alzheimer's disease and attempted to discern the risk factors and found that women have a greater lifetime risk of developing Alzheimer's disease and burden of the disease impacts women more significantly than men. They also pointed out that women generally live longer than men and have a higher risk of having disease morbidities, including coronary heart disease, depression, and myocardial infarction, which are all risk factors for cognitive decline or impairment (46, 47). Furthermore, as a possible attribute of the sex effects in risk for cognitive decline, they suggest evidence that older women (aged 65 years or above) who received estrogen-containing hormone therapy had a doubled risk for dementia (48, 49), while those who began receiving hormone therapy early in the menopausal transition had a lower dementia risk, compared with those who did not. In addition to those risk factors, evidence indicates that the genetic component "APOE ε4" interacts with sex to affect the risk of cognitive decline. For instance, in a study of analyzing 5,400 ordinary people, women with APOE ε4 had a higher risk of dementia based on the Clinical Dementia Rating Score as compared to APOE ε4-negative women as well as men with APOE ε4 (50). Furthermore, other sex-/gender- differing factors (i.e., body size, stress, socioeconomic status, and gendered activities, including cleaning, cooking, etc.) could be potential attributes of the sex/gender difference in the relationship between air pollution exposure and cognitive function (51).

This study has some limitations. First, the 1,484 subjects of an elderly Korean cohort, including the 569 men and 915 women, could have increased by involving and encouraging more participation. Probably, a relatively small number of male subjects may explain why, overall, no significant results were found for men in our stratified models. Further, there could be other aspects attributed to limitations in our results, including small effect size related to a unit increase in air pollution exposure and the air pollutant prediction model with less resolution to estimate air pollution exposure precisely (52). Second, merely three air pollutants (PM_{10}, $PM_{2.5-10}$, and NO_2) were explored in this study. However, to our knowledge, many more studies had already examined other pollutants, including $PM_{2.5}$, ozone (O_3), carbon monoxide (CO), etc. (12, 19, 21, 23, 53–55). Third, our findings may not be applied to particular outdoor air pollution, given the fact that air pollution, mainly as traffic-related, is a composite of particles and gases that could also be correlated one another (2). Fourth, there may be a possibility of social desirability bias concerning a high proportion of missing information about smoking status in women. It is likely because traditionally in Korea, people had considered female smoking more negatively compared to male smoking. Also, given the questionnaire for smoking status "whether a participant smoked more than five packs of cigarettes in each entire life," it is possible that women than men could have been more reluctant to answer the question appropriately. Next, in spite of adjusting for related confounding factors indicated by the prior studies, there may be other factors that we did not control for. For example, we were unable to adjust for genetic component (i.e., APOE ε4) due to a lack of genetic information in our data. Also, there could be other related environmental factors, including noise, weather, etc. Nevertheless, in consideration of the number of subjects, it is believed that the study adjusted for associated factors to the greatest extent.

In spite of the limitations, the present study has several strengths. First, the study was conducted based on the four different regions in Korea, which allowed us to examine the effects of different air pollution exposure levels on cognitive function in elderly in Korea so that our findings may be applied to other elderly living in similar environments. Second, to our knowledge, this study was one of the first, based on a Korean older adult cohort, to explore the extent of gender difference in the relation of cognitive function associated with outdoor air pollution. Furthermore, this study used different regression models (i.e., penalized logistic regression, negative binomial regression, and generalized linear mixed models) and strategies, including subgroup analyses, as well as sensitivity analyses to investigate the extent of gender difference mentioned. Finally, our study adjusted not only for the known risk factors but also for other probable confounding factors, including geographic location, physical activity, and quality of daily life, which other studies less considered for adjustment.

In summary, we found the presence of gender differences in decreased cognitive function associated with increased exposure to outdoor air pollution in an elderly population without known neurological diseases. Despite some limitations in our results, the study findings suggest that the adverse effects of increased exposures to air pollutants such as PM_{10} and $PM_{2.5-10}$ on cognition seemed to be higher among women than men, particularly among those in the metropolitan areas, the adverse effect of NO_2 exposure on cognitive function appeared to be greater in women than men. Our study results were confirmed by the sensitivity analyses, which also suggest that women with higher levels of air pollution exposure than men were found to have lower MMSE scores. In spite of our findings, additional research is warranted to further explore gender differences in the effects of air pollution on cognition and the mechanism in consideration of a possibility of the potentially related factors and probable bias source. Furthermore, more critical, if the adverse effect of outdoor air pollution on cognitive function is collectively found to be stronger in women than men, developing and implementing prevention programs or interventions tailored on older women, particularly those with increased risk for air pollution exposure, should be necessarily considered.

DATA AVAILABILITY STATEMENT

The datasets for this manuscript are not publicly available because of the government policy that regulates the disclosure of the datasets concerning this study. Requests to access the datasets should be directed to CK (PREMAN@yuhs.ac).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board of the Yonsei University Health System with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Yonsei University Health System Clinical Trial Centre (IRB Approval No. 4-2014-0359).

AUTHOR CONTRIBUTIONS

HK planned the study, performed statistical analyses, and prepared the manuscript, including the revision. JN, YN, S-BK, and SO involved in the collection of the cohort data. CK supervised the study, critically reviewed the manuscript, and contributed to revising the paper.

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Air Quality Characterization at Three Industrial Areas in Southern Italy

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Outdoor air pollution is responsible for more than 4 million premature deaths worldwide and its contribution is particularly severe in industrial contaminated sites, where epidemiological studies highlight often mortality rate larger than the national average. In the framework of the CISAS project, this study investigates spatial and temporal variability of air pollution across three industrial contaminated sites in southern Italy classified as "High Risk Area of Environmental Crisis": Crotone, Milazzo, and Priolo. The environmental analysis employed three investigation approaches:—meteorological characterization of the sites with analyses of local air mass circulation;—statistical evaluation of the continuous measurements of gaseous pollutants and PM concentration;—determination of mass concentration and detailed speciation of the chemical components of atmospheric particulate matter during intensive field campaigns. Continuous trace gases and aerosol measurements (including NOx, SO_2, O_3, NMHC, PM_{10}, and $PM_{2.5}$) over the period 2016–2018 were analyzed, and specific intensive field experiments (2016–2017), representative of winter and summer conditions, were carried out in order to determine PM_{10} and $PM_{2.5}$ chemical composition. The analyses of PM components (ions, elemental composition, trace organic pollutants, organic carbon, and elemental carbon) show concentrations typical of rural and urban areas. The results concerning gaseous pollutants and PM concentration showed a general compliance of the concentrations of some regulated species with the limits set by the EU Ambient Air Quality (AAQ) Directive. However, in particular in the industrial areas of Milazzo and Priolo, the analysis here reported highlights the need for a stringent regulation on NMHC ambient concentration and composition, further investigation of fine particle composition and atmospheric processing, and a deeper understanding of the role of anthropogenic emissions on ozone formation, also considering the World Health Organization (WHO) limits.

Keywords: atmosphere, environment, industrial contaminated sites, air quality, pollution

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INTRODUCTION

Air pollutants are substances able to harm human health, vegetation, animals, and cultural heritage. The potential impact of atmospheric compounds on humans and the environment is based on toxicological, epidemiological, and exposure studies. Air pollutants include gas phase species, like nitrogen oxides and ozone, as well as chemical species that compose particulate matter in the atmosphere, including metals and black carbon. Moreover, some air pollutants, such as polycyclic aromatic hydrocarbons and dioxins partition between the gas and the condensed phase, depending on ambient temperature.

The toxicological mechanisms through which air pollutant harm human health, as well as the health outcomes can be very different. Air pollutants enter the human body though inhalation and digestion (Kampa and Castanas, 2008). Fine particles and gas can penetrate deep into the lungs and reach the alveoli. In addition, gas and particulate pollutants can deposit on the ground and contaminate water and food. Through the respiratory system and the digestion system, chemicals can induce local inflammatory and oxidative stress or impact other organs (Kelm, 1999; WHO, 2005; Pope and Dockery, 2006). Epidemiological studies highlight a causal link between short-term and long-term exposure to air pollutants and different health outcomes, including nausea, difficulties in breathing, asthma, allergy, pre-term birth, reduced-activity of the immune system, stroke, nervous system damages, pulmonary and cardiovascular morbidity, and mortality (Kampa and Castanas, 2008 and references therein).

Based on the evident link between air pollution exposure and health damages, air pollution is mainly investigated in urban areas, especially in developed countries, where the largest fraction of population lives (WHO Europe, 2006; WHO, 2018). More recently special attention has been dedicated to contaminated areas, where the highest concentrations of pollutants are expected. According to the World Health Organization, contaminated areas are those that host or have hosted industrial activities for production of chemicals and petrochemicals products, energy, cements, as well as plants for waste disposal or treatment (WHO, 2012). Political concerns arise from the fact that human health can be under threat in these areas, both due to residential and professional exposure. Linking health outcomes to specific pollutant or contamination pathways is particularly challenges since hazards are multiple, exposure data are sparse, and proximity of urban areas can increase the complexity of pollution sources (Martuzzi et al., 2014). In particular, characterization of atmospheric air pollutants in contaminated areas is generally limited, compared to soil and water (Zona et al., 2019).

In this study we investigate air quality at three industrial contaminated sites located in southern Italy and classified as "High Risk Area of Environmental Crisis" (AERCA—*Area ad Elevato Rischio di Crisi Ambientale*): Priolo, Milazzo, and Crotone. The health outcome in these three areas is investigated in details by the national programme of epidemiological surveillance in contaminated sites (Zona et al., 2019), highlighting a higher standardized mortality rate compared to the national average. In particular, in Priolo and Crotone, the largest difference from the national average mortality rate is due to specific causes like pleural mesothelioma and respiratory deceases, which can be directly linked to inhalation of air pollutants.

Time series analysis of air pollution metrics regulated by the national and European legislation (daily concentration of PM_{10}, 8-h running average of ozone and carbon monoxide, and hourly average of sulfur dioxide and nitrogen dioxide concentrations) classifies Priolo air quality as "low polluted" (Monforte and Ragusa, 2018). The lack of markers typical of industrial emissions in this kind of analysis, like non-methane hydrocarbons, might bias the results (Abita et al., 2019). Fazzo et al. (2016) investigated the incidence of cancer in Priolo and suggested a link between health effects and high concentration of contaminants in soil and water. Nevertheless, the lack of data on harmful pollutants in the atmosphere limited the interpretation of the results.

In this work, we analyse the concentrations of regulated and non-regulated atmospheric pollutants in the three AERCA sites with the goal to characterize the air quality and to support the analysis of population exposure to these specific air pollutants. To the authors' best knowledge this is the first study that integrates the analysis gas-phase concentration and detailed aerosol chemical composition in multiple contaminated sites in southern Italy. This work moves away from urban-focused research and aims at addressing the gap related to the limited knowledge on atmospheric pollutant in industrial areas in order to identifying priority and non-traditional pollutants (e.g., organic vapors) that should be monitored in contaminated areas, to better understand the link between air quality and human health in compliance with the WHO guidelines rather than current air quality guidelines.

The approach here described includes:—modeling and meteorological analyses;—statistical interpretation of the data obtained by the regional networks;—field determination of the concentration and chemical composition of particulate matter (PM). The non-hydrostatic meteorological model MOLOCH, developed by CNR-ISAC, was used to calculate high spatial resolution wind fields on annual basis for the region of interest. For the main gaseous pollutants and for PM (concentration only), we carried out a detailed evaluation and interpretation of the existing data from the regional networks. Such dataset was analyzed both to support the design of the experimental field campaigns at Priolo, Milazzo, and Crotone, and to better understand the atmospheric measurements carried out during this study. Field characterization of PM was focused on its chemical composition because the existing regional air monitoring networks record only gaseous pollutants, including NOx, SO_2, O_3, and NMHC and, in some cases PM mass concentration. The determination of a high number of species in the particulate phase, instead, requires a complex and articulated analytical procedure that, up to now, had never been implemented in the investigated areas.

This study is part of the national research project CISAS (International Centre of advanced study in environment, ecosystem and human health), which aims at understanding processes and mechanisms responsible for the transfer of

conventional and emerging contaminants from the environment to the ecosystem and to humans, in contaminated areas.

MATERIALS AND METHODS

Sites Description

The study was carried out at three polluted sites located in southern Italy and classified by the Italian Ministry of the Environment as Site of National Interest—High Risk Area of Environmental Crisis AERCA (Area ad Elevato Rischio di Crisi Ambientale). These sites, Crotone, Milazzo, and Priolo, shown in **Figure 1**, are defined as large and contaminated areas where environmental pollution may cause an important impact in terms of health and ecological risk, as well as a detriment to cultural and environmental heritage. These three AERCA sites are also considered in the "SENTIERI Project: Mortality study of residents in Italian polluted sites", aiming at evaluating the epidemiological evidence on the association between 63 different causes of death and environmental exposures (Zona et al., 2019).

The three areas have a millennial history, proved by many important archaeological evidences, and have seen an important industrial development during the last century.

Crotone is a coastal city of about 65,000 inhabitants, sited in the Calabria region. The industrial development took place between the two World Wars, with a chemical industry, a metallurgical plant producing zinc alloys and other minor plants. The two main industries were at their maximum activity during the sixties but suffered from a deep crisis during the eighties and were closed at the end of the century, leaving a number of unsolved remediation problems.

The AERCA of Comprensorio del Mela (in the following denoted Milazzo) and of Siracusa are located in Sicily. The Milazzo AERCA includes Milazzo and also Condrò, Gualtieri Sicaminò, Pace del Mela, S. Filippo del Mela, Santa Lucia del Mela, San Pier Niceto. Milazzo is a city of about 30,000 inhabitants sited on the north-eastern coast of Sicily. The industrial development sharply increased during the fifties and sixties with a steel industry, energy production plants and, most of all, the widest oil refinery in Europe, which is still in business.

The AERCA of Siracusa (in the following denoted Priolo), with the municipalities of Priolo Gargallo (12,000 inhabitants), Augusta (36,000 inhabitants), Melilli (13,000 inhabitants), Siracusa (122,000 inhabitants), Floridia (23,000 inhabitants), and Solarino (8,000 inhabitants), covers an area of 550 km^2. This AERCA includes the huge Siracusa petrochemical district (the widest in Europe, about 27 Km^2) that extends from the north of Siracusa to Augusta, where the main industrial activities include oil refinery, processing of oil-related products and energy production. Born in the fifties and in the two following decades, many of the industrial plants reduced their activity from the eighties, causing serious remediation problems.

Based on 2012 Sicily emission inventory, industrial activities account for 67% of NMHC and 96% of SO_2 emissions in the Sicilian industrial areas. NOx, which on regional average is mainly due to traffic emissions (55%), at the industrial areas combustion and non-combustion industrial emissions account for 57% of local NOx emissions and the contribution of traffic is less significant (24%) (Abita et al., 2016).

Continuous Atmospheric Observations

Selection of Measurement Sites

In this study, continuous atmospheric measurements in the period 2016–2018 have been considered, thanks to long term measurements of atmospheric gas phase species and particulate matter mass concentration recorded in Sicily and Calabria regions by Regional Environmental Protection Agencies, ARPA (Agenzia Regionale Protezione Ambiente). **Table 1** describes the monitoring sites, grouped by AERCA areas, considered during the present study for air quality continuous observations. Sites were selected based on data availability and their representativeness of different environments inside each AERCA. Santa Lucia del Mela (SLM), contrada Gabbia (GAB), and Termica (TER) are representative of the Milazzo AERCA; Melilli (MEL), Priolo (PRI), and Scala Greca (SCG) are representative of the AERCA of Priolo, while Rocca di Neto (RDN) and Giovanni da Fiore (GDF) are representative of the Crotone area. For Priolo and Milazzo AERCAs we identified a site that might be affected by processed/aged industrial emissions, and hereafter indicated as "downwind." In addition, we consider a station located nearby plants and representative of the industrial area, and a site representative of urban conditions. In the Crotone area we present data exclusively from an urban site (GDF) and a downwind location (RDN).

Instrumental Setup

Gas phase species here investigated include nitrogen oxides (NO_x), sulfur dioxide (SO_2), total non-methane hydrocarbons (NMHC), and ozone (O_3). These gas phase pollutants are monitored continuously by the monitoring network of the ARPA Sicily and Calabria. NO_x measurements are performed by a Teledyne API 200E NO_X Chemiluminescent analyzer, while SO_2 is monitored by a Teledyne API Model 100E analyzer, based on fluorescence signal induced by UV energy absorption. NMHC are quantified by gas chromatography with a NIRA analyzer model 301. O_3 is monitored based on UV light absorption signal with a Teledyne API ozone analyzer model 400E. Hourly average concentrations are stored after validation, based on the number of valid measurements within each hour (minimum data coverage is set at 75%) and on the difference between daily span measurements and calibration values. Every 6 months, certification tests are applied to verify the consistency with long-term data records and among sites. Daily $PM_{2.5}$ and PM_{10} mass concentration is measured through beta absorption with a FAI Instrument SWAM dual channel. Particulate matter samples are collected from midnight to midnight at a flow rate of 2.3 Nm^3/h.

In the Priolo and Crotone AERCAs the data coverage is larger than 93% at all sites and during all seasons. In the Milazzo AERCA, due to lack of measurements during the second part of 2018, concentration of gas phase species in summer and fall are under-represented; nevertheless, seasonal variability is well-captured during 2016 and 2017 for NO_x, O_3, and SO_2. NMHC data coverage at GAB ranges between 30 and 38% (April 2017–April 2018), while at TER it ranges between 58 and 60% (January

FIGURE 1 | Map of the investigated AERCA sites generated from google map (https://www.google.it/maps).

2016–December 2017). Since all seasons are represented by the two time-series, even if limited to 1 and 2 years, respectively, these data were considered in further analysis.

Intensive Field Experiments

Site Selection

Since the CISAS project was aimed at investigating the link between air quality and health of the people living in the study areas, it was decided to locate the PM sampling stations in central, residential areas characterized by a high number of exposed individuals, instead of considering the areas of maximum impact of the industrial emissions. The following three sampling sites were selected (see **Table 1**). Crotone: close to a traffic street, at about 2 Km from the industrial area; Milazzo: on the terrace of a school, at about 3 Km from the oil refinery; Priolo: close to the Municipality building, at about 1.5 Km from the petrochemical district.

Sampling Periods

In order to evaluate the average concentration affecting the citizens and, at the same time, keep the economic budget to a reasonable level, we designed a schedule characterized by long-duration samplings and by the determination of many chemical components, instead of carrying out daily samplings that would necessarily be few in number of samples or in number of chemical determinations. It was decided to monitor the air concentration of both PM_{10} and $PM_{2.5}$ and of their chemical components for a time period of 6 months over 1 year. Each sampling period lasted for 1 month and was simultaneously performed at the three AERCAs; three of the six samplings periods were during the winter (from December 15th, 2016 to March 14th, 2017) and three during the summer (from July, 5th to October 4th, 2017). With a relatively small number of PM characterizations and affordable costs it was thus possible to obtain a reliable evaluation of the concentration and chemical composition of particles of different size and during different and characteristic period of the year.

Experimental Design

The choice of two size fractions (PM_{10} and $PM_{2.5}$) allowed a distinction between anthropic and natural source contributions. Anthropic sources (combustion processes and industries) emit almost exclusively in the fine fraction of PM, the same fraction where the species produced by atmospheric reactions (secondary pollutants) are found. Natural sources (sea-salt, soil, bio-aerosol), instead, release particles mainly in the coarse fraction (difference between PM_{10} and $PM_{2.5}$).

Two groups of analyses were carried out on the sampled filters, for a total of more than 100 chemical determinations on each sample. The first group of analyses were carried out on both PM_{10} and $PM_{2.5}$, in duplicate (two side-by-side samplings, analyzed individually), for a total of 72 samples (three sites, 6 months, two size fractions, two replicates). They include: elements, inorganic ions, elemental carbon, and organic carbon. The analysis in the second group were carried out on PM_{10} only, on two 15-day samplings for each month, for a total of 36 samples (three sites, 6 months, two samples per month). They include micro-organics: polycyclic aromatic hydrocarbons (PAH),

TABLE 1 | List of monitoring sites included in the present study.

AERCA	Acronym	Activity	Coordinates (Lat N, Lon E)	Altitude (m asl)	Type of station
MILAZZO AERCA					
Santa Lucia del Mela	SLM	Air quality continuous observations	38.1605 15.275	210	Downwind
Pace del Mela - Gabbia	GAB	Air quality continuous observations	38.2055 15.3101	13	Industrial
Termica	TER	Air quality continuous observations	38.1906 15.2592	20	Urban
Milazzo	MIL	Intensive field experiments	38.2219 15.2375	1	Urban
PRIOLO AERCA					
Melilli	MEL	Air quality continuous observations	37.18223 15.12882	245	Downwind
Priolo	PRI	Air quality continuous observations	37.15623 15.19099	18	Industrial
Siracusa–Scala Greca	SCG	Air quality continuous observations	37.1024 15.26566	52	Urban
Priolo	PRU	Intensive field experiments	37.1596 15.1864	30	Urban
CROTONE AERCA					
Rocca di Neto	RDN	Air quality continuous observations	39.18752 17.01034	71	Downwind
via G. da Fiore	GDF	Air quality continuous observations	39.06808 17.11417	37	Urban
Crotone	CRT	Intensive field experiment	39.0794 17.1231	8	Urban

The sites are grouped by AERCA—Area ad Elevato Rischio di Crisi Ambientale (High Risk Area of Environmental Crisis).

polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyl (dl-PCB), polybrominated diphenyl ethers (PBDEs).

The choice of the analyses to be carried out followed two main criteria: species that are toxic/carcinogen (micro-organics and some elements) and species that are major components of PM. PM major components (typically constituting more than 1% of the PM mass) include some elements (Al, Si, Fe, Na, K, Mg, Ca), inorganic ions (Cl^-, NO_3^-, $SO_4^=$, Na^+, NH_4^+, K^+, Mg^{++}, Ca^{++}), elemental carbon (EC) and organic carbon (OC, including the whole of organic species). When all these components are detected it is possible to obtain the mass closure, that is the coincidence between the measured mass and the sum of the measured PM components (reconstructed mass). Obtaining the mass closure proves the good quality of the analytical phase. Moreover, the individual chemical determinations can be grouped in order to obtain a picture of PM macro-sources, that is, the main, ubiquitous sources of the atmospheric aerosol (soil, sea, atmospheric reactions, combustion, biomass/biosphere) (Perrino et al., 2014).

PM Sampling

At each site the samplings were carried out simultaneously, by using three PM samplers: two for ions, elements and EC/OC in duplicate, one for micro-organics. The instruments are shown in **Supplementary Material**.

For PM mass, ions, elements and EC/OC we used instruments operating at the flow rate of 10 L min^{-1} (Silent Sampler, FAI Instruments) equipped with 4-position sequential units. The sequential units were arranged as follows: (1) PM_{10} impactor and Teflon filter; (2) PM_{10} impactor and quartz filter; (3) $PM_{2.5}$ impactor and Teflon filter; (4) $PM_{2.5}$ impactor and quartz filter. Teflon filters (filters with polyethylene ring, 47 mm in diameter, 2.0 μm pore size, Whatman) were used to determine ions and elements; quartz filters (47 mm in diameter, QM-A Whatman) were used to determine elemental and organic carbon. These samplings were carried out in duplicate to compensate for possible malfunctions and to increase the accuracy of the analytical determinations. In order to carry out 1-month samplings without overloading the filters but keeping time representativeness, every 2 h we activated the sampling for 30 min and stopped it for 90 min (duty cycle 1:4).

Given the very low air concentration of micro-organics, we sampled PM_{10} by using high volume samplers operated at the flow rate of 100 L min^{-1} (Echo High Volume Sampler, TCR Tecora). We used quartz filters 102 mm in diameter and downstream polyurethane foam cartridges, aimed to recover semi-volatile species. Before each sampling,

the filters were spiked with a known amount of 13C-labeled standard (EN 1948SS for PCDD/F, P48-SS for dl-PCB, Wellington Laboratories), in order to evaluate the collection efficiency.

Analysis of PM and PM Components

PM mass was determined on Teflon filters by gravimetric analysis, using an automated microbalance (1 μg sensitivity, mod. ME5, Sartorius AG), after conditioning at 50% R.H. and 20°C for 48 h.

The collected filters were then analyzed by for their elemental content (Al, Ca, Cl, Cr, Fe, K, Mg, Mn, Na, Ni, Pb, Si, S, Ti, V) by energy dispersive X-ray fluorescence (ED-XRF; Xepos, Spectro Analytical Instruments); then the polyethylene ring was removed and the filters were US extracted in deionized water (20 min).

A first aliquot of the solution was analyzed for its ionic content (Cl^-, NO_3^-, $SO_4^=$, Na^+, NH_4^+, K^+, Mg^{++}, Ca^{++}) by ion chromatography (IC; ICS1000, Dionex).

The residual amount was added with acetate buffer (0.01 M; pH 4.5), filtered by using nitrate cellulose filters (0.45 μm pore size, Millipore) and analyzed for the soluble fraction of micro- and trace- elements (As, Be, Cd, Ce, Co, Cu, Cs, Fe, La, Li, Mn, Mo, Ni, Pb, Rb, Sb, Se, Sn, Sr, Tl, U, V, Zr) by inductively coupled plasma mass spectroscopy (ICP-MS; Brucker 820-MS). The solid left on both the Teflon filter and the NC filtration filter was then transferred to a PTFE vessel and HNO_3/H_2O_2 2:1 solution was added to mineralize the residual solid matter. After digestion in microwave oven (Ethos 1 Touch Control, Milestone), the solution was filtered again at 0.45 μm and analyzed by ICP-MS for the residual fraction of the same elements. The chemical fractionation of the total elemental content into a water-soluble and a residual fraction adds useful information for the identification of PM sources and enhances the estimation of the elemental bio-accessibility (Canepari et al., 2009a, 2014).

Forty seven millimeter quartz filters were analyzed for their organic carbon (OC) and elemental carbon (EC) content by thermo-optical analysis (OCEC Carbon Aerosol Analyzer, Sunset Laboratory, OR, USA), applying the NIOSH-quartz temperature protocol.

Further information about the whole analytical procedure, previously validated, are available in Canepari et al. (2009b), Perrino et al. (2011, 2014). A scheme of the method is reported in **Figure 2**.

For the determination of micro-organics, 102-mm quartz filters and PUF cartridges were Soxhlet extracted in toluene for 36 h. PCDD/F, dl-PCB and PBDE 13C- labeled standards were added before the extraction to evaluate the efficiency of the extraction, purification, and enrichment phases. The extract was concentrated to about 10 ml; a 1/10 aliquot was used for the analysis of Polycyclic Aromatic Hydrocarbons (PAH), the residual solution was used for the determination of PCDD/PCDF, dl-PCB, and PBDE.

For the analysis of PAH the extracts were spiked with perdeuterated PAHs used as internal standard (L429-IS, Wellington Laboratories), then they were concentrated under a gentle stream of nitrogen and purified by using a silica/alumina micro-column. The aliphatic fraction was eliminated through elution with hexane; PAHs were eluted by hexane:dichloromethane (1:1 v/v) and analyzed by GC-MS, operating in single ion monitoring mode (Ultra Trace gas chromatograph coupled to TSQ mass spectrometer, Thermo Scientific). Recovery standards (L429-RS, Wellington Laboratories) were added before injection.

The aliquot for the analysis of PCDD/F, PCBs and PBDE was transferred quantitatively on multi-layer chromatographic column (packed manually with anhydrous Na_2SO_4, silica, acidic silica, and silica containing $AgNO_3$); the sample was eluted with 150 ml hexane, then with 100 ml of a mixture hexane: methylene chloride (1:1) and concentrated. The eluate, brought to a volume of a few μL, was analyzed in GC/MS (MS/MS mode) after addition of PBDE internal injection standards. Finally, the sample was transferred on glass micro-column containing alumina. The fraction containing the PCBs was collected by eluting with a mixture of hexane with 4% methylene chloride; the fraction containing PCDD and PCDF was subsequently collected by eluting with a mixture hexane: methylene chloride (1:1). Both fractions were concentrated, made up with mixtures of internal standards for recovery calculation and analyzed by GC/MS (MS/MS mode).

The list of the individual PAH, PCDD, PCDF, dl-PCB, and PBDE determined in this study is reported in **Supplementary Material**.

RESULTS

Meteorological Characterization and Air Masses Circulation

Seasonal Regimes

Meteorological measurements performed at the three AERCA sites were analyzed for the 3-year period 2016–2018. Such analyses are based on data made available by the Regional Environmental Protection Agencies of Sicily and Calabria. The measurement sites are located on coastal areas, close to the see, as shown in **Figure 3**, where seasonal wind roses for the above-mentioned sampling sites are reported.

Wind roses point out that the dominant regimes, in terms of occurrences of wind direction and related wind magnitude, are strongly associated with sea and land breezes for the three sampling sites. Typically, for the coastal sites, the sea-breeze frequency is more pronounced in summer during the daytime, and land-breeze in winter during nighttime. The polar plots (not shown), elaborated for the measurements collected at Priolo, Milazzo and Crotone, confirm such features.

Local Circulation Description

In the absence of synoptic flow, during the day the sea breeze front progresses toward inland normal to the coastline, at speeds of 1–5 m/s and can reach 20–50 km inland by the end of the day. On contrary, at night-early morning, cold air from land flows out to sea in analogous manner to the sea breeze, although there have been insufficient observations of its propagation across the water to be conclusive (Stull, 2012). For instance, for Priolo sampling site, the literature (i.e., Brusasca, 2004) reports that the frequency of breeze regime is of about 60% on annual basis.

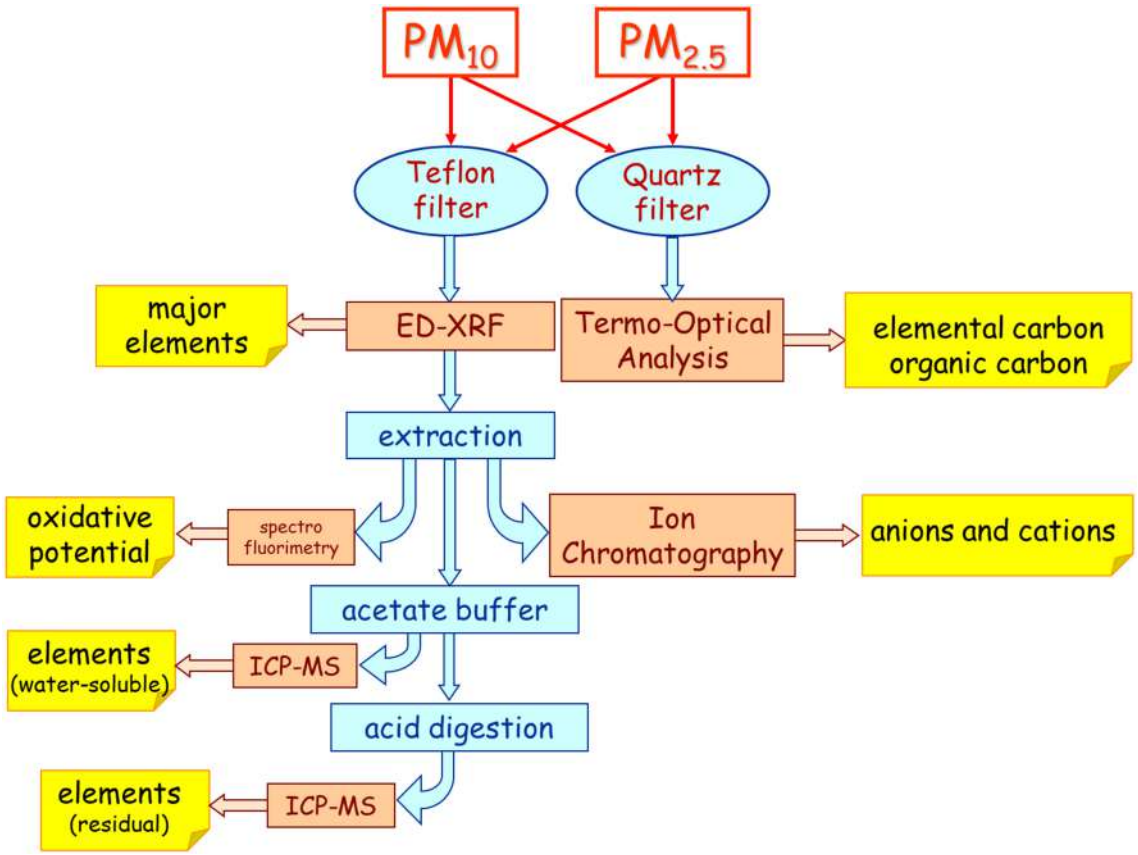

FIGURE 2 | Schematic diagram of the analytical procedure for determining elements, ions, elemental carbon, organic carbon, oxidative potential.

Based on the analysis of local wind data, the plots of day hours as function of wind speed and direction (**Figure 4**), allows to determine the diurnal and nocturnal wind patterns associated to sea/land breeze circulation system. Synoptic circulation associated with stronger winds (e.g., wind speed larger than 6 m/s) are sporadic (frequency <5% of hours per year). As expected, sea breeze is dominant during the warmer part of the day and it results in stronger winds during the spring and summer. At Priolo we observe a persistent sea breeze from North-East (NE) during the central hours of the day (11–16 UTC). Such feature is present at the sampling site in Milazzo, from North-North-East (NNE), and in Crotone from East-South-East (ESE), as reported in the **Figure 4** where the frequency of hourly wind direction is reported for the three sampling sites. In Crotone, however, it is not so pronounced as in the other two locations. On the other hand, land breeze is stronger and more frequent than sea breeze during the colder seasons at all the sampling sites: at Priolo from West-South-West (WSW), at Milazzo from South-South-East (SSE), and at Crotone from West (SW), as reported in the **Figure 4**. It is important to keep in mind that, in the complex orography conditions, in many real situations, ambient synoptic or mesoscale winds can modify, or even eliminate the weak geographical circulation. For instance, if the background synoptic flow is in the same direction of sea breeze, its front speed is well-approximated by a linear sum of the imposed wind component perpendicular to the front, and the speed of the front in still air.

Thus, the characterization of the typical local circulation is actually important for pollutants dispersion and dilution within the planetary boundary layer. Basically, the industrial areas and the major traffic sources span along the coastal line for the three sites. During the warmer seasons the high densely populated areas are located down-wind from the industrial and traffic areas, which results in pollutants transport originating mainly from industrial activities toward the urbanized parts. Such situation is associated with the people exposure to the poor air quality conditions for Priolo, Milazzo, and Crotone as well.

Continuous Atmospheric Observations

In order to characterize the seasonal variation of regulated and non-regulated trace gases NO_x, SO_2, NMHC, and O_3 and atmospheric aerosol PM2.5 and PM10 for every AERCA, we consider three different areas classified as Industrial, Urban, and Downwind, as reported in **Table 1**.

For the considered pollutants, means of daily average concentrations are reported in **Table 2**, where for O_3 the daily maximum of 8-h running average is used instead of daily average.

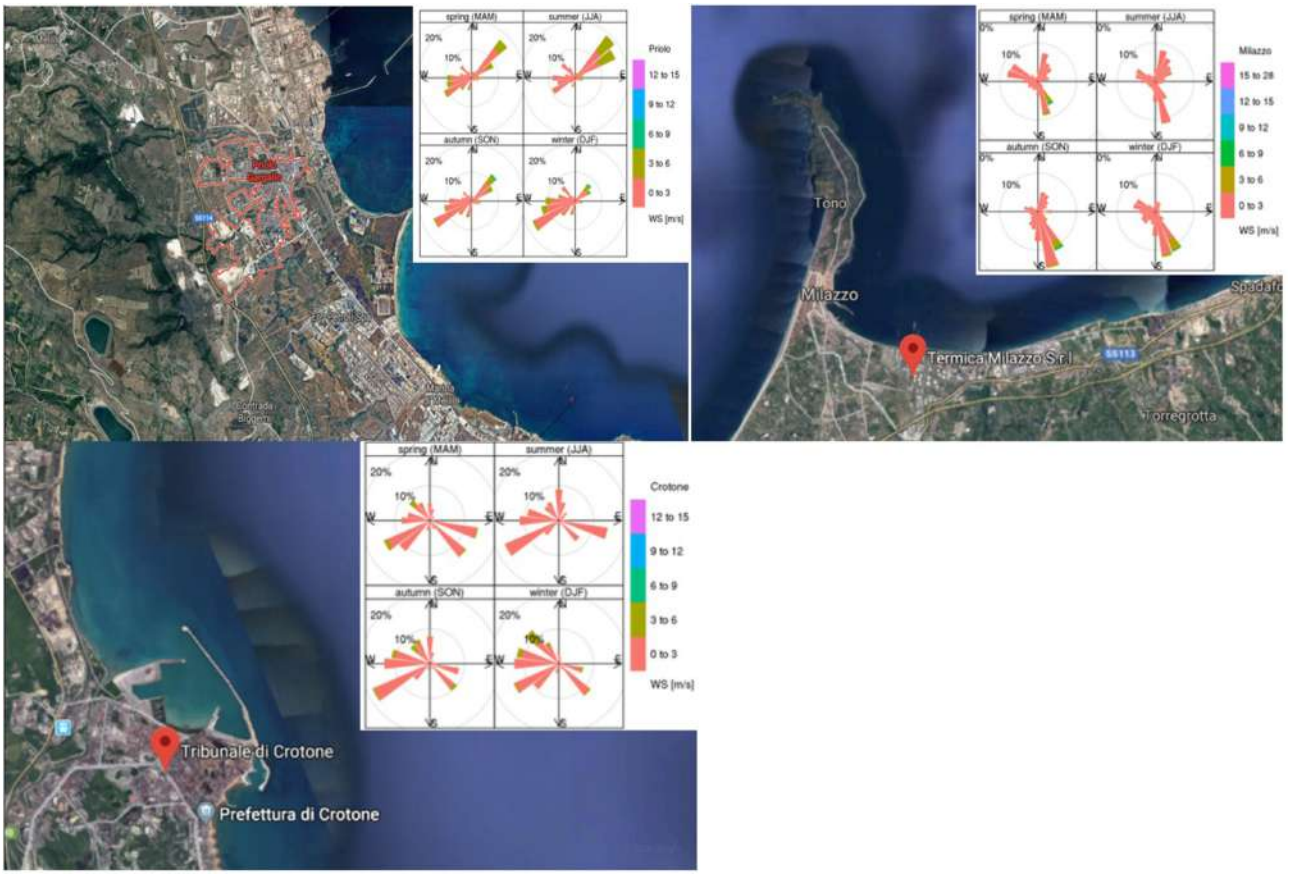

FIGURE 3 | Wind roses of observed seasonal wind speed and direction are shown for Priolo (upper panel), Milazzo (middle panel), and Crotone (lower panel). The integration period ranges between January 2016 and December 2018. The colorbar indicates the wind speed [m/s], keeping in mind that 5 m/s is the max wind intensity for wind breeze regimes. The concentric circumferences represent the occurrence (%) of wind direction calculated over all period (maps were generated by superimposing the wind roses and the google map - https://www.google.it/maps - screenshots of the regions of interest).

The seasonal variability of each trace-gas pollutant at the investigated sites is shown in **Figure 5** while the box plots of $PM_{2.5}$, PM_{10}, and $PM_{2.5}/PM_{10}$ daily means are shown in **Figure 7.**

Gas-Phase Pollutants

NO_x is emitted during combustion processes at high temperature, and main emission sectors are, based on regional inventory data (2012) of Sicily, road transport (55%) and energy production (15%) (Regione Siciliana, 2018). In addition, low temperature combustion, such as open-fire biomass burning and domestic heating, could represent an additional source of NOx not included in conventional emission inventories (Schultz et al., 2015). In each AERCA, NO_x concentrations are higher at the urban sites, followed by the industrial areas. Comparing NO_X concentrations at the urban sites, they are lower in the Milazzo AERCA due to the small size of the urban agglomerate where the monitoring station is placed. Seasonal behavior shows higher values during the cold season and minimum during the warmer period, due to the lower atmospheric lifetime (Gilge et al., 2010; Schultz et al., 2015). This result agrees with observations reported by Cristofanelli et al. (2017), who showed similar seasonal cycle for other location in southern Italy.

According to the emission inventory of the Sicily region, the main SO_2 anthropogenic sources are energy production (65%) and industrial processes (26%); however, the central Mediterranean represents a European hotspot for the impact of ship emissions on air chemistry (Becagli et al., 2012) with a special emphasis on SO_2. In the Milazzo and Priolo AERCAs, SO_2 concentrations at the industrial and downwind areas are comparable, indicating well-mixed anthropogenic emission (industrial activities and ship emissions) from emitting sources to surrounding regions.

The term non-methane hydrocarbons (NMHC) refers to low molecular weight hydrocarbon composed by 2–12 carbon atoms, including linear and branched alkanes, alkenes, alcohols, ketones, and aromatic compounds (Bustaffa et al., 2016).

The sources of atmospheric NMHC include anthropogenic and natural emitters so that that different sources are characterized by different chemical fingerprints. Main anthropogenic sources are thermal plants, traffic, and industrial processes, while natural sources are vegetation, marine, and soil

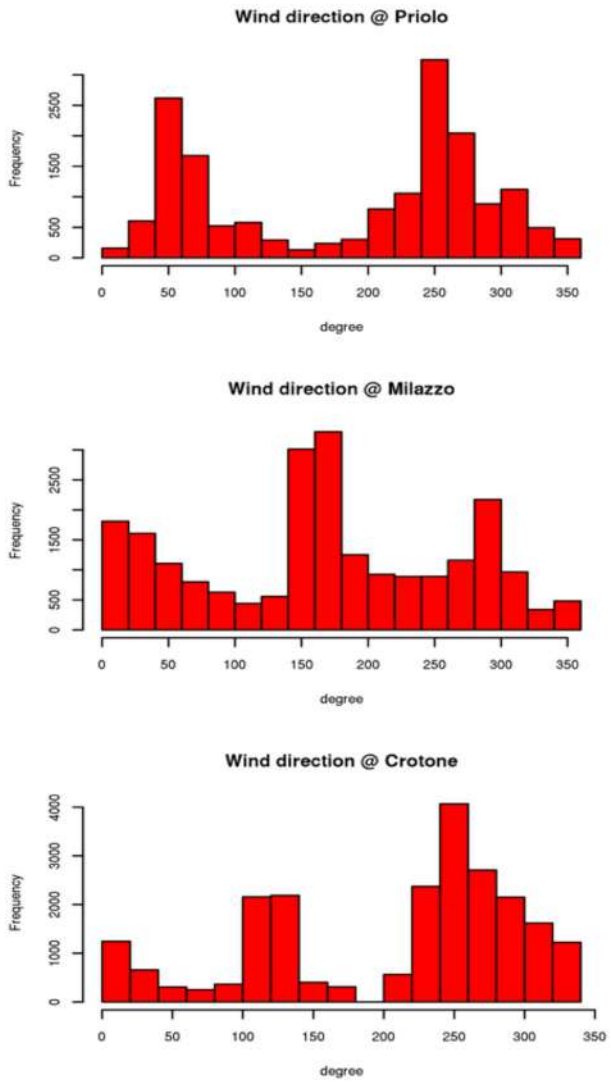

FIGURE 4 | Histograms of frequency of hourly wind direction at the sampling sites as described in **Figure 3**.

TABLE 2 | Averaged concentration of investigated gas-phase species over the entire study period in $\mu g/m^3$ (standard deviation is indicated in brackets) (January 2016–December 2018).

AERCA		NO_x	SO_2	NMHC	O_3	$PM_{2.5}$	PM_{10}
Milazzo	Downwind	5.7 (4.5)	5.1 (8.7)	30.1 (17.8)	–	–	–
	Industrial	11.4 (5.9)	2.6 (5.3)	218 (246)	–	–	–
	Urban	13.5 (6.1)	–	67.9 (50.7)	82.4 (14.8)	–	20.4 (12.7)
Priolo	Downwind	7.8 (4.1)	3.4 (3.7)	18.9 (35.6)	98.2 (21.4)	9.4 (4.6)	18.9 (35.3)
	Industrial	14.9 (8.5)	2.4 (3.8)	95.6 (49.8)	93.8 (20.6)	11.6 (5.0)	22.8 (17.1)
	Urban	54.0 (32.5)	0.8 (1.0)	61.8 (36.7)	70.3 (18.5)	10.8 (5.0)	25.7 (18.9)
Crotone	Downwind	27.0 (13.8)	2.6 (3.1)	–	86.8 (25.2)	14.7 (9.2)	21.4 (12.3)
	Urban	39.5 (28.3)	1.8 (0.7)	–	82.2 (19.0)	15.5 (8.4)	25.7 (14.8)

emissions (Seinfeld and Pandis, 1998). Nevertheless, NMHC data collected in the AERCA areas do not give information about their chemical speciation, and the term NMHC here refers to the sum of the different chemical species. At the Milazzo and Priolo AERCAs, where NMHC are recorded, a distinctive spatial pattern can be observed, with the highest concentrations in the industrial areas, and one to two orders of magnitude lower levels at the downwind sites. The spatial pattern suggests that anthropogenic emissions are dominating over natural ones in these regions. NMHC ambient concentration was regulated by the DPCM 28/03/1983, setting at 200 $\mu g/m^3$ the limit of hourly measurements. After the abrogation of such legislation, there is no limit to NMHC ambient levels. The reference value of 200 $\mu g/m^3$ of hourly measurements (DPCM 28/03/1983), is exceeded for a significant fraction of time at the two industrial sites: 31% of the time at Milazzo AERCA and 14% of the time at Priolo. The NMHC at the industrial sites show often peak events that last from a few hours up to a day, with hourly maximum concentration higher than 1,000 $\mu g/m^3$. The NMHC seasonal behavior, similar at the two AERCAs, shows higher concentration in summer, in agreement with a likely contribution of industrial fugitive emissions, favored by higher summer temperatures.

Ozone is a secondary pollutant formed in the troposphere by NOx, CO, and NMHC, which react in the presence of sunlight (Lelieveld and Dentener, 2000). Long-term objective for O_3 sets a maximum daily 8-h mean concentration at 120 $\mu g/m^3$ (Ambient Air Quality AAQ EU Directive[1]) and 100 $\mu g/m^3$ (WHO). At the Priolo and Crotone AERCAs, where measurements are available at multiple sites, O_3 concentrations are the highest at the downwind sites and the lowest at the urban sites, showing a spatial pattern opposite to that of NOx. O_3 pattern can be attributed to ozone titration by nitrogen oxides and photochemical production in the polluted air masses. The mean O_3 level for downwind and industrial areas ranges between 88 and 98 $\mu g/m^3$, confirming the high concentrations measured in these coastal areas of Sicily, where the seasonal trend shows maxima in summer and minima in winter and fall in agreement with atmospheric photochemical production rate (**Figure 5**). In the AERCAs, the AAQ and WHO limits are exceeded most of the time in spring and summer, both at the downwind and nearby the industrial areas. In fact, over the investigated period, O_3 8-h mean was higher than 100 $\mu g/m^3$ (120 $\mu g/m^3$) 42% (14%) of the days at the downwind and 39% (9%) at the industrial sites of Priolo, while at urban area of Milazzo AERCA, the limit was exceeded 11% (1%) of the time. In the Crotone AERCA, O_3 exceeded the limits 32% (10%) of the days at downwind and 19% (2%) at urban areas.

In view of this result, the contribution of anthropogenic activities on O_3 levels in the Priolo AERCA during the four

[1,2]Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on Ambient Air Quality and Cleaner Air for Europe, OJ L 152, 11.6.2008

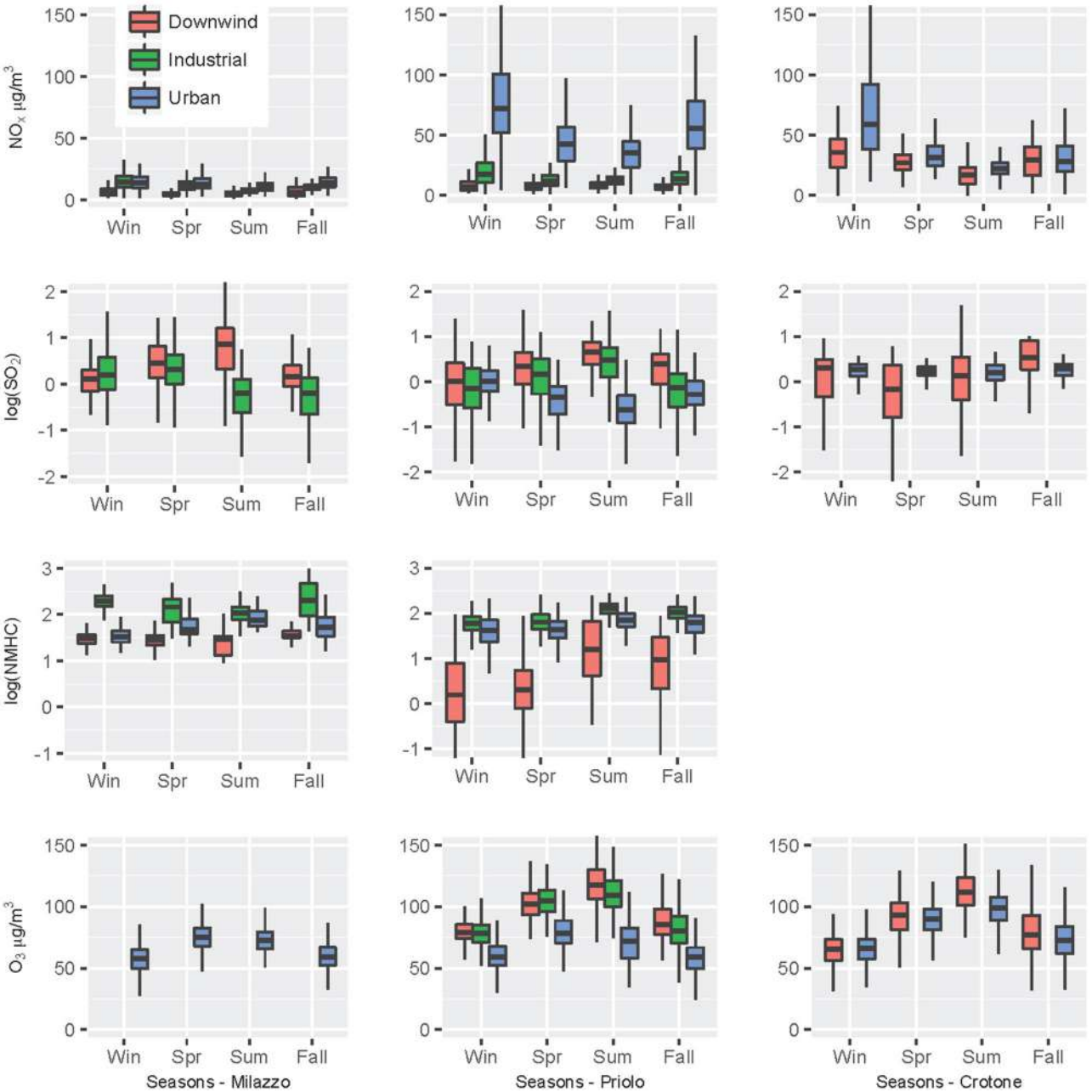

FIGURE 5 | Box plot of NOx, SO_2, and NMHC daily means and O_3 8-h means grouped by AERCA and season. Each colored column corresponds to a different AERCA site, according to **Table 1**.

seasons was investigated, considering the diurnal behavior of O_3 concentrations at industrial and downwind areas under sea breeze conditions, i.e., when the wind direction measured at downwind site ranged between 45 and 135 degree and wind speed was below 5 m s^{-1} (**Figure 6**). These conditions allow the selection of time-periods when downwind area is reached by air masses from the sea that sweep the industrial areas along the coast. Interestingly, the O_3 diurnal variations registered at industrial and downwind areas overlap most of the time with a lower O_3 concentration at the polluted industrial site in the morning, concomitant with the rush hour increase in NOx concentration, as reaction of NO leads to O_3 titration. Typical O_3 diurnal variation shows a broad maximum in the early afternoon, due to the combined effect of local photochemical production, enhanced vertical air mass mixing with boundary layer and entrainment zone, and transport of air masses from off-shore. During winter, spring, and fall the maximum is less pronounced than in summer.

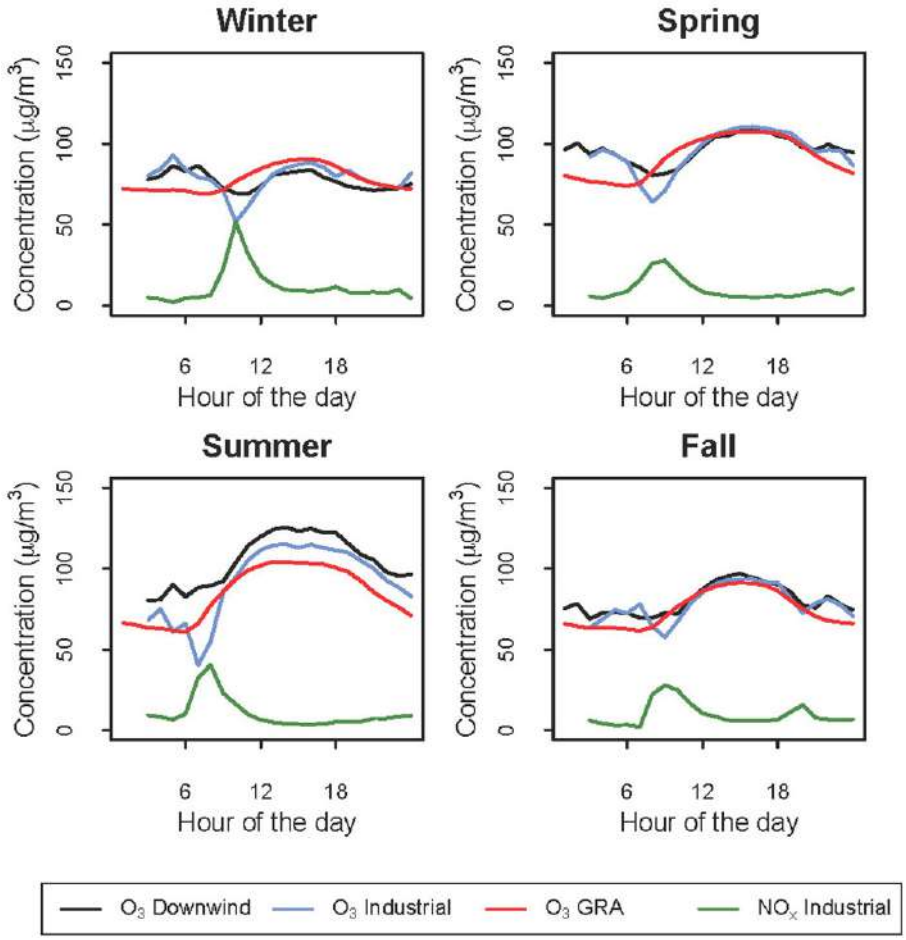

FIGURE 6 | Ozone seasonal diurnal variation under sea breeze conditions at the Priolo AERCA sites: industrial (blue lines), downwind (black lines) and at the regional background coastal site of Capo Ganitola (red lines), together with NO_X diurnal trend in industrial area (green lines).

In order to better understand the high annual O_3 mean as well as the high O_3 diurnal concentrations registered at this AERCA site, the O_3 measured at Capo Granitola (GRA), a WMO-GAW regional station representative of a background coastal site in Sicily (Cristofanelli et al., 2017), is considered and reported. The average O_3 concentration (calculated from daily maximum of 8-h running averages) was 82.2 $\mu g/m^3$. The comparison among downwind and industrial AERCA Priolo sites, and GRA suggest that the high background O_3 concentration, which typically affect the Mediterranean basin, contributes to the high O_3 levels observed in southern Italy, also during night-time, for great part of the year (Lefohn et al., 2018). However, the comparison points out higher O_3 night-time at the AERCA sites, probably indicating the impact of recirculation of O_3 photochemically produced during day-time. The diurnal variations in **Figure 6** show a clear increase at the industrial site and, although less pronounced, at downwind in the afternoon hours during the summer season, when O_3 concentrations are higher than night-time levels. In particular, the higher downwind O_3 values in respect to industrial areas, confirm the contribution of anthropogenic activities to O_3 formation during the warmer season in the industrial/urban where also the highest concentration of NO_x and NMHC are observed: under sea breeze regime, the air masses are enriched with O_3 in presence of high solar radiation and transported toward downwind areas, where O_3 concentrations remain higher due to the absence of compounds capable of destroying it.

Particulate Matter (PM)

The seasonal trend of PM mass concentration at the three AERCAs is shown in **Figure 7**. Milazzo AERCA is characterized by only PM_{10} measurements at the urban site, where large day-to-day variability is observed, although daily concentrations never exceed the WHO limit of 50 $\mu g/m^3$. At the Priolo AERCA, PM_{10} is characterized by a clear spatial pattern with increasing concentrations moving from downwind to industrial and then to urban area. The urban area is also the site characterized by the lowest $PM_{2.5}$ to PM_{10} ratio, often lower than 0.5, indicating a significant contribution of coarse particles, likely due to traffic re-suspension, in agreement with the observed high NO_x values.

Intensive Field Experiments

PM_{10} and $PM_{2.5}$ concentrations during the 6-month intensive study period is reported in **Figure 8**. Monthly mean

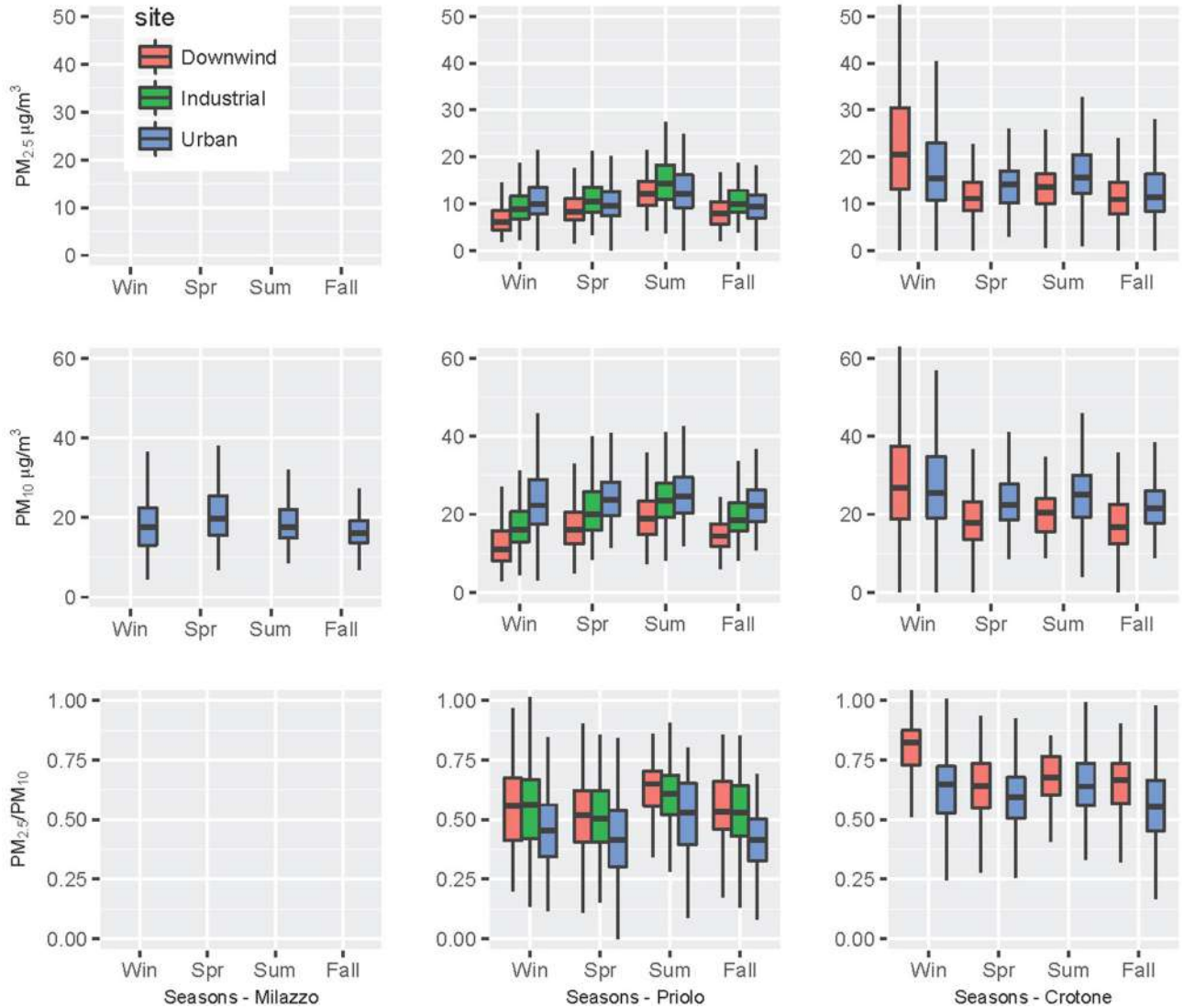

FIGURE 7 | Box plot of $PM_{2.5}$, PM_{10}, and $PM_{2.5}$ to PM_{10} ratio daily means grouped by AERCA and season. Each column corresponds to a different AERCA site, according to **Table 1**.

concentration of PM_{10} at the three sites ranged between 15 and 35 $\mu g/m^3$ and never exceeded the 1-year concentration limit set by the European Union (40 $\mu g/m^3$; EU Directive 2008/50/EC[2]). $PM_{2.5}$ concentration ranged between 8 and 19 $\mu g/m^3$, also in this case below the target concentration value of 25 $\mu g/m^3$. The more restrictive concentration values set by the WHO, 20 $\mu g/m^3$ for PM_{10} and 10 $\mu g/m^3$ for $PM_{2.5}$, were instead exceeded in all three sites: the mean 6-month PM_{10} concentration was 23.0 $\mu g/m^3$ in Crotone, 20.3 $\mu g/m^3$ in Milazzo, and 22.9 $\mu g/m^3$ in Priolo (mean value of the two duplicate measurements during the six periods), while the concentration recorded for $PM_{2.5}$ was 13.8 $\mu g/m^3$ in Crotone, 10.6 $\mu g/m^3$ in Milazzo and 11.3 $\mu g/m^3$ in Priolo.

In order to check the robustness of our sampling schedule, we compared PM_{10} concentrations measured in this study with the values recorded daily by ARPA Sicilia, at the monitoring station of TER, immediately adjacent to the oil refinery, at 3.7 Km from the measurement site of this study. Daily PM_{10} concentration measured at TER during the six considered periods and the comparison between the calculated ARPA mean values and the mean concentration obtained in this study are shown in **Figure 9**. It is worth noting that daily PM_{10} values showed a limited range of variability: relative standard deviation was in the range 15–45%, with the only exceptions of a desert dust event of February 24–27 and of a local event of August 6th, which was responsible for a sharp increase of PM_{10} concentration up to more than 90 $\mu g/m^3$. The differences in the mean values obtained by ARPA daily measurements and by the cumulate samplings of this study were very small. In the worst case (period 14/12–16/01) the difference was 22%, and it was probably due to the very low 24-h

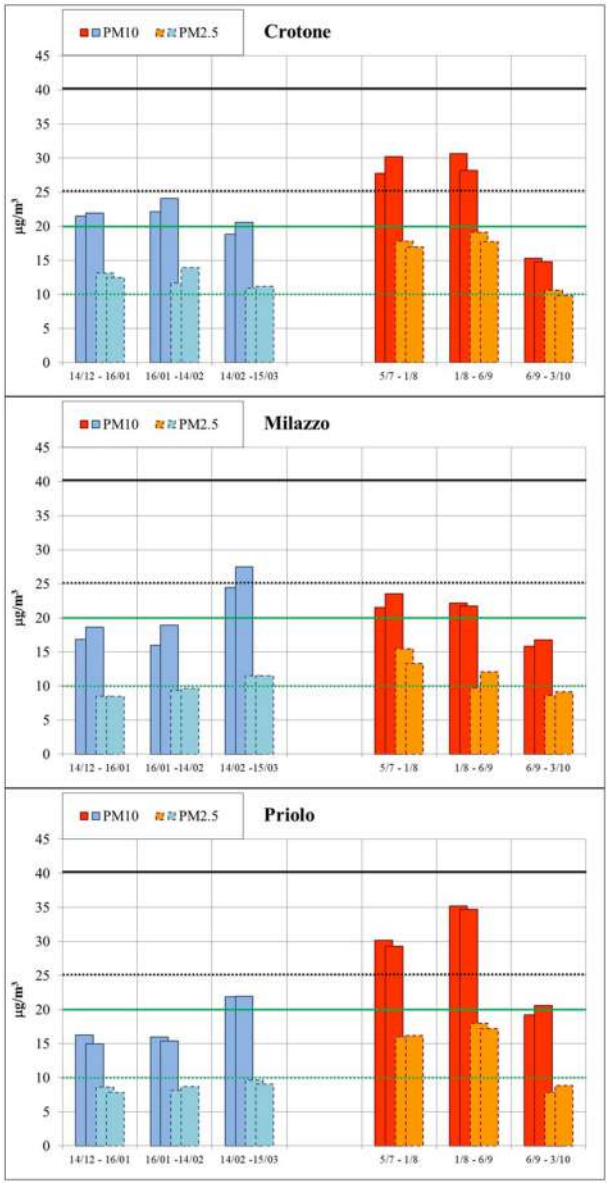

FIGURE 8 | Mass concentration of PM_{10} and $PM_{2.5}$ during the winter (blue bars) and the summer (orange bars) periods at the three AERCAs. EU concentration limits are shown in black, WHO concentration limits are shown in green (PM_{10}: solid lines; $PM_{2.5}$: dashed lines).

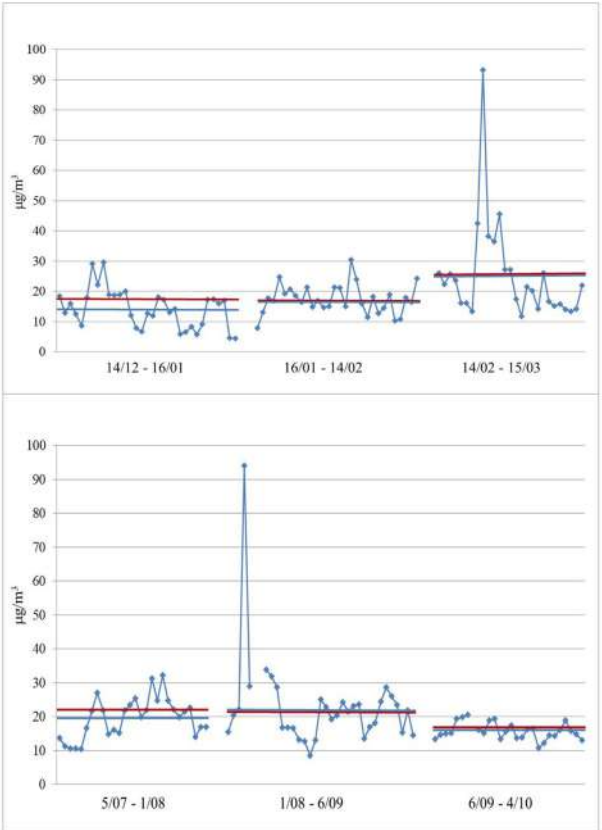

FIGURE 9 | Time pattern of PM_{10} concentration measured daily by the local environmental agency at the monitoring station of Termica Milazzo (TER), in winter (upper panel) and summer (lower panel); mean values referring to the six sampling periods (blue line) and monthly mean concentration measured by this study (red line).

concentration (below 10 µg/m^3) recorded during 16 of the 33 days characterizing this sampling period.

Figure 10 shows an overall chemical composition of PM_{10} and $PM_{2.5}$ at the three considered AERCAs. Considered components were: elemental carbon, organic matter (OC multiplied by 1.8 to take into account non-C atoms), ammonium, nitrate, non-sea-salt sulfate, sea-salt (calculated from Na^+ and Cl^- concentration multiplied by 1.176, in order to take into account minor sea-water components) and crustal matter (calculated by summing the concentration of Al, Si, and Fe, as metal oxides, the insoluble fractions of K, Mg, and Ca and calcium and magnesium carbonate) (Perrino et al., 2014).

As expected, sea-salt and crustal matter, natural components of primary origin, were mostly found in the coarse fraction of PM. The concentration of sea-salt in PM_{10} was higher in winter (2.5–4 µg/m^3), characterized by more frequent strong advection episodes, than during the summer (0.9–2.5 µg/m^3). This contribution to PM_{10} was particularly important in Milazzo, located in a very narrow peninsula with the sea at the distance of <500 m on both sides (E and W). The concentration of crustal components, instead, was higher during the summer than during the winter period (4.4–7.5 vs. 4.1–4.9 µg/m^3), due to the easier re-suspension of soil dust promoted by dry weather conditions. Being re-suspension mostly due to traffic, this concentration increase was not observed in Milazzo, where the sampling site was located on a terrace at about 10 m from the ground.

Elemental carbon, released by combustion processes, was found in the fine fraction of PM and was in the range 0.6–1.1 µg/m^3. Organic matter, mostly produced by photochemical reactions of anthropogenic and biogenic NMHC, also showed

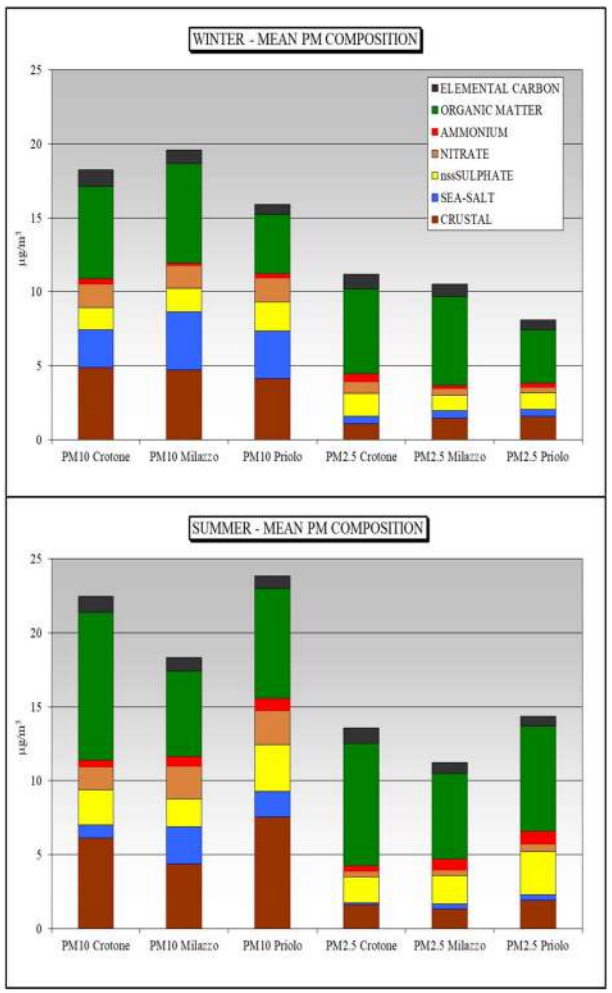

FIGURE 10 | Chemical composition of PM_{10} and $PM_{2.5}$ at the three considered AERCAs.

a size distribution predominantly in the fine range and higher concentration were recorded during summer (5.8–10 vs. 4–6.7 $\mu g/m^3$ in PM_{10}).

Ammonium sulfate, of photochemical origin, shared the same seasonal pattern as OM, with higher summer values (2.1–3.4 vs. 1.7–2.1 $\mu g/m^3$). Most of the nitrate amount, instead, was found in the coarse fraction (78–82% during the summer, 49–77% during the winter). This indicates that ammonium is fully neutralized by sulfate and most nitrate was associated with dust and sea salt in coarser particles.

Table 3 reports the average concentration of micro-organics during the winter and the summer study periods. Total concentration of the 14 considered PAH congeners was in the range 5.4–20 ng/m^3 during the winter and 2.9–7.0 ng/m^3 during the summer, a seasonal difference that could be ascribed to the contribution of heating appliances.

The concentration of total PCDD/F (sum of 17 congeners) and total dioxin-like-PCB (sum of 12 congeners) are reported in **Table 3** in terms of toxic equivalency (TEQ). TEQ expresses the toxicity of a mixture of dioxins and dioxin-like compounds in a single number, which results from the product of the concentration of each congener by its individual Toxic Equivalence Factor (TEF). TEF expresses the toxicity of the congeners in terms of the most toxic form of dioxin (2,3,7,8-TCDD), which has TEF = 1. The most recent revision of TEFs was published in 2005 by WHO (Van den Berg et al., 2006).

Total PCDD/F concentration was in all cases below 10 fg TEQ/m^3, with the only exception of the first summer month in Priolo, when an increase up to 38 fg TEQ/m^3 was recorded, probably as the result of a short-duration local episode.

Regarding PCBs, the WHO guidelines contain information only about the typical air concentration of total PCBs (3 pg/m^3 in rural areas and 3 ng/m^3 in industrial/urban areas), but there are no indications about the concentration of dl-PCB, that is, PCB of particular interest because of their toxicological properties similar to PCDD. For these congeners, however, TEF values have been set, making possible the calculation of their toxic equivalence. In this study, the sum of the TEQ concentrations for PCDD/F and dl-PCB was in all cases very low: between 2.9 and 8.6 during the winter and between 6.1 and 59 during the summer.

The concentration of PBDE (sum of 44 congeners), organobromine compounds widely used as flame retardants, was in the range 14–99 pg/m^3 and was mostly due to decabromodiphenylethane (DBDPE). This compound is often used as a replacement for decaBDE, which was assigned a classification of "suggestive evidence of carcinogenic potential" by USA-EPA and was banned both in Europe and in the United States.

DISCUSSIONS

This study, deploys modeling activities and experimental characterization of atmospheric compounds. In this context, continuous trace gases and aerosol measurements (including NOx, SO_2, O_3, NMHC, PM_{10}, and $PM_{2.5}$) performed during 3 years (2016–2018) were analyzed, and specific intensive field experiments (2016–2017), representative of winter and summer conditions, were carried out in order to determine PM_{10} and $PM_{2.5}$ chemical composition.

In each AERCA, none of the sites exceeded the annual AAQ Directive NO_2 limit, although at the urban site of the Priolo the hourly concentration was sporadically (<0.05% of the time) higher than 200 $\mu g/m^3$.

Generally, SO_2 concentrations at the AERCA industrial and downwind areas were comparable, indicating a well-mixed of emission sources in the considered areas. The SO_2 exposure limit set by WHO (20 $\mu g/m^3$ on daily basis) was exceeded <5% of the days at the Milazzo and Priolo, while at the Crotone this limit was exceeded <0.2% of the time. The AAQ Directive SO_2 limit was never exceeded in the investigated sites, with the exception of the downwind area in Milazzo, where limit was exceeded once over the entire investigated period.

NMHCs, measured at the Milazzo and Priolo, showed a distinctive spatial pattern with the highest concentrations in

TABLE 3 | Air concentration of micro-organics at the three AERCAs during the study period.

		PAH	B(a)P	PCDD/F	dl-PCB	PBDE
Aerca	**Period**	**(ng/m^3)**		**(fg WHO-TEQ/m^3)**		**(pg/m^3)**
CROTONE	14/12–16/01	6.2	0.094	4.2	0.67	69
CROTONE	16/01–14/02	9.3	0.14	5.1	1.1	43
CROTONE	14/02–15/03	6.2	0.10	2.8	1.0	28
CROTONE	5/7–1/8	4.1	0.032	8.9	6.8	70
CROTONE	1/8–6/9	3.3	0.036	5.9	7.2	23
CROTONE	6/9–3/10	2.9	0.030	3.7	2.5	17
MILAZZO	14/12–16/01	13	0.15	3.4	0.90	49
MILAZZO	16/01–14/02	20	0.18	7.8	0.73	99
MILAZZO	14/02–15/03	14	0.10	6.9	0.71	36
MILAZZO	5/7–1/8	7.0	0.041	10	5.5	14
MILAZZO	1/8–6/9	6.9	0.044	6.3	6.0	17
MILAZZO	6/9–3/10	5.3	0.055	4.4	5.1	32
PRIOLO	14/12–16/01	5.6	0.072	1.8	1.1	51
PRIOLO	16/01–14/02	5.6	0.039	4.2	1.3	85
PRIOLO	14/02–15/03	5.4	0.029	3.8	0.94	27
PRIOLO	5/7–1/8	3.7	0.030	38	21	40
PRIOLO	1/8–6/9	3.4	0.036	7.4	14	17
PRIOLO	6/9–3/10	4.1	0.032	9.5	8.6	33

the industrial areas, and one to two orders of magnitude lower levels at the downwind sites, indicating that in these regions anthropogenic emissions are dominating over natural ones. The reference concentration of 200 $\mu g/m^3$(DPCM 28/03/1983) was exceeded for about one third of the investigate period at the industrial site of Priolo, and one tenth of the time at the Milazzo industrial area. NMHC promotes O_3 production, eventually leading to secondary organic aerosol formation. In addition to their impact on air quality, some NMHC, such as 1–3 butadiene and benzene, are recognized cancer-causing agents (Group 1, IARC) while emissions of several NMHC are also perceived as unpleasant odor by most of the population. A detailed speciation of the most relevant NMHC is needed to better understand their impacts on environment and health. In addition, the implementation of specific legislation for industrial areas controling the ensemble of NMHC is recommended.

The different emission mechanisms and atmospheric reactivity of NMHC and SO_2 are well-traced in the Priolo by their different spatial pattern. In this area, where SO_2 is mainly emitted by thermal plants and industrial processes through many chimneys, which are tens of meters high, relatively homogeneous concentrations are observed. Although associated with the burning of fossil fuels by cars and trucks, NMHC are released into the atmosphere mainly through fugitive emissions near the ground, thus significant different concentrations are recorded nearby the industrial site and in downwind area. In fact, NMHC, in presence of solar radiation, react quickly in the atmosphere leading to O_3 formation, while SO_2 oxidation to sulfate is rather slow unless cloud processing occurs. Assuming a OH radical concentration of about 5×10^6 radicals/cm^3, typical of moderately polluted environment, and p-xylene or n-hexane kinetic constants (14×10^{-12} cm^3 molecules^{-1} s^{-1} and 5×10^{-12} cm^3 molecules^{-1} s^{-1} at 25°C, respectively), the atmospheric lifetime of most of low molecular weight aliphatic and aromatic hydrocarbons would be a few hours. It follows that NMHC emitted in the industrial area are oxidized in the atmosphere before reaching downwind sites located at a few kilometers away. On the contrary, typical SO_2 atmospheric residence time is a few days and a more homogenous spatial concentration is observed across the AERCAs.

The Mediterranean basin is characterized by high ozone background levels, significantly higher than those registered in continental Europe (Kalabokasa et al., 2000), as confirmed by the high O_3 values recorded at the a WMO-GAW regional station of Capo Granitola (GRA), a site representative of background coastal conditions in Sicily. The highest O_3 concentration across the Mediterranean Sea are observed during subsidence conditions, pointing toward local O_3 formation from anthropogenic and biogenic precursors (Velchev et al., 2011). O_3 concentrations in downwind and industrial areas exceeded the AAQ EU Directive and the WHO limits most of the time in spring and summer, ranging between 88 and 98 $\mu g/m^3$ (average values for the entire period), stressing on the critical points raised by the WHO which considers O_3 as one of the main factors in morbidity and mortality due to asthma. O_3 is also a Short Lived Climate Forcer and Pollutant, able to alter the Earth's energy balance by absorbing outgoing radiation from the Earth's surface and therefore warm the atmosphere.

At Priolo, the WHO and AAQ Directive limits of 100 and 120 $\mu g/m^3$ were exceeded for the 42 and 14% of the days at the industrial site, and 39 and 9% at the downwind site. In the urban area of Milazzo, the limits were exceeded 11 and 1% of the time while in the Crotone, O_3 exceeded the limits 32 and 10% of the days at the downwind site and 19 and 2% at the urban areas.

$PM_{2.5}$ and PM_{10} average concentrations over the entire periods were below the annual limits set by the AAQ Directive,

while Priolo and Crotone AERCA were characterized by urban PM_{10} and urban and industrial $PM_{2.5}$ concentrations higher than the WHO annual limits (20 and 10 $\mu g/m^3$, respectively). Concerning the daily averages, at the Crotone AERCA daily PM_{10} and $PM_{2.5}$ values exceeded WHO daily limits in winter (50 and 25 $\mu g/m^3$, respectively), while the number of PM_{10} daily exceedances were well below the threshold set by the AAQ Directive (35 days per year larger than 50 $\mu g/m^3$) at all the investigated sites. The $PM_{2.5}$ to PM_{10} ratio, higher in the industrial and downwind areas, was generally larger than 0.5, suggesting a significant contribution of secondary organic and inorganic species to fine particle mass.

The chemical characterization of PM, carried out during two 3-month experimental campaigns in summer and in winter, included the determination of elements, inorganic ions, elemental carbon, organic carbon in PM_{10} and $PM_{2.5}$, polycyclic aromatic hydrocarbons (PAH), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyl (dl-PCB), polybrominated diphenyl ethers (PBDEs) in PM_{10}.

During summer PM_{10} and $PM_{2.5}$ concentrations were higher than in winter, particularly in Priolo, and, at a less extent, in Crotone. This seasonal pattern is typical for coastal sites in southern Italy, where the winter season is characterized by dilution of atmospheric pollutants due to atmospheric mixing and frequent advections, while the higher temperature and lower precipitation in summer promotes soil dust re-suspension and to photochemical formation of new particles.

The evaluation of the main PM macro-sources (sea, soil, traffic, secondary formation, organics) showed the prevalence of sea-salt and crustal matter, natural components of primary origin, in the coarse fraction of PM, with higher values during winter for sea-salts and during summer for soil. Traffic components, organics and ammonium sulfate were mostly in the fine fraction of PM. Organics and ammonium sulfate, of photochemical origin from anthropogenic and biogenic NMHC and from NH_3 and SO_2, respectively, showed higher values during the warmer period.

PAH concentration (sum of 14 congeners) was higher during winter, likely due to the contribution of heating emissions. Nevertheless, the concentration of benzo[a]pyrene was in all cases very far from the target value set by the AAQ Directive at 1 ng/m^3 (1-year average). Total PCDD/F concentration was of the order of 10 fg TEQ/m^3; all the recorded values were well-below the concentration indicated by WHO as acceptable value in urban areas (100 fg TEQ/m^3) and two orders of magnitude lower than the concentration considered by the same organization as indicative of the presence of local sources that have to be identified and evaluated (>300 fg TEQ/m^3); (WHO, 2000). Seasonal variations of these micro-organics were negligible.

When adding also dl-PCB, TEQ concentrations remained very low (2.9–8.6 fg TEQ/m^3 during the winter and 6.1–59 fg TEQ/m^3 during the summer) and was mostly due to PCDD/F during the winter and equally distributed between PCDD/F and dl-PCB during the summer The increase in the atmospheric concentration of PCB during the warm period has been widely reported in the literature and was attributed to the easier volatilization from the soil (Gasser et al., 2008). The concentration of PBDE (sum of 44 congeners) was in the range 14–99 pg/m^3 and was mostly due to decabromodiphenylethane (DBDPE).

CONCLUSIONS

At the three AERCA sites of Crotone, Milazzo and Priolo, during all the considered periods, the measurements of PM mass concentration and of the main gaseous regulated pollutants, except O_3, indicate air quality compliance with the AAQ Directive, which is specifically designed for urban areas and does not consider specific markers for contaminated sites. Nevertheless, when stricter and safer guidelines are considered, critical issues arise particularly for O_3 and NMHC. The observations reported in this work suggest that air quality legislation in contaminated areas should follow lower standards, to meet the WHO guidelines, and should include non-traditional pollutants tackling specific anthropogenic emissions, in order to efficiently protect public health.

The results obtained in this work highlight the need for a deeper chemical characterization of organic vapors (NMHC), some of which are carcinogenic and mutagenic, in contaminated sites hosting industrial activities. Organic species in the gas phase are also responsible for unpleasant odors. As suggested by the result of our study, the regulation of NMHC atmospheric concentration should also consider the occurrence of short-lasting peak events and not only metrics based on daily or annual time scales.

In addition, $PM_{2.5}$ measurements, highlight that fine particles dominated over coarse ones in these regions. The high concentration of NMHC and atmospheric oxidants like O_3 indicates that volatile organic species can contribute significantly to fine particle mass in industrially contaminated sites, and a deeper characterization if this organic fraction can support a better understand of the impact of particle chemical composition on health.

Finally, the results of this study will be considered with an integrated approach, addressing together with the atmosphere compartment the other environmental matrices, i.e., soil, sediments, inland and sea water, and food, to better understand the impacts of anthropogenic pollution on environment and health.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the first and corresponding authors.

AUTHOR CONTRIBUTIONS

CP, SG, TL, and PB analyzed the data and wrote the paper. CP designed the field experiment. MC, SD, GE, MG, SM, SP, ER, and TS carried out sampling and chemical analysis during intensive field experiments. PC, GT, MB, and FC collected gas

phase measurements at the WMO-GAW site. AA, IF, and SO provided gas phase and PM mass databases and contributed to data discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00196/full#supplementary-material

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Indoor Particulate Matter From Smoker Homes Induces Bacterial Growth, Biofilm Formation, and Impairs Airway Antimicrobial Activity. A Pilot Study

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Background: Particulate matter (PM) air pollution causes deleterious health effects; however, less is known about health effects of indoor air particulate matter (IAP).

Objective: To understand whether IAP influences distinct mechanisms in the development of respiratory tract infections, including bacterial growth, biofilm formation, and innate immunity. Additionally, we tested whether IAP from Iowa houses of subjects with and without recent respiratory exacerbations recapitulated the National Institute of Standards and Technology (NIST) IAP findings.

Methods: To test the effect of NIST and Iowa IAP on bacterial growth and biofilm formation, we assessed *Staphylococcus aureus* growth and *Pseudomonas aeruginosa* biofilm formation with and without the presence of IAP. To assess the effect of IAP on innate immunity, we exposed primary human airway surface liquid (ASL) to NIST, and Iowa IAP. Lastly, we tested whether specific metals may be responsible for effects on airway innate immunity.

Results: NIST and Iowa IAP significantly enhanced bacterial growth and biofilm formation. NIST IAP (whole particle and the soluble portion) impaired ASL antimicrobial activity. IAP from one Iowa home significantly impaired ASL antimicrobial activity ($p < 0.05$), and five other homes demonstrated a trend ($p \leq 0.18$) of impaired ASL antimicrobial activity. IAP from homes of subjects with a recent history of respiratory exacerbation tended ($p = 0.09$) to impair ASL antimicrobial activity more than IAP from homes of those without a history respiratory exacerbation. Aluminum and Magnesium impaired ASL antimicrobial activity, while copper was bactericidal. Combining metals varied their effect on ASL antimicrobial activity.

Conclusions: NIST IAP and Iowa IAP enhanced bacterial growth and biofilm formation. ASL antimicrobial activity was impaired by NIST IAP, and Iowa house IAP from subjects with recent respiratory exacerbation tended to impair ASL antimicrobial activity. Individual metals may explain impaired ASL antimicrobial activity; however, antimicrobial activity in the presence of multiple metals warrants further study.

Keywords: particulate matter, indoor environment, COPD - chronic obstructive pulmonary disease, airway surface liquid (ASL), innate host defense

INTRODUCTION

Health impacts of acute ambient particulate matter (PM) exposure, especially fine ($\leq$2.5 μm) PM, are well-documented and include an increased risk of respiratory infections, especially in susceptible populations (1–7).

Airway innate immune mechanisms, such as airway surface liquid (ASL) antimicrobial activity, play an important role in the development of respiratory infections, as well as the mechanism of chronic lung diseases such as cystic fibrosis (8, 9). The ASL is a thin layer of liquid coating the lungs and is responsible for immediately killing incoming pathogens. It is composed of various antimicrobial peptides and proteins (AMPs), such as β-defensins, lactoferrin, lysozyme, and surfactant proteins, among others, and is instrumental in airway innate immunity (10–13).

Air contaminants, such as PM, can impair airway innate immunity and reduce immediate ASL bacterial killing (14, 15). Additionally, transition metals in PM, including iron, can affect airway innate immunity, which can lead to enhanced bacterial growth and biofilm formation (16). Our laboratory has studied the effect of outdoor ambient PM on ASL antimicrobial activity as well as its effect on bacterial growth and biofilm formation (15, 17).

Additionally, a recent area of interest in the environmental science community is the effect of indoor PM on lung health, especially since we spend the majority of our time in buildings (18, 19). Chronic exposure to indoor air pollution could have a disproportionately high impact on overall daily PM exposure. Indoor air quality can be affected by outdoor pollutants entering the home, as well as from indoor sources such as building materials, cleaning and cooking products, or combustion bi-products (20). However, there is a paucity of data regarding how indoor PM may influence mechanisms of airway innate defense mechanisms, despite the disproportionate amount of time we spend indoors.

To this end, we hypothesize that National Institute of Standards and Technology (NIST) standard indoor air particulate matter (IAP) will enhance bacterial growth and biofilm formation (*Staphylococcus aureus*, *Pseudomonas aeruginosa*), and impair bacterial clearance in ASL from airway epithelial cells (AECs). Additionally, we hypothesize that these results will translate using IAP collected from Iowa homes. To test this, we carried out a pilot study in 21 homes of current and former smokers, with and without a history of respiratory exacerbations, to assess whether IAP collected from their homes would affect *in vitro* bacterial growth, biofilm formation, and ASL antimicrobial activity.

METHODS

IAP's Effect on *S. aureus* Growth in Minimal Media

To test if the NIST Standard Reference Material® 2584 IAP enhances bacterial growth, we suspended 190 μL of log-phase bioluminescent *S. aureus* (Xen29, Caliper Lifesciences Bioware®) isolated from a human strain and modified [single-copy of *Photorhabdus luminescens* luxABCDE operon on the chromosome (17)], in a 10 mM $NaPO_4$ buffer with 1% TSB in a 96-well plate (OptiPlate™, PerkinElmer, USA) maintained at 37°C, for details see **Supplementary Material**. We used two concentrations of the IAP (10 and 50 μg/mL), suspended in minimal media, which were plated aside vehicle control. Relative light units (RLUs) were read hourly for 3 h. Hourly growth was quantified as percent growth relative to RLUs at time 0, per condition. Upon termination of the bacterial growth assay, bacteria were aspirated and killed with bleach in a biosafety compliant manner. Any disposable materials used in the assay were sanitized with bleach and disposed of in a biosafety container.

IAP's Effect on *P. aeruginosa* Biofilm Formation

We used *P. aeruginosa* [PA01, (21)] that expresses the pMRP9-1 plasmid constitutively to test for biofilm formation, adapting methods previously described (22–24). We cultured *P. aeruginosa* (PA01, $OD_{i600} = 0.01$) using MBEC™ Calgary (Innovotech, Edmonton, Alberta, CA) 96-well biofilm inoculator plates in M63 minimal media supplemented with 0.4% arginine for 24 h, then exposed the inoculum to challenge conditions containing doses of 10 or 50 μg/mL of NIST IAP ($n = 12$). We then stained inoculated pegs with 0.1% crystal violet and eluted them in 30% glacial acetic acid. We read the OD (550 nm) for each sample and compared to the control condition (*P. aeruginosa* suspended in UltraPure™ distilled water, Life Technologies™) to quantify percent biofilm formation. Upon termination of the biofilm assay, bacteria were aspirated and killed with bleach and disposed of in a biosafety compliant manner. Any disposable materials used in the assay were sanitized with bleach and disposed of in a biosafety container.

ASL Antimicrobial Activity

ASL was collected from primary human airway epithelium (HAE) cell cultures, from the University of Iowa cell culture core, grown at the air–liquid interface, as previously described (with modifications) (25). Briefly, we serially washed cell apical surfaces

(four wells per donor) three times with 120 μL of 10 mM $NaPO_4$ buffer (Ca and Mg free) every two days, and froze the samples at −80°C. Cell media (USG with antibiotics) was changed every 3 days to maintain cell viability (25).

We tested immediate bacterial killing, as defined by percent live bacteria within 20 min. We provide the specific timepoint analyzed for each of the experiments. Ten microliter of bacteria, suspended in minimal media (10 mM $NaPO_4$, TSB $concentration_{Final} = 1\%$), was injected into a 10 μL solution of ASL combined with 1 μL of the particle solution, field blank (FB) solution, or water. The 96-well plate was maintained at 37°C and bacterial luminescence, quantified as RLUs, was read at a 527 nm wavelength after 16 min.

For NIST IAP, we calculated the percentage of live *S. aureus* bacteria remaining (within 10 min) to the RLUs at $t = 0$ for each condition. For the IAP from Iowa homes, the percentage of live bacteria remaining was calculated by comparing ASL in the presence of each sample to the ASL treated with the FB control. ASL controls (12 from three donors) with 1 μL H_2O were interspersed throughout the plate to account for any potential evaporation. Bacterial killing, as measured by RLUs, has been previously validated using colony forming units (17).

To test the effect of the soluble portion of the particles to inhibit bacterial killing, we used the previous method. However, before applying the particle mixture to the ASL, we centrifuged the particles (RPM = 12,000) for 4 min, then added the soluble portion of the particle mixture to the ASL and compared the percent of live bacteria within 10 min to live bacteria at the initial reading (0 min), as the soluble portion did not have physical particles to interfere with RLUs.

Study Population

We recruited subjects from an NIH funded cohort (COPDGene, http://www.copdgene.org/) who reside in Iowa. All methods were performed in accordance with the relevant guidelines and regulations, informed consent was obtained, and no human tissue samples were used. Subjects were selected based on respiratory exacerbation history and their house location (within a 30-mile radius of the University of Iowa Hospital). Our study radius facilitated study adherence with elderly subjects and allowed for timely device transfer/maintenance. Selection aims were: similar GOLD (Global Initiative for Obstructive Lung Disease Criteria for COPD) stage and 50% male/female ratio. We recruited a total of 13 female and 8 male subjects who are current ($n = 2$) and former smokers ($n = 19$), with and without respiratory exacerbations based on history prior to enrollment (within the previous 3 years-−2015–2017). To be considered within the exacerbator group, participants were required to experience ≥1 exacerbation per year within the previous 3 years. Non-exacerbators had not experienced any exacerbations within the same time-frame. These subjects were followed from November 2016 to April 2017. Participants were provided with a survey (**Supplemental Table 1**) which inquired about potential IAP generating sources, and personal habits that may affect exposures, such as cleaning methods, cooking materials and combustion sources (20).

Aerosol Collection

Particles were collected over 1 month during winter, to reduce infiltration from ambient particles and isolate particles generated in the home. We collected particles ≥20 nm using electrostatic precipitator (ESP) devices (OION B-1000, ©OION Technologies). We requested that the ESP be placed in the room most frequently occupied by study participants, typically the living room. ESP particle collection efficiency using our method varies depending on the type and size of a particle, with water soluble particles preferentially recovered; however, the OION ESP particle collection efficiency for a typical outdoor contaminant (Arizona road dust) is 65% (26).

The ESP generated 0.036 mg/min of ozone, and at a ventilation rate of 230 L/min the ESP would not exceed NAAQS exposure limits. All homes participating had active ventilation systems in place (26).

Removing Particles From ESP and Filters

ESP collection plates are flat, which enables easy PM recovery. Twenty-one ESPs were wiped with eight wet (DI H_2O) PVC filters (26). These filters were allowed to dry at least 72 h, then weighed and chemically analyzed using a microwave digestion methodology, with modified parameters to increase the recovery of metal analytes (27, 28). FBs were treated identically, where a wet PVC filter was used to wipe a clean ESP. The filter with a mass closest to 5 mg was submerged in a 5 mL conical tube. In one home, four of the five filters masses were >15 mg, in which case the filter with the lowest of the four masses was chosen. We used 1 mg/mL (mass/water) to normalize for particle dose. The mean volume of water used in all samples was added to the FB filter (3.7 mL).

To recover the soluble portion of PM, preferentially selected in our PM collection method, we cut PVC filters into strips, and submerged in distilled water (UltraPure™ Distilled Water, Invitrogen, Life Technologies, Grand Island, NY, USA). Conical tubes containing the 22 filters (21 homes, one FB) and H_2O were placed overnight (18 h) on a titer-plate shaker (Lab-line instruments, Inc. Melrose Park, IL, USA) at a setting of 6 (∼750 RPM) at room temperature. The following morning, the soluble, "supernatant" portion of the remaining suspension was isolated and frozen. Any remaining solution was also frozen at −4°C. Although the indoor PM samples represent only the soluble component of the IAP, we hereafter refer to it as IAP for consistency.

Bacterial Colonization of Filter

The soluble portion of the IAP was tested for biological organisms by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry, as previously described (29). Briefly, the sample was mixed uniformly in a large matrix, which then absorbs ultraviolet light (nitrogen laser light, wavelength 337 nm) and converts it to heat energy. A small portion heats in nanoseconds and is vaporized with the sample. Time of flight differs according to the value of ionic mass-to-charge ratio. This value is compared to a strain-identification database.

Participant IAPs' Effect on Bacterial Growth, Biofilm Formation

To assess whether IAP from homes of Iowa smokers influenced *S. aureus* growth, we used the previously described method; however, added only the soluble portion of IAP from homes to the bacterial suspension at a $dose_{initial}$ of 50 μg/mL.

To test the effect of Iowa smokers' IAP on *P. aeruginosa* biofilm formation, we exposed inoculator lids to challenge conditions containing each indoor PM sample for 24 h. Following the particle challenge, inoculated pegs were stained using 0.1% crystal violet and eluted in 30% glacial acetic acid. OD_{550} was read for each sample as a measure of biofilm formation.

Effects of Metals on ASL Antimicrobial Activity

The two most abundant metals, by mass fraction, present in the Iowa samples (aluminum and magnesium) were selected and analyzed for their effect on immediate ASL antimicrobial activity (30). We then combined $AlCl_3$ and $MgCl_2$ (>98% purity, Fischer Scientific, Pittsburgh, PA, USA) in an aqueous solution using DI water, to match their average IAP proportion and analyzed their effect on ASL antimicrobial activity. Furthermore, we investigated the effect of copper on antimicrobial killing. Because Cu is a divalent cation, it also has the potential to inhibit ASL killing; however, Cu is also a known antimicrobial agent, therefore its effect on ASL bacterial killing was of interest. We tested $CuCl_2$ (>98% purity, Fischer Scientific, Pittsburgh, PA, USA) with human large airway ASL and *S. aureus*. Lastly, individual metal mass fractions were compared to bacterial killing in the presence of IAP.

Statistical Analysis

We used Graph Pad Prism software, version 8.2.0. Significance was determined at $\alpha \leq 0.05$. To compare bacterial growth for each condition, we used unpaired two-tailed *t*-tests comparing growth under each condition to the control ($n = 4$ per condition). We tested for differences in biofilm growth from vehicle control (OD_{550}) using unpaired two-tailed *t*-tests. For Iowa household IAP, bacterial growth at 4 h using a field-blank control was compared to each condition using Brown-Forsythe and Welch ANOVA tests, and biofilm formation was assessed in the same manner.

To analyze significance between immediate bacterial killing per condition we compared the percent of live *S. aureus* remaining within 10 min in the presence of ASL with and without exposure to whole-particle NIST IAP (50 μg/mL dose), compared to percent live bacteria in vehicle (SPB) with particle control at the same time-point. ASL control was compared to ASL+IAP with a paired two-tailed *t*-test. To test for differences between the percent of live bacteria between the control and soluble portion of the particle mixtures, live bacteria within 10 min was compared to $t = 0$ min for ASL alone, and live bacteria within 10 min in the presence of the soluble portion of NIST IAP (50 μg/mL $dose_{initial}$) was compared to $t = 0$ min at the same condition. Paired, two-tailed *t*-tests were used to compare the percent of live bacteria within 10 min to live bacteria at the initial reading (0 min), as the soluble portion did not have physical particles to interfere with RLUs.

Bacterial growth and biofilm formation were assessed using a Brown-Forsythe and Welch ANOVA test where bacterial growth and biofilm formation from each home was compared to their respective vehicle control. To test for differences between each house's IAP and ASL with the field-blank (filter) control, live bacteria at 16 min per condition was compared to initial RLUs (0 min.) and converted to a percentage of live bacteria. The percentage of live bacteria remaining after ASL was treated with each participants' IAP was compared to the ASL control's (percentage of live bacteria) using paired *t*-tests. Bacterial killing in the presence of metals was calculated the same way, but live bacteria at 6 min was per condition was compared to initial RLUs (0 min.), using paired *t*-tests to compare untreated ASL to each dose of each metal. ASL antimicrobial activity between groups (exacerbators vs. non-exacerbators) was assessed using unpaired *t*-tests.

To test whether Global Obstructive Lung Disease (GOLD) status modifies the effect of percent live bacteria on exacerbation status we fit a logistic regression model with an interaction term for GOLD status and percent live bacteria using Stata/SE (v15.1, StataCorp., LLC, College Station, Texas, USA). Briefly, a "low" GOLD score was defined as GOLD zero ($n = 13$), and a "high" score was defined as GOLD one or two ($n = 7$). The GOLD status for one participant was not available, and this participant was therefore not included in the aforementioned analysis.

RESULTS

IAP's Effect on Bacterial Growth and Biofilm Formation

In order to test whether IAP influences bacterial growth, we added two doses (10 and 50 μg/mL) of NIST IAP to *S. aureus* suspended in minimal media (10 mM $NaPO_4$ buffer with 1% TSB), and assessed bacterial growth over time (RLUs at one, 2 and 3 h compared to RLUs at inoculation). After 2 and 3 h, NIST IAP significantly enhanced *S. aureus* growth ($p = 0.03$, 0.02; $p = 0.04$, 0.02, respective to dose and time) relative to bacteria without particles, **Figure 1A**, recapitulating previous observations of *S. aureus* growth in the presence of the same dose of an ambient pollutant [50 μg/mL, Coal Fly Ash (CFA)] (17). We used *P. aeruginosa* to assess whether IAP influenced biofilm growth by culturing *P. aeruginosa* in minimal media (M63 + 0.4% arginine) for 24 h, then exposing the inoculum to 10 or 50 μg/mL NIST IAP. We compared OD for each sample with *P. aeruginosa* suspended in vehicle control. As seen in **Figure 1B**, the percent of biofilm formation was increased using NIST IAP in a dose-dependent manner.

ASL Antimicrobial Activity

Because we have demonstrated CFA can inhibit ASL antimicrobial activity in human AECs (17), we were interested whether IAP would recapitulate this effect. To test this, we treated human AEC ASL with NIST IAP (dose = 50 μg/mL) for 45 min and then injected *S. aureus* and calculated immediate bacterial killing. The percentage of live *S. aureus* bacteria

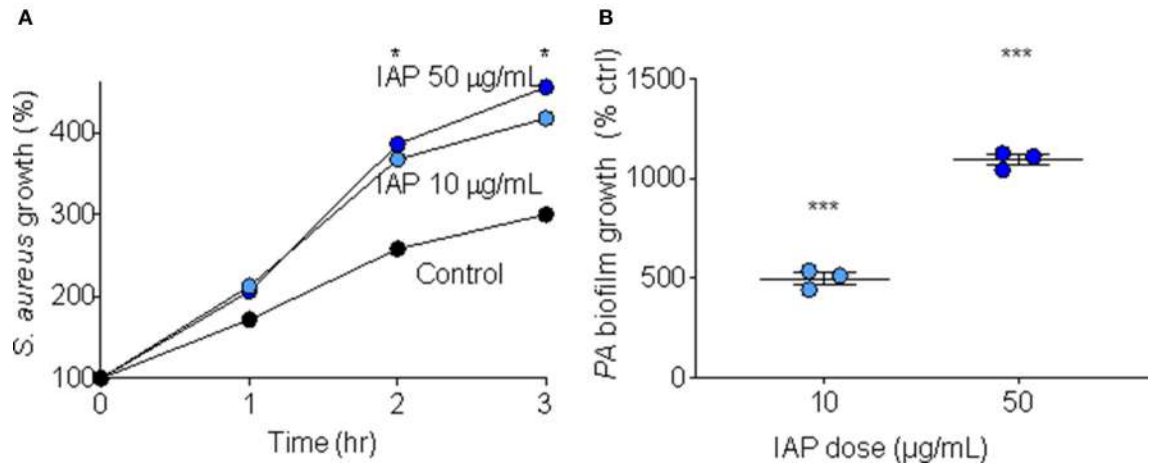

FIGURE 1 | Bacterial growth and biofilm formation in presence of NIST IAP. **(A)** IAP enhances *S. aureus* bacterial growth in minimal media after 2 and 3 h, unpaired twotailed *t*-tests compared growth per condition to control ($n = 4$ per condition), $^*p < 0.05$ **(B)** *P. aeruginosa* biofilm growth in the presence of IAP particles at two doses (10 and 50 µg/mL). Error bars represent means and standard error of the means. Differences in OD_{550} of biofilm growth compared to vehicle control were compared using an unpaired two-tailed *t*-test $^{***}p < 0.001$.

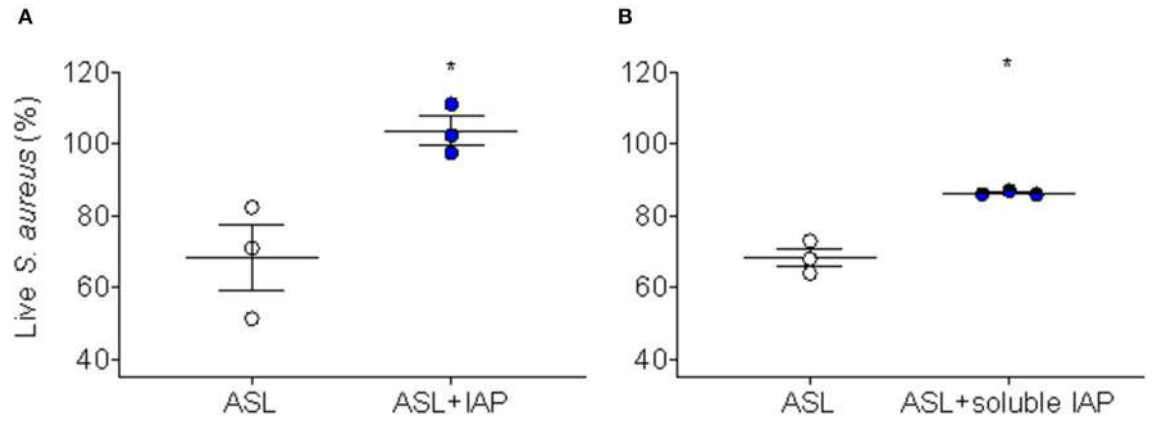

FIGURE 2 | Inhibition of ASL antimicrobial activity using NIST IAP. **(A)** Percent of live *S. aureus* remaining within 10 min in the presence of ASL with and without exposure to whole-particle NIST IAP (50 µg/mL dose), compared to percent live bacteria in vehicle (10 mM $NaPO_4$ buffer) with particle control at the same time-point; the whole IAP significantly inhibited large HAE cell ASL bacterial killing ($p < 0.05$), $n = 3$ from three different donors, ASL control was compared to ASL + IAP with a paired two-tailed *t*-test and error bars represent means and standard error of the means. **(B)** Live bacteria within 10 min compared to $t = 0$ min for ASL alone, and live bacteria within 10 min in the presence of the soluble portion of NIST IAP (50 µg/mL $dose_{initial}$) was compared to $t = 0$ min at the same condition; the soluble portion of IAP significantly inhibited large HAE cell ASL bacterial killing ($p < 0.05$), $n = 3$ from three different donors. Error bars represent means and standard error of the means. Paired, two-tailed *t*-tests were used to compare the percent of live bacteria within 10 min to live bacteria at the initial reading (0 min), as the soluble portion did not have physical particles to interfere with RLUs, $^*p < 0.05$.

remaining after 2 min was compared to vehicle with particle control per condition ($n = 3$), **Figure 2A**.

We have reported that the soluble component of CFA does not inhibit human ASL antimicrobial activity (17); therefore, we decided to test whether the soluble portion of NIST IAP would inhibit human ASL antimicrobial activity. We therefore tested whether the soluble portion of the particle mixture (NIST IAP) may also inhibit bacterial killing using ASL from large human AECs. To test the effect of the soluble portion of IAP (whole-particle dose = 50 µg/mL), we treated human ASL with the supernatant of the particle mixture, then calculated the percentage of live *S. aureus* remaining within 10 min to the bacteria RLUs at $t = 0$ per condition, **Figure 2B**.

ASL in the presence of the whole-particle mixture inhibited ASL antimicrobial activity, **Figure 2A** (17). The soluble portion of NIST IAP also inhibited ASL immediate *S. aureus* bacterial killing ($p < 0.05$) (**Figure 2B**). However, the soluble portion of NIST IAP inhibited ASL antimicrobial activity less than the whole-particle mixture, **Figures 2A,B**.

Study Population

We next assessed IAP from Iowa homes of subjects with and without active respiratory exacerbations. Subjects participating

TABLE 1 | Study demographics.

Demographics	Exacerbators (SD)	Non-exacerbators (SD)	*P* value
Age (y/o)	70 (8.1)	67 (6.6)	ns
Female	6	7	ns
GOLD status 1-2	5	2	ns
Exacerbations per year	2	0	***<0.01***
Current smoker	1	1	ns
Pack years	41 (19.7)	28 (9.2)	0.07

Number of study participants within exacerbator and non-exacerbator groups by demographics (n = 21); a GOLD status one or greater indicates COPD stage. Significance was assessed using unpaired t-tests (exacerbators vs. non-exacerbators). Bold font indicates significance, italicized font indicates a trend ($p < 0.15$), and significance was defined as $p \leq 0.05$, $^{**}p < 0.01$.

in our study who had experienced respiratory exacerbations over a 3-year period ("exacerbator group"), ($n = 10$) experienced a total of 64 exacerbations in the previous 3 years (mean = 2.1 exacerbations/year). Subsequent analyses were carried out to test for differences in the samples between the two groups. **Table 1** describes study participant demographics.

As seen in **Table 2**, the group without respiratory exacerbations had significantly more pet ownership, specifically cats ($p \leq 0.05$). Additionally, the *p*-value for aluminum pan use was 0.11, with exacerbators reporting use of aluminum pans more than non-exacerbators.

We tested whether any bacteria could be found in the soluble portion of the filter samples from each home, and whether there was a difference between exacerbators vs. non exacerbators (**Supplemental Table 2**). Upon matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis, ten IAP samples (48%) produced bacterial growth (**Supplemental Table 2**). Pathogens identified from the samples include: *Paenibacillus rhizosphaerae*, *Bacillus pumilus*, *Bacillus subtilis*, *Kocuria carniphila*, *Kocuria marina*, *Kocuria palustris*, *Micrococcus luteus*, *Micrococcus flavus*, *Microbacterium testaceum*, *Staphylococcus saprophyticus*, *Staphylococcus pettenkoferi*, *Pantoea calida*, *Pseudomonas fulva,* and 18 unknown bacterial species (not shown). Colony forming units ranged from 20–600,000 CFUs/mL. In addition, no significant difference in total bacterial CFUs was found between exacerbation groups ($p = 0.22$).

Participant IAPs' Effect on Bacterial Growth, Biofilm Formation

Since NIST IAP enhanced bacterial growth and biofilm formation, we assessed whether IAP from Iowa homes would also consistently enhance *S. aureus* bacterial growth and *P. aeruginosa* biofilm formation. To test bacterial growth, we added IAP from each home to *S. aureus* and measured percent growth at 4 h, compared to the field blank control. To test biofilm formation, we exposed inoculator lids to challenge conditions containing each indoor PM sample for 24 h, then read OD_{550} compared to control.

IAP from all houses enhanced bacterial growth after 4 h compared to the FB control. As shown in **Figure 3A**, bacterial growth significantly increased in all samples, except for in homes 1, 11, 16, 17, and 19. In homes whose IAP did not significantly promote bacterial growth, $p \leq 0.18$.

As shown in **Figure 3B**, biofilm growth was greater than that of the FB control in all samples. The FB did not result in significant biofilm formation (data not shown), however all indoor samples demonstrated statistically significant biofilm formation ($p < 0.05$). We confirmed these findings using stereoscopic images (data not shown). We also looked at differences in bacterial growth and biofilm formation based on exacerbator status, but there was no difference ($p = 0.78$ and 0.93, for bacterial growth and biofilm formation, respectively), **Supplemental Figures 1A,B**.

TABLE 2 | Survey responses regarding potential IAP generating sources.

Survey response[†]	Exacerbators (SD)	Non-exacerbators (SD)	*P*-value
Time cleaning/week (h)	3.5 (3.2)	3.6 (2.6)	ns
Vacuum	9	9	ns
Dust	8	7	ns
Sweep	4	5	ns
Other cleaning	2	4	ns
Time cooking/week (h)	10 (5.9)	8.1 (4.7)	ns
Cook with gas	4	4	ns
Cook using aluminum pan[‡]	*4*	*1*	0.11
Cook using non-stick pan	6	4	ns
Cook using cast-iron	3	4	ns
Burn candles	3	2	ns
House heated by gas (inc gas fireplace)	8	10	ns
Garage attached to house	6	7	ns
Has pets*	***3***	***8***	***≤0.05***
Owns dogs	3	3	ns
Owns cats*	***0***	***4***	***≤0.05***
Total pets in home (#)*	***4***	***22***	***≤0.05***

[†] Always indicates number of subjects in each category, unless otherwise noted.
[‡] Some pans counted in both categories, i.e., metal and non-stick, if information available.
Study-participant survey reponses (n = 21) related to life-style and housing conditions, significance was defined as $p \leq 0.05$. Significance was assessed using unpaired t-tests (exacerbators vs. non-exacerbators). Bold font indicates significance (*), defined as $p \leq 0.05$ and italicized font indicates a trend ($p < 0.15$).

ASL Antimicrobial Activity

IAP differed from CFA in that the soluble component of NIST IAP inhibited ASL antimicrobial activity, unlike CFA (17). We next tested the effect of the soluble portion of IAP from houses of Iowa smokers on ASL antimicrobial activity. **Figure 4** shows percent live bacteria of the ASL control, and percent live bacteria

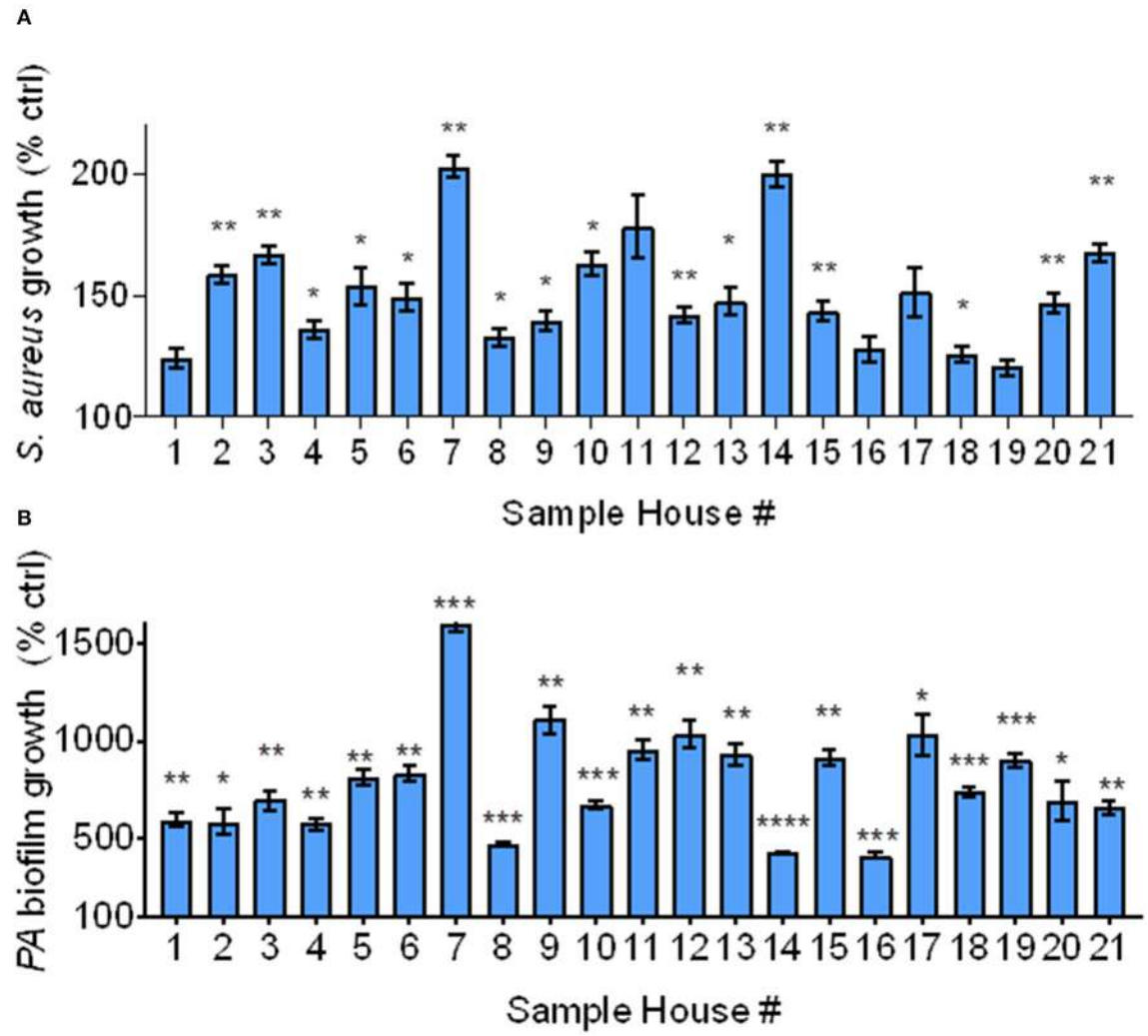

FIGURE 3 | Bacterial growth and biofilm formation using Iowa house IAP. **(A)** *S. aureus* bacterial growth relative to the FB control at 4 h in the presence of soluble IAP from all homes, error bars represent means and standard error of the means and differences between FB control were compared to each condition using Brown-Forsythe and Welch ANOVA tests *indicates $p < 0.05$, **$p < 0.01$. **(B)** *P. aeruginosa* biofilm formation was higher in the presence of all IAP, error bars represent means and standard error of the means and differences between control compared to each condition were assessed using Brown-Forsythe and Welch ANOVA tests *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

after ASL was treated with each participants' IAP samples. We report $p < 0.20$.

IAP from one home significantly inhibited ASL antimicrobial activity, and there was a trend ($p \leq 0.18$) of inhibited ASL antimicrobial activity in five other homes (#2, 7, 9, 12, 13). In addition, there was a trend of enhanced ASL antimicrobial activity in two homes (#20 and 21).

We also looked at ASL antimicrobial activity using IAP from homes of respiratory exacerbators compared to bacterial killing in the presence of IAP of non-exacerbators, **Figure 5**.

We found a trend ($p = 0.09$) of inhibited ASL antimicrobial activity in the exacerbator group, compared to non-exacerbators, **Figure 5**. Additionally, IAP from subjects with a mild GOLD status appeared to greater influence ASL antimicrobial activity than IAP from subjects with a higher GOLD status (low GOLD status OR 1.32 [95% CI 0.93–1.88]; $p = 0.12$, High GOLD status OR 0.95 [95% CI 0.45–1.16]; $p = 0.18$).

ASL Antimicrobial Activity in the Presence of Metals

The two most abundant metals (by mass fraction) in the Iowa samples were aluminum and magnesium (30). While we don't know the dose of Al or Mg in the soluble component of the NIST IAP, our 50 μg/mL dose contained 1.16 μg/mL Al, and 0.80 μg/mL Mg.

Divalent cations such as magnesium, have been reported to inhibit ASL antimicrobial activity (13). Copper is also a divalent cation, suggesting it may inhibit ASL bacterial killing; however, it is also a known antimicrobial agent, making it a metal of interest, especially given that IAP from the two houses had a trend of

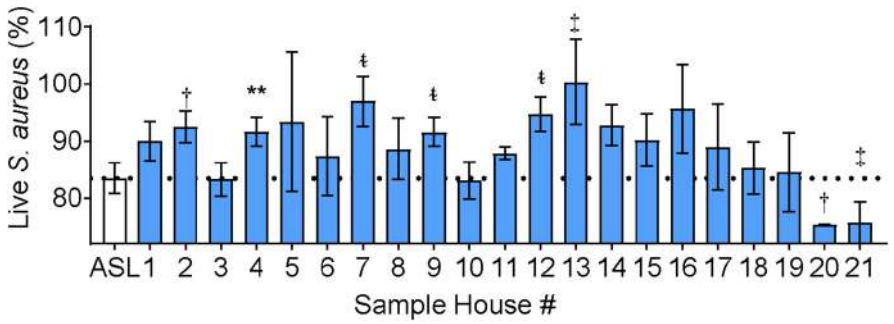

FIGURE 4 | ASL antimicrobial activity in presence of Iowa house IAP. Percent live bacteria after treatment with ASL control (dotted line), and percent live bacteria after ASL was treated with indoor samples from all study participants. Error bars represent means and standard error of the means. IAP from house #4 significantly inhibited *S. aureus* bacterial killing, while IAP from five houses resulted in a trend of inhibited *S. aureus* killing, and two houses had a trend of enhanced *S. aureus* killing, ($p \leq 0.18$). To test for differences between each house's IAP and ASL with the field-blank (filter) control, percent live bacteria after treatment with ASL control was compared using paired *t*-tests to the percentage of live bacteria remaining after ASL was treated with each participants' IAP, $^{**}p < 0.01$, $^{\dagger}p = 0.06$–0.10, $^{\ddagger}p = 0.11$–0.15, and $^{\text{ł}}p = 0.16$–0.20.

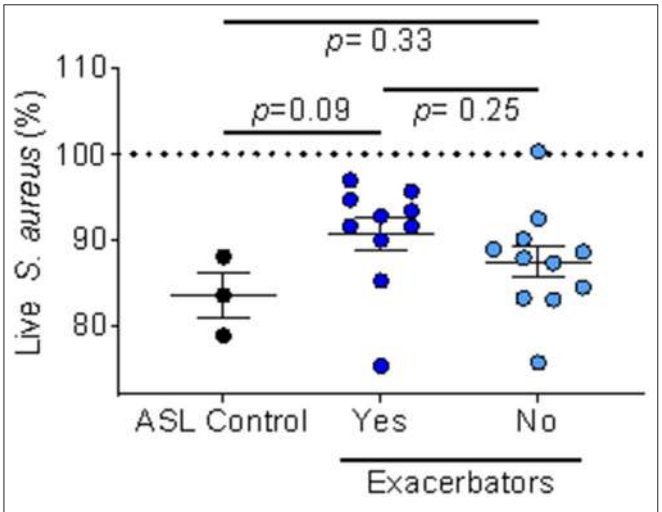

FIGURE 5 | IAP's effect on ASL antimicrobial activity based on exacerbation history. Percent of live *S. aureus* at 16 min in the presence of IAP from exacerbators vs. non-exacerbators—values greater than the ASL control indicate inhibition of bacterial killing, while values lower than ASL alone indicate increased bacterial killing. Error bars represent means and standard error of the means. To test for differences between ASL with the field-blank control and the respiratory exacerbator group (and non-exacerbator group) we used unpaired *t*-tests.

enhanced killing (#20 and 21). These homes had both the lowest amount of copper of any of the houses (#20), and the second highest amount of copper (#21), so we were therefore interested in copper's ability to kill bacteria.

We tested if adding Al^{3+}, Mg^{2+}, and Cu at six doses to the ASL altered immediate antimicrobial activity, compared to baseline ASL antimicrobial activity (**Figures 6A–C**).

As shown in **Figure 6A**, $AlCl_2$ appeared to inhibit ASL antimicrobial activity in a dose-response manner; however, $AlCl_2$ only significantly ($p < 0.01$) inhibited ASL antimicrobial activity at 1 mM. $MgCl_2$ did not consistently affect ASL antimicrobial activity—it aided bacterial killing at 100 nM; however, inhibited ASL antimicrobial activity at 1 μM ($p < 0.05$) (**Figure 6B**). $CuCl_2$ consistently had an antimicrobial effect on *S. aureus*, **Figure 6C**. At 100 nM, $CuCl_2$ had a trend of enhanced killing ($p \leq 0.18$), and at higher doses (≥100 μM), $CuCl_2$ significantly enhanced bacterial killing, **Figure 6C**.

However, in our Iowa samples, we found that IAP from different homes had variable effects. Since Al and Mg are the most abundant metals (30), and are combined in the Iowa IAP samples, we decided to test whether a combination of these metals at similar proportions (60/40 of $AlCl_2$ and $MgCl_2$, respectively) would have different effects. When $AlCl_2$ and $MgCl_2$ are combined to the average proportion present in Iowa house IAP, the dose-response profile is dissimilar to either individual metal (enhanced killing at 10 nM and inhibition of killing at 1 μM), **Figure 6D**. There was no clear dose-response pattern influencing ASL antimicrobial activity for any specific metal analyzed, indicating the difficulty in predicting toxicity **Figures 6A–D**.

To test whether individual metal content explains the differences in modulation of ASL antimicrobial activity by IAP, we compared the weighted mass-fraction of each metal (30) to bacterial killing using a simple linear regression analysis. The mass fraction of Al, Mg, and Cu present in the IAP samples was not statistically significantly associated with the enhancement, nor inhibition, of bacterial killing ($R^2 = -0.00$, 0.03, and 0.00, respectively). Therefore, the metal content of the particles was not enough to predict its biological effect on ASL antimicrobial activity.

DISCUSSION

The indoor environment can be a key source of pollution exposure. Indoor air pollution is linked to a multitude of negative human health outcomes (31, 32). We found that NIST IAP increases bacterial growth (**Figure 1A**) and biofilm formation (**Figure 1B**) in a dose response manner.

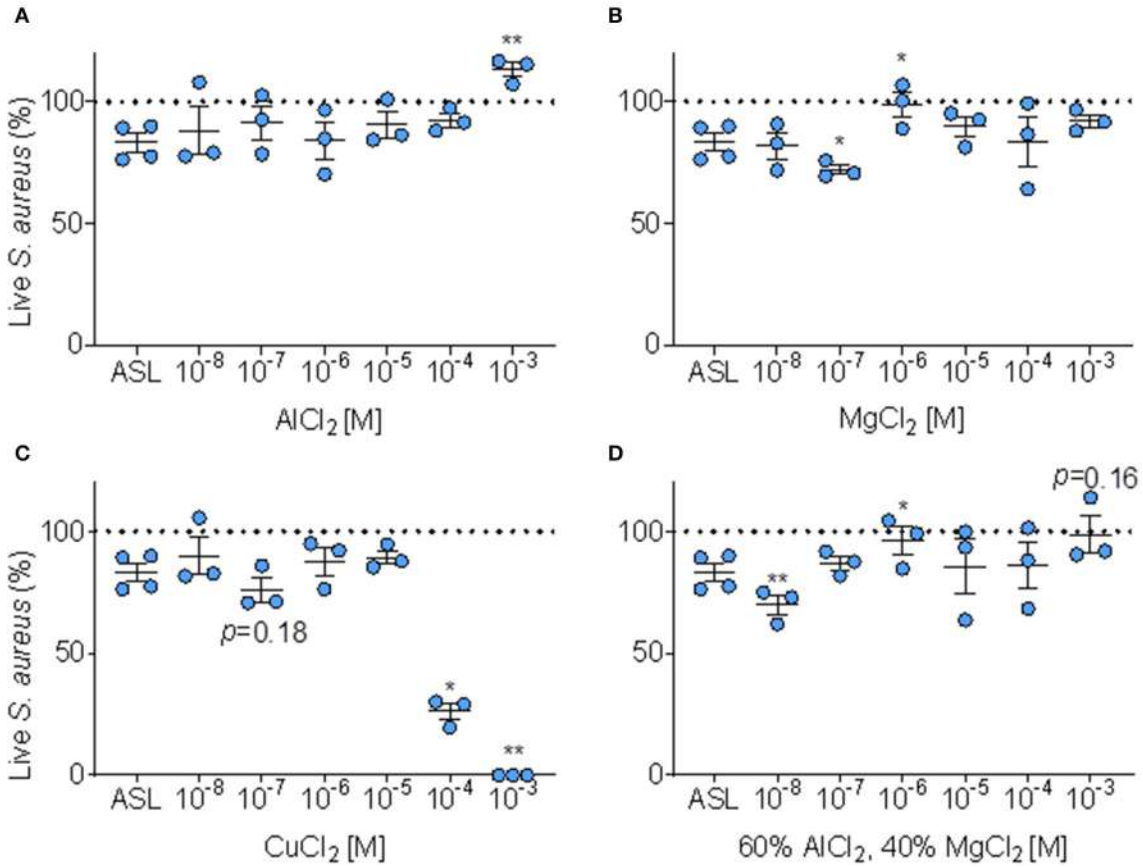

FIGURE 6 | Effect of metals on ASL antimicrobial activity. **(A)** Percent of *S. aureus* bacteria alive immediately (≤10 min.) after exposure to ASL combined with six doses of $AlCl_2$, error bars represent means and standard error of the means. **(B)** Percent *S. aureus* bacteria alive immediately (≤10 min.) after exposure to ASL combined with six doses of $MgCl_2$, error bars represent means and standard error of the means. **(C)** Percent of *S. aureus* alive immediately (≤10 min.) after exposure to six doses of $CuCl_2$ (proportions derived from sample means) relative to untreated ASL, error bars represent means and standard error of the means. **(D)** Percent *S. aureus* bacteria alive immediately (≤10 min.) after exposure to ASL combined with six doses of $MgCl_2$ combined with $AlCl_2$ in their average Iowa IAP proportions, error bars represent means and standard error of the means. *P*-values are generated from paired *t*-tests of percent live bacteria of each metal dose + ASL (at the same timepoint for each metal, ≤10 min) compared to ASL control at the same timepoint. **(A–D)** $^{*}p \leq 0.05$ and $^{**}p < 0.01$.

Prior work has implicated the insoluble portion of coarse PM as inducing toxic, proinflammatory responses to lung tissue (33); and our laboratory has previously demonstrated that the physical particle of coal fly ash adsorbs cationic antimicrobial peptides, reducing the bioavailability of these proteins to bind to bacteria, while the soluble component did not inhibit human large ASL antimicrobial activity; however, research in this field is limited (17, 34). We were therefore interested in assessing whether the soluble portion of the IAP mixture may also inhibit ASL antimicrobial activity. Not only did the whole-particle mixture of IAP inhibit ASL antimicrobial activity (**Figure 2A**), but the soluble portion of the particle mixture also significantly inhibited ASL antimicrobial activity (**Figure 2B**), suggesting the involvement of other mechanisms of ASL antimicrobial activity impairment.

COPD subjects with moderate to severe COPD have previously been shown to experience increased respiratory symptoms, medication use, and risk of severe exacerbations alongside increased $PM_{2.5}$ levels in the primary household living area (35). Also, it was recently reported that symptomatic current and former smokers with preserved lung function have respiratory exacerbations (36). We were therefore interested in determining whether IAP from houses of smokers with and without a history of respiratory exacerbations would differentially affect bacterial growth (*S. aureus*) biofilm formation (*P. aeruginosa*), and ASL antimicrobial activity. No differences were found between participants experiencing respiratory exacerbations and those without—IAP promoted bacterial growth and biofilm formation independent of exacerbation status (**Figures 3A,B**). Bacterial growth was variable but consistent in all cases. Enhanced bacterial growth in the presence of IAP implies that, upon exposure to pathogens, the potential for bacteria to remain viable in subjects who have inspired IAP may be greater.

While we found no differential effect of IAP on bacterial growth and biofilm formation between groups (those

experiencing recent respiratory exacerbation and those without respiratory exacerbation), there was a differential effect of ASL antimicrobial activity. Iowa house IAP samples either impaired, had no effect, or had a trend of enhanced ASL antimicrobial activity. We also found a trend indicating that home IAP of respiratory exacerbators may preferentially inhibit ASL antimicrobial activity compared to IAP from homes of non-exacerbators (**Figure 5**). The *p*-value may be explained by the low power of the sample size. It is known that host innate immunity is compromised in COPD subjects due to a number of factors including impaired mucociliary clearance and reduced AMPs, among others (37). IAP from houses of exacerbators may preferentially attenuate the airway's natural defense system, potentially playing an important role in innate immunity. Additionally, there appeared to be a more pronounced effect of IAP on the mild GOLD status subjects. While not statistically significant, it suggests a differential effect of air pollution depending on underlying COPD severity. We speculate that this result reflects that the home environment of smokers (ex and current) *without* established COPD (GOLD 0) may impair airway antimicrobial activity more so than the home environment of those with established COPD (GOLD $\geq$ 1), thus contributing to an increased risk of developing respiratory exacerbations.

Our cohort of subjects was relatively homogenous in terms of age, gender, smoking status, geographical location, and typical time spent performing house-hold activities. However, two distinct differences emerged between the non-exacerbator and exacerbator groups. Subjects without a recent history of respiratory exacerbation more frequently reported owning pets, and had significantly more pets (cats), than those with recent respiratory exacerbation. This finding may reflect health status, e.g., those not currently experiencing exacerbations are more capable of handling the demands of a domestic animal but could also be indicative of previous pet-ownership. The link between cat ownership and sensitization (allergy, asthma) is uncertain, but dog ownership has been shown to either have no effect, or a positive effect on reducing sensitization (38). More research on respiratory exacerbations and household pet ownership is warranted. Additionally, respiratory exacerbators reported cooking with aluminum pots/pans, **Table 2,** while only one participant in the non-exacerbator group noted using aluminum. Although not statistically significant, the difference is noteworthy considering our findings regarding the inhibitory effect of aluminum on airway innate immunity.

Non-exacerbator samples had twice as many micro-organism species identified in filters from their homes compared to the exacerbator group, **Supplemental Table 2**. This finding is not surprising considering non-exacerbators owned more animals. When comparing total CFUs based on exacerbation status, there was no significant difference between the two groups. *Pseudomonas* and *Staphylococcus species* (2) were found in IAP samples (House #13 and 18). Both are important pathogens in the progression of lung disease. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common pathogens (gram positive and negative) found in immunocompromised patients (39–41). In addition, *P. aeruginosa* growth is enhanced in the presence of ambient air pollution, and *S. aureus* bacterial growth is enhanced in the bronchoalveolar lavage of smokers (15, 16).

Antimicrobial peptide activity in the ASL is known to be disrupted by certain divalent cations in the mM range (13, 42). Divalent cations are thought to bind to negatively charged bacteria, rendering bacteria less capable of binding to cationic antimicrobial peptides present in the ASL (17). Interestingly, only IAP from COPD house number 4 statistically inhibited bacterial killing (**Figure 4**). Although some IAP appeared to have a trend of enhanced ASL antimicrobial activity, this is not necessarily indicative of a positive health outcome, as $PM_{2.5}$ can be pro-inflammatory (induce cytokine release) and cytotoxic, which is in part mediated by transition metals (43, 44).

Previous work has shown in-home endotoxin exposures are not the causative agent in worsening of COPD symptoms and, because iron can alter bacterial growth, we hypothesized that metals may play an important role in respiratory exacerbations (16, 45). However, no statistically significant association between specific metals (mass fraction nor soluble component) in the samples, such as iron, and bacterial growth was found. This may be due to the small sample size, or the wide variety of metals present in the samples and distinct combinations of metals potentially affecting bacteria in unique ways.

We analyzed Al^{3+} and Mg^{2+} in a dose-response manner to test their effect on bacterial killing and found that at 1 mM ($AlCl_2$), and 1 μM ($MgCl_2$), these metals inhibited antimicrobial activity, **Figures 6A,B**. However, at 100 nM $MgCl_2$, ASL bacterial killing was enhanced, **Figure 6B**. Polyvalent cations, Al^{3+} and Mg^{2+} have a complex effect on ASL bacterial killing when combined (**Figure 6D**). Aluminum, a polyvalent cation, seems to have a trend of inhibiting in a dose-response manner. When combined to their average indoor PM proportion, Al^{3+} and Mg^{2+} resulted in enhanced killing at a low dose (10 nM), yet there was more inhibition at higher doses (1 μM and 1 mM). This presents a novel dose-response profile compared to the individual metals, and confirms the metals behave distinctly when combined.

Interestingly, Copper, which is commonly found in building furnishings and generated during cooking (46) has been shown to be an independent antimicrobial agent (47–49) and, as expected, at doses >10 μM it was sufficient to kill bacteria independent of ASL activity (**Figure 6C**). The half maximal inhibitory concentration 50% (IC_{50}) of ASL with $CuCl_2$ occurred at ~194 μM (not shown), while $CuCl_2$ combined with water also immediately killed *S. aureus* independently of ASL antimicrobial activity (**Supplemental Figure 2**). However, bacterial killing occurred at lower doses of copper combined with ASL (IC_{50} ~ 58), compared to copper alone (IC50 ~ 194 μM), suggesting the antimicrobial effect of copper appears to be synergistic with ASL compared to water alone.

The volume of ASL used in our assay was 10 μL, healthy human lung has an ASL height of ~40 μm (50) given the surface of a human lung is ~70 m^2 (51), this would translate to an approximate ASL volume of 28 * 10^{-4} m^3. Soluble metal concentrations in the house samples for Al^{3+}, Cu^{2+}, and Mg^{2+} in our study ranged from 0 to 29 μg/mL. These appear to be biologically relevant doses, as reports of lung tissue loads

(analyzed from deceased males) from two continents ($n = 36$), were 43–67 mg/kg, and rat serum Al^{3+} levels were ∼21 μg/L (52). Magnesium levels remain fairly constant across all species, as all mammalian cells contain free cytosolic Mg^{2+} from 0.25 to 1 mM (53).

One of the limitations of our study is that all our experimental media had a pH of 7.4–7.54 and had identical ionic concentration at baseline. Therefore, our results are evaluating only antimicrobial peptides and proteins in the ASL, and not assessing other variables present in the ASL that could potentially affect antimicrobial activity. Additionally, our study is a pilot study, consisting of *in vitro* data and our sample size is a limitation in determining statistical significance.

Our study confirmed that NIST standard PM from the indoor environment affected mechanisms demonstrably culpable in the development of airway infection, including enhanced bacterial growth, biofilm formation and impaired ASL antimicrobial activity. Additionally, these results were translated in a high-risk study population (smokers) and we demonstrated that two of the same mechanisms (bacterial growth and biofilm formation) were recapitulated using IAP from homes of smokers, and additionally, ASL antimicrobial activity may be preferably compromised using the IAP from homes of subjects with history of frequent respiratory exacerbations. This finding was supported by the result that subjects with a mild GOLD status had a much higher OR of impairing innate immunity than those with a high GOLD status. We consider these mechanistic insights in a translational setting our study strengths.

CONCLUSION

We demonstrated that NIST IAP and Iowa house IAP enhanced bacterial growth and biofilm formation. NIST IAP significantly impaired ASL antimicrobial activity, while we found a trend of impaired ASL antimicrobial activity using Iowa house IAP from homes of subjects with a history of frequent respiratory exacerbations. We found that although specific metals may help explain mechanisms of impaired airway innate immunity, the effect of combined metals on airway innate immunity remains to be explored.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Iowa IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ESta, RM, JN, GP, ESto, OC, TH, and RB contributed to the data acquisition and analysis. ESta, TP, ESto, PP, JZ, and AC contributed to the concept and design of the work. ESta and AC drafted. XL, ESta, AC, and JZ revised the manuscript. All authors agree to be accountable for the work.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Environmental and Health Impacts of Air Pollution: A Review

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One of our era's greatest scourges is air pollution, on account not only of its impact on climate change but also its impact on public and individual health due to increasing morbidity and mortality. There are many pollutants that are major factors in disease in humans. Among them, Particulate Matter (PM), particles of variable but very small diameter, penetrate the respiratory system via inhalation, causing respiratory and cardiovascular diseases, reproductive and central nervous system dysfunctions, and cancer. Despite the fact that ozone in the stratosphere plays a protective role against ultraviolet irradiation, it is harmful when in high concentration at ground level, also affecting the respiratory and cardiovascular system. Furthermore, nitrogen oxide, sulfur dioxide, Volatile Organic Compounds (VOCs), dioxins, and polycyclic aromatic hydrocarbons (PAHs) are all considered air pollutants that are harmful to humans. Carbon monoxide can even provoke direct poisoning when breathed in at high levels. Heavy metals such as lead, when absorbed into the human body, can lead to direct poisoning or chronic intoxication, depending on exposure. Diseases occurring from the aforementioned substances include principally respiratory problems such as Chronic Obstructive Pulmonary Disease (COPD), asthma, bronchiolitis, and also lung cancer, cardiovascular events, central nervous system dysfunctions, and cutaneous diseases. Last but not least, climate change resulting from environmental pollution affects the geographical distribution of many infectious diseases, as do natural disasters. The only way to tackle this problem is through public awareness coupled with a multidisciplinary approach by scientific experts; national and international organizations must address the emergence of this threat and propose sustainable solutions.

Keywords: air pollution, environment, health, public health, gas emission, policy

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APPROACH TO THE PROBLEM

The interactions between humans and their physical surroundings have been extensively studied, as multiple human activities influence the environment. The environment is a coupling of the biotic (living organisms and microorganisms) and the abiotic (hydrosphere, lithosphere, and atmosphere).

Pollution is defined as the introduction into the environment of substances harmful to humans and other living organisms. Pollutants are harmful solids, liquids, or gases produced in higher than usual concentrations that reduce the quality of our environment.

Human activities have an adverse effect on the environment by polluting the water we drink, the air we breathe, and the soil in which plants grow. Although the industrial revolution was a great success in terms of technology, society, and the provision of multiple services, it also introduced the production of huge quantities of pollutants emitted into the air that are harmful to human health. Without any doubt, the global environmental pollution is considered an international public health issue with multiple facets. Social, economic, and legislative concerns and lifestyle habits are related to this major problem. Clearly, urbanization and industrialization are reaching unprecedented and upsetting proportions worldwide in our era. Anthropogenic air pollution is one of the biggest public health hazards worldwide, given that it accounts for about 9 million deaths per year (1).

Without a doubt, all of the aforementioned are closely associated with climate change, and in the event of danger, the consequences can be severe for mankind (2). Climate changes and the effects of global planetary warming seriously affect multiple ecosystems, causing problems such as food safety issues, ice and iceberg melting, animal extinction, and damage to plants (3, 4).

Air pollution has various health effects. The health of susceptible and sensitive individuals can be impacted even on low air pollution days. Short-term exposure to air pollutants is closely related to COPD (Chronic Obstructive Pulmonary Disease), cough, shortness of breath, wheezing, asthma, respiratory disease, and high rates of hospitalization (a measurement of morbidity).

The long-term effects associated with air pollution are chronic asthma, pulmonary insufficiency, cardiovascular diseases, and cardiovascular mortality. According to a Swedish cohort study, diabetes seems to be induced after long-term air pollution exposure (5). Moreover, air pollution seems to have various malign health effects in early human life, such as respiratory, cardiovascular, mental, and perinatal disorders (3), leading to infant mortality or chronic disease in adult age (6).

National reports have mentioned the increased risk of morbidity and mortality (1). These studies were conducted in many places around the world and show a correlation between daily ranges of particulate matter (PM) concentration and daily mortality. Climate shifts and global planetary warming (3) could aggravate the situation. Besides, increased hospitalization (an index of morbidity) has been registered among the elderly and susceptible individuals for specific reasons. Fine and ultrafine particulate matter seems to be associated with more serious illnesses (6), as it can invade the deepest parts of the airways and more easily reach the bloodstream.

Air pollution mainly affects those living in large urban areas, where road emissions contribute the most to the degradation of air quality. There is also a danger of industrial accidents, where the spread of a toxic fog can be fatal to the populations of the surrounding areas. The dispersion of pollutants is determined by many parameters, most notably atmospheric stability and wind (6).

In developing countries (7), the problem is more serious due to overpopulation and uncontrolled urbanization along with the development of industrialization. This leads to poor air quality, especially in countries with social disparities and a lack of information on sustainable management of the environment. The use of fuels such as wood fuel or solid fuel for domestic needs due to low incomes exposes people to bad-quality, polluted air at home. It is of note that three billion people around the world are using the above sources of energy for their daily heating and cooking needs (8). In developing countries, the women of the household seem to carry the highest risk for disease development due to their longer duration exposure to the indoor air pollution (8, 9). Due to its fast industrial development and overpopulation, China is one of the Asian countries confronting serious air pollution problems (10, 11). The lung cancer mortality observed in China is associated with fine particles (12). As stated already, long-term exposure is associated with deleterious effects on the cardiovascular system (3, 5). However, it is interesting to note that cardiovascular diseases have mostly been observed in developed and high-income countries rather than in the developing low-income countries exposed highly to air pollution (13). Extreme air pollution is recorded in India, where the air quality reaches hazardous levels. New Delhi is one of the more polluted cities in India. Flights in and out of New Delhi International Airport are often canceled due to the reduced visibility associated with air pollution. Pollution is occurring both in urban and rural areas in India due to the fast industrialization, urbanization, and rise in use of motorcycle transportation. Nevertheless, biomass combustion associated with heating and cooking needs and practices is a major source of household air pollution in India and in Nepal (14, 15). There is spatial heterogeneity in India, as areas with diverse climatological conditions and population and education levels generate different indoor air qualities, with higher $PM_{2.5}$ observed in North Indian states (557–601 $\mu g/m^3$) compared to the Southern States (183–214 $\mu g/m^3$) (16, 17). The cold climate of the North Indian areas may be the main reason for this, as longer periods at home and more heating are necessary compared to in the tropical climate of Southern India. Household air pollution in India is associated with major health effects, especially in women and young children, who stay indoors for longer periods. Chronic obstructive respiratory disease (CORD) and lung cancer are mostly observed in women, while acute lower respiratory disease is seen in young children under 5 years of age (18).

Accumulation of air pollution, especially sulfur dioxide and smoke, reaching 1,500 mg/m^3, resulted in an increase in the number of deaths (4,000 deaths) in December 1952 in London and in 1963 in New York City (400 deaths) (19). An association of pollution with mortality was reported on the basis of monitoring of outdoor pollution in six US metropolitan cities (20). In every case, it seems that mortality was closely related to the levels of fine, inhalable, and sulfate particles more than with the levels of total particulate pollution, aerosol acidity, sulfur dioxide, or nitrogen dioxide (20).

Furthermore, extremely high levels of pollution are reported in Mexico City and Rio de Janeiro, followed by Milan, Ankara, Melbourne, Tokyo, and Moscow (19).

Based on the magnitude of the public health impact, it is certain that different kinds of interventions should be taken into account. Success and effectiveness in controlling air pollution,

specifically at the local level, have been reported. Adequate technological means are applied considering the source and the nature of the emission as well as its impact on health and the environment. The importance of point sources and non-point sources of air pollution control is reported by Schwela and Köth-Jahr (21). Without a doubt, a detailed emission inventory must record all sources in a given area. Beyond considering the above sources and their nature, topography and meteorology should also be considered, as stated previously. Assessment of the control policies and methods is often extrapolated from the local to the regional and then to the global scale. Air pollution may be dispersed and transported from one region to another area located far away. Air pollution management means the reduction to acceptable levels or possible elimination of air pollutants whose presence in the air affects our health or the environmental ecosystem. Private and governmental entities and authorities implement actions to ensure the air quality (22). Air quality standards and guidelines were adopted for the different pollutants by the WHO and EPA as a tool for the management of air quality (1, 23). These standards have to be compared to the emissions inventory standards by causal analysis and dispersion modeling in order to reveal the problematic areas (24). Inventories are generally based on a combination of direct measurements and emissions modeling (24).

As an example, we state here the control measures at the source through the use of catalytic converters in cars. These are devices that turn the pollutants and toxic gases produced from combustion engines into less-toxic pollutants by catalysis through redox reactions (25). In Greece, the use of private cars was restricted by tracking their license plates in order to reduce traffic congestion during rush hour (25).

Concerning industrial emissions, collectors and closed systems can keep the air pollution to the minimal standards imposed by legislation (26).

Current strategies to improve air quality require an estimation of the economic value of the benefits gained from proposed programs. These proposed programs by public authorities, and directives are issued with guidelines to be respected.

In Europe, air quality limit values AQLVs (Air Quality Limit Values) are issued for setting off planning claims (27). In the USA, the NAAQS (National Ambient Air Quality Standards) establish the national air quality limit values (27). While both standards and directives are based on different mechanisms, significant success has been achieved in the reduction of overall emissions and associated health and environmental effects (27). The European Directive identifies geographical areas of risk exposure as monitoring/assessment zones to record the emission sources and levels of air pollution (27), whereas the USA establishes global geographical air quality criteria according to the severity of their air quality problem and records all sources of the pollutants and their precursors (27).

In this vein, funds have been financing, directly or indirectly, projects related to air quality along with the technical infrastructure to maintain good air quality. These plans focus on an inventory of databases from air quality environmental planning awareness campaigns. Moreover, pollution measures of air emissions may be taken for vehicles, machines, and industries in urban areas.

Technological innovation can only be successful if it is able to meet the needs of society. In this sense, technology must reflect the decision-making practices and procedures of those involved in risk assessment and evaluation and act as a facilitator in providing information and assessments to enable decision makers to make the best decisions possible. Summarizing the aforementioned in order to design an effective air quality control strategy, several aspects must be considered: environmental factors and ambient air quality conditions, engineering factors and air pollutant characteristics, and finally, economic operating costs for technological improvement and administrative and legal costs. Considering the economic factor, competitiveness through neoliberal concepts is offering a solution to environmental problems (22).

The development of environmental governance, along with technological progress, has initiated the deployment of a dialogue. Environmental politics has created objections and points of opposition between different political parties, scientists, media, and governmental and non-governmental organizations (22). Radical environmental activism actions and movements have been created (22). The rise of the new information and communication technologies (ICTs) are many times examined as to whether and in which way they have influenced means of communication and social movements such as activism (28). Since the 1990s, the term "digital activism" has been used increasingly and in many different disciplines (29). Nowadays, multiple digital technologies can be used to produce a digital activism outcome on environmental issues. More specifically, devices with online capabilities such as computers or mobile phones are being used as a way to pursue change in political and social affairs (30).

In the present paper, we focus on the sources of environmental pollution in relation to public health and propose some solutions and interventions that may be of interest to environmental legislators and decision makers.

SOURCES OF EXPOSURE

It is known that the majority of environmental pollutants are emitted through large-scale human activities such as the use of industrial machinery, power-producing stations, combustion engines, and cars. Because these activities are performed at such a large scale, they are by far the major contributors to air pollution, with cars estimated to be responsible for approximately 80% of today's pollution (31). Some other human activities are also influencing our environment to a lesser extent, such as field cultivation techniques, gas stations, fuel tanks heaters, and cleaning procedures (32), as well as several natural sources, such as volcanic and soil eruptions and forest fires.

The classification of air pollutants is based mainly on the sources producing pollution. Therefore, it is worth mentioning the four main sources, following the classification system: Major sources, Area sources, Mobile sources, and Natural sources.

Major sources include the emission of pollutants from power stations, refineries, and petrochemicals, the chemical and fertilizer industries, metallurgical and other industrial plants, and, finally, municipal incineration.

Indoor area sources include domestic cleaning activities, dry cleaners, printing shops, and petrol stations.

Mobile sources include automobiles, cars, railways, airways, and other types of vehicles.

Finally, *natural sources* include, as stated previously, physical disasters (33) such as forest fire, volcanic erosion, dust storms, and agricultural burning.

However, many classification systems have been proposed. Another type of classification is a grouping according to the recipient of the pollution, as follows:

Air pollution is determined as the presence of pollutants in the air in large quantities for long periods. Air pollutants are dispersed particles, hydrocarbons, CO, CO_2, NO, NO_2, SO_3, etc.

Water pollution is organic and inorganic charge and biological charge (10) at high levels that affect the water quality (34, 35).

Soil pollution occurs through the release of chemicals or the disposal of wastes, such as heavy metals, hydrocarbons, and pesticides.

Air pollution can influence the quality of soil and water bodies by polluting precipitation, falling into water and soil environments (34, 36). Notably, the chemistry of the soil can be amended due to acid precipitation by affecting plants, cultures, and water quality (37). Moreover, movement of heavy metals is favored by soil acidity, and metals are so then moving into the watery environment. It is known that heavy metals such as aluminum are noxious to wildlife and fishes. Soil quality seems to be of importance, as soils with low calcium carbonate levels are at increased jeopardy from acid rain. Over and above rain, snow and particulate matter drip into watery ' bodies (36, 38).

Lastly, pollution is classified following type of origin:

Radioactive and nuclear pollution, releasing radioactive and nuclear pollutants into water, air, and soil during nuclear explosions and accidents, from nuclear weapons, and through handling or disposal of radioactive sewage.

Radioactive materials can contaminate surface water bodies and, being noxious to the environment, plants, animals, and humans. It is known that several radioactive substances such as radium and uranium concentrate in the bones and can cause cancers (38, 39).

Noise pollution is produced by machines, vehicles, traffic noises, and musical installations that are harmful to our hearing.

The World Health Organization introduced the term DALYs. The DALYs for a disease or health condition is defined as the sum of the Years of Life Lost (YLL) due to premature mortality in the population and the Years Lost due to Disability (YLD) for people living with the health condition or its consequences (39). In Europe, air pollution is the main cause of disability-adjusted life years lost (DALYs), followed by noise pollution. The potential relationships of noise and air pollution with health have been studied (40). The study found that DALYs related to noise were more important than those related to air pollution, as the effects of environmental noise on cardiovascular disease were independent of air pollution (40). Environmental noise should be counted as an independent public health risk (40).

Environmental pollution occurs when changes in the physical, chemical, or biological constituents of the environment (air masses, temperature, climate, etc.) are produced.

Pollutants harm our environment either by increasing levels above normal or by introducing harmful toxic substances. Primary pollutants are directly produced from the above sources, and secondary pollutants are emitted as by-products of the primary ones. Pollutants can be biodegradable or non-biodegradable and of natural origin or anthropogenic, as stated previously. Moreover, their origin can be a unique source (point-source) or dispersed sources.

Pollutants have differences in physical and chemical properties, explaining the discrepancy in their capacity for producing toxic effects. As an example, we state here that aerosol compounds (41–43) have a greater toxicity than gaseous compounds due to their tiny size (solid or liquid) in the atmosphere; they have a greater penetration capacity. Gaseous compounds are eliminated more easily by our respiratory system (41). These particles are able to damage lungs and can even enter the bloodstream (41), leading to the premature deaths of millions of people yearly. Moreover, the aerosol acidity ([H+]) seems to considerably enhance the production of secondary organic aerosols (SOA), but this last aspect is not supported by other scientific teams (38).

CLIMATE AND POLLUTION

Air pollution and climate change are closely related. Climate is the other side of the same coin that reduces the quality of our Earth (44). Pollutants such as black carbon, methane, tropospheric ozone, and aerosols affect the amount of incoming sunlight. As a result, the temperature of the Earth is increasing, resulting in the melting of ice, icebergs, and glaciers.

In this vein, climatic changes will affect the incidence and prevalence of both residual and imported infections in Europe. Climate and weather affect the duration, timing, and intensity of outbreaks strongly and change the map of infectious diseases in the globe (45). Mosquito-transmitted parasitic or viral diseases are extremely climate-sensitive, as warming firstly shortens the pathogen incubation period and secondly shifts the geographic map of the vector. Similarly, water-warming following climate changes leads to a high incidence of waterborne infections. Recently, in Europe, eradicated diseases seem to be emerging due to the migration of population, for example, cholera, poliomyelitis, tick-borne encephalitis, and malaria (46).

The spread of epidemics is associated with natural climate disasters and storms, which seem to occur more frequently nowadays (47). Malnutrition and disequilibration of the immune system are also associated with the emerging infections affecting public health (48).

The Chikungunya virus "took the airplane" from the Indian Ocean to Europe, as outbreaks of the disease were registered in Italy (49) as well as autochthonous cases in France (50).

An increase in cryptosporidiosis in the United Kingdom and in the Czech Republic seems to have occurred following flooding (36, 51).

As stated previously, aerosols compounds are tiny in size and considerably affect the climate. They are able to dissipate sunlight (the albedo phenomenon) by dispersing a quarter of the sun's rays back to space and have cooled the global temperature over the last 30 years (52).

AIR POLLUTANTS

The World Health Organization (WHO) reports on six major air pollutants, namely particle pollution, ground-level ozone, carbon monoxide, sulfur oxides, nitrogen oxides, and lead. Air pollution can have a disastrous effect on all components of the environment, including groundwater, soil, and air. Additionally, it poses a serious threat to living organisms. In this vein, our interest is mainly to focus on these pollutants, as they are related to more extensive and severe problems in human health and environmental impact. Acid rain, global warming, the greenhouse effect, and climate changes have an important ecological impact on air pollution (53).

Particulate Matter (PM) and Health

Studies have shown a relationship between particulate matter (PM) and adverse health effects, focusing on either short-term (acute) or long-term (chronic) PM exposure.

Particulate matter (PM) is usually formed in the atmosphere as a result of chemical reactions between the different pollutants. The penetration of particles is closely dependent on their size (53). Particulate Matter (PM) was defined as a term for particles by the United States Environmental Protection Agency (54). Particulate matter (PM) pollution includes particles with diameters of 10 micrometers (μm) or smaller, called PM_{10}, and extremely fine particles with diameters that are generally 2.5 micrometers (μm) and smaller.

Particulate matter contains tiny liquid or solid droplets that can be inhaled and cause serious health effects (55). Particles <10 μm in diameter (PM_{10}) after inhalation can invade the lungs and even reach the bloodstream. Fine particles, $PM_{2.5}$, pose a greater risk to health (6, 56) (**Table 1**).

Multiple epidemiological studies have been performed on the health effects of PM. A positive relation was shown between both short-term and long-term exposures of $PM_{2.5}$ and acute nasopharyngitis (56). In addition, long-term exposure to PM for years was found to be related to cardiovascular diseases and infant mortality.

Those studies depend on $PM_{2.5}$ monitors and are restricted in terms of study area or city area due to a lack of spatially resolved daily $PM_{2.5}$ concentration data and, in this way, are not representative of the entire population. Following a recent epidemiological study by the Department of Environmental Health at Harvard School of Public Health (Boston, MA) (57), it was reported that, as $PM_{2.5}$ concentrations vary spatially, an exposure error (Berkson error) seems to be produced, and the relative magnitudes of the short- and long-term effects are not yet completely elucidated. The team developed a $PM_{2.5}$ exposure model based on remote sensing data for assessing short- and long-term human exposures (57). This model permits spatial resolution in short-term effects plus the assessment of long-term effects in the whole population.

Moreover, respiratory diseases and affection of the immune system are registered as long-term chronic effects (58). It is worth noting that people with asthma, pneumonia, diabetes, and respiratory and cardiovascular diseases are especially susceptible and vulnerable to the effects of PM. $PM_{2.5}$, followed by PM_{10}, are strongly associated with diverse respiratory system diseases (59), as their size permits them to pierce interior spaces (60). The particles produce toxic effects according to their chemical and physical properties. The components of PM_{10} and $PM_{2.5}$ can be organic (polycyclic aromatic hydrocarbons, dioxins, benzene, 1-3 butadiene) or inorganic (carbon, chlorides, nitrates, sulfates, metals) in nature (55).

Particulate Matter (PM) is divided into four main categories according to type and size (61) (**Table 2**).

Gas contaminants include PM in aerial masses.

Particulate contaminants include contaminants such as smog, soot, tobacco smoke, oil smoke, fly ash, and cement dust.

TABLE 1 | Penetrability according to particle size.

Particle size	Penetration degree in human respiratory system
>11 μm	Passage into nostrils and upper respiratory tract
7–11 μm	Passage into nasal cavity
4.7–7 μm	Passage into larynx
3.3–4.7 μm	Passage into trachea-bronchial area
2.1–3.3 μm	Secondary bronchial area passage
1.1–2.1 μm	Terminal bronchial area passage
0.65–1.1 μm	Bronchioles penetrability
0.43–0.65 μm	Alveolar penetrability

TABLE 2 | Types and sizes of particulate Matter (PM).

Type		PM diameter [μm]
Particulate contaminants	Smog	0.01–1
	Soot	0.01–0.8
	Tobacco smoke	0.01–1
	Fly ash	1–100
	Cement Dust	8–100
Biological Contaminants	Bacteria and bacterial spores	0.7–10
	Viruses	0.01–1
	Fungi and molds	2–12
	Allergens (dogs, cats, pollen, household dust)	0.1–100
Types of Dust	Atmospheric dust	0.01–1
	Heavy dust	100–1000
	Settling dust	1–100
Gases	Different gaseous contaminants	0.0001–0.01

Biological Contaminants are microorganisms (bacteria, viruses, fungi, mold, and bacterial spores), cat allergens, house dust and allergens, and pollen.

Types of Dust include suspended atmospheric dust, settling dust, and heavy dust.

Finally, another fact is that the half-lives of PM_{10} and $PM_{2.5}$ particles in the atmosphere is extended due to their tiny dimensions; this permits their long-lasting suspension in the atmosphere and even their transfer and spread to distant destinations where people and the environment may be exposed to the same magnitude of pollution (53). They are able to change the nutrient balance in watery ecosystems, damage forests and crops, and acidify water bodies.

As stated, $PM_{2.5}$, due to their tiny size, are causing more serious health effects. These aforementioned fine particles are the main cause of the "haze" formation in different metropolitan areas (12, 13, 61).

Ozone Impact in the Atmosphere

Ozone (O_3) is a gas formed from oxygen under high voltage electric discharge (62). It is a strong oxidant, 52% stronger than chlorine. It arises in the stratosphere, but it could also arise following chain reactions of photochemical smog in the troposphere (63).

Ozone can travel to distant areas from its initial source, moving with air masses (64). It is surprising that ozone levels over cities are low in contrast to the increased amounts occuring in urban areas, which could become harmful for cultures, forests, and vegetation (65) as it is reducing carbon assimilation (66). Ozone reduces growth and yield (47, 48) and affects the plant microflora due to its antimicrobial capacity (67, 68). In this regard, ozone acts upon other natural ecosystems, with microflora (69, 70) and animal species changing their species composition (71). Ozone increases DNA damage in epidermal keratinocytes and leads to impaired cellular function (72).

Ground-level ozone (GLO) is generated through a chemical reaction between oxides of nitrogen and VOCs emitted from natural sources and/or following anthropogenic activities.

Ozone uptake usually occurs by inhalation. Ozone affects the upper layers of the skin and the tear ducts (73). A study of short-term exposure of mice to high levels of ozone showed malondialdehyde formation in the upper skin (epidermis) but also depletion in vitamins C and E. It is likely that ozone levels are not interfering with the skin barrier function and integrity to predispose to skin disease (74).

Due to the low water-solubility of ozone, inhaled ozone has the capacity to penetrate deeply into the lungs (75).

Toxic effects induced by ozone are registered in urban areas all over the world, causing biochemical, morphologic, functional, and immunological disorders (76).

The European project (APHEA2) focuses on the acute effects of ambient ozone concentrations on mortality (77). Daily ozone concentrations compared to the daily number of deaths were reported from different European cities for a 3-year period. During the warm period of the year, an observed increase in ozone concentration was associated with an increase in the daily number of deaths (0.33%), in the number of respiratory deaths (1.13%), and in the number of cardiovascular deaths (0.45%). No effect was observed during wintertime.

Carbon Monoxide (CO)

Carbon monoxide is produced by fossil fuel when combustion is incomplete. The symptoms of poisoning due to inhaling carbon monoxide include headache, dizziness, weakness, nausea, vomiting, and, finally, loss of consciousness.

The affinity of carbon monoxide to hemoglobin is much greater than that of oxygen. In this vein, serious poisoning may occur in people exposed to high levels of carbon monoxide for a long period of time. Due to the loss of oxygen as a result of the competitive binding of carbon monoxide, hypoxia, ischemia, and cardiovascular disease are observed.

Carbon monoxide affects the greenhouses gases that are tightly connected to global warming and climate. This should lead to an increase in soil and water temperatures, and extreme weather conditions or storms may occur (68).

However, in laboratory and field experiments, it has been seen to produce increased plant growth (78).

Nitrogen Oxide (NO_2)

Nitrogen oxide is a traffic-related pollutant, as it is emitted from automobile motor engines (79, 80). It is an irritant of the respiratory system as it penetrates deep in the lung, inducing respiratory diseases, coughing, wheezing, dyspnea, bronchospasm, and even pulmonary edema when inhaled at high levels. It seems that concentrations over 0.2 ppm produce these adverse effects in humans, while concentrations higher than 2.0 ppm affect T-lymphocytes, particularly the CD8+ cells and NK cells that produce our immune response (81).It is reported that long-term exposure to high levels of nitrogen dioxide can be responsible for chronic lung disease. Long-term exposure to NO_2 can impair the sense of smell (81).

However, systems other than respiratory ones can be involved, as symptoms such as eye, throat, and nose irritation have been registered (81).

High levels of nitrogen dioxide are deleterious to crops and vegetation, as they have been observed to reduce crop yield and plant growth efficiency. Moreover, NO_2 can reduce visibility and discolor fabrics (81).

Sulfur Dioxide (SO_2)

Sulfur dioxide is a harmful gas that is emitted mainly from fossil fuel consumption or industrial activities. The annual standard for SO_2 is 0.03 ppm (82). It affects human, animal, and plant life. Susceptible people as those with lung disease, old people, and children, who present a higher risk of damage. The major health problems associated with sulfur dioxide emissions in industrialized areas are respiratory irritation, bronchitis, mucus production, and bronchospasm, as it is a sensory irritant and penetrates deep into the lung converted into bisulfite and interacting with sensory receptors, causing bronchoconstriction. Moreover, skin redness, damage to the eyes (lacrimation and

corneal opacity) and mucous membranes, and worsening of pre-existing cardiovascular disease have been observed (81).

Environmental adverse effects, such as acidification of soil and acid rain, seem to be associated with sulfur dioxide emissions (83).

Lead

Lead is a heavy metal used in different industrial plants and emitted from some petrol motor engines, batteries, radiators, waste incinerators, and waste waters (84).

Moreover, major sources of lead pollution in the air are metals, ore, and piston-engine aircraft. Lead poisoning is a threat to public health due to its deleterious effects upon humans, animals, and the environment, especially in the developing countries.

Exposure to lead can occur through inhalation, ingestion, and dermal absorption. Trans- placental transport of lead was also reported, as lead passes through the placenta unencumbered (85). The younger the fetus is, the more harmful the toxic effects. Lead toxicity affects the fetal nervous system; edema or swelling of the brain is observed (86). Lead, when inhaled, accumulates in the blood, soft tissue, liver, lung, bones, and cardiovascular, nervous, and reproductive systems. Moreover, loss of concentration and memory, as well as muscle and joint pain, were observed in adults (85, 86).

Children and newborns (87) are extremely susceptible even to minimal doses of lead, as it is a neurotoxicant and causes learning disabilities, impairment of memory, hyperactivity, and even mental retardation.

Elevated amounts of lead in the environment are harmful to plants and crop growth. Neurological effects are observed in vertebrates and animals in association with high lead levels (88).

Polycyclic Aromatic Hydrocarbons(PAHs)

The distribution of PAHs is ubiquitous in the environment, as the atmosphere is the most important means of their dispersal. They are found in coal and in tar sediments. Moreover, they are generated through incomplete combustion of organic matter as in the cases of forest fires, incineration, and engines (89). PAH compounds, such as benzopyrene, acenaphthylene, anthracene, and fluoranthene are recognized as toxic, mutagenic, and carcinogenic substances. They are an important risk factor for lung cancer (89).

Volatile Organic Compounds(VOCs)

Volatile organic compounds (VOCs), such as toluene, benzene, ethylbenzene, and xylene (90), have been found to be associated with cancer in humans (91). The use of new products and materials has actually resulted in increased concentrations of VOCs. VOCs pollute indoor air (90) and may have adverse effects on human health (91). Short-term and long-term adverse effects on human health are observed. VOCs are responsible for indoor air smells. Short-term exposure is found to cause irritation of eyes, nose, throat, and mucosal membranes, while those of long duration exposure include toxic reactions (92). Predictable assessment of the toxic effects of complex VOC mixtures is difficult to estimate, as these pollutants can have synergic, antagonistic, or indifferent effects (91, 93).

Dioxins

Dioxins originate from industrial processes but also come from natural processes, such as forest fires and volcanic eruptions. They accumulate in foods such as meat and dairy products, fish and shellfish, and especially in the fatty tissue of animals (94).

Short-period exhibition to high dioxin concentrations may result in dark spots and lesions on the skin (94). Long-term exposure to dioxins can cause developmental problems, impairment of the immune, endocrine and nervous systems, reproductive infertility, and cancer (94).

Without any doubt, fossil fuel consumption is responsible for a sizeable part of air contamination. This contamination may be anthropogenic, as in agricultural and industrial processes or transportation, while contamination from natural sources is also possible. Interestingly, it is of note that the air quality standards established through the European Air Quality Directive are somewhat looser than the WHO guidelines, which are stricter (95).

EFFECT OF AIR POLLUTION ON HEALTH

The most common air pollutants are ground-level ozone and Particulates Matter (PM). Air pollution is distinguished into two main types:

Outdoor pollution is the ambient air pollution.

Indoor pollution is the pollution generated by household combustion of fuels.

People exposed to high concentrations of air pollutants experience disease symptoms and states of greater and lesser seriousness. These effects are grouped into short- and long-term effects affecting health.

Susceptible populations that need to be aware of health protection measures include old people, children, and people with diabetes and predisposing heart or lung disease, especially asthma.

As extensively stated previously, according to a recent epidemiological study from Harvard School of Public Health, the relative magnitudes of the short- and long-term effects have not been completely clarified (57) due to the different epidemiological methodologies and to the exposure errors. New models are proposed for assessing short- and long-term human exposure data more successfully (57). Thus, in the present section, we report the more common short- and long-term health effects but also general concerns for both types of effects, as these effects are often dependent on environmental conditions, dose, and individual susceptibility.

Short-term effects are temporary and range from simple discomfort, such as irritation of the eyes, nose, skin, throat, wheezing, coughing and chest tightness, and breathing difficulties, to more serious states, such as asthma, pneumonia, bronchitis, and lung and heart problems. Short-term exposure to air pollution can also cause headaches, nausea, and dizziness.

These problems can be aggravated by extended long-term exposure to the pollutants, which is harmful to the neurological, reproductive, and respiratory systems and causes cancer and even, rarely, deaths.

The long-term effects are chronic, lasting for years or the whole life and can even lead to death. Furthermore, the toxicity of several air pollutants may also induce a variety of cancers in the long term (96).

As stated already, respiratory disorders are closely associated with the inhalation of air pollutants. These pollutants will invade through the airways and will accumulate at the cells. Damage to target cells should be related to the pollutant component involved and its source and dose. Health effects are also closely dependent on country, area, season, and time. An extended exposure duration to the pollutant should incline to long-term health effects in relation also to the above factors.

Particulate Matter (PMs), dust, benzene, and O_3 cause serious damage to the respiratory system (97). Moreover, there is a supplementary risk in case of existing respiratory disease such as asthma (98). Long-term effects are more frequent in people with a predisposing disease state. When the trachea is contaminated by pollutants, voice alterations may be remarked after acute exposure. Chronic obstructive pulmonary disease (COPD) may be induced following air pollution, increasing morbidity and mortality (99). Long-term effects from traffic, industrial air pollution, and combustion of fuels are the major factors for COPD risk (99).

Multiple cardiovascular effects have been observed after exposure to air pollutants (100). Changes occurred in blood cells after long-term exposure may affect cardiac functionality. Coronary arteriosclerosis was reported following long-term exposure to traffic emissions (101), while short-term exposure is related to hypertension, stroke, myocardial infracts, and heart insufficiency. Ventricle hypertrophy is reported to occur in humans after long-time exposure to nitrogen oxide (NO_2) (102, 103).

Neurological effects have been observed in adults and children after extended-term exposure to air pollutants.

Psychological complications, autism, retinopathy, fetal growth, and low birth weight seem to be related to long-term air pollution (83). The etiologic agent of the neurodegenerative diseases (Alzheimer's and Parkinson's) is not yet known, although it is believed that extended exposure to air pollution seems to be a factor. Specifically, pesticides and metals are cited as etiological factors, together with diet. The mechanisms in the development of neurodegenerative disease include oxidative stress, protein aggregation, inflammation, and mitochondrial impairment in neurons (104) (**Figure 1**).

Brain inflammation was observed in dogs living in a highly polluted area in Mexico for a long period (105). In human adults, markers of systemic inflammation (IL-6 and fibrinogen) were found to be increased as an immediate response to PNC on the IL-6 level, possibly leading to the production of acute-phase proteins (106). The progression of atherosclerosis and oxidative stress seem to be the mechanisms involved in the neurological disturbances caused by long-term air pollution. Inflammation comes secondary to the oxidative stress and seems to be involved in the impairment of developmental maturation, affecting multiple organs (105, 107). Similarly, other factors seem to be involved in the developmental maturation, which define the vulnerability to long-term air pollution. These include birthweight, maternal smoking, genetic background and socioeconomic environment, as well as education level.

However, diet, starting from breast-feeding, is another determinant factor. Diet is the main source of antioxidants, which play a key role in our protection against air pollutants (108). Antioxidants are free radical scavengers and limit the interaction of free radicals in the brain (108). Similarly, genetic background may result in a differential susceptibility toward the oxidative stress pathway (60). For example, antioxidant supplementation with vitamins C and E appears to modulate the effect of ozone in asthmatic children homozygous for the GSTM1 null allele (61). Inflammatory cytokines released in the periphery (e.g., respiratory epithelia) upregulate the innate immune Toll-like receptor 2. Such activation and the subsequent events leading to neurodegeneration have recently been observed in lung lavage in mice exposed to ambient Los Angeles (CA, USA) particulate matter (61). In children, neurodevelopmental morbidities were observed after lead exposure. These children developed aggressive and delinquent behavior, reduced intelligence, learning difficulties, and hyperactivity (109). No level of lead exposure seems to be "safe," and the scientific community has asked the Centers for Disease Control and Prevention (CDC) to reduce the current screening guideline of 10 μg/dl (109).

It is important to state that impact on the immune system, causing dysfunction and neuroinflammation (104), is related to poor air quality. Yet, increases in serum levels of immunoglobulins (IgA, IgM) and the complement component C3 are observed (106). Another issue is that antigen presentation is affected by air pollutants, as there is an upregulation of costimulatory molecules such as CD80 and CD86 on macrophages (110).

As is known, skin is our shield against ultraviolet radiation (UVR) and other pollutants, as it is the most exterior layer of our body. Traffic-related pollutants, such as PAHs, VOCs, oxides, and PM, may cause pigmented spots on our skin (111). On the one hand, as already stated, when pollutants penetrate through the skin or are inhaled, damage to the organs is observed, as some of these pollutants are mutagenic and carcinogenic, and, specifically, they affect the liver and lung. On the other hand, air pollutants (and those in the troposphere) reduce the adverse effects of ultraviolet radiation UVR in polluted urban areas (111). Air pollutants absorbed by the human skin may contribute to skin aging, psoriasis, acne, urticaria, eczema, and atopic dermatitis (111), usually caused by exposure to oxides and photochemical smoke (111). Exposure to PM and cigarette smoking act as skin-aging agents, causing spots, dyschromia, and wrinkles. Lastly, pollutants have been associated with skin cancer (111).

Higher morbidity is reported to fetuses and children when exposed to the above dangers. Impairment in fetal growth, low birth weight, and autism have been reported (112).

Another exterior organ that may be affected is the eye. Contamination usually comes from suspended pollutants and may result in asymptomatic eye outcomes, irritation (112), retinopathy, or dry eye syndrome (113, 114).

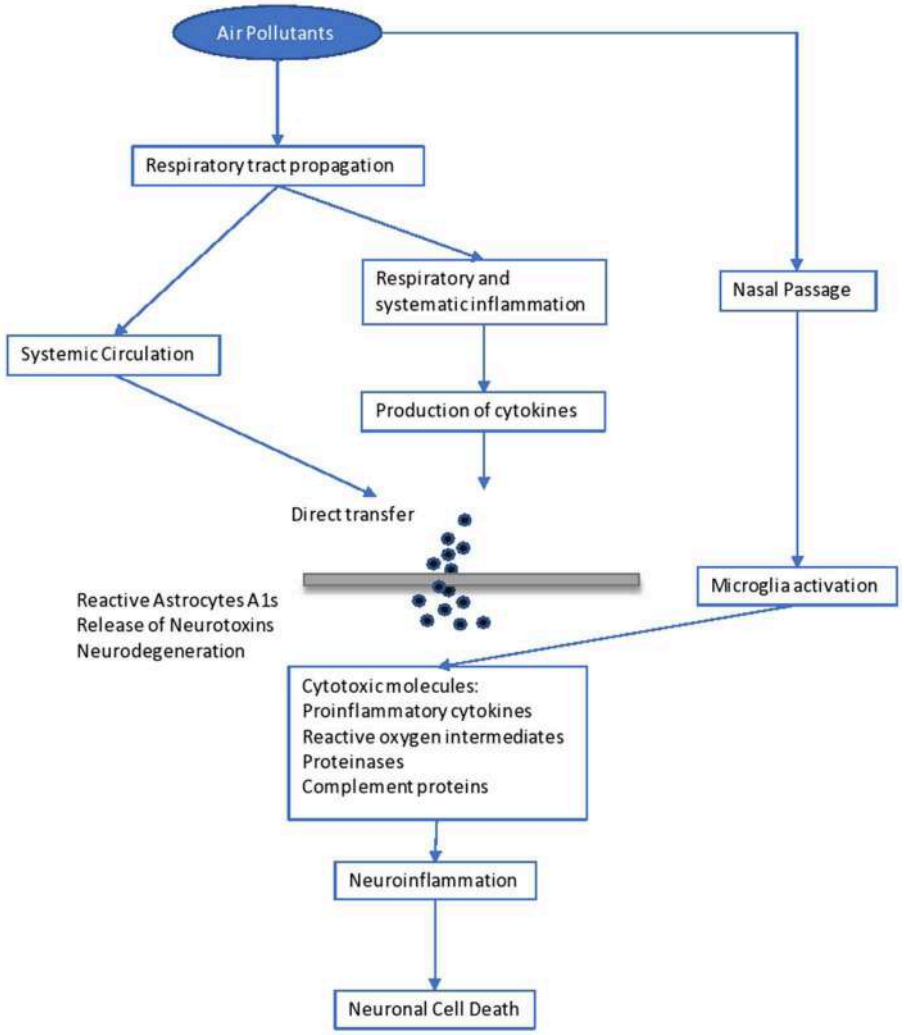

FIGURE 1 | Impact of air pollutants on the brain.

ENVIRONMENTAL IMPACT OF AIR POLLUTION

Air pollution is harming not only human health but also the environment (115) in which we live. The most important environmental effects are as follows.

Acid rain is wet (rain, fog, snow) or dry (particulates and gas) precipitation containing toxic amounts of nitric and sulfuric acids. They are able to acidify the water and soil environments, damage trees and plantations, and even damage buildings and outdoor sculptures, constructions, and statues.

Haze is produced when fine particles are dispersed in the air and reduce the transparency of the atmosphere. It is caused by gas emissions in the air coming from industrial facilities, power plants, automobiles, and trucks.

Ozone, as discussed previously, occurs both at ground level and in the upper level (stratosphere) of the Earth's atmosphere. Stratospheric ozone is protecting us from the Sun's harmful ultraviolet (UV) rays. In contrast, ground-level ozone is harmful to human health and is a pollutant. Unfortunately, stratospheric ozone is gradually damaged by ozone-depleting substances (i.e., chemicals, pesticides, and aerosols). If this protecting stratospheric ozone layer is thinned, then UV radiation can reach our Earth, with harmful effects for human life (skin cancer) (116) and crops (117). In plants, ozone penetrates through the stomata, inducing them to close, which blocks CO_2 transfer and induces a reduction in photosynthesis (118).

Global climate change is an important issue that concerns mankind. As is known, the "greenhouse effect" keeps the Earth's temperature stable. Unhappily, anthropogenic activities have destroyed this protecting temperature effect by producing large amounts of greenhouse gases, and global warming is mounting, with harmful effects on human health, animals, forests, wildlife, agriculture, and the water environment. A report states that global warming is adding to the health risks of poor people (119).

People living in poorly constructed buildings in warm-climate countries are at high risk for heat-related health problems as temperatures mount (119).

Wildlife is burdened by toxic pollutants coming from the air, soil, or the water ecosystem and, in this way, animals can develop health problems when exposed to high levels of pollutants. Reproductive failure and birth effects have been reported.

Eutrophication is occurring when elevated concentrations of nutrients (especially nitrogen) stimulate the blooming of aquatic algae, which can cause a disequilibration in the diversity of fish and their deaths.

Without a doubt, there is a critical concentration of pollution that an ecosystem can tolerate without being destroyed, which is associated with the ecosystem's capacity to neutralize acidity. The Canada Acid Rain Program established this load at 20 kg/ha/yr (120).

Hence, air pollution has deleterious effects on both soil and water (121). Concerning PM as an air pollutant, its impact on crop yield and food productivity has been reported. Its impact on watery bodies is associated with the survival of living organisms and fishes and their productivity potential (121).

An impairment in photosynthetic rhythm and metabolism is observed in plants exposed to the effects of ozone (121).

Sulfur and nitrogen oxides are involved in the formation of acid rain and are harmful to plants and marine organisms.

Last but not least, as mentioned above, the toxicity associated with lead and other metals is the main threat to our ecosystems (air, water, and soil) and living creatures (121).

DISCUSSION

In 2018, during the first WHO Global Conference on Air Pollution and Health, the WHO's General Director, Dr. Tedros Adhanom Ghebreyesus, called air pollution a "silent public health emergency" and "the new tobacco" (122).

Undoubtedly, children are particularly vulnerable to air pollution, especially during their development. Air pollution has adverse effects on our lives in many different respects.

Diseases associated with air pollution have not only an important economic impact but also a societal impact due to absences from productive work and school.

Despite the difficulty of eradicating the problem of anthropogenic environmental pollution, a successful solution could be envisaged as a tight collaboration of authorities, bodies, and doctors to regularize the situation. Governments should spread sufficient information and educate people and should involve professionals in these issues so as to control the emergence of the problem successfully.

Technologies to reduce air pollution at the source must be established and should be used in all industries and power plants. The Kyoto Protocol of 1997 set as a major target the reduction of GHG emissions to below 5% by 2012 (123). This was followed by the Copenhagen summit, 2009 (124), and then the Durban summit of 2011 (125), where it was decided to keep to the same line of action. The Kyoto protocol and the subsequent ones were ratified by many countries. Among the pioneers who adopted this important protocol for the world's environmental and climate "health" was China (3). As is known, China is a fast-developing economy and its GDP (Gross Domestic Product) is expected to be very high by 2050, which is defined as the year of dissolution of the protocol for the decrease in gas emissions.

A more recent international agreement of crucial importance for climate change is the Paris Agreement of 2015, issued by the UNFCCC (United Nations Climate Change Committee). This latest agreement was ratified by a plethora of UN (United Nations) countries as well as the countries of the European Union (126). In this vein, parties should promote actions and measures to enhance numerous aspects around the subject. Boosting education, training, public awareness, and public participation are some of the relevant actions for maximizing the opportunities to achieve the targets and goals on the crucial matter of climate change and environmental pollution (126). Without any doubt, technological improvements makes our world easier and it seems difficult to reduce the harmful impact caused by gas emissions, we could limit its use by seeking reliable approaches.

Synopsizing, a global prevention policy should be designed in order to combat anthropogenic air pollution as a complement to the correct handling of the adverse health effects associated with air pollution. Sustainable development practices should be applied, together with information coming from research in order to handle the problem effectively.

At this point, international cooperation in terms of research, development, administration policy, monitoring, and politics is vital for effective pollution control. Legislation concerning air pollution must be aligned and updated, and policy makers should propose the design of a powerful tool of environmental and health protection. As a result, the main proposal of this essay is that we should focus on fostering local structures to promote experience and practice and extrapolate these to the international level through developing effective policies for sustainable management of ecosystems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Environmental Status of Italian Coastal Marine Areas Affected by Long History of Contamination

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In the first decades of 2000s, several Italian sites affected by strong anthropogenic impact were recognized as Sites of National Interest (SINs) for a successive reclamation project, some of which also including marine sectors. These coastal areas are characterized by high complexity and diversity as regards the natural setting as well as for extent, history, type, and degree of contamination. For this, the Italian Ministry of Environment charged its scientific research Institute (earlier ICRAM, now ISPRA) with planning a flexible, adaptable, and large-scale environmental characterization. In this context, the investigation of marine sediments was identified as the primary target to assess the environmental status, because of their conservative capacity with respect to contaminants and their role in the exchange processes with other environmental matrices, such as water column and aquatic organisms. A multidisciplinary, chemical–physical, and ecotoxicological survey was identified as the most appropriate and objective criterion for assessing the sediment quality associated, when necessary, with integrative studies. The results derived from this multidisciplinary approach highlighted the main sources of contamination, together with size and extent of the environmental impact on the coastal marine areas, strictly correlated with the kind of anthropogenic activities and coastal morphology. In order to underline how the different environmental setting influences the degree of anthropogenic impact, four different case studies, selected among the more complex by geochemical and geomorphological viewpoints and more extensively studied, were considered. A comprehensive evaluation of these case studies allowed to deduce some general principles concerning the effects of anthropogenic impact, which can be applicable to other transitional and marine coastal areas.

Keywords: sediment contamination, Sites of National Interest, coastal areas, anthropogenic impact, Italy

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INTRODUCTION

In the last century, part of the Italian territory has been subjected to a strong impact due to the increasing presence of several industrial, mining, and agricultural activities, which gradually determined its environmental decay. Consequently, the Italian Government adopted a national program (D.M. 471/99), similar to that defined by the U.S. Government "Comprehensive Environmental Response, Compensation and Liability Act" (National Research Council 2007), to protect human health and terrestrial and marine environment from hazardous substances

released in the most impacted areas. This program, based on several environmental criteria (i.e., site characteristics, quantity, and hazard of pollutants), identified many contaminated areas (d.lgs. 22/97; d.lgs. 152/2006), which required cleanup actions, that were defined as Sites of National Interest (SINs). They were initially 57, 26 of which included marine or transitional areas; successively, the overall number was gradually reduced to 39 in response to improvements in environmental characterization or change of the managing administration (Regional Relevance Site, D.M. 11 January 2013); among the sites including marine areas, the declassed ones were as follows: Basso Bacino del Fiume Chienti, Fiume Saline Alento, Grado e Marano, Litorale Domizio Flegreo, La Maddalena, Litorale Vesuviano, Massa Carrara, Pitelli, and Venezia. At the moment, only 17 SINs including marine or transitional areas are still active (**Figure 1** and **Table 1**).

The marine areas included in the SINs are representative of sandy and rocky coast lines, lagoons, harbors, and river mouths. They are all subjected to a combination of impacts derived from industrial activities (i.e., chemical, petrochemical, metallurgical, steel, mechanical, pharmaceutical, cement, thermal, or thermoelectric plants), incinerators, uncontrolled landfills, intensive agricultural activities, military arsenals, shipyards, and high maritime traffic, which altered the natural conditions. In some cases, the high naturalistic, economic, and touristic values of the area coexist with the anthropogenic activities (Lenzi et al., 2005; Frontalini et al., 2009; Cardellicchio et al., 2015; Romano et al., 2015, 2016, 2018b; Cannata et al., 2016). The supply of contaminants to the aquatic environment may occur for direct discharge to the sea, contribution of runoff from contaminated lands, atmospheric deposition, and so on. In this context, marine and transitional sediments play a fundamental role, as they are the final sink of most of the toxic, persistent, and bioaccumulative (Kowalewska et al., 2011). In most cases, although the supply of contaminants ceased owing to the closure of the plants or the implementation of environmental legislation and accompanying treatment technologies, the contamination persists as a legacy (Croudace et al., 2015). In other cases, the supply is still active for both the continuation of polluting activities and the presence of waste materials as secondary source, for example, in dismissed mining sites where large of mine residues are still subjected to processes of weathering (Romano et al., 2017).

TABLE 1 | Details on the current SINs including a marine costal area.

Region	ID	SIN	Legislative reference	Total area (ha)	
				Sea	Land
Friuli VG	24	Trieste	D.M. 468/2001	1,196	506
Liguria	27	Cogoleto	D.M. 468/2001	167	45
Toscana	9	Piombino	L. 426/1998	2,117	931
	36	Livorno	D.M. 468/2001	577	206
	47	Orbetello	L. 179/2002	2,645	204
Marche	44	Falconara Marittima	L. 179/2002	1,165	108
Campania	2	Napoli	L. 426/1998	1,433	834
	17	Bagnoli	L. 388/2000	1,453	249
Puglia	5	Manfredonia	L. 426/1998	855	303
	6	Brindisi	L. 426/1998	5,597	5,851
	7	Taranto	L. 426/1998	7,006	4,383
Calabria	21	Crotone	D.M. 468/2001	1,448	884
Sicilia	3	Gela	L. 426/1998	4,583	795
	4	Priolo	L. 426/1998	10,129	5,814
	53	Milazzo	L. 266/2005	2,198	549
Sardegna	34	Sulcis – Iglesiente	D.M. 468/2001	32,416	19,751
	49	Porto Torres	L. 179/2002	2,748	1,874

The main contaminants are metals and trace elements, heavy hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), organochlorine pesticides, dioxins and furans, chlorinated organic solvents, and organotin compounds (TBTs). Most of them, owing to their hazardous and/or persistent characteristics, belong to the priority list of the European Water Framework Directive (2000/60/EC); in fact, they were demonstrated to be a threat for the aquatic environment, having adverse effects on organisms and also bioaccumulating in their organs and tissues, so representing a potential risk for human health (Suedel et al., 1994; Atkinson et al., 2007).

In addition to the chemical contamination, some sites underwent also physical changes, like as strong modification of the coastline due to the construction of industrial and harbor facilities, which influenced water circulation, sediment transport, and deposition as well as contaminant distribution (Romano et al., 2009a).

In order to establish an adequate investigation strategy for the marine coastal areas, not yet provided for by Italian legislation, in 2001, the Ministry of Environment (MoE) tasked its scientific research institute (earlier ICRAM, now ISPRA) with designing a flexible and large-scale monitoring program aimed at obtaining an integrated assessment of environmental quality, taking into account extension, history, type, and degree of contamination, and also natural features such as morphology, geology, geochemistry, and hydrodynamics.

This review presents the methodological approach used to characterize these areas and the obtained results. It then explains how this approach was successfully applied to elucidate the different responses to the anthropogenic impact as a function of geological/geomorphological setting and hydrodynamic conditions. A special focus was committed to four case studies because of their complex environmental scenario and long-lasting anthropogenic impact, in order to deduce criteria of general interest concerning the effects of anthropogenic impact in transitional and marine coastal areas and the approach to be adapted according to specific conditions.

INVESTIGATION APPROACH

The investigation strategy was planned taking into account the main European legislations on the environmental topics in force at that time, as Water Framework Directive (2000/60/EC), Barcelona (1976), OSPAR (1992), and HELCOM (1992) Conventions. The environmental assessment consisted on

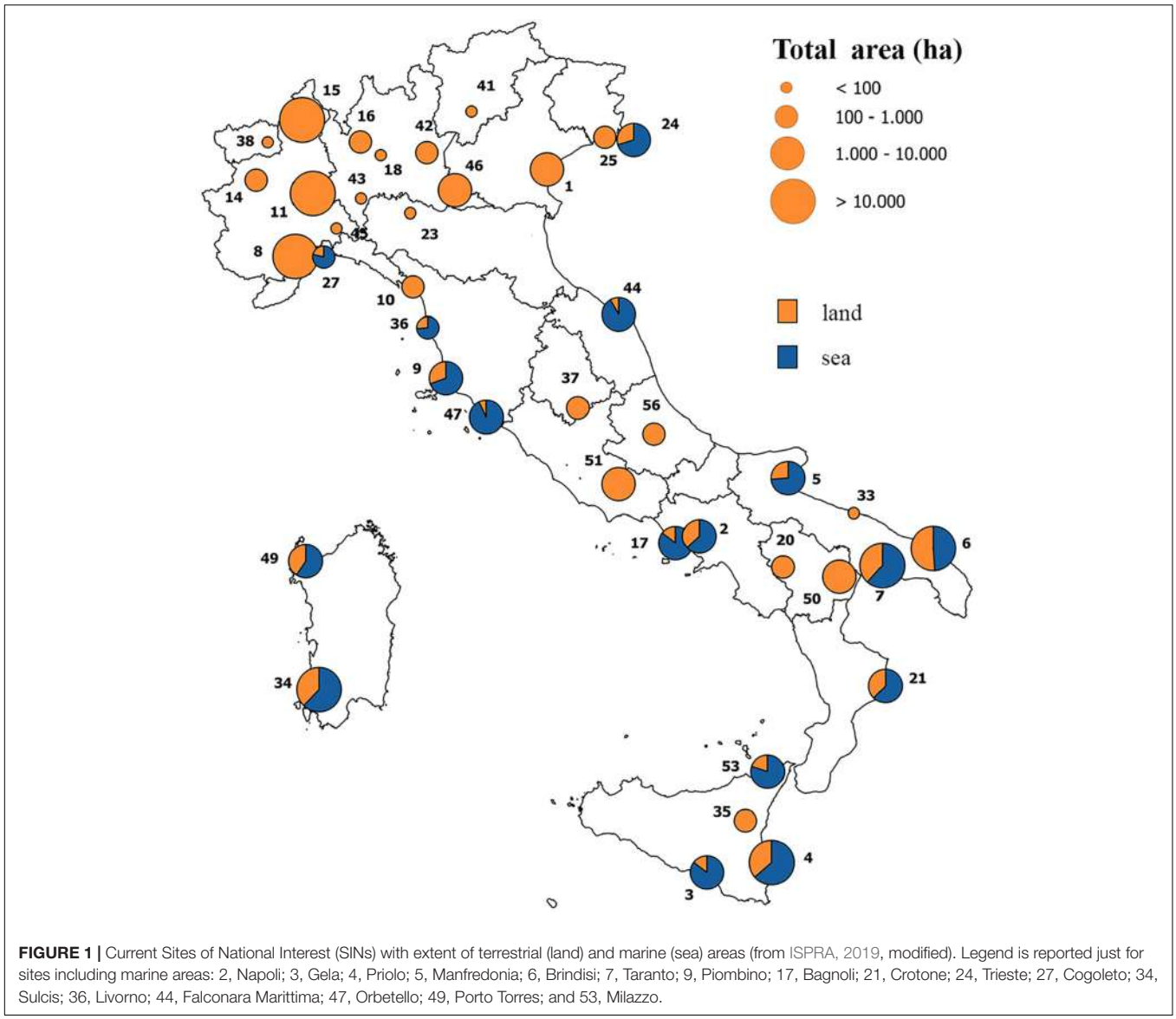

FIGURE 1 | Current Sites of National Interest (SINs) with extent of terrestrial (land) and marine (sea) areas (from ISPRA, 2019, modified). Legend is reported just for sites including marine areas: 2, Napoli; 3, Gela; 4, Priolo; 5, Manfredonia; 6, Brindisi; 7, Taranto; 9, Piombino; 17, Bagnoli; 21, Crotone; 24, Trieste; 27, Cogoleto; 34, Sulcis; 36, Livorno; 44, Falconara Marittima; 47, Orbetello; 49, Porto Torres; and 53, Milazzo.

summarizing the distribution of contaminants in the marine areas, taking into account all the environmental matrices (sediments, water, and biota) and identifying the potential risk for the aquatic environment and/or human health in relation to their uses (fish life, fishing, aquaculture, bathing, recreational uses, etc.). Among the three matrices, priority was given to the study of sediments because they represent the final sink of most anthropogenic contaminants and are considered the most conservative matrix playing a primary role in the accumulation of contaminants through various types of processes, from the simple deposition of suspended particles to adsorption by clay minerals, as well as in their redistribution, through biotic and abiotic exchange processes at the interface with water column (Li et al., 2000; Santschi et al., 2001; Spencer and Macleod, 2002). Water and biota were considered in a second phase of the investigation owing to the highly dynamic and variable property of the water column and the different responses of the biota that can vary significantly according to seasonal variations, type of contamination and species, gender, tissue, size, and reproductive status considered (Birch, 2017).

A regular and systematic sampling strategy for the sediment characterization, according to the same principle used for geochemical mapping (Reimann et al., 2010, 2015), adaptable to the features (type and extension of the study area) and environmental settings (i.e., industrial plants), was adopted (**Figure 2**). The sampling scheme was organized in grids of variable size (from 50 × 50 m to 450 × 450 m), according to the complexity of the site, and transects to cover the remaining areas. A sediment core (from 2 up to 5 m, according to the distance from the contamination source) in each grid and a variable number of superficial samples on the transects were planned (**Figure 2**).

Standardized levels (0–10, 10–30, 30–50, 100–120, 180–200, 280–300, 380–400, and 480–500 cm) from sediment cores were defined and finalized to physical, chemical, and ecotoxicological

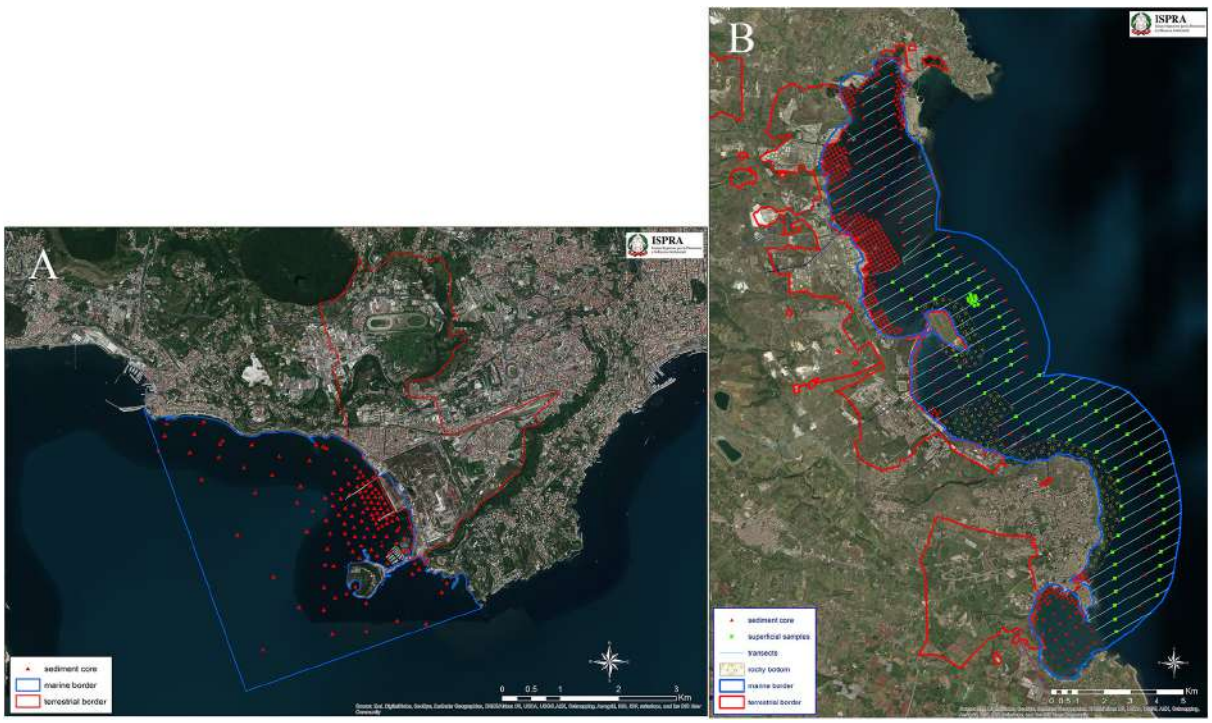

FIGURE 2 | Examples of sediment sampling srategy. Sites of National Interest (SINs) Bagnoli **(A)** and Priolo **(B)**.

analyses. Also, standard chemical–physical analyses [such as grain size, total organic carbon (TOC), metals and trace elements, PAHs, PCBs, heavy hydrocarbons, and chlorinated pesticides] were planned for all the samples; a number of additional chemical analyses (chloroaromatics, phenols, aromatic solvents, TBTs, dioxins, and furans) were also included in the monitoring scheme, together with ecotoxicological and microbiological analyses, provided that they were directly connected to the known industrial activities in each area.

In geochemically anomalous areas, where high metal and trace element concentrations can be naturally enriched in the sediments, it was considered necessary to discriminate the geogenic contribution from the anthropogenic one (Xu et al., 2014; Romano et al., 2015, 2018a; Guo and Yang, 2016; Birch, 2017, among the most recent ones). For this purpose, some sediment cores, sampled at high resolution (2 cm levels) and representative of the whole site, were used for reconstructing the evolution of contamination and determining the local background values (BGVs) (Matschullat et al., 2000; Apitz et al., 2009; Romano et al., 2015, 2018a).

In order to highlight the bioavailability of contaminants and assess the potential risk of contamination in the aquatic environment, specific bioaccumulation (Ausili et al., 2008; Signa et al., 2017), and biomarker (Losso et al., 2004; Annicchiarico et al., 2007) investigations on marine organisms, such as bivalves and some nekton and benthic species, were included in the investigation plans.

Owing to the lack of reference values for assessing environmental status in anthropized marine coastal areas, with the only exception of the Environmental Quality Standards (EQS) for sediments (D.M. 56/09, D.M. 260/10), as defined by the Italian Government according to 2000/60/CE, but not applicable for impacted areas, ICRAM defined site-specific "action levels" according to the current scientific literature (Burton, 2002; Wenning et al., 2005; den Besten, 2007) and the main approaches already applied by the USA (U. S. Environmental Protection Agency, 1996, 1997), Canada (CCME, 2001; Macfarlane and MacDonald, 2002), and Europe (van de Meent et al., 1990; Ospar Commission, 2004a,b), taking into account the peculiar geochemical characteristics along the Italian coast.

A chemical–physical and ecotoxicological approach was identified as the most appropriate and objective criterion for assessing these values. Specifically, the "effect level approach," which statistically associates chemical and biological data to define sediment concentration levels with rare toxic effects [threshold effect level (TEL)] and levels above which the effects are frequently encountered [probable effect level (PEL)], was adopted (Long et al., 1995; MacDonald et al., 1996, 2004). After that, action levels were determined by taking into account site-specific chemical and ecotoxicological data for each SIN, according to standardized procedures by national and/or international bodies such as ISO, USEPA, ASTM, and UNI (**Table 2**).

The analytical data were processed by applying geostatistical methodologies in order to reconstruct the vertical and spatial distribution of contaminants, allowing an easier data managing aimed at the three-dimensional

TABLE 2 | Site-specific action levels for metals and trace elements (mg kg^{-1} d.w.), as determined by ICRAM.

Metals/Action levels	Pitelli	Massa-Carrara	Livorno	Piombino	Orbetello	Bagnoli	Priolo	Crotone	Brindisi	Taranto	Venezia
As	45	42	37	42	18	50	32	42	20	20	22
Cd	1.0	0.8	0.8	1.0	1.3	1.0	1.0	1.0	1.0	1.0	1.4
Cr	250	250	180	175	69	160	150	160	100	70*/160**	90
Cu	65	65	90	95	50	65	75	52	45	45	45
Hg	0.8	0.8	0.5	1.7	n.d.	0.7	1.0	0,7	0.4	0.8	1.4
Ni	130	110	90	90	50	60	63	63	50	40*/100**	50
Pb	130	105	90	95	140	160	80	112	50	50	80
Zn	230	192	210	200	230	300	165	166	110	110	150

(*) pelitic fraction ≤ 20% (**) pelitic fraction > 20% n.d. not determined.

reconstruction of the extent of contaminant concentrations (Innocenti et al., 2005).

ENVIRONMENTAL STATUS OF MARINE COASTAL AREAS

Almost all the coastal marine areas included in the SINs were completely characterized between 2004 and 2014. The results highlighted the presence in most sites of high concentrations of contaminants attributable to current or previous human activities, and lower concentrations in the other cases (Gabellini et al., 2011; Ausili et al., 2012). In all sites characterized by industrial and harbor activities, the spectrum of contaminants in the marine environment was fairly homogeneous; in fact, metals and trace elements, PAHs, and heavy hydrocarbons were prevalently and ubiquitously present, although the concentrations varied from site to site depending on the type of activity and the harbor's vocation (commercial, industrial, military, tourism, etc.). Only two SINs were characterized by a single source of pollution, that is, Cogoleto, where a factory produced Cr compounds for decades, and Manfredonia, where nitrogenous fertilizers were prevalent (**Figure 1**).

Based on the all results, it was possible to group SINs according to the impacts and types of anthropogenic activities. The ones characterized by long-lasting and past activities of iron and steel plants like as Bagnoli, Piombino, Pitelli, and Crotone, or still active, such as Taranto, were mainly contaminated by metals and trace elements (Cd, Pb, and Zn and, secondly, As, Cu, and Hg), according to the type of processing and raw materials used (**Figure 1**). The highest concentrations exceeding one or two order of magnitude the action levels for Cd, Hg, Pb, and Zn were recorded on the sea bottom closest to the plants. The concentrations showed a very wide range, generally decreasing from coast to offshore (**Figure 3**), and with the highest values in the superficial sediments (up to 1–1.5 m), even if in some sites like Taranto, Piombino, and Bagnoli the contamination attained a depth of 3–3.5 m, ascribable to the past maximum industrial activities and/or the absence of adequate environmental legislation (**Table 3**). In the same sites, also high concentrations of PAHs (>1,000 mg kg^{-1}), benzo(*a*)pyrene (>100 mg kg^{-1}), and TBTs (12 mg kg^{-1}) were recorded.

The SINs characterized by both industrial and petrochemical activities (Napoli, Brindisi, Livorno, Priolo, and Gela; **Figure 1**) showed a contamination mainly due to metals and heavy hydrocarbons (**Table 4**). In spite of the similar activities, the magnitude and extension of sediment contamination were very different between sites. These differences are well evident between Priolo, in particular the Augusta harbor, and Gela sites, where the concentrations of Cu, Hg, Pb, Zn, PAHs, and heavy hydrocarbons differ by up to three orders of magnitude. This can be due to the location and morphology of the two sites, a natural bay closed by artificial dams, which facilitates the accumulation of contaminants in fine sediments for Augusta harbor, and an open coastal site, subjected to strong coastal currents, for Gela. Similar differences can be noticed in Napoli (**Figure 4**) and Livorno harbors, where the contaminant distribution is strongly different, diffuse in the first one, and concentrated only close to the piers in the second one, according to the different morphological and harbor settings.

In the other sites, the contaminants showed always lower concentrations (Litorale Vesuviano, Massa Carrara, and Falconara Marittima) except for two sites, both of them impacted by past mining activity (Orbetello and Sulcis) and also characterized by geochemical enrichment (Hg for Orbetello, Romano et al., 2015; Cd, Pb, and Zn for Sulcis, Romano et al., 2017).

In almost all the investigated sites, the ecotoxicological response showed a toxic effect due to the complex mixtures of contaminants, strictly correlated with the concentration recorded in the sediments; only in a few cases (i.e., Sulcis) was this correspondence not recognized.

The studies carried out on marine organisms generally showed a tendency to bioaccumulate those elements and compounds that had been identified as contaminants of concern by the environmental characterization. In the Napoli, Brindisi, and Taranto SINs, Pb is the metal that most accumulates in the organisms, as evidenced by the increase of concentrations in transplanted mussels (mussel watch) and the higher concentrations recorded in fish species than those of control areas; in particular, the most significant concentrations were recorded in the liver of fishes, testifying current availability of these contaminants, even if the concentrations never exceeded the limit values reported

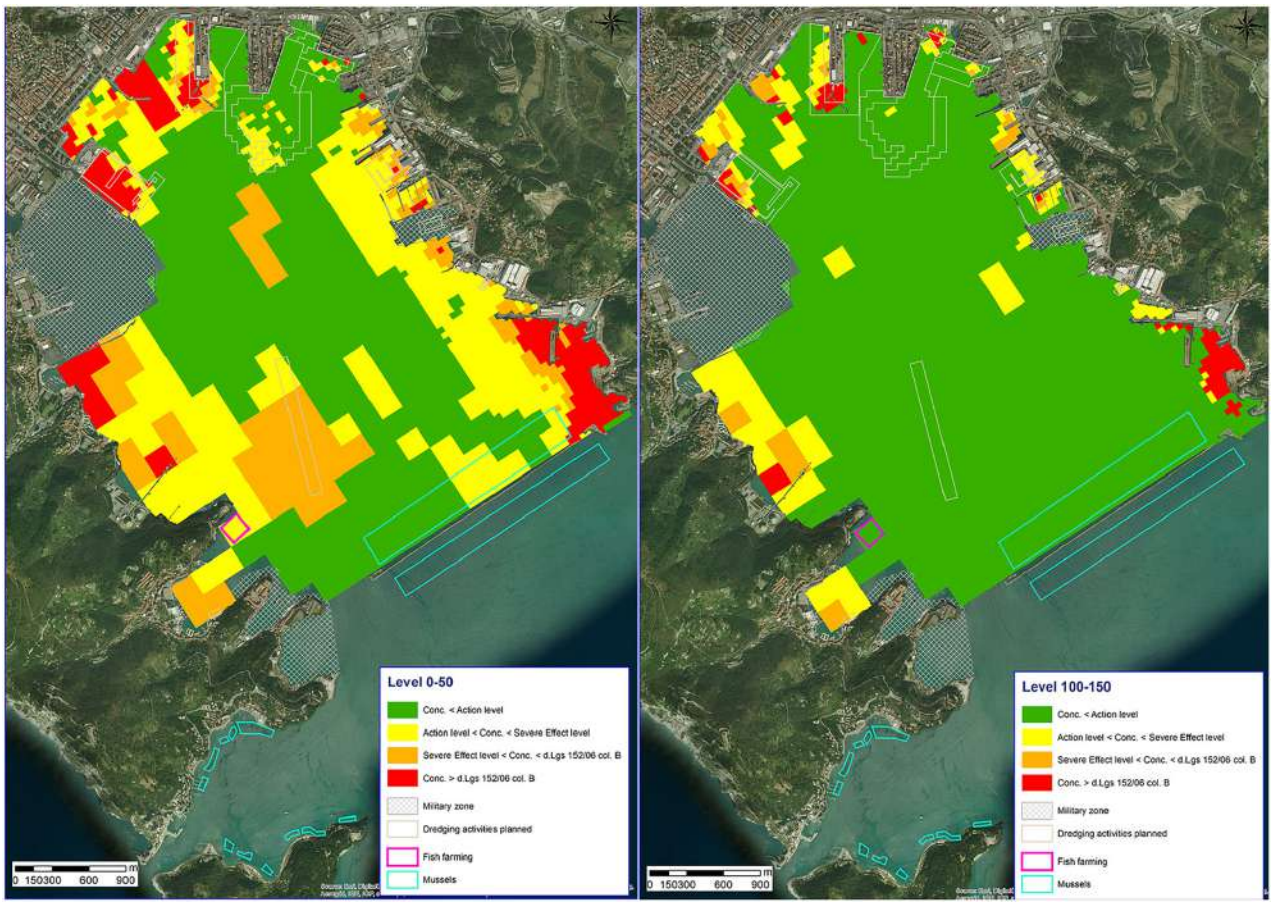

FIGURE 3 | Environmental status of Site of National Interest (SIN) Pitelli at different depths (0–50 and 100–150 cm).

TABLE 3 | Descriptive statistics of contaminant concentrations (mg kg^{-1} d.w.) in SINs characterized by long activity of large iron and steel plants.

Contaminant	Pitelli	Piombino	Bagnoli	Crotone	Taranto
As	10–945	2.1–816	1.4–73	<1.5–82	<1–165
Mean ± SD	20 ± 32	42 ± 50	10 ± 7	14 ± 9	11 ± 8
Cd	<0.1–485	<0.5–26	<0.01–44	<0.01–82	<0.01–9
Mean ± SD	0.9 ± 12.5	1.2 ± 2.9	1.3 ± 2.8	3.8 ± 7.9	0.2 ± 0.4
Cu	10–657	<1–2,800	<3–408	<5–960	<1–1831
Mean ± SD	68 ± 115	32 ± 88	48 ± 51	20 ± 42	26 ± 51
Hg	<0.1–61	<0.5–10.4	<0.01–8.3	<0.05–474	<0.001–107
Mean ± SD	1.0 ± 2.6	0.4 ± 0.9	0.7 ± 1.1	1.6 ± 15.4	0.8 ± 2.7
Pb	50–48,515	1–3,711	21–3,446	15–2,659	<1–1,560
Mean ± SD	194 ± 1551	170 ± 401	329 ± 383	75 ± 194	54 ± 114
Zn	14–37,772	10–16,000	90–5,185	3–22,027	<1–3,750
Mean ± SD	270 ± 1,420	550 ± 1366	690 ± 753	403 ± 1027	137 ± 128
PAHs	0.001–176	<0.01–3.23	<0.01–2,947	<0.01–1.76	0.01–1,490
Mean ± SD	2.5 ± 2.0	0.05 ± 0.22	166 ± 423	0.16 ± 0.15	6.3 ± 53.1
Heavy hydrocarbons	<1–8,293	<1–10,441	<1–23,346	<5–236.3	<1–5,480
Mean ± SD	88 ± 415	340 ± 997	71 ± 1052	8.8 ± 18.2	113 ± 302

in the annex EC Regulation 1881/2006, which defines the maximum levels of some contaminants in products for human consumption. Only in SIN Taranto were high PCB and PAH concentrations, among which benzo(*a*)pyrene with higher values, found in the mussels with higher values than those reported in the EC Regulation.

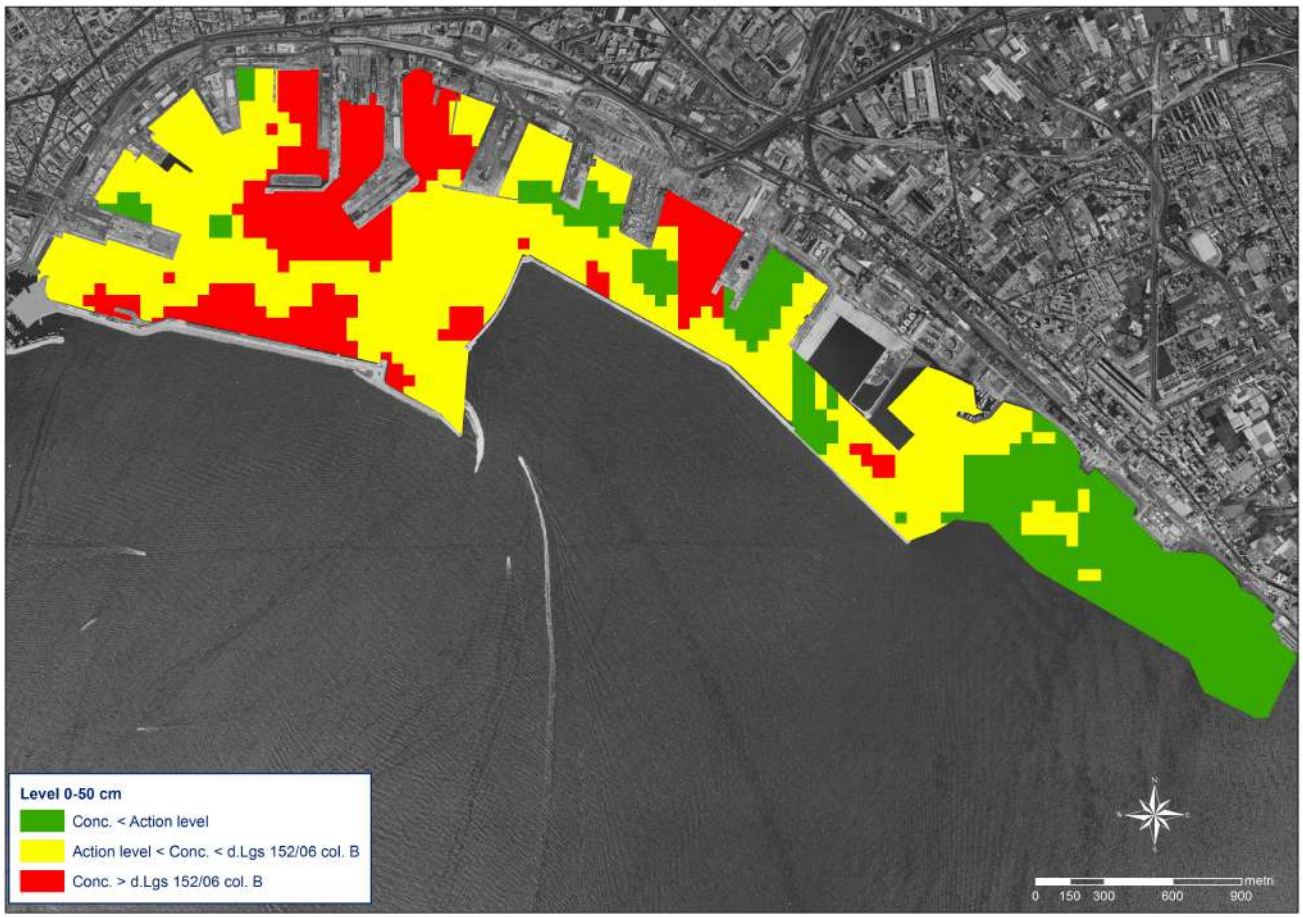

FIGURE 4 | Environmental status of Site of National Interest (SIN) Napoli, with particular focus on the internal harbor.

TABLE 4 | Descriptive statistics of contaminant concentrations (mg kg^{-1} d.w.) in SINs characterized by industrial and petrochemical activities.

Contaminant	Napoli	Brindisi	Livorno	Augusta bay (Priolo)	Gela
As	1.8–1,121	2–60	<1–74	1–98	3–162
Mean ± SD	21 ± 43	12 ± 7	11 ± 8	17 ± 7	14 ± 7
Cd	0.01–5.34	0.01–5.13	0.01–152.9	0.02–2.9	0.01–1.04
Mean ± SD	0.47 ± 0.61	0.04 ± 0.28	0.86 ± 6.05	0.23 ± 0.26	0.10 ± 0.07
Cu	6.7–5,743	1–284	3.6–1,339	1–18,338	1–696
Mean ± SD	106 ± 245	8 ± 30	39 ± 71	48 ± 464	6 ± 18
Hg	<0.001–139.2	0.01–5.7	0.02–22.1	0.02–788.3	<0.001–4.80
Mean ± SD	1.99 ± 7.01	0.12 ± 0.42	0.28 ± 1.00	13.61 ± 49.69	0.04 ± 0.18
Pb	7.1–3,083	2.5–1,131	0.3–4,902	1–5,393	1–486
Mean ± SD	147 ± 238	4.0 ± 66.4	56 ± 233	32 ± 140	6 ± 16
Zn	5.2–8,818	2.1–846	1. 9–4,360	1 -7,320	0.01–133
Mean ± SD	286 ± 625	7.5 ± 50.1	118 ± 261	75 ± 201	26 ± 14
PAHs	0.08–39.02	0.01–48.72	0.3–548	0.004–121.18	0.01–2,394
Mean ± SD	2.51 ± 3.54	0.01 ± 2.00	3.42 ± 21.12	1.16 ± 4.96	12 ± 74
Heavy hydrocarbons	<1–17,138	1–4,825	<1–540	2.5–61,882	2.5–4,849
Mean ± SD	461 ± 1524	2 ± 335	8.7 ± 30.4	793 ± 2,951	5 ± 113

The general results of the environmental characterization highlighted for all the SINs how the degree and extension of contamination were directly correlated with the morpho-bathymetric and sedimentological characteristics, as well as the coastal morphology where the site is positioned (enclosed bay, lagoon, open sea, etc.).

SELECTED CASE STUDY AND THEIR GENERAL INTEREST

Some SINs, characterized by different environmental settings (lagoon, bay, coastal site) and types of impact (mining, iron and steel plant, and chemical and petrochemical factories), but with common features of geochemical and geomorphological complexity and long-lasting and multi-source contamination, are considered of particular significance and described below. The comprehensive evaluation of results derived from the characterization and the integrative studies was useful for deducing general principles applicable on the environmental characterization of contaminated areas with similar characteristics.

Orbetello (Tuscany)

The SIN Orbetello includes the homonymous lagoon, located close to Monte Argentario (southern Tuscany, **Figure 1**). It is an enclosed brackish-water coastal basin, divided into West and East sub-basins by a dune cordon, connected with the sea by means of few narrow canals (Nassa, Fibbia, and Ansedonia) and characterized by very shallow water (mean depth 1 m). It was classified as Site of Community Importance (*SIC* IT51A0026), Special Protection Area (SPA IT51A0026), and protected area under the Ramsar Convention, classifying it as site of high naturalistic value (**Figure 5**). From a geological viewpoint, it is included in the sector of the southern Tuscany where, starting from Miocene (20 My–5 My ago), the uplift of magmas into carbonate Mesozoic rocks determined widespread mineralization, particularly abundant in the Monte Argentario and, more consistently, in the close Monte Amiata district (Protano et al., 1998; Grassi and Netti, 2000). In the East basin, mining over large scale was carried out from 1873 to 1958 with the exploitation of mainly pyrite (FeS_2), chalcopyrite ($CuFeS_2$), and cinnabar (HgS). Industrial activity affected the West basin, owing to a factory (ex-SITOCO) that produced, from 1908 to 1985, granular fertilizers and, as waste products, sulfuric acid and copper sulfate, as well as pyrite-rich ashes in As, Cd, Zn, and Pb. Moreover, the lagoon has been affected by sewage effluents from the Orbetello town, and wastewater from agriculture and aquaculture, which increased considerably in the second half of the 20th century (Lenzi et al., 2003).

The environmental characterization, which was preceded by preliminary environmental studies, carried out by different bodies (Regional Environmental Protection Agency, ISPRA, University of Siena), involved the collection of both surface samples and sediment cores. Sediments of the lagoon were mostly silty, with considerable percentages of sand, mainly constituted by bioclastic fraction. Chemical analyses indicated considerable enrichment for some trace elements (As, Cd, Cr, Cu, Hg, Pb, and Zn); Hg, in particular, showed very high concentrations (up to 70 mg kg^{-1}), not only in the surface samples but also in depth (ISPRA, 2009b). Concentration levels were the highest in the east lagoon, close to the mine site, indicating the exploiting activities as the main source of contamination. Another sector, characterized by significant contamination, was recognized close to the ex-SITOCO plant, where higher concentrations of some trace elements (As, Cd, Cu, Pb, and Zn) were recorded (ISPRA, 2008, 2009a).

The high Hg concentrations recorded in this area, characterized by geochemical anomaly (Protano et al., 1998), made necessary to carry out integrative studies aimed at the determination of local BGVs for a reliable assessment of environmental status. For this purpose, a total of 10 sediment cores were collected and subsampled at high resolution (2 cm levels), to investigate the concentration profiles of metals and trace elements. On four of these cores, a geochronological study was conducted to determine the sedimentation rates by means of radiometric methods (^{210}Pb and ^{137}Cs), according to GAU (2010). The application of a statistical approach on the unpolluted levels of these cores allowed to determine the natural concentration variability for trace elements and, consequently, their BGVs, which were found to be abnormally high for As and, more markedly, for Hg (Ausili et al., 2011; Romano et al., 2015).

Additional investigations, carried out from 2004 to 2006 on biological matrices (macroalgae, phanerogams, crustaceans, fish, and mollusks), showed high Hg concentrations mainly in the East lagoon, exceeding in some cases the regulatory limits for food consumption (ARPAT, 2007). A specific study carried out on the total mercury (Hg_{tot}) and methyl mercury (MeHg) concentrations in two fish species (*Dicentrarchus labrax* and *Sparus aurata*) from the East basin highlighted that approximately 90% of the concentrations measured exceeded Hg_{tot} regulatory maximum level of 0.5 mg kg^{-1} w.w. (Miniero et al., 2013).

A specific research, based on environmental indicators and aimed at the evaluation of the ecological status in relation to the recorded contamination, was applied. Benthic foraminifera were used for this purpose owing to their high sensitivity and fast responses to natural and anthropogenic environmental changes, abundance in sediments, and high species diversity. The results indicated a strong influence (negative correlation) on species diversity and density by the anthropogenic chemical enrichment, which favored the abundance of pollution-tolerant species and the inclusion of anomalous elements in the crystal lattice of their carbonate shell (Succi, 2015). These results were confirmed by another study on sediment geochemistry and benthic foraminifera, which classified the sediments of the Orbetello lagoon as strongly contaminated by Cd, Cu, Ni, Pb, Zn, and Hg, whereas benthic foraminifera showed increased number of abnormal specimens attributable to increased metal concentrations (Frontalini et al., 2009).

General considerations can be deduced form the different studies carried out on the SIN Orbetello: areas affected by geochemical anomalies need the determination of local BGVs for the definition of actual anthropogenic contribution and a

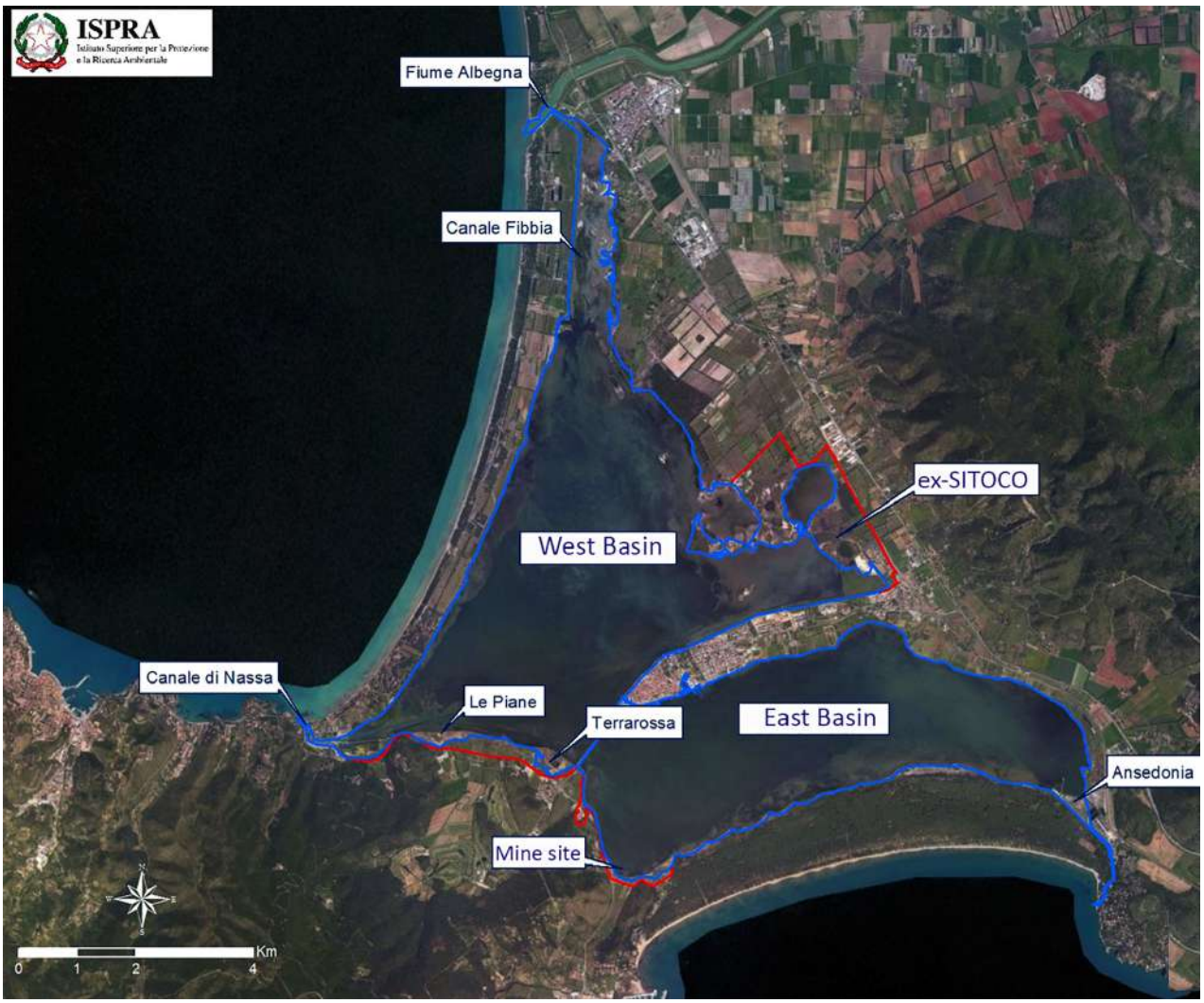

FIGURE 5 | Site of National Interest (SIN) Orbetello. Main anthropogenic activities (ex-SITOCO factory and mine site).

reliable environmental assessment; surface sediment samples are not suitable for this aim, while the study of concentration profiles in dated sediment cores produces reliable results; ecological studies on autochthonous organisms should take into account the confined conditions, naturally occurring in lagoon systems, which may determine environmental stress, in addition to the one due to contamination.

Bagnoli (Campania)

The SIN Bagnoli (**Figure 6**) is located on the western coast of the Pozzuoli Gulf (Napoli, southern Italy), which represents, by a geological viewpoint, the eastern edge of the Campi Flegrei, an active volcanic complex consisting of main ∼8 km caldera originated by the eruptions of Neapolitan Yellow Tuff (∼15 cal. ka BP; Deino et al., 2004) and Campanian Ignimbrite (39 cal. ka BP; Rolandi et al., 2003). The area is affected by bradiseismic motions accompanied by degassing and low magnitude seismicity (Barberi et al., 1984; Del Gaudio et al., 2010), resulting in structurally controlled major vertical ground deformation (Passaro et al., 2013). Presently, magmatic activity is evident from bradiseismic and As-rich hydrothermal vents, particularly common in the coastal sectors of the Pozzuoli Bay (Celico et al., 1992).

The Bagnoli industrial district included an important steel plant, which started the activity in 1910 determining, by both physical and chemical viewpoints, a strong environmental impact on the marine area. Two long piers were built in 1930 for unloading raw materials and loading finished products and a fill, between the two piers, which was discovered to be using contaminated material in the early 1960s to increase the surface of the industrial area. The steel production ended in 1990, and the industrial facilities were totally dismantled at the beginning of 2000s (Romano et al., 2004).

At the first stage, the environmental survey, limited to the coastal area close to the plant, recognized high concentration for Fe (3–60%), Pb (52–896 mg kg^{-1}), Zn (91–2,313 mg kg^{-1}),

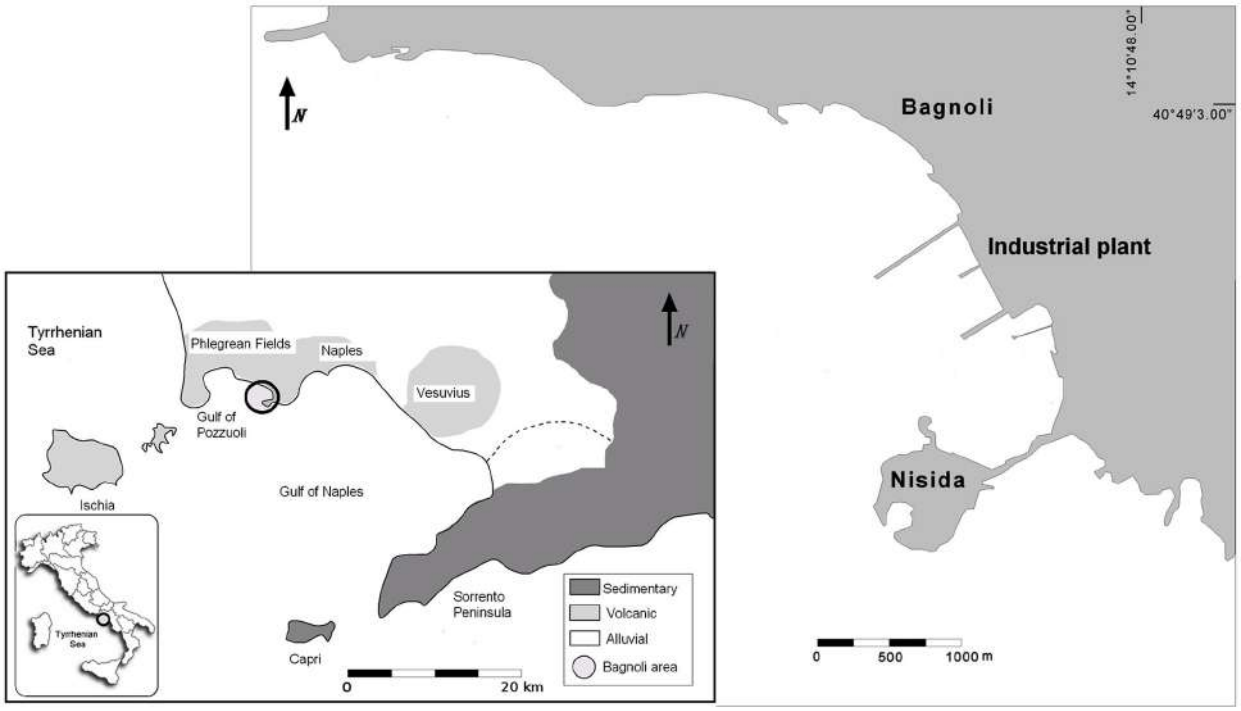

FIGURE 6 | Site of National Interest (SIN) Bagnoli with main geological elements.

and Hg (up to 9.27 mg kg^{-1}); high values were also found for Zn (1,162–2,834 mg kg^{-1}), Cd (1.17–3.65 mg kg^{-1}), and Hg (1.47–2.83 mg kg^{-1}) in some deep layers of sediment cores. The statistical analysis on environmental data highlighted how the contaminant distribution was strictly correlated with the filling area (Romano et al., 2004). This was also confirmed by successive surveys, which took place in front of the southern sector of the plant and identified the industrial area as the origin of recent contamination; in addition, a strong correlation between contaminants and silty sediment fraction and TOC was found. The anthropogenic impact on the marine area was also confirmed by the application of benthic foraminifera as environmental indicators, which recognized deformed specimens positively correlated to metal concentrations (Romano et al., 2008).

The successive steps of characterization, including a wider marine area and carried out up to 2008, were based on a huge number of both superficial samples and sediment cores (**Figure 2**). The grain size analyses highlighted a gradient from coast to offshore with sandy to silty sediments; the only exception was recognized in the sector comprised between the two piers, where finer sediments were found, owing to a change of water circulation and sedimentation conditions. The qualitative study of different grain size fractions revealed a considerable contribution of anthropogenic grains (coal fragments and blast furnace slag) in proximity of the plant, which became dominant close to the piers.

Chemical analyses on sediments revealed, on the whole, very high concentrations mainly of Pb and Zn (up to 3,446 and 5,185 mg kg^{-1}, respectively) and PAHs (up to 3,000 mg kg^{-1}) in front of the plant (**Figure 7**), clearly indicating the industrial area as the main source of contamination; the particularly high benzo(*a*)pyrene levels (>100,00 mg kg^{-1}) in some samples classified sediments as "dangerous" according to national legislation d.lgs. 152/06. These contaminants were recognized as bioavailable by means of studies of bioaccumulation carried out on mussels (*Mytilus edulis*) collected close to the piers, which showed 2–4 times and 4–7 times higher Pb and PAHs concentrations, respectively, than those registered in the control area (ICRAM, 2006; ICRAM and Stazione Zoologica Anton Dohrn, 2006; Romano et al., 2009a).

More recently, a study carried out on three sediment cores, subsampled at high definition (2 cm levels), was conducted in order to reconstruct the historical evolution of the contamination identifying the metal background concentrations. The concentration profiles and enrichment factor (EF) of metals and trace elements, together with the profiles of PAHs and PCBs as chronological constrain, were interpreted also taking into account the historical information on the industrial activity. Several elements (Cd, Cu, Hg, Pb, and Zn) were recognized as responsible for contamination of the marine area, whereas generalized high As concentrations were primarily attributed to hydrothermal submarine springs. The highest EFs of Cu, Pb, and Zn, with depth of between 30 and 100 cm, were associated with the time interval of 1950s–1980s, when the highest production levels were attained; nevertheless, contaminants maintained considerable concentrations up to the top of the core, indicating recent contribution to the marine environment (Romano et al., 2018b).

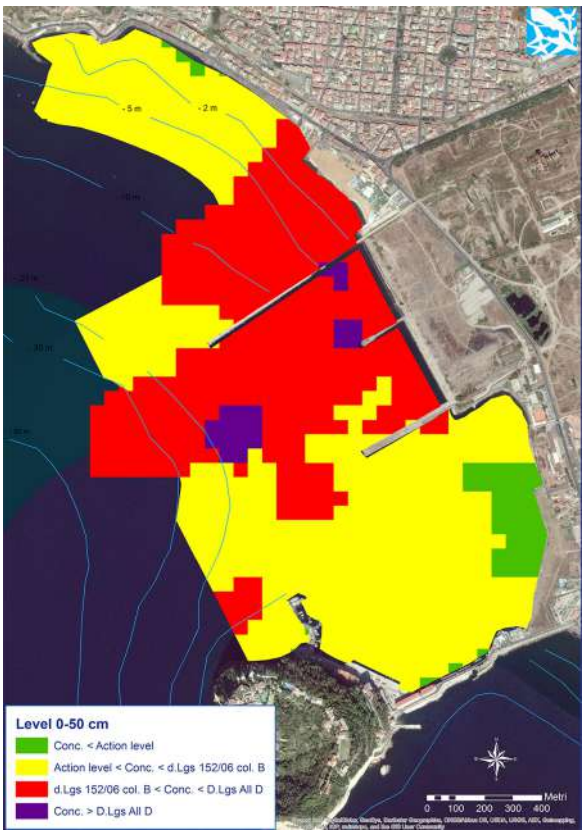

FIGURE 7 | Site of National Interest (SIN) Bagnoli. Concentration of all analyzed contaminants in superficial (0–50 cm) sediments (from ICRAM, 2006, modified).

A recent study, which took into account all the environmental datasets derived from the characterizations carried out between 1999 and 2013, integrated with geomorphology of the sea bottom, allowed to reconstruct the temporal changes of contamination attributable to the steel plant activity, but also recent contaminant supply occurred several years after the dismantling of the industrial area (Sprovieri et al., 2019).

The anthropogenic impact, mainly due to metals and trace elements and PAHs, deriving from the past activities of the industrial site was recognized by Arienzo et al. (2017) and Trifuoggi et al. (2017) in the sediments of outermost and deepest areas of the Gulf of Pozzuoli.

It is evident how this type of industrial facilities can modify the coastal morphology and hydrodynamic and natural sedimentation conditions. This consequently can influence the physical characteristics (texture and composition) of sediments as well as the accumulation and distribution of contaminants. Moreover, the industrial areas may release considerable amounts of contaminants even after the closing and dismantling of industrial facilities.

Augusta Bay (Priolo, Sicily)

The SIN Priolo includes a very large marine coastal area, from Augusta Bay to Siracusa, extending up to 3 km offshore (**Figure 2**). This case study is focused on a limited sector of the SIN, the Augusta Bay, because of the complexity of the area by geomorphological and environmental viewpoint, and the many and diversified anthropogenic activities operating in the area with the related environmental impact.

From a geological viewpoint, the Meso-Caenozoic carbonates and basalts of the Hyblean plateau outcrop in the mainland, whereas the main lithotypes along the coast are characterized by Pliocene clays and Quaternary biocalcarenites (Carbone, 2011). The Augusta harbor is located within the natural bay, bordered by breakwaters built in the early 1960s and characterized by an intense commercial and industrial maritime activity as well as a huge chemical and petrochemical pole, which has been in operation for several decades. The industrial activities started in the 1950s and quickly developed until the 1980s, making the site the most important hub in Europe. Subsequently, some industries closed, whereas some others are still active. In particular, a chlor-alkali plant, based on mercury cell technology and in operation from 1958 to 2003, was considered as an element of high environmental concern for the marine area owing to the discharge of over 500 tones of Hg directly in the sea in a historical period during which no environmental legislation was active (Croudace et al., 2015).

During the environmental characterization, performed through some implementation phases, a total of 530 sediment cores (2–3 m long) and 39 surface samples were collected. Furthermore, six cores were collected and subsampled at high definition (2 cm levels) for recognizing reference conditions (ICRAM, 2008). The results showed exceptionally high concentrations (**Figure 8**), mainly located in the southern sector, of Hg (up to 198 mg kg^{-1} in the surface samples and to 728 mg kg^{-1} in the deeper ones), associated with very high levels of Ba, hexachlorobenzene (HCB), and PCBs (up to 0.83 mg kg^{-1} in the surface samples and to 14 mg kg^{-1} in the deeper ones).

In addition, 10 sediment cores were collected from the whole harbor area and sampled at high resolution (3 cm levels) for a more detailed spatial and vertical reconstruction of contamination patterns and the identification of pre-industrial conditions. Along the core depth, the concurrent increase of contaminant concentrations and of fine sediments was interpreted as the effect of the establishment of dams, which limited water circulation. Three of these cores were dated by means of radiometric methods (^{210}Pb and ^{137}Cs), according to Croudace et al. (2015), allowing to reconstruct the chronology of contamination. The results highlighted that the exceptionally high concentrations of Hg, Ba, and PCBs were attributable to the historical activity of the chlor-alkali plant (Croudace et al., 2015; Romano et al., 2019a).

The bioassays, carried out on three species (*Dunaliella tertiolecta*, *Vibrio fischeri*, and *Phaeodactylum tricornutum*) and two matrices (solid and elutriate), demonstrated toxic and/or hormetic (eutrophic) effects in at least one species and in at least one environmental matrix, highlighting low to high toxicity of the sediments, widespread throughout the bay. The studies carried out on native and transplanted bivalves (*Mytilus galloprovincialis*) and fishes (*Mullus barbatus*, *Boops boops*, *Diplodus* sp.) highlighted a considerable Hg contamination of

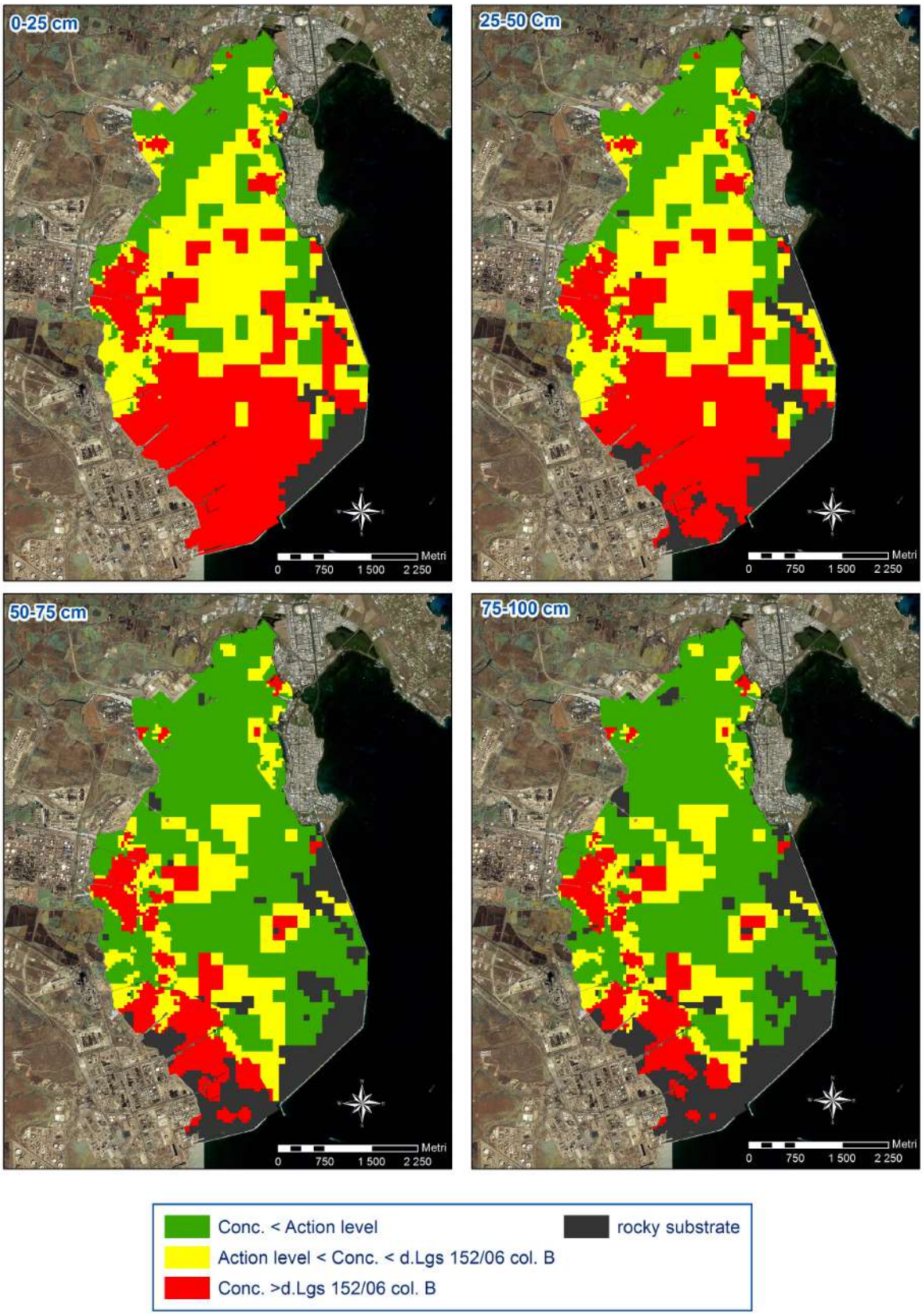

FIGURE 8 | Site of National Interest (SIN) Priolo. Distribution of all analyzed contaminants in four depth intervals (from ICRAM, 2008, modified).

tissues (up to 0.45 mg kg^{-1} w.w. in mussels; up to 0.58 and 1.01 mg kg^{-1} w.w. in muscle of *M. barbatus* and *Diplodus* sp., respectively) and liver (up to 0.94, 2.0, and 5.9 mg kg^{-1} w.w., respectively), demonstrating the bioavailability of this contaminant (ICRAM, 2008). Besides chemical accumulation, there was also evidence of toxicological effects of Hg in both mussels (*M. galloprovincialis*) and mullets (*M. barbatus*). The incidence of micronuclei (MN) in the hemocytes and gills revealed a frequency up to 11‰ in mussels and 9‰ in fish. Similar values have never been previously reported, especially for mussels (Bolognesi et al., 2006), and clearly demonstrate a marked biological reactivity of environmental mercury (Ausili et al., 2008).

Adverse effects due to Hg and PCBs contamination were recognized in benthic foraminifera, applied as environmental indicators in surface samples and sediment cores. They showed, in the most contaminated area, decreased species diversity and increased abundance of pollution-tolerant species. Faunal density was particularly affected by contamination, determining the disappearance of foraminifera in correspondence of the most contaminated subsurface sediments. After the time interval in the 1970s–1980s, benthic foraminifera re-appeared at the sediment–water interface despite the high levels of contamination in the underlying sedimentary layer (Romano et al., 2009b, 2013, 2015). Subsequent research demonstrated that the highly contaminated sediments of the Augusta bay represent an important source of Hg for the Mediterranean Sea basin (Sprovieri et al., 2011). Successive studies, carried out on benthic and demersal fishes, confirmed the Hg bioavailability inside and off the Augusta Bay (Bonsignore et al., 2013, 2015). Further evidence of contamination derived from the Augusta harbor on the coastal zone was obtained by the study of distribution patterns of some contaminants (mainly Hg and PAHs) in sediments collected in neighboring areas and off the Augusta bay (Di Leonardo et al., 2014a,b).

From the environmental results, it can be deduced that both chemical and textural characteristics of sediments may be considered as proxies of the anthropogenic impact; for this reason, the identification of the source of contamination should be based not only on spatial distribution but also on the historical reconstruction. In addition, because of the known persistence of most of contaminants examined here, their sedimentary store should be considered in the evaluation of future potential impacts on the marine environment.

Sulcis (Sardinia)

The SIN Sulcis includes a large sector of southwestern Sardinia, from Piscinas to Sant'Antioco lagoon where mining activities strongly impacted the marine areas, and the eastern side close to Cagliari, where the industrial activity is prevalent (Sarroch industrial plant). This case study is focused on the southwestern sector, characterized by volcanism of Tertiary age, which affected Cambrian dolostones and limestones, producing widespread mineralization, which was used since pre-Roman times (**Figure 9**). The mining district includes over 40 dismissed mines, which exploited mainly base metal (Zn, Pb, Ag, and Ba) and calamine (Zn and Pb) deposits, constituting the main economic resource of the area between the 19th and 20th centuries. Nowadays, large outcrops of sulfide and oxide ores, as well as the products of long-lasting mining activity, are responsible for strong contamination of soil, streams, and groundwater (Cidu, 2009, 2011).

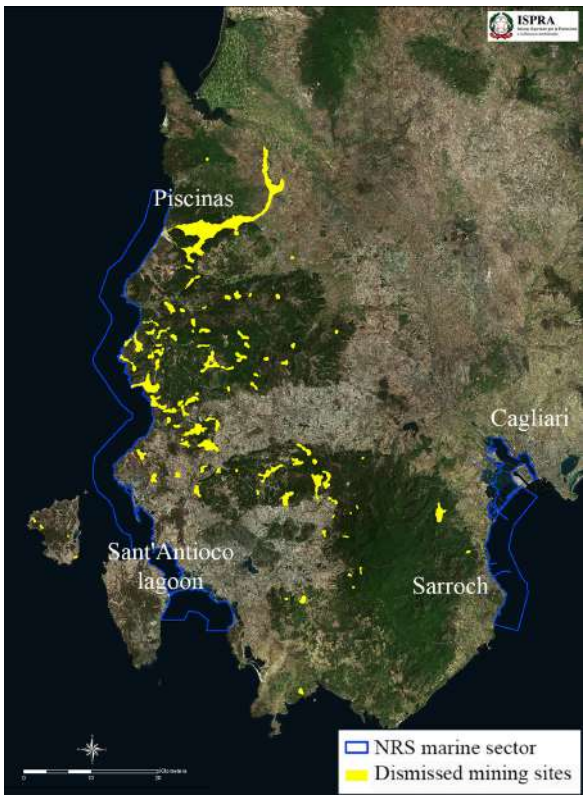

FIGURE 9 | Site of National Interest (SIN) Sulcis with mining area and investigated marine sectors.

Other kinds of anthropogenic impact, which may affect the environmental status of the marine area, are due to the activities carried out in Sant'Antioco (harbor, municipal wastes, tourist facilities, and a disused factory for the production of magnesium oxide) and those of the industrial sites of Sarroch and Portoscuso-Portovesme; the first one includes mainly petrochemical industries, whereas iron and steel industries and an electric power plant are present in the second one. In the framework of the environmental characterization, carried out in 2015, a total of 415 surface and subsurface sediment samples were analyzed, among the other parameters, for grain size and mineralogical content, metals and trace elements, PAHs, and PCBs (ISPRA, 2015). The analytical results highlighted almost exclusively sandy sediments and a mineralogical composition reflecting the types of rocks outcropping inland also including processing residues derived from exploiting activities (**Figure 10**).

Metal and trace element concentrations confirmed the presence of very high levels of Cd (up to 164 mg kg^{-1}), Pb (up to 3,423 mg kg^{-1}), and Zn (up to 42,764 mg kg^{-1}) over the entire area and, more locally, significant concentrations of As (up to

FIGURE 10 | View under stereo microscope of sediment. Mining residues in the sediments (ISPRA, 2015).

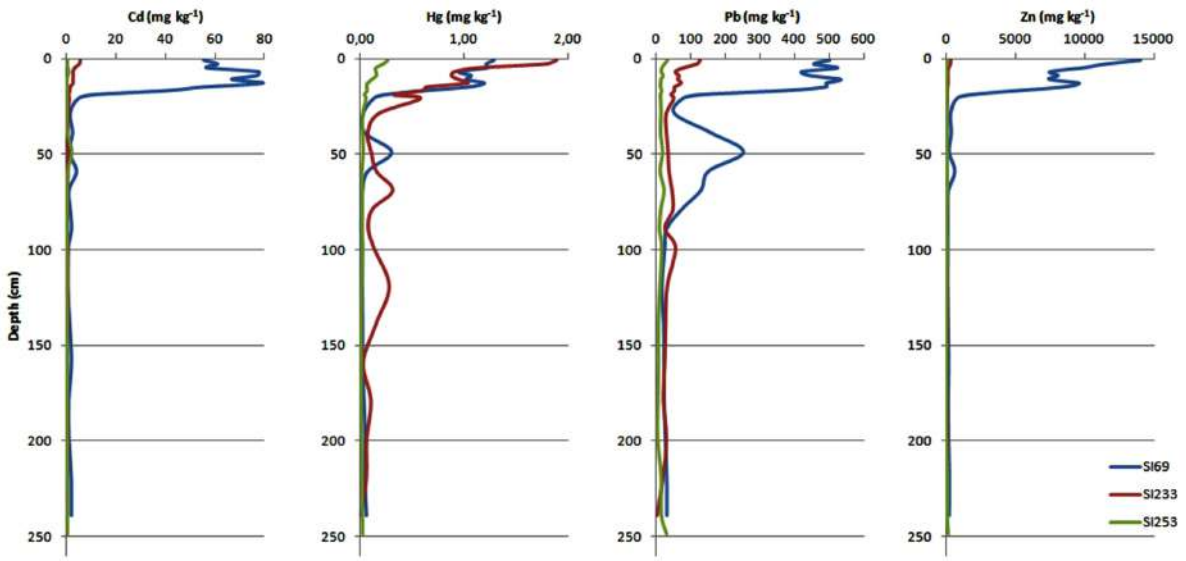

FIGURE 11 | Concentration profiles for main elements in the three cores from mining (SI69), lagoonal (SI233), and industrial area (SI253) (ISPRA, 2015).

72 mg kg^{-1}) and Hg (up to 12 mg kg^{-1}). Most of the Cd, Pb, and Zn concentrations exceeded considerably local BGVs, pointing out an anthropogenic enrichment for these elements, which is attributable to the past exploiting activity. This enrichment is well documented in the deeper sediment levels, whereas the still high concentration recorded in the surface ones are due to the weathering of mine waste residues, still present in the mainland (ISPRA, 2015; Romano et al., 2017).

Differently from the northern coastal belt, sediments of the Sant'Antioco lagoon resulted widely variable as regards texture, mainly sandy with a significant presence of the fine fraction and, locally, of gravel; anthropogenic grains were abundant in several stations. For most of the trace elements, the concentrations were fairly homogeneous, with the exception mainly of Cd, but also Zn, Pb, and Hg, which showed, in the upper 50 cm of some stations, higher concentrations than the average values of the area.

Marine sediments of the eastern sector of SIN, in front of the industrial site of Sarroch, were characterized mainly by sand with significant pelitic fraction and grains of potential anthropogenic origin in some superficial samples. As regards chemical parameters, the results did not show any environmental criticality.

A specific study was conducted on three sediment cores as representative of the different areas (mining, lagoonal, and industrial areas), subsampled at high resolution (2 cm levels), for reconstructing the concentration profiles of metals and trace elements and recognizing the reference conditions. The analytical results highlighted different concentration profiles and range of values for each area; in particular, the profiles of core collected in the mining area showed the strongest anthropogenic enrichment for all the analyzed parameters (Cd, Hg, Pb, and Zn) with higher and variable concentrations in the superficial levels and

their significant reduction, with steady values, in the underlying ones (**Figure 11**). This behavior, associated with the determined BGVs and the presence of mine waste minerals, confirmed the correspondence, in the upper core, of anthropogenic trace metal enrichment due to the exploiting over industrial scale, whereas the still high metal enrichment in sediment surface levels suggested a present impact due to mine dumps and tailings weathering (Romano et al., 2017). The improvement of core dating by means of luminescence method confirmed, for the strong metal enrichment in the upper 20 cm of the core, the attribution to historical mining activity (Sechi et al., 2018).

Also, the mobility Cd, Pb, and Zn was investigated in an integrative study by means of a sequential extraction procedure (five steps). The results displayed the high mobility of Cd and Pb, with higher concentrations in the first phase, of metals physically adsorbed and bound to the carbonate, and in the second one, referable to metals present in the reductive phase bound to manganese–iron oxides. Cd was extracted in percentages ranging from 15 to 87% and from 6 to 81% in the first and second steps, respectively, whereas, as regards Pb, the percentages of the first and second phases were 26–72% and 11–39%, respectively. Also, Zn was found to be mobile, because it was mainly extracted in the second step, in percentages ranging between 22 and 99%. In spite of this, bioassays (*V. fischeri*, *D. tertiolecta*, *Brachionus plicatilis*) carried out on both solid and elutriate did not highlight toxic response. These apparently conflicting outcomes were attributed to the fact that biogeochemical features of sea bottom and its aquatic/biotic interface constitute a dynamic and complex system, including an ensemble of variables, which in some cases, make it difficult to obtain univocal response by different lines of evidence (Romano et al., 2019b).

The study of this SIN established a milestone for the environmental assessment in marine areas close to mine districts, that is, the essential need to determine local BGVs for metals and trace elements in order to assess their actual anthropogenic contribution. This is because the mined contaminants are enriched not only in mining residues but also throughout the geological landscape. The study of this SIN also revealed that contamination from mine waste deposits could persist after the end of exploiting activities.

CONCLUSION

This review examines the environmental surveys carried out in the early 2000s on transitional and marine coastal areas included in the SINs, when a specific legislation was missing. The wide variability that characterizes both the natural environmental setting and type/degree of anthropogenic impact of the Italian SINs imposed the adoption of an investigative approach based on general scientific-based principles, but also adaptable to large scale. This approach, highly flexible as regards sampling strategy and investigated parameters, considered sediments as the preferential matrix, owing to their conservative feature, for the assessment of marine contamination; nevertheless, ecotoxicological and biological tests were also considered. Integrative analyses, such as the study of dated sediment cores for the determination of local BGVs or sequential extractions to verify metal mobility, were also included, according to specific conditions of studied sites.

The synthesis of results reported in this review allows us to draw some general conclusions on the environmental quality status of the Italian marine-coastal and transitional areas included in SINs. In all cases, the characterization activities recognized a direct correlation of organic and inorganic contaminants with past or present activities in the area, identifying the actual anthropogenic contribution of metals and trace elements naturally occurring in the sediments. It was highlighted that the extent of the contamination was directly correlated not only with type of activity and amount of the released contaminants but also with geomorphological, bathymetric, and sedimentological characteristics of the area.

The special focus on four different sites allowed us to develop a set of general criteria for environmental characterization of contaminated marine areas:

- Grain size analysis is a basic part of environmental characterization both because sediment texture influences contaminant accumulation and because it may be a proxy of the anthropogenic impact.
- Although sediments are the eligible matrix for contamination assessment, studies of bioaccumulation and adverse effects on biota are necessary to recognize the actual environmental risk.
- The environmental characterization has to be based not only on surface samples but also on sediment cores; the latter can be used to reconstruct the spatial and vertical distribution of contaminants, as well as the historical evolution of contamination and the reference conditions in pre-impacted times.
- Local BGVs must be calculated in areas affected by natural geochemical enrichment in order to isolate the actual anthropogenic contribution. The determination of these values must be based on sediment cores that allow for the collection of undisturbed and uncontaminated sediments in the deeper levels.
- The SINs may also be considered as laboratories for studying the effects of contaminants on marine environment and identifying reliable new environmental indicators, not traditionally considered.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. Each author contributed equally to all sections of the manuscript.

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Birth Cohorts in Highly Contaminated Sites: A Tool for Monitoring the Relationships Between Environmental Pollutants and Children's Health

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Industrial areas are characterized by the dispersion of environmental stressors that could possibly have long-term detrimental effects on both human health and the environment. Environmental contamination has been indicated to be one of the major risks for reproductive health. In this context, the effects of environmental pollution on pregnant women living in heavily polluted areas is of special interest. In fact, fetal development is a crucial phase due to the dynamic interaction between the maternal/external environments and the developing organs and tissues. Moreover, following Barker's postulate of the intrauterine origin of health and disease, the events occurring in this time window could affect future health. Birth cohorts provide the most suitable design for assessing the association between early-life and possible long-term health outcomes in highly contaminated sites. By providing an assessment of the early life environment throughout the collection of biological samples, birth cohorts offer the opportunity to study in-depth several possible confounders and outcomes by means of questionnaires and follow-ups based on clinical evaluations and bio-specimen samplings. The exposome comprises the totality of exposures from conception onwards; the birth cohort approach allows the integration of the exposures as a whole, including those related to socioeconomic status, with "omics" data from biological samples collected at birth and throughout life. In the characterization of the "fetal exposome," the placenta represents a highly informative and scarcely considered organ. For this purpose, the "Neonatal Environment and Health Outcomes" (NEHO) birth cohort has been established by enrolling pregnant women residing in contaminated sites and in surrounding areas.

Keywords: birth cohorts, highly contaminated sites, fetal exposome, DOHaD postulate, placenta, child growth

INTRODUCTION

Health Effects of Environmental Exposures in Highly Contaminated Sites

Contaminated sites can be defined as "areas hosting or having hosted human activities which have produced or might produce environmental contamination of soil, surface or groundwater, air, food-chain, resulting or being able to result in human health impacts" (1). Contaminants such as heavy metals (HMs) and persistent organic pollutants (POPs) may transfer from one environmental matrix to another and, depending on their chemical-physical properties, are able to infiltrate the human body through different exposure pathways and routes (2). Living near environmental hazards contributes to a poorer general health status (3). Moreover, contaminated sites are often located in socially deprived neighborhoods; this makes exposure patterns more complex and results in interactions with other health determinants (4). Environmental pollution is one of the largest causes of death and disability in the world. In 2015, about 16% of premature deaths worldwide were caused by exposure to chemicals released into the environment (5). The WHO highlighted that 26% of deaths among children under five are due to modifiable environmental factors, and therefore can be prevented (6). Moreover, many childhood morbidities and disabilities are attributable to environmental causes and to gene-environment interaction starting from the fetal development period (7).

Environmental contamination is one of the major risk factors for reproductive health (8). Indeed, causal relationships between parental or prenatal environmental exposures and several adverse pregnancy and childhood outcomes have been clearly documented (9–13). Moreover, toxicants were associated with intrauterine growth restriction (14–16), inadequate birth weight (17), and premature births (18). These birth outcomes are of special interest for their double significance: they represent both an adverse outcome *per se* and could be considered as risk factors for future childhood pathologies.

Many chemicals released into the environment due to industrial processes are able to disrupt the programming of endocrine signaling. Thus, gametes, pregnant women, and developing fetuses are particularly vulnerable to the harmful impact of these environmental toxicants (19–21). For example, cadmium (Cd) has been identified as an endocrine disruptor and is released by industrial plants, negatively influencing both male and female reproductive health, acting at the level of the hypothalamic-pituitary-gonadal intercommunication axis (22). Lastly, human studies have shown an association between Cd exposure during pregnancy and low birth weight (23).

There is growing evidence supporting the hypothesis that prenatal exposure to toxicants is associated with long-term effects on children's neurological development (24, 25), respiratory and cardiovascular systems (26), and metabolic signaling (27). For instance, children born to women exposed to organochlorine pesticides have a higher risk of developing neurodevelopmental, neurodegenerative, and neurobehavioral disorders (28, 29). Prenatal exposure to methylmercury has also been associated with the development of autism spectrum disorders (30). Similarly, a significant increase in the incidence of "bronchitis" was reported in Taiwanese children born to women exposed to polychlorinated biphenyls (PCBs) during pregnancy (31). Moreover, prenatal exposure to perfluoroalkyl substances and postnatal exposure to copper, ethylparaben, and household crowding were associated with poorer lung function in 6- and 12-year-old children (32). *In-utero* exposure to hexachlorobenzene (HCB) and dichlorodiphenyldichloroethylene (DDE) was associated with childhood obesity and higher blood pressure levels at 4 years (33). In this study, an obesogenic effect of DDE and HCB was hypothesized through sex steroid dysregulation. Moreover, in a French birth cohort, an association was found between high maternal Cd and lead (Pb) blood levels and increased risk of gestational diabetes (34). Fetal exposure to maternal gestational diabetes was further associated with an altered glucose-induced hypothalamic activity in children and, as a consequence, with increased risk of obesity later in life (35).

Scientific data on the long-term effects of developmental exposures provide new insight into the importance of preventing the negative effects of environmental chemicals on the residents of highly polluted sites. There are different methodologies to assessing pollutant impacts on human health through studies with both ecological and etiological design. One example is the SENTIERI Project, which works toward multiple endpoints, including hospital discharges during infancy and congenital anomalies, in all the main contaminated sites in Italy, implementing an a-priori identification of health endpoints linked with pollution sources (36). This approach is of undeniable value for public health monitoring, even though any demonstration of pathophysiological links between environmental pollutants and health effects requires further research.

It is generally recognized that prospective pregnancy or birth cohort studies, incorporating exposure biomarkers during sensitive windows, are required to examine the potential health effects of developmental exposure to chemicals. Birth cohort studies provide the most suitable design for assessing the association of early-life adversities occurring at critical developmental windows with their possible long-lasting effects on postnatal health and well-being. Cohort populations living in highly contaminated sites have been studied mainly in occupational settings; in contrast, their use in the general population is not well-represented in scientific literature, though remarkable examples are available, especially in cases of accidental events, such as the Seveso disaster (37) or Minamata disease (38).

Fetal Development and Environmental Epidemiology

Starting from Barker's postulate of the "intrauterine origins of health and disease susceptibility" (39–41), growing evidence has highlighted how environmental stressors can interfere with the early stages of fetal development leading to diseases later in life. Chemical compounds, social stress, and lifestyle can lead to the permanent alteration of fetal development, possibly resulting in increased susceptibility to adverse health outcomes over a

person's lifetime (42, 43). Homeostatic processes during fetal life allow the organism to dynamically adapt to changes in the intrauterine environment in order to obtain an immediate survival chance and to have future adaptive advantages in adulthood (44). However, changes that turn out to be adaptive for one endpoint, such as surviving an acute stressful condition, may be maladaptive in other life stages, thus producing a higher risk of non-communicable disease occurrence (45).

In recent years, the role of epigenetic mechanisms (e.g., nucleotide and histone chemical modifications and small non-coding RNAs) has been recognized in regulating fetal development and its adaptation to changing environmental conditions through changing gene expression (45), while growing evidence has drawn attention to epigenetic alterations induced by environmental contaminants (46). Epigenetic alterations that affect the trajectories of fetal development may maintain their effects over generations (47). Industrial activities and power plants are known sources of many chemicals which can induce epigenetic effects (2, 48–52). DNA methylation is a potential mechanism by which environmental exposures may contribute to the etiology of complex diseases (53). Epigenetic changes have been observed in pregnant women, placentas, and cord blood after exposure to various environmental contaminants, such as phthalate and bisphenol A (54), but also to maternal smoking (55) and psychological stress (56). In a large-scale epigenome-wide meta-analysis, the authors found a significant association of PM_{10} and $PM_{2.5}$ exposures during pregnancy with methylation differences in newborns' genes relevant to respiratory health, such as FAM13A and NOTCH4 (57).

Pregnant women living in highly contaminated sites can be exposed via multiple pathways, including food, inhalation, and dermal contact. The exposure of the developing fetus to environmental contaminants may lead to multi-organ alterations producing organ dysfunction and diseases. Toxicants influence fetal development in different ways. The influence can be direct, as in the case of arsenic (As), Pb, and mercury (Hg) as these substances can readily pass through the placenta into the fetal environment (58), or indirect, as in the case of Cd, by interfering with maternal and placental homeostatic functions leading in turn to altered signaling with the fetus (**Figure 1**). For other environmental toxicants, such as PCBs, their ability to pass through the placenta is related to congener specific chemical-physical properties, such as molecular weight and lipophilicity (59).

Many studies performed in highly contaminated areas have evaluated residential proximity to pollution sources or air pollutant exposures (11, 60–63). On the contrary, studies on the contribution of soil and water contamination to human exposure, as well as those related to the food chain and human biomonitoring, are less represented in scientific literature (64–66).

Recently, Heindel and colleagues published a comprehensive review of epidemiological studies evaluating associations between *in-utero* and early post-natal life exposure to environmental chemicals and adverse health outcomes (67). They examined 425 papers, showing that most of the publications were related to neurological/cognitive outcomes, followed by cancer and respiratory diseases. Only in recent years have researchers focused on metabolic outcomes (including obesity) and second generation reproductive health (67). Similarly, studies in highly contaminated sites have indicated a greater incidence and prevalence of a variety of health conditions, including cancer, respiratory diseases, diabetes, obesity, and negative reproductive health outcomes (68, 69). Moreover, in Heindel's review, more than 60 different chemical compounds were identified, most of which are known to be related to environmental contamination due to power plants and industrial/petrochemical emissions. The most frequently studied chemicals are PCBs, often associated with incineration and power generation processes (70). Regarding heavy metals, Hg, Pb, and As were the most represented in the review. In **Table 1**, a short list of studies on environmental pollutants present in highly polluted areas is reported along with the relevant health outcomes in pediatric age (71–98).

However, people living in heavily contaminated areas present a different exposure profile as compared to the general population, in terms of both level of exposure and number of contaminated environmental matrices. In this respect, multipollutant models are designed to overcome the difficulty of identifying effects of multiple pollutants in epidemiological studies which also try to effectively capture the health impact of pollution mixtures observed under real-life conditions. These models are of particular interest, though scarcely represented, especially in the context of highly contaminated sites. Moreover, the evaluation of real-life exposure represents a methodological challenge for the overall integration of exposure measures obtained from different matrices (e.g., ambient air, blood, tissues).

The Fetal Exposome and the Placenta

Omics, including genome, epigenome, transcriptome, metabolome, and microbiome, have widened our ability to investigate complex biological processes. The possibility of considering multiple molecular pathways at once gives us the opportunity to have a more holistic and comprehensive understanding of an organism's development and functions. Along the same line, Christopher Paul Wild coined the term "exposome" in order to promote the use of an omics approach in the field of environmental epidemiology (99).

Consistent advances have been made in "measuring" the levels of environmental contaminants in biological tissues; however, a delineated exposome approach has not been applied in clinical settings. The exposome not only concerns toxic chemicals but also includes three domains: (1) a general external domain including the social and economic context and stress factors; (2) a specific external domain including environmental pollutants, diet, and drugs; and (3) a specific internal domain including biomarkers of exposure, effects, and susceptibility (100). Another key concept in defining the exposome is its dynamic nature (100): for example, changes in household, school, occupation, socioeconomic profile, social interactions and stress, course of medical treatment, exposure profile (even for a short period), and migration flows may all produce changes in the exposome

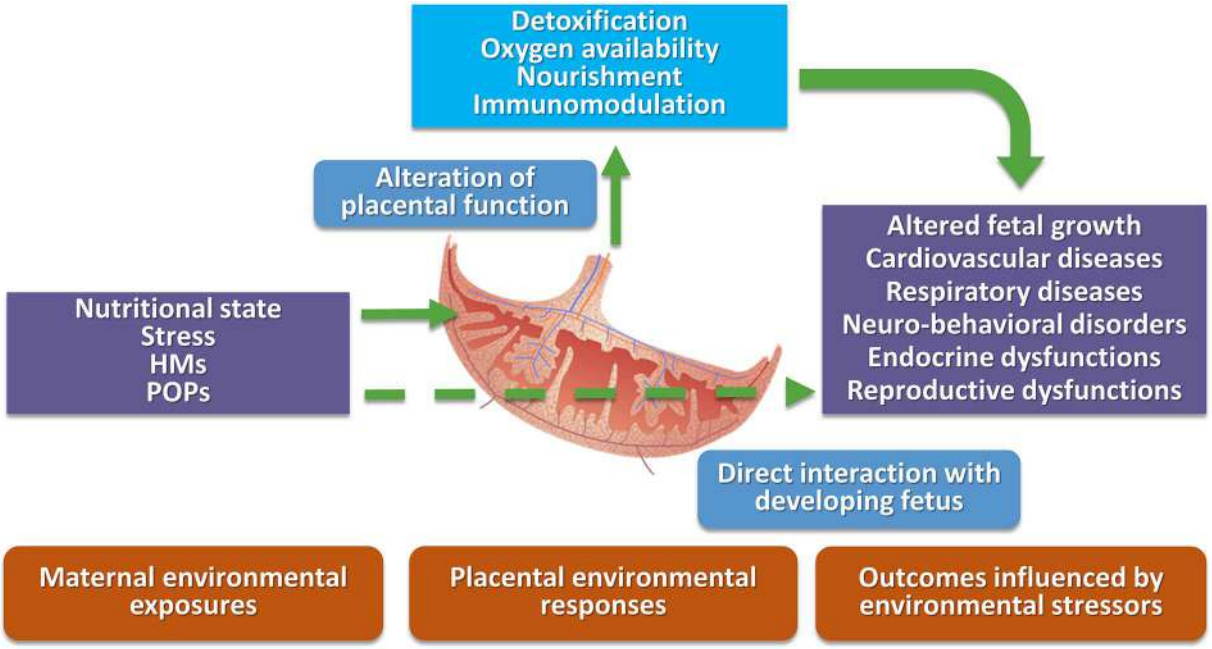

FIGURE 1 | Schematic representation of direct and indirect interactions among environmental stressors, placental domain, and possible negative health outcomes. HMs, heavy metals; POPs, persistent organic pollutants.

during a lifetime and should be measured over time. Therefore, the full characterization of an individual's exposome requires a number of measures able to capture exposure during their lifespan. However, individual susceptibility changes with age, and specific time windows can be identified.

As discussed above, fetal life is one of the crucial time windows during which future health takes shape through a dynamic interaction between the maternal/external environments and developing organs and tissues (**Figure 2**). In this context, the effort to characterize the fetal exposome is a priority for determining future health and disease predisposition.

The placenta, as a sort of gestational logbook, is a useful organ for defining the exposome, as well as a valid target organ for molecular biomarkers. In fact, the placenta plays a key role in the maintenance of an adequate intrauterine environment as well as in signal transmissions from the fetus to the mother and vice versa (101). Nutrition supplies, gas exchange, endocrine, and immune regulation are guaranteed by the placenta. At the same time, it has a pivotal role in minimizing the quantity of environmental contaminants, toxins, pathogens, and maternal stress hormones reaching the fetus (102). Placental development begins during the first few weeks after fertilization; from this moment, the success of fetal development is dependent on an appropriate placentation and on the remodeling of maternal circulation to ensure its perfusion (103). Moreover, environmental toxicants from maternal blood can reach the fetus only by passing through the placenta, which is known to be a selective barrier (104). Placental cells express detoxification enzymes and antioxidant molecules which are involved in fetal protection against toxicants and free oxygen radicals (105). On the other hand, those chemicals which do not pass the placental barrier may accumulate in placental tissue, thus modifying its functions and indirectly affecting the fetus. The placenta is usually discarded after birth and can be sampled in a non-invasive procedure. It has been previously defined as a "black-box" event recorder due to its highly informative potential for summarizing the *in-utero* experience (106). From this point of view, placental multi-omics investigation (e.g., epigenomics, transcriptomics, and proteomics) could be considered an essential step for simultaneously testing exposure, effects, and susceptibility biomarkers in a single biological matrix—i.e., a valid proxy for the internal fetal exposome.

For example, with respect to placental exposure biomarkers, studies have shown that placental levels of Cd correlate to the expression levels of the Metallothionein gene (107). Another study found that placental expression of the arsenic transporter AQP9 was positively associated with maternal urinary As levels during pregnancy (108). However, these studies consider placental gene expression for exposure to a single pollutant. Only a few studies have tried to investigate two (109) or more co-exposures and their potential effects on placental physiology or functions (110). Deyssenroth and colleagues proposed an exposure regression analysis to derive metal mixture indices associated with placental networks, which in turn are associated with small-for-gestational age (SGA) status. They found that, among 19 metals tested, As and Cd levels are associated with SGA, and the effects of these metals persist even after accounting for the presence of correlated co-pollutants (110).

With regards to biomarkers of possible effects, in a study conducted by Ahmed and colleagues, a reduction in levels

TABLE 1 | Selected list of studies on environmental pollutants performed in pediatric age in highly polluted areas, along with the relevant health outcomes.

Exposure	Type of study	Sample characteristics and compounds measurements	Outcomes	References
As	Retrospective cohort study	*in utero* and childhood exposure to As. Standard mortality rates calculated for populations living in contaminated areas compared to those of the rest of Chile.	Exposure through drinking water during early childhood or *in utero* increases mortality rate in young adults due to both malignant and non-malignant lung disease.	Smith et al. (71)
	Case-control study	339 women having children with congenital heart defects (CHDs) and 333 women with normal live births in China. As levels were measured in maternal hair samples.	Maternal exposure to As had a significant association with the risk of CHDs in offspring.	Jin et al. (72)
	Case-control study	435 women having children affected by oro-facial clefts and 1,267 mothers of unaffected children. As levels were estimates by questionnaire (occupational, drinking water, and dietary As exposure) along with a subsample of subjects with measures of individual exposure levels to As.	Positive association was observed for maternal occupational As exposure and cleft palate.	Suhl et al. (73)
	Cross-sectional study	Concentration of As in cord blood samples collected in 892 births.	Prenatal exposure to As was associated with poor neurobehavioral performance of newborns, particularly among those born to older mothers.	Wang et al. (74)
	Meta-analysis	Including 18 reports from cross-sectional, case-control and cohort studies of As exposure.	Authors reported that 50% increase of As levels in child urine would be associated with a 0.4 decrease in the intelligence quotient of children aged 5–15.	Rodríguez-Barranco et al. (75)
Hg	Meta-analysis	Meta-analysis was conducted for two major exposure sources: thimerosal vaccines that contain ethylmercury (clinical exposure) and environmental sources, using relevant literature published before April 2014.	Moderate adverse effects were observed only between environmental inorganic or organic Hg exposures and autism spectrum and attention deficit hyperactivity disorders. No effect of vaccine-derived Hg was observed.	Yoshimasu et al. (76)
	Cohort study	The Mediterranean (Italy, Slovenia, Croatia, and Greece) cohort study included 1,308 mother-child pairs. Hg levels were measured in different maternal biological samples and cord blood.	Inverse relation between Hg levels and child developmental motor scores at 18 months. No evidence of detrimental effects of Hg was found for cognitive and language outcomes.	Barbone et al. (77)
	Cohort study	Including 458 mother/infant pairs. Blood Hg levels were measured in cord blood at early and late pregnancy and at 2 and 3 years of age.	Blood Hg levels at late pregnancy and early childhood were associated with more severe autistic behaviors.	Ryu et al. (78)
	Cohort study	Maternal Hg blood concentration at 17th gestational week analyzed in 2,239 women of a Norwegian cohort.	A small but significant adverse association between children above the 90th percentile dietary Hg exposure and childhood language skills.	Vejrup et al. (79)
Cd	Cohort study	300 mothers in China. Maternal blood Cd concentration.	A 10-fold increase in maternal Cd levels was associated with a 5.7-point decrease in social domain developmental quotient and a 4.3-point decrease in circulating brain-derived neurotrophic factor levels.	Wang et al. (80)
	Cohort study	575 mother-child pairs from the prospective "Rhea" cohort on Crete, Greece. Exposure was estimated by maternal urine Cd concentrations during pregnancy.	Elevated urinary Cd concentrations ($\geq$0.8 μg/L) were inversely associated with children's general cognitive score.	Kippler et al. (81)
	Cohort study	515 mother-child pairs from the "Rhea" cohort on Heraklion, Greece. Urinary Cd concentrations measured during early pregnancy.	Elevated prenatal Cd levels were significantly associated with a slower weight trajectory and a slower height trajectory in girls and in children born to mothers who smoked during pregnancy.	Chatzi et al. (82)
	Cohort study	Cd exposure was assessed by urinary concentrations during early pregnancy (n = 1,299), 5 (n = 1,453), and 10 years of age (n = 1,498).	Childhood Cd exposure was associated with lower intelligence in boys, and there were indications of altered behavior in girls for both prenatal and childhood exposures.	Gustin et al. (83)
	Cohort study	185 participants from the ELEMENT birth cohorts in Mexico City with complete data on urinary Cd exposures, anthropometry and covariates.	Prenatal Cd exposure was negatively associated with measures of both abdominal and peripheral adiposities in girls, but not in boys.	Moynihan et al. (84)
Pb	Cohort study	4,285 pregnant women from the ALSPAC cohort. Pb levels were analyzed in blood samples from pregnant women and from 235 children at age of 30 months.	Prenatal Pb exposure was not significantly associated with child IQ at 4 or 8 years. However, some evidence suggests that boys are more susceptible than girls to prenatal exposure to Pb.	Taylor et al. (85)

(Continued)

TABLE 1 | Continued

Exposure	Type of study	Sample characteristics and compounds measurements	Outcomes	References
	Cohort study	965 pregnant women. Information about dietary intake, and maternal and cord blood levels were collected for Pb exposure assessment.	Maternal late pregnancy Pb was marginally associated with deficits in mental development index of children at 6 months.	Shah-Kulkarni et al. (86)
	Cohort study	Pb was measured in 334 mid-pregnancy women, in 362 late-pregnancy women and in umbilical cord blood, in a cohort of full-term infants in rural northeastern China.	Auditory brainstem response (ABR) and grating visual acuity (VA) maturation appears delayed in infants with higher prenatal Pb exposure during late-pregnancy, even at relatively low levels.	Silver et al. (87)
	Cohort study	Pb and As were measured in 257 maternal toenail samples collected at 28 weeks gestation and/or in 285 samples 6 weeks postpartum.	*in utero* toxic metal exposures may be associated with early life increases in blood pressure in children, which could have consequences for long-term health.	Farzan et al. (88)
	Cohort study	Pb levels were measured between 20 to 24 weeks of pregnancy and in cord blood, in 402 children from the Polish Mother and Child Cohort (REPRO_PL).	Fetal exposure to very low Pb levels might affect early cognitive domain, with boys being more susceptible than girls.	Polanska et al. (89)
PCBs	Case-control study	Southern California births, including 545 children with autism spectrum disorders (ASD) and 181 with intellectual disability (ID), as well as 418 healthy children. Concentrations of 11 PCB congeners and 2 OCPs measured in second-trimester maternal serum samples.	Higher levels of organochlorine compounds during pregnancy are associated with ASD and ID.	Lyall et al. (90)
	Cohort study	PCB and DDE were measured in maternal serum and breast milk in 656 women.	Association of PCD and DDE levels with body-mass index of girl aged 5–7 years in relation to maternal body weight.	Tang-Péronard et al. (91)
	Cohort study	Concentration of 17 PCB congeners analyzed in umbilical blood cord samples, in a total of 40 healthy term pregnancies.	Association between PCB 118 concentration and fixation pattern examined by the upright and inverted biological motion (BM) test at 4-months after birth, as a measure of social functioning.	Doi et al. (92)
	Meta-analysis	Pooled data from seven European birth cohorts with biomarker concentrations of PCB-153 and DDE in 2,487 and 1,864 samples respectively.	Significant increase in growth associated with DDE, seemingly due to prenatal exposure, and significant decrease in growth was associated with postnatal PCB-153 exposure.	Iszatt et al. (93)
	Cohort study	Concentrations of PCBs and OH-PCBs were determined in cord blood samples of 97 mother-infant pairs.	Associations between PCB and OH-PCB levels and motor optimality score, including detailed aspects of the early motor development, measured at 3-month-old infants.	Berghuis et al. (94)
PAH	Cohort study	727 Dominican or African American women living in Northern Manhattan or the South Bronx were enrolled during pregnancy. Prenatal PAH exposure was measured from 48-h personal air monitoring, and children's PAH exposure at 5 to 6 years of age was measured from residential indoor monitoring.	Repeated high exposure to pyrene was positively associated with the development of asthma, ever wheeze, asthma medication use, and emergency department visits for asthma.	Jung et al. (95)
	Cohort study	727 Dominican or African American women living in Northern Manhattan or the South Bronx were enrolled during pregnancy. Prenatal PAH exposure was measured from 48-h personal air monitoring.	Higher prenatal PAH exposures were significantly associated with higher risk for obesity at 5 and 7 years of age.	Rundle et al. (96)
	Cohort study	353 women enrolled in Krakow, Poland with valid airborne PAH data. To assess exposure to PAHs, the women were personally monitored over a 48-h period during the second ($n = 253$) or third ($n = 100$) trimester of pregnancy.	Higher prenatal exposure to airborne PAHs was found associated with a statistically significant reduction in scores on a test of non-verbal child intelligence in 5-year-old children.	Edwards et al. (97)
	Cohort study	151 children from a birth cohort study conducted by the Columbia Center for Children's Environmental Health (CCCEH) residing in Krakow, Poland. Prenatal airborne PAH exposure was measured by personal air monitoring.	PAH measures from prenatal personal air monitoring was positively associated with adverse neurodevelopment in children.	Genkinger et al. (98)

As, arsenic; Hg, mercury; Cd, cadmium; Pb, lead; PCB, polychlorinated biphenyls; PAH, polycyclic aromatic hydrocarbons.

of placental T cells and alterations of cord blood cytokine concentrations were observed in a Bangladeshi population associated with high maternal As exposure (111). Along the same line, Lambertini et al. (112) detected a placental-specific imprinted gene expression panel associated with both maternal psychosocial stress during pregnancy and birthweight. The same authors, in a previous work, also showed that alterations of placental imprinted gene expression were associated with

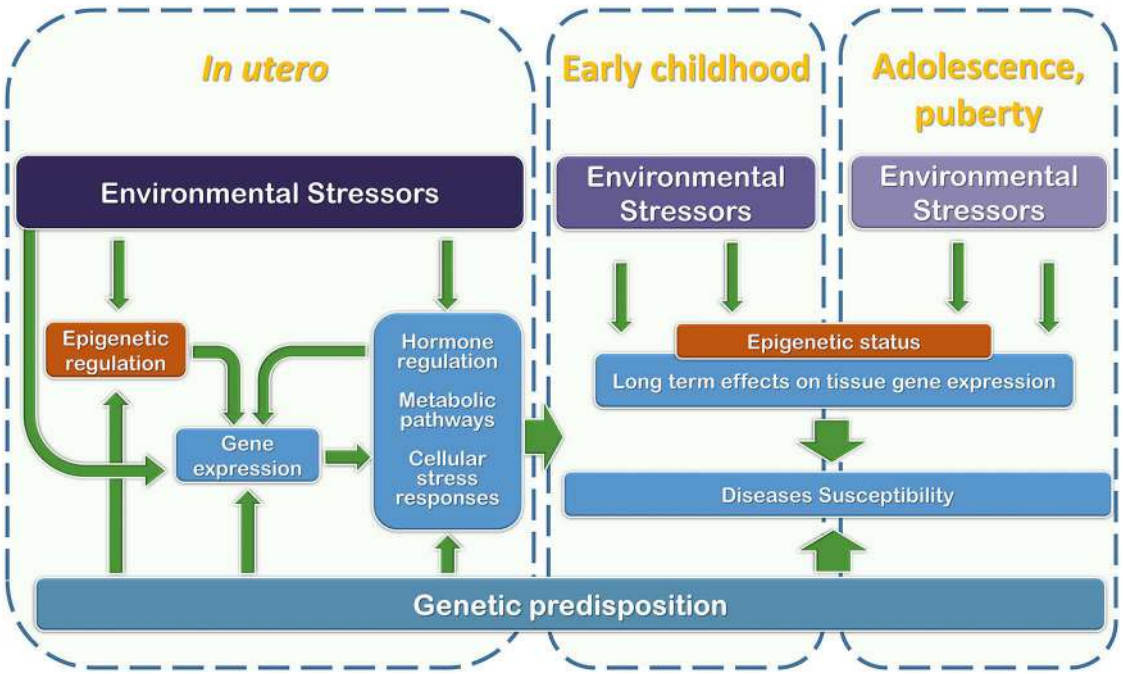

FIGURE 2 | Graphical representation of the relationships among development phases, genetic predisposition, and environmental stressor factors that may interfere with regulatory mechanisms during the early stages of life (from *in utero* development to puberty).

suboptimal perinatal growth and responsive to exposure to PCBs and DDE (113). Finally, in a recent epigenome-wide study, Maccani et al. identified evidence of hypomethylation of the EMID2 gene in association with *in-utero* Hg exposure. This altered methylation status was also found to be linked to adverse neurobehavioral outcomes during infancy (114).

In addition to data from placental examination and the bio-monitoring of multiple pollutants in maternal and cord blood (internal exposome), maternal data collected through questionnaires (diet, physical activity, lifestyle, stress factors, socio-demographic characteristics, and use of medication during pregnancy) and geo-spatial data associated with environmental monitoring stations could be used to define the external fetal exposome (both general and specific) and its association with postnatal health outcomes.

In the context of a highly polluted site, cohort studies concerning the fetal exposome may be useful for describing the complexity of chronic multi-toxicant exposure, socioeconomic determinants, and maternal life-style habits and their combined effects on the derived population of children. In the case of heavily contaminated sites, social and physical environmental toxicants tend to cluster in the most socially disadvantaged populations (115); thus, a better understanding of these complex interdependencies may help to prevent health disparities. Socioeconomic status during childhood has been found to have greater influence on adult DNA methylation profiles than socioeconomic status during adult life (116). Moreover, dietary lifestyle and micronutrient supplementation may play a role in maintaining DNA stability (117). Nevertheless, the studies on the combined effects of lifestyles/socioeconomic determinants and environmental pollutants are poorly represented in the literature. Indeed, it is well-established that maternal stress increases blood cortisol levels. The placenta is able to reduce the amount of cortisol that can reach the fetus through the 11β-HSD2 enzyme (118). At the same time, exposure to Cd influences the expression and activity of placental 11β-HSD2 (119). Thus, the contemporary presence of stressful conditions during pregnancy and Cd exposure can irreversibly affect the hypothalamic-pituitary-adrenal axis via fetal exposure to cortisol. Another example of the joint effects of the social and physical environment includes the interaction between NO_2 air exposure and elevated social stress on increasing risk for childhood asthma (120).

Finally, it is essential to produce consistent data to uncover the fetal developmental windows and molecular pathways most vulnerable to the negative influence of toxicants. Discovering specific biomarkers of prenatal exposure, which may be predictive for the child health outcomes, will increase our ability to develop early diagnostic and prophylactic/therapeutic tools to be applied in pregnant women residing in highly contaminated areas.

EXISTING BIRTH COHORTS ON PRENATAL EXPOSOME AND METHODOLOGICAL ASPECTS

Highly contaminated sites often present serious environmental contamination scenarios, where pollutants can persist in the environment for decades even after pollution sources

are removed. Moreover, in these areas, interaction between environmental pollutants and other health determinants, such as social stress, poverty, and limited access to medical services, may coexist (121). To date, the need to reduce environmental exposures has been widely highlighted, focusing on the close link between human health and the environment, as well as the possible large-scale economic return (122). Birth cohorts are an approach allowing the integration of the exposures as a whole, including those related to socioeconomic status, with "omics" data from biological samples collected at birth and throughout life. One of the major advantages provided by birth cohort design is the assessment of the early life environment, studying in-depth several possible confounders and outcomes by means of questionnaires, follow-ups based on clinical evaluation, and biological samples collected at different time points.

The Project on *Human Early Life Exposome*—HELIX is the first attempt at developing a multistep statistical analysis approach based on different tools and methods, also integrating "omics" into the exposome. To do this, HELIX pooled six existing longitudinal population-based birth cohort studies in Europe, measuring the external exposome (individual and outdoor exposures), integrating the external and internal exposome (integrating molecular exposure signatures), and evaluating the impact of the early-life exposome on child health (also including the effect of multiple exposures) (32). HELIX measured over 200 single environmental exposures of concern for child health, allowing a detailed analysis of the structure of the early life exposome, including its correlations, patterns, and variability (123). On the same emerging line, the Project *Health and Environment-wide Associations based on Large Population Surveys*—HEALS was aimed at identifying the complex links among genes, environment, and many human diseases by means of a large collaborative action among existing cohorts in Europe (124).

Recently, Sarigiannis and Karakitsios, in the context of COST Action IS1408 on "Industrially Contaminated Sites and Health Network," developed a model for the characterization of the exposome in children living close to a very large landfill area (125). With a design especially developed for a highly contaminated area, this project is an attempt to use the exposome paradigm to better understand the relationships that exist among the co-determinants of exposures and its effects on the health of exposed individuals and their progeny.

The exposure of pregnant women to environmental contaminants present in highly contaminated areas can severely impact the wellbeing of future generations. The Italian *International Centre of Advanced Study in Environment, Ecosystem and Human Health* (CISAS) project is aimed at understanding the chemical-physical and biological processes that regulate the distribution of contaminants in various environmental matrices, as well as their transfer to the ecosystem and the human body and consequent health *sequelae* (126). In the context of the CISAS project, the "*Neonatal Environment and Health Outcomes*" (NEHO) birth cohort has been established by enrolling pregnant women residing in these contaminated sites and in surrounding areas. The CISAS project evaluates pollutants in all the environmental matrices (air, soil, sediment, inland waters, and sea) as well as the food chain (fish, meat, eggs, milk, and dairy products, sampled from local producers of each evaluated area) within three heavily contaminated sites in southern Italy: two widely industrialized coastal sites characterized by petrochemical complexes and power plants and one disused industrial area. Environmental data will be linked to georeferenced maternal residences, also taking into account possible daily commuting to work. The NEHO questionnaires collect information on maternal pre-pregnancy and gestational health status, lifestyle, and socio-demographic characteristics, along with smoking habits and possible chemical exposures. The protocol includes the collection of maternal and cord blood, along with placental tissue at delivery (126). In the context of the NEHO cohort, measurements of the levels of toxicants will be taken from maternal and cord blood as well as the placenta. Because the placenta has an active role in fetal development, and the impairment of placental formation, differentiation, and/or function may affect fetal development, in the context of the NEHO cohort we will investigate the relationship between exposure to environmental toxic compounds (both HMs and POPs) and shifts in gene expression by means of a whole transcriptome analysis. Finally, follow up of the offspring will be conducted to uncover the possible consequences of specific toxicants. The follow-up on children will allow the evaluation of the possible relationship between the fetal exposome and long-term health outcomes. To this aim, after delivery, information is collected on newborns regarding use of medicine, characteristics of home environments, breastfeeding and nutritional outcomes (including growth), respiratory disease, metabolic disorders, neurocognitive development, infections and injuries, and hospitalizations (also collecting hospital discharge records). The main objectives of the NEHO cohort are: (1) to understand processes and mechanisms for the transfer of HMs and POPs from the environment and the ecosystem to the developing fetus, (2) discover specific placental biomarkers informative of fetal exposure, and (3) identify possible primary intervention strategies aimed at reducing fetal exposure. The implementation of these milestones could have an impact on the early detection of negative outcomes during childhood based on placental omics, as well as on our ability to prioritize intervention strategies.

CONCLUSIONS

The fourth session of the United Nations Environment Assembly of the UN Environment Programme, Nairobi 2019, recommended increasing efforts to overcome common health-related challenges as well as addressing the role of pollution as a cause of disease (127). Accordingly, the health of pregnant women in heavily polluted areas is an absolute priority for public health strategies.

To this end, in our opinion, the use of birth cohorts in heavily polluted areas represents a great opportunity for a better comprehension of the mechanisms underlying the relationship between environment and human health, adopting the *in-utero* developmental phase as a useful time window for identifying the origin of health and disease in childhood and adult life.

In this context, the human placenta represents a useful matrix for exploring fetal exposure to environmental contaminants and possible predisposition to adverse health effects later in life.

AUTHOR CONTRIBUTIONS

SR, GD, FB, SS, and FC made substantial contributions to the conceptualization and design of the study. SR, GD, FB, and FC are involved in study monitoring. All authors drafted and critically revised the manuscript for its content, and gave final approval of the version to be published.

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Occurrence of Halogenated and Organophosphate Flame Retardants in Sediments and Eels (*Anguilla anguilla*) From Bizerte Lagoon, Tunisia

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Contamination by classic (polybromodiphenyl ethers, PBDEs) and emerging halogenated flame retardants (HFRs) like pentabromobenzene (PBEB), hexabromobenzene (HBB), decabromodiphenyl ethane (DBDPE), and halogenated norbornenes (HNs), as well as organophosphate flame retardants (OPFRs) were investigated in sediment and eel (*Anguilla Anguilla*) samples from Bizerte Lagoon, northern Tunisia. For sediment samples, HFR levels ranged from 3.30 to 28.5 ng/g dry weight (dw), with a mean value of 10.6 ± 4.36 ng/g dw, while OPFR levels ranged between 9.77 and 164 ng/g dw, with a mean value of 53.6 ± 10.5 ng/g dw. As regards levels in fish, concentration of HFRs ranged between 4.72 and 151 ng/g lipid weight (lw) (mean value of 36.5 ± 28.5 ng/g lw), and OPFR levels ranged between 19.7 and 2,154 ng/g lw (mean value of 404 ± 367 ng/g lw). This is the first time that OPFR levels have been reporting in this area, being higher than those for HFRs. Statistical analysis of the relationship between OPFR and HFR concentrations in sediment and total organic carbon (TOC) was examined. Results suggested that OPFR levels were significantly correlated with TOC, whereas no correlation was found for HFRs and TOC. The health risk associated by the consumption of eel in Bizerte city was assessed and posed no threat to public health concerning PBDE and OPFR intakes.

Keywords: Bizerte Lagoon, HFRs, OPFRs, sediment, eel, TOC, risk assessment

INTRODUCTION

The growth of the human industrial activities has resulted in the contamination of many ecosystems. An increasing advance in technology and global population lead to increase of chemical production, and therefore, their release into the environment. The production and use of chemical additives known as flame retardants (FRs) have also increased. FRs are applied to plastics, electronics, vehicles, textiles, etc. (De Wit, 2002; Alaee et al., 2003). There are several types of FRs: halogenated flame retardants (HFRs) including brominated (BFRs) and chlorinated (CFRs), and organophosphorus flame retardants (OPFRs) which represent 20% of FRs consumption in

2006 in Europe (Van der Veen and de Boer, 2012). The most widely used group of HFRs were polybromodiphenyl ethers (PBDEs). PBDEs have been found in numerous environmental and biological matrices, such as sediment (Hu et al., 2010; Barón et al., 2014a; Herrero et al., 2018), air (Castro-Jiménez et al., 2017; Reche et al., 2019; Yadav and Nevi, 2019), biota (McHugh et al., 2010; Ben Ameur et al., 2013; Barhoumi et al., 2014a; Polder et al., 2014) and human samples (Norén and Meironyté, 2000; Zhou et al., 2002; Ben Hasine et al., 2015). Due to their adverse effect on the environment and human health, PBDEs were banned and added to the Stockholm Convention on Persistent Organic Pollutants (POPs) (SC, 2008).

Special consideration was given to new FRs which have been considered as an alternative to PBDEs, such as emerging BFRs, hexabromobenzene (HBB), pentabro-moethylbenzene (PBEB) and decabromodiphenyl ethane (DBDPE), and halogenated norbonenes (HNs), such as Dechlorane 602 (Dec 602), Dechlorane 603 (Dec 603), Dechlorane 604 (Dec 604), and Dechlorane plus (DP). HNs seem to have similar properties as PBDEs, such as persistence, high hydrophobicity and toxicity (Santín et al., 2016). In addition, many reports showed their presence in environmental and biological matrices, such as sediment (Sverko et al., 2008; Yu et al., 2015; Giulivo et al., 2017), fish (Santín et al., 2013; Widelka et al., 2016; Guo et al., 2017), bird eggs (Gauthier et al., 2007; Barón et al., 2014b).

The family of FRs encompasses OPFRs, which are organic compounds used as FRs but also as plasticizers, antifoaming agents and hydraulic fluids (Wei et al., 2015). OPFRs are proposed as alternative for PBDEs and they used in plastics, textile, electronic equipment, and furniture (Van der Veen and de Boer, 2012; Ma et al., 2017). Moreover, OPFRs are resistant to degradation and can stay persistently in the environment (Wei et al., 2015; Zhang et al., 2016). The occurrence of OPFRs was observed in various environmental compartments like air (Van der Veen and de Boer, 2012), sediment (Cao et al., 2012; Cristale and Lacorte, 2013; Wu et al., 2016) and biota (Giulivo et al., 2016; Greaves et al., 2016; Sala et al., 2019).

One example of an ecologic area submitted by several anthropogenic pressures is the Bizerte Lagoon, located in northern Tunisia, and placed in a heavily industrialized area. It supported many anthropogenic pressures, such as industrial, urbanization and agricultural activities (metallurgical industry, cement works, tire pollution factories, etc.). Previous research described the occurrence of different POPs in this area: polychlorinated biphenyl (PCBs) (Derouiche et al., 2004; Barhoumi et al., 2014b), polycyclic aromatic hydrocarbons (PAHs) (Trabelsi and Driss, 2005; Barhoumi et al., 2014c, 2016), and PBDEs and their methoxylated analogs (MeO-PBDEs) (Ben Ameur et al., 2013; Mekni et al., 2019). In this area, POPs appear with high concentration in surface sediment ($\sum$PAHs from 16.9 to 394 ng/g dw) (Barhoumi et al., 2014c), in fish ($\sum$PBDEs: 171 ng/g lw) (Ben Ameur et al., 2013), and clams ($\sum$PBDEs: 95.6 ng/g lw) (El Megdiche et al., 2017).

European eels (*Anguilla Anguilla*) were chosen as bioindicator species for organic pollution due to their specific physical and behavioral characteristics (McHugh et al., 2010). Eels are long-lived and they migrate for a large distance to reach their spawning sites (Malarvannan et al., 2014). Moreover, eels have benthic live style. PBDE, HN, and OPFR occurrence in eel was previously reported in some works (Sühring et al., 2016; Zacs et al., 2016). However, to date, there is no information on contamination levels of FRs in eels from Bizerte Lagoon. The aim of this study is to evaluate the occurrence of HFRs and OPFRs in both abiotic (sediment) and biotic (eel) samples from Bizerte Lagoon (northern Tunisia).

MATERIALS AND METHODS

Standards and Reagents

Native standards of OPFRs, including tris(2-butoxyethyl) phosphate (TBOEP), tris(chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TClPP), trihexyl phosphate (THP), and tris(2-ethylhexyl) phosphate (TEHP), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tris(phenyl) phosphate (TPHP), triphenylphosphine oxide (TPPO) and tris(1,3-dichloro-2-propyl) phosphate (TDClPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isodecyldiphenyl phosphate (IDPP) and 2-ethylhexyldiphenyl phosphate (EHDPP) were purchased from AccuStandard (New Haven, CT, USA). Diphenyl cresyl phosphate (DCP), tri-n-butyl phosphate (TNBP) and tricresyl phosphate (TMCP) were provided by Dr. Ehrenstorfer (Augsburg, Germany). Tris(isopropyl-phenyl) phosphate (IPPP) was purchased from Chiron (Trondheim, Norway). d_{15}-TDClPP, d_{27}-TNBP, d_{12}-TCEP, and $^{13}C_2$-TBOEP were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). d_{15}-TPHP was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

PBEB, HBB, DBDPE were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Native and ^{13}C-labeled standards mixtures of PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183, and BDE-209), *syn*- and *anti*-DP isomers and ^{13}C-*syn*-DP were obtained from Cambridge Isotope Laboratories Inc. Dec 602 (95%), Dec 603 (98%), and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada).

Dichloromethane (DCM), hexane, toluene, sulfuric acid, alumina (0.063–0.2 mm) and copper (<63 μm) were provided from Merck (Darmstadt, Germany). Al-N cartridges were obtained by Biotage (Uppsala, Sweden).

Sampling

Bizerte lagoon is the second largest lagoon in Tunisia. It is located in an important very economically part of northern Tunisia (latitude 37°80′-37°14′N; longitude 9°46′-9°56′E), and it covers an area of 138 km^2 with a mean depth of 7 m (**Figure 1**). Menzel Abderahmen (S1), Menzel Jemil (S2), Menzel Bourguiba (S3), Faroua (S4), Maghraoua (S5), and Channel (S6) were chosen to be sediment sampling stations, whereas S1, S2, S3 and Sea Mediterranean (SM) sites were selected for eel samples. They were selected based on possible differences in contamination levels, as well as depending on the availability of fish. Three contaminated zones (S1, S2 and S3) clearly exposed to anthropogenic pressure, as well as a non-urbanized area (SM),

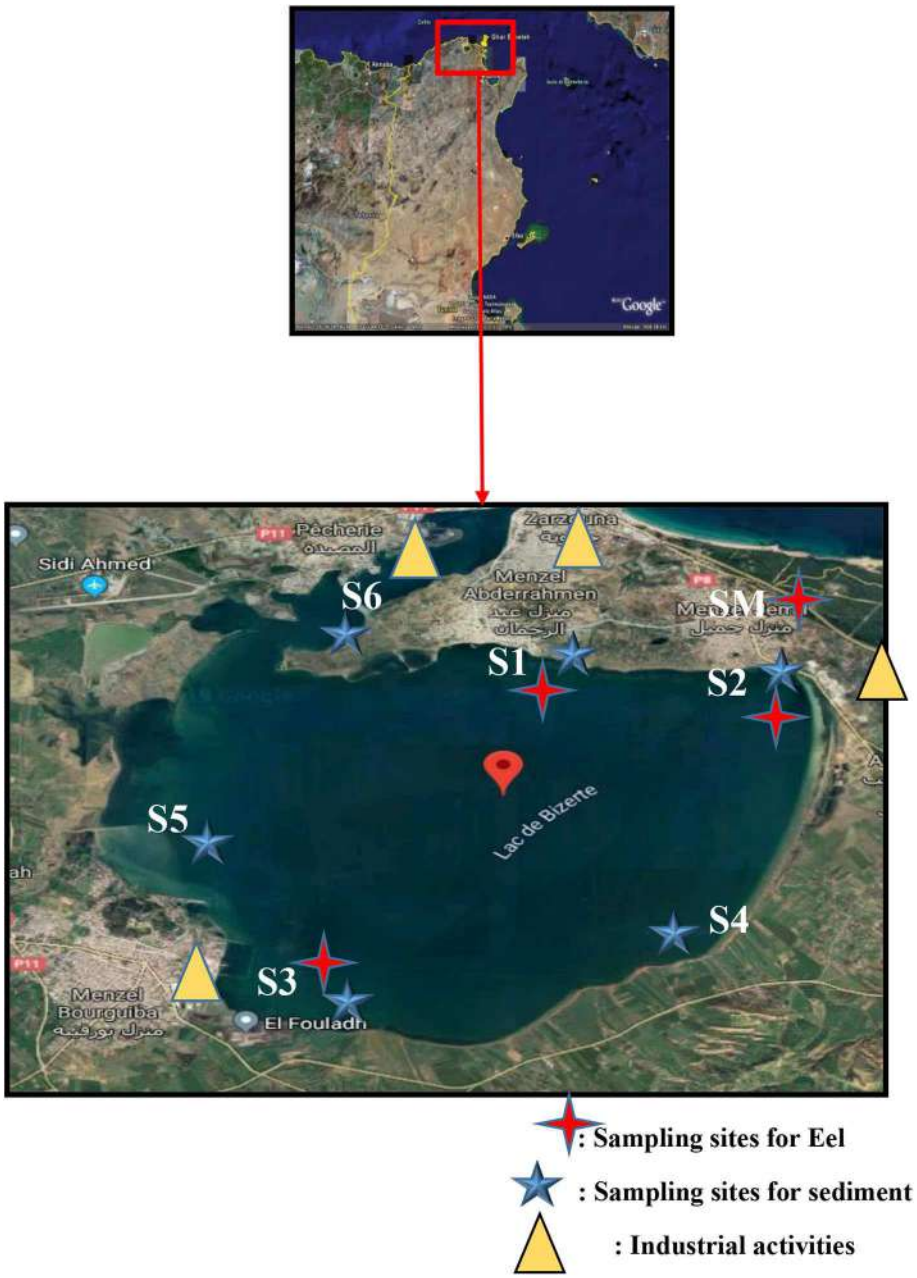

FIGURE 1 | Sediment and eel sampling locations.

were selected for eel sampling. S1 is the most populated station (10,000 inhabitants) receiving chronic inputs from urban runoff, and it is also surrounded by industrial units, such as electronic industries, textile fabrics, etc… S2, located near to industrial and mussel farming area, receives massive effluents from many industrial units, such as textile fabrics and electronic industries located in the city. Also, it receives chronic discharges from sewerage and urban runoff from the town of Menzel Jemil. S3 is influenced by the discharge from Ichkeul Lake. This site is characterized by the intensive activity, such as naval construction, tire production and metallurgical industry, and also it receives industrial effluents from the steel complex "El fouladh." S4, situated in southeast part of Bizerte Lagoon, is far from industrial source of pollution, but it is influenced by agricultural inputs. S5, situated in the northwestern part of the lagoon, is also characterized by the intensive agricultural activities. Finally, S6 is characterized by industrial activities including the passage of ships and cement manufacturing. This area is affected by the urban effluents from the Bizerte city.

Sediment and eel (*Anguilla anguilla*) samples were collected in March 2017. Two replicates of surface sediments were collected at the six stations mentioned above, reaching 12 samples. The

top 0–10 cm of sediment was collected using a stainless steel grab sampler. Sediments were freeze-dried, filtered and sieved using a 2-mm mesh and stored at 4°C until their analysis. Physicochemical parameters of sediment samples are described in **Table 1**. As regards eel samples, the sampling procedure and handling was largely based on *European Union guidelines* (OJEU, 2010). They were euthanized by cervical dislocation, and the muscle tissues of each fish were rapidly dissected and stored separately at −20°C until they were freeze-dried and homogenized with a blender. Only four stations were selected, and six specimens were sampled at each site, reaching a total of 24 eel samples. The average ± SD length (centimeter) and weight (gram) of eel collected from Bizerte lagoon were, respectively: S1 (59.6 ± 11.1; 762 ± 395), S2 (54.6 ± 6.18; 260 ± 93.7), S3 (60.4 ± 6.06; 464 ± 110), and SM (57.8 ± 13.9; 328 ± 229).

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of European Union guidelines (OJEU, 2010). The fish sampling procedures and protocols have been used previously by Barhoumi et al. (2016). Before taking any sample we took the authorization from "National Agency for Navigation and Fishing Bizerte Tunisia." The sampling was done respecting and following the instructions of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals for specific purposes "articles 24 and 25." The number of collected samples was 24 fish and that was the exact number needed for our experiences and it was preapproved by the "National Agency for Navigation and Fishing Bizerte Tunisia" to collect fish from four different sampling sites to not affect the number of fish in the area. In addition, the number of eel (*Anguilla anguilla*) is high during the period of our sampling (March of every year) knowing that it's its period of immigration of Bizerte Lagoon.

Sample Preparation

HFR analyses in sediments were carried out with a selective pressurized liquid extraction (PLE) method, in which extraction and purification were done simultaneously (De la Cal et al., 2003). One gram dry weight (dw) of sediment was spiked with internal standards (^{13}C-PBDE mixture and ^{13}C-*syn*-DP). Spiked samples were kept overnight to equilibrate. Spiked samples were ground with alumina and copper (1:2:2) and loaded into 22 mL cell extraction. Solvent extraction was DCM:hexane (1:1). Extracts were concentrated to incipient dryness and re-dissolved with toluene until a final volume of 40 μL prior to instrumental analysis.

For OPFRs, sediment extraction was also carried out by PLE. One gram dw of sample was loaded in 22 mL extraction cell with hydromatrix and copper and extracted with hexane: acetone (1:1). Extracts were concentrated to incipient dryness and re-dissolved with methanol for obtaining 500 μL as a final volume. Prior to instrumental analysis, labeled compounds (d_{12}-TCEP, d_{15}-TDClPP, d_{27}-TNBP, d_{15}-TPHP, and $^{13}C_2$-TBOEP) were added as internal standards.

Regarding HFR analyses in eel samples, 1.5 g dw was spiked with ^{13}C-PBDE mixture and ^{13}C-*syn*-DP. Samples were kept overnight in the fridge to equilibrate. Spiked samples were loaded into 11 mL extraction cell and ground with copper (1:2). A PLE was performed out using a previous optimized method (Barón et al., 2012, 2014c). After extraction, extracts were concentrated to dryness, and kept in the oven at 95°C for 2 h. Then, lipid content was determined gravimetrically. Extracts were re-dissolved in hexane and threated with sulfuric acid to remove lipids. Afterwards the organic phase was cleaned by solid phase extraction (SPE) using alumina cartridges (5g) eluted with hexane:DCM (1:1). The extracts were concentrated and reconstituted with toluene for a final volume of 40 μL.

Extraction of OPFRs from eel samples was performed by ultrasound-assisted extraction method according to Giulivo et al. (2017). 0.5 g dw were extracted twice with 15 mL of an hexane:acetone mixture (1:1). Extracts were combined and evaporated to dryness under a purified nitrogen stream, and reconstituted with hexane:methanol (1:3) up to a volume of 5 mL. Then, the solution was centrifuged and 200 μL were collected and spiked with a mixture of labeled compound (d_{12}-TCEP, d_{15}-TDClPP, d_{27}-TNBP, d_{15}-TPHP, and $^{13}C_2$-TBOEP) prior to the instrumental analysis.

Total organic carbon (TOC) is an important parameter to evaluate the environmental status of aquatic ecosystems in marine sediment (Hu et al., 2008). Properties, such as TOC or the presence of other substances in the sediment are able to decrease or increase the bioavailability of the contaminants (Santín et al., 2016). Thus, the TOC value can partly influence the distribution of organic pollutants in a coastal environment. In this work, sediment TOC was calculated by colorimetry using a 702 coulomat after decarbonisation of sediment with HCl-2N at 60°C (Ouertani et al., 2006). The percentage of fine

TABLE 1 | Sampling site locations and physico-chemical properties in costal superficial sediments of Bizerte lagoon, Tunisia.

Station	Latitude	Longitude	Depth (m)	Temperature (°C)	pH	TOC (%)	%<63 μm
S1	37°13.598′N	9°52.073′E	3.11	21.7	8.29	0.98	50.6
S2	37°12.722′N	9°55.784′E	5.2	21.9	8.19	1.87	38.7
S3	37°09.041′N	9°49.209′E	4.01	21.01	8.01	1.36	99.1
S4	37°09.207′N	9°52.744′E	5.38	20.2	8.00	1.13	96.0
S5	37°11.277′N	9°47.411′E	5.20	21.9	8.14	0.76	13.0
S6	37°15.555′N	9°51.330′E	13.4	20.1	8.07	4.24	59.1

TOC (%), percentage of total organic carbon; %<63 μm, percentage of fine grain size fraction.

grain fraction sizes (%<63 μm) for each sediment sample was determined gravimetrically after wet sieving (Savinov et al., 2000) (see **Table 1**).

Chemical Analysis

Analysis of PBDEs and emerging BFRs (PBEB, HBB, and DBDPE) was carried by an Agilent 7890 gas chromatograph coupled to an Agilent 7000A GC/MS triple quadrupole. Chromatographic separation was performed with a DB-5ms column (15 m × 0.25 mm × 0.1 μm film thickness). Gas chromatography coupled to tandem mass spectrometry (GC-MS-MS) using electron ionization (EI) was applied. More details on instrumental conditions were previously published (Eljarrat et al., 2002, 2007; Barón et al., 2014c). Due to the low sensibility obtained for decabrominated compounds, BDE-209 and DBDPE were determined by GC-NCI-MS using an Agilent 6890A gas chromatograph connected to an Agilent 5975A network mass spectrometry (Eljarrat et al., 2004). HNs were also analyzed by GC-MS-MS, but using negative chemical ionization (NCI) with CH_4^+ as reagent gas (Barón et al., 2012).

For OPFRs, an online sample purification and analysis by turbulent flow chromatography (TFC) coupled to tandem mass spectrometry (MS-MS) was performed with a Thermo Scientific TurboFlow™ system (Giulivo et al., 2016). Cyclone™−P (0.5 × 50 mm) and C18-XL (0.5 × 50 mm) columns were used for purification, whereas Purosphere Star RP-18 (125 × 0.2 mm) column was used for chromatographic separation. Spectrometric analysis was carried with a triple quadrupole with a heated-electrospray ionization source.

Selective reaction monitoring (SRM) mode was used with two transitions monitored for each compound. The most intense transitions were used for quantification purposes, and the second ones for confirmation criteria.

Quality Control

Identification of analytes was based on the following criteria: (i) simultaneous responses for the two monitored transitions must be obtained at the same retention time than those of available standards; (ii) signal-to-noise ratios must be >3; and (iii) relative peak intensity ratio must be within ±20% of the theoretical values obtained with standard solutions.

Analytical parameters, such as recoveries, limits of detection (LODs) and limits of quantification (LOQ) are summarized in **Table SI 1**.

Estimated Daily Intake and Risk Evaluation

The human exposure to HFRs and OPFRs through the consumption of eels was evaluated by calculating the estimated daily intake (EDI), expressed in ng/kg body weight/day. The calculation was based on a body weight (BW) of 60.0 kg (Ben Ameur et al., 2013) and a daily fish consumption (DFC) of 27 g/day, a value obtained from the International Institute of Nutrition and Food Technology (http://www.institutdenutrition.rns.tn). The hazard quotient (HQ) and the cancer risk (CR) were calculated for the target compounds using the methodology described by Staskal et al. (2008) and by Ni et al. (2012).

Statistical Analysis

Statistical treatment of obtained data was performed with STATISTICA (version 6). Pearson's correlation coefficient was calculated to examine the possible relationship between parameters; statistical significance was accepted at $p < 0.05$. In box plot figures, outliers (×) were calculated as values above Q3−1.5 IQR and below Q1−1.5 IQR (Q3 = third quartile, IQR = interquartile range, Q1 = first quartile). Two sample *t*-tests were carried out, using the GraphPad prism 5 program, to determine significant difference ($p \leq 0.05$).

RESULTS AND DISCUSSION

Sediment Samples

Table SI 2 summarizes the concentration levels, expressed in ng/g dw, of HFRs and OPFRs detected in sediment samples from Bizerte Lagoon, Tunisia. For results of individual compounds in each sediment sample, see **Table SI 3**.

HFRs

HFRs were detected in all sediment samples at concentration levels ranging from 3.30 to 28.5 ng/g dw, being S5 and S6 the most contaminated sites. PBDEs were detected in all analyzed samples, between 1.77 and 19.1 ng/g dw, whereas emerging BFRs were not detected in any sample. HNs were also detected in all the samples, except for one, at contamination levels between not detected (nd) and 14.2 ng/g dw.

PBDE concentrations

Mean value of ∑PBDEs in sediment samples was 7.86 ng/g dw. The highest concentration value corresponded to S5 (mean value of 14.0 ng/g dw), followed by S6 (mean value of 8.04 ng/g dw), and then by S3, S2 and S1 (mean values of 7.55, 7.19, and 5.38 ng/g dw, respectively). Relatively low concentration was detected at the selected station far from urban and industrial areas, S4 (mean value of 5.05 ng/g dw). Of the 8 different PBDE congeners included in the study, 7 were detected in samples: BDE-28, −47, −99, −100, −154, −153, and −209, with ∑PBDE levels ranging from 5.05 to 14.0 ng/g lw. PBDE congener pattern was dominated by BDE-209, with an average contribution of 83% over total PBDEs. Similar PBDE pattern in sediment samples has been previously described in different works (Lee et al., 2018; Zhu et al., 2018).

There is a lack of information regarding occurrence of PBDEs in sediments from the Bizerte Lagoon. There are some published studies focused on other pollutants, such as PCBs (Derouiche et al., 2004) or PAHs (Barhoumi et al., 2014c), from the same area. PCB levels varied from 0.89 to 6.63 ng/g dw being similar to those obtained in our study for PBDEs (1-79-19.1 ng/g dw), whereas concentrations of PAHs were higher ranging from 16.9 to 394 ng/g dw. In our work, the analysis of PBDEs in surface sediment show a difference on concentration values with a recent published study reported by Mekni et al. (2019), which focused on the analysis of the same compound in the same sampling points. The concentration levels obtained in the previous work (∑PBDEs mean value, 5.51 ng/g dw), were similar but slightly lower than those obtained in this study (∑PBDEs mean value, 7.86 ng/g

dw). The increase of level of PBDEs is probably explained by the appearance of other source of pollution, such as urban or industrial in the studied area.

If we compare our results with those recently reported in sediments from other locations around the world, similar levels were found in Danshui river basin (Taiwan) (2.30–10.5 ng/g dw) and Awash river basin (Ethiopia) (3.71–19.0 ng/g dw) (Cheng and Ko, 2018; Chai et al., 2019). However, our results are lower compared to samples collected in South China sea, with levels between 8.09 and 596 ng/g dw (Zhu et al., 2018), and in Peal river estuary, with levels ranging from 1.25 to 206 ng/g dw (Zhang et al., 2015).

HN concentrations

Dec 602, Dec 603, Dec 604, and both DP isomers (*syn*-DP and *anti*-DP) were detected in all sediment, with ∑HNs mean value of 2.69 ng/g dw. Similarly to PBDEs, S4 was the least contaminated site by HNs confirming that this station is located far of industrial and agricultural sources of pollution. S6 represented the most polluted site (mean value of 9.06 ng/g dw) followed by S1 (mean value of 3.16 ng/g dw), S2 and S3 (mean values of 1.60 and 1.57 ng/g dw, respectively) and S5 (mean value of 0.54 ng/g dw). Dec 602 was the most dominant compound, with levels up to 3.99 ng/g dw. In previous studies, Dec 602, Dec 603, Dec 604, and DP were also detected in all surface sediment samples from the Great Lakes (Shen et al., 2010) and from the Arctic Circle (Na et al., 2015). In case of both DP isomers were detected, F_{anti} defined as concentration of *anti*-DP divided by the sum of concentration of *syn*-DP and *anti*-DP, was calculated. F_{anti} values ranged from 0.23 to 0.64, being lower than value in commercial DP products, between 0.64 and 0.80 (Hoh et al., 2006) and suggesting a stereo selective enrichment of *syn*-DP isomer in the environment (Zhu et al., 2018).

Our ∑HNs ranged between nd and 14.2 ng/g dw, being comparable to values obtained in sediment from Chenab River, Pakistan (0.1–12.5 ng/g dw) (Mahmood et al., 2015), and slightly lower than in surface sediment from Jiulong River Estuary, southeast China (9.3–36.2 ng/g dw) (Chen et al., 2018).

OPFRs

OPFRs were also detected in all sediment samples at concentration levels ranging from 9.77 to 164 ng/g dw, with a mean value of 53.6 ng/g dw. S6 is the most contaminated site (mean value of 136 ng/g dw), followed by S2 (mean value of 52.0 ng/g dw), and then by S1, S5, S3, and S4 (mean values of 41.0, 38.6, 28.1, and 26.3 ng/g dw, respectively).

The fourteen OPFRs included in our analytical methodology were detected in sediment samples, with the exception of IDPP which was not detected in any sample. Moreover, THP was detected, but always at concentration levels below limit of quantification. TPPO presented the highest concentration values (mean value of 15.3 ng/g dw), followed by TPHP (mean value of 12.3 ng/g dw) and then TClPP, TNBP, and EHDPP (mean value of 8 ng/g dw, each one).

Until now, only limited studies have reported OPFR levels in sediments. Our results were higher than those published in sediments from Hainan Island, south China (0.74–60 ng/g dw) (Mo et al., 2019) or sediments from Bohai and Yellow Seas, China (0.08–4.55 ng/g dw) (Zhong et al., 2018). Lower OPFR values were found in ocean sediments from the North Pacific to the Arctic Ocean (0.16–0.46 ng/g dw) (Ma et al., 2017).

If we compare our sediments FR results, we observed that OPFR levels were higher than HFR concentrations (**Figure 2**). Moreover, student's *t*-test showed significant statistical differences between OPFRs and HFR ($t = 3.83$, $df = 17$, $p < 0.05$). There are not many published studies with HFR and OPFR values in the same series of samples. Similar findings were observed by Brandsma et al. (2015) in their study on the Western Scheldt estuary (The Netherlands). In the abiotic compartments (sediment and suspended particular matter) they found that OPFR concentrations were often higher than those of PBDEs. Similarly, Giulivo et al. (2017) analyzed sediments collected from three European river basins, Evrotas, Adige and Sava. HFR levels ranged between 0.25 and 34.0 ng/g dw, whereas OPFR concentrations were between 0.31 and 549 ng/g dw.

Sediment Geochemistry and HFR and OPFR Accumulation

Some sediment parameters, such as TOC and fine fraction percentages can influence the concentration levels of organic contaminants. The TOC and fine fraction percentages in surface sediments from the Bizerte Lagoon ranged from 0.76 to 4.24% and from 13.0 to 99.1%, respectively (**Table 1**). S6 showed the highest content of TOC (4.24%), which can be related to the massive occurrence of industrial and urban wastewater discharges, that are rich in organic matter. Regression analysis was carried out to examine the relationship between the percentage of TOC and the concentrations of ∑HFRs and ∑OPFRs. No significant correlation was observed between ∑HFRs and TOC ($p > 0.05$), suggesting that HFR distribution is more influenced by direct industrial discharge rather than organic matter content in sediment.

As regards OPFRs, significant correlation was observed between ∑OPFRs and TOC (p-values < 0.05), suggesting that OPFR distribution is influenced by the organic matter content in sediment, or/and the distribution of OPFRs may be controlled mainly by the distribution of organic matter in the sediments, which is in agreement with previous reports (He et al., 2019).

Eel Samples

Table SI 4 summarizes the concentration levels, expressed in ng/g lw, of HFRs and OPFRs obtained in eel sample from Bizerte Lagoon, Tunisia. For results of individual compounds in each eel sample, see **Table SI 5**.

HFRs

HFRs were detected in all eel samples at concentration levels ranging from 4.72 to 151 ng/g lw, being S1 the most contaminated site. The high concentration in this site can be attributed to the total length and weight of eel, which was significantly greater in this station. PBDEs were detected in all analyzed samples, between 3.03 and 59.0 ng/g lw, whereas emerging BFRs were not detected in any sample. HNs were also detected in some samples, at contamination levels between nd and 103 ng/g lw.

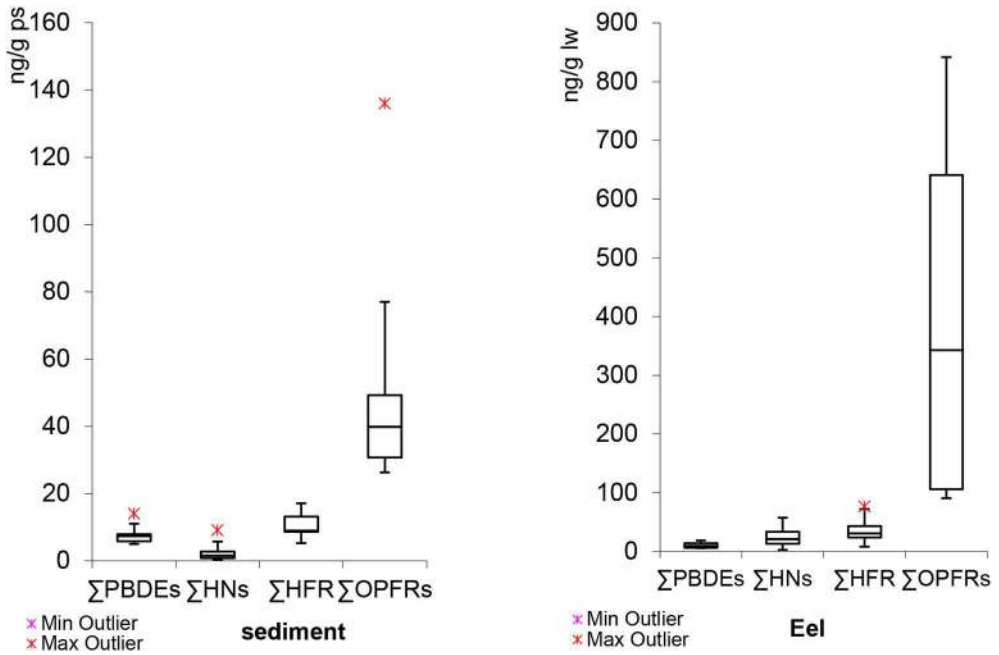

FIGURE 2 | Concentration of FRs in Eels and sediment. Outliers shown (×).

PBDE concentrations

Mean concentration of ∑PBDEs was 10.8 ng/g lw. Eight different PBDEs (BDE-28, BDE-47, BDE-100, BDE-99, BDE-154, BDE-153, BDE-183, and BDE-209) were detected in eel samples. BDE-47 was the predominant congener, followed by BDE-99 and BDE-100. This PBDE congener pattern was consistent with Penta-BDE commercial mixture contamination. The predominance BDE-47 is consistent with the general pattern found in other published works with eel samples (see references in **Table 2**), with the exception of eel samples from Irish waters (McHugh et al., 2010), which were exposed to Octa-BDE mixture rather than Penta-BDE. BDE-209, which represented the most prevalent PBDE congener in sediments, was not detected or present at low levels (from nq to 4.58 ng/g lw) in eel samples due to their very low bioavailability (log $K_{OW} \approx 10$). However, some studies reported the predominance of this high brominated congener in bivalves from Korean coastal waters (Moon et al., 2007), or high levels (from 10 to 87 ng/g lw) in eels from Flanders (Belgium) (Roosens et al., 2010).

∑PBDE levels observed in this study were compared with previous data in eel samples collected around the world (**Table 2**). Our results were similar to those found in samples collected in Loire estuary (between 25.9 and 46.2 ng/g lw) (Couderc et al., 2015), whereas they were lower than those reported by Guhl et al. (2014) in North Rhine-Westphalia rivers, with values up to 242 ng/g lw, or those published by Malarvannan et al. (2014) in Belgium, with a mean value of 94 ng/g lw.

HN concentrations

As regards HNs, their frequency of detection was slightly lower than that of PBDEs: PBDEs were detected in the 100% of analyzed samples, whereas HNs were found in the 88% of samples. The four compounds included in the analytical work were detected being Dec 604 the most times detected (75%), followed by DP (67%) and finally Dec 602 and Dec 603 (54%). However, the highest concentrations corresponded to Dec 602, with values between nd and 85.4 ng/g lw. HN concentrations varied depending on the sampling station, with S1 and S2 presenting the higher concentrations with mean values of 57.4 and 25.6 ng/g lw, respectively. Levels in SM were lower, with a mean value of 16.7 ng/g lw, corresponding with the situation of this site located far from industrial and agricultural areas. On the other hand, and despite his position near to urban and industrial areas, S3 has the lowest HN levels with a mean value of 2.41 ng/g lw, proving the limited use of HNs in this area.

There are few available studies reporting HN levels in eel samples collected in different locations of the world (**Table 2**). However, our results were always much higher than those previously published for samples collected in Germany (mean values between 0.04 and 0.13 ng/g lw) (Sühring et al., 2013a, 2016) or samples from Latvian lakes (mean value of 0.62 ng/g lw) (Zacs et al., 2018).

In addition for sediment samples, F_{anti} values have been also calculated for eel samples. F_{anti} values ranged from 0.50 to 0.53, being lower than values in commercial DP products, between 0.64 and 0.80 (Hoh et al., 2006) and suggesting that *anti*-DP can be easily degradable or that *syn*-DP is more bioaccumulative than *anti*-DP.

OPFRs

The average ± SD (∑OPFRs) concentration in eel samples from Bizerte lagoon was 404 ng/g lw (± 367). OPFRs were detected

TABLE 2 | PBDE, HN, and OPFR range of concentrations or mean values (expressed in ng/g lw) found in European eel samples collected around the world.

Location	∑PBDEs	∑HNs	∑OPFRs	References
Irish Waters	1.01–7.05[a]	nr	nr	McHugh et al., 2010
Flanders, Belgium	94	nr	673	Malarvannan et al., 2014
River Elbe, Germany	8.90	0.041	nr	Sühring et al., 2013a
River Elbe, Germany	1.8[a]	0.13–0.5[a]	nr	Sühring et al., 2013b
Latvian Lakes	0.28–26.7[a]	nr	nr	Zacs et al., 2016
Latvian Lakes	nr	0.62	nr	Zacs et al., 2018
North Rhine-Westphalia rivers, Germany	9.2–242[c]	nr	nr	Guhl et al., 2014
Loire estuary, France	25.9–46.2	nr	nr	Couderc et al., 2015
Western French, Mediterranean coast	0.08–1.74[a]	nr	nr	Labadie et al., 2010
French estuaries	11.3[b]	nr	nr	Bragigand et al., 2006
Across Scotland	13,12[c]	nr	nr	MacGregor et al., 2010
Bizerte Lagoon, Tunisia	3.90–59.0	nd–103	32.5–2,161	This study

nr, not reported; nd, below limit of detection.
[a]*Concentrations expressed in ng/g ww.*
[b]*Concentration expressed in ng/g fresh w.*
[c]*Concentration expressed in μg/kg ww.*

in all eel samples at concentration levels ranging from 19.7 to 2,154 ng/g lw, being S2 the most contaminated site (mean value of 842 ng/g lw), followed by S1 (mean value of 574 ng/g lw) and S3 (mean value of 111 ng/g lw). Finally, the lower concentrations were found at site SM (mean value of 90.7 ng/g lw).

Thirteen of the 14 OPFRs included in our analytical methodology were detected in at least some eel sample. However, THP was not detected in any sample. TClPP, TDClPP, TPHP, TNBP and DCP were detected in all analyzed eel samples. However, TMCP presented the highest levels (mean value of 436 ng/g lw), followed by EHDPP (mean value of 309 ng/g lw), IDPP (mean value of 290 ng/g lw), DCP (mean value of 206 ng/g lw), IPPP (mean value of 111 ng/g lw), TPHP (mean value of 106 ng/g lw), and TPPO (mean value of 88.2 ng/g lw).

Until now, only one study has reported OPFR levels in eel samples collected in Flanders, Belgium (Malarvannan et al., 2015), with values slightly higher than those found in our study, with a mean value of 673 ng/g lw.

Similarly to sediment samples, OPFRs were significantly higher than those HFRs in eel ($t = 3.47$, $df = 17$, $p < 0.05$) (**Figure 2**). The ratio between mean values of OPFRs and HFRs in eels from different sites ranged between 3.1 and 26, whereas for sediments these ratios were slightly lower, between 2.7 and 8.0. Higher bioaccumulation potential of HFRs vs. OPFRs has been previously described (Giulivo et al., 2017), as well as limited OPFR biomagnification through food web (Hallanger et al., 2015) probably due to biotransformation processes (Strobel et al., 2018). Thus, the higher ratio observed in eel samples could be a result of an additional OPFR source of exposure. Recently, two other studies in marine environment showed the same behavior, with OPFR levels higher than those of PBDEs in dolphin tissue samples from the Mediterranean (Sala et al., 2019) and Indian Ocean (Aznar-Alemany et al., 2019). One hypothesis would be related to the fact that OPFRs are also used as plasticizers, antifoaming agents and performance additives in consumer products. Precisely, its use as plasticizers as well as the large amount of microplastics both in continental and marine waters could also contribute to the OPFR levels found in eel samples. Microplastics come from their manufacturing for various applications, such as their use in cosmetics, toothpaste, hand soap and cleaning products. On the other hand, when plastics reach the sea, they are fragmented into microplastics by the action of sunlight and waves. Once the microplastics are ingested by the organism, the animal can accumulates the chemical compounds associated with the plastic in their tissues. However, additional studies are needed to evaluate the extent to which plastics transfer additives to organisms after ingestion and to verify if high OPFR levels in biota are due to this source of contamination.

Risk Assessment of Human Exposure

In order to understand the magnitude of the contamination in eel samples through consumption, we calculated the EDI values for the target compounds and for the general population in Bizerte. EDI values of 0.92, 2.18, and 34.5 ng/kg/day for PBDE, HNs, and OPFRs, respectively, were obtained (**Table 3**). In order to put the results in a more concrete context, it is important to calculate HQ and CR to screen the potential health risk associated to the consumption of eels from the Bizerte Lagoon.

The literature about the dietary intakes of the selected pollutants are very scarce. In this study, dietary intakes for PBDEs was 55.2 ng/day, moderately lower than those obtained for fish (*Solea solea*) consumption in the same area of study (64.3 ng/day) (Ben Ameur et al., 2013). However, EDI of PBDEs in Bizerte are higher than those from USA, 8.94–15.7 ng/day (Schecter et al., 2006), Italy 20.9 ng/day (Martellini et al., 2016), or Finland, 23 ng/day (Kiviranta et al., 2004). On the other hand, a previous published work also reported lower dietary intake in swordfish from Spain (0.06 ng/day) (Domingo et al., 2006). Regarding HNs, our dietary intake of 131 ng/day was higher than that reported by Mekni et al. (2019) in sea urchin (42 ng/day)

TABLE 3 | Calculated of estimated daily intakes (EDI) and hazard quotient (HQ) by consumption of eel (*Anguilla Anguilla*) from Bizerte Lagoon.

	EDI (ng/kg/day)	RfD (mg/kg bw/day)	HQ
BDE-47	0.38	1.0 E-04	3.8 E-03
BDE-99	0.27	1.0 E-04	2.7 E-03
BDE-100	0.17	1.0 E-04	1.7 E-03
BDE-153	0.06	2.0 E-04	3.0 E-04
BDE-154	0.007		
BDE-209	0.04	7.0 E-03	5.7 E-06
PBDE	0.92		
Dec 602	1.42		
Dec 603	0.18		
Dec 604	0.15		
Syn-DP	0.19		
Anti-DP	0.23		
HNs	2.18		
HFRs	3.10		
TCEP	0.12	22 E-03[a]	5.4 E-06
TPPO	1.88		
TCLPP	0.16	8.0 E-2[a]	2.0 E-06
TDCLPP	0.49	15 E-03[a]	3.2 E-06
TPHP	2.27	7.0 E-02[a]	3.2 E-05
TNBP	0.41	24 E-03[a]	1.7 E-05
DCP	4.41		
TBOEP	0.25		
TMCP	9.31		
EHDP	6.59		
IDPP	6.19		
IPPP	2.37		
TEHP	0.09	35 E-03[b]	2.5 E-06
OPFRs	34.5		

[a]*Ali et al. (2012).*
[b]*Kim et al. (2013).*

from the same area of Bizerte lagoon, Tunisia, and in fish (144 pg/day) from Japan (Kakimoto et al., 2012). Finally, our dietary intake for OPFRs was 2,070 ng/day, a value higher than that obtained by Soumis et al. (2003) in fish from Amazon River (990 ng/day).

The RfD is an estimate dose of a daily exposure to the human population that is likely to be without an appreciable risk of deleterious effects, and it is expressed in mg/kg/day units. EDI values obtained in this investigation ranged between 7.0 E-03 and 0.38 ng/kg/day for PBDEs, between 0.15 and 1.42 ng/kg/day for NHs and between 9.0 E-02 and 9.31 ng/kg/day for OPFRs. The U.S. EPA (2008) proposed an oral RfD between 2.0 E-04 and 7.0 E-03 mg/kg/day, for BDE-47, BDE-99, BDE 100, BDE-153, and BDE-209 congeners (**Table 3**). As regards OPFRs, oral RfD lying between 35 E-03 and 8.0 E-02 mg/kg/day for TCEP, TCLPP, TDCLPP, TPHP, TNBP and TEHP congeners. Oral RfD values are much higher than our EDI values, suggesting that the consumption of eels will not likely pose any health risks to consumers.

As far as, when the HQ value >1, indicate the potential risk of exposure for humans (Asante et al., 2011; Ni et al., 2012). In this present study, the HQ results are all below 1 suggesting a low deleterious risk to PBDEs and OPFRs through the consumption of eel from Bizerte city. Similar results were recently obtained by Mekni et al. (2019) for sea urchin (*Paracentrotus lividus*), clams (*Ruditapes decussatus*) (El Megdiche et al., 2017), and in fish (*Solea solea*) (Ben Ameur et al., 2013) from the Bizerte Lagoon, Tunisia. Around the word, our HQ values are also similar to those reported in fish and mussels from Italy (Martellini et al., 2016) and in seafood from chine (Miyake et al., 2008; Ni et al., 2012).

BDE-209 presented the only congener for which information is available to evaluate its carcinogenicity. In this case, our CR values ranged from 4.7×10^{-9} and 6.3×10^{-8}, being slightly lower than those obtained in sea urchin (Mekni et al., 2019), and in clam (El Megdiche et al., 2017) from the Bizerte Lagoon. According to CR values obtained in this study, PBDEs in eel from Bizerte Lagoon had no threat to the human health [acceptable increased cancer risk ranges between 1×10^{-6} and 1×10^{-4}, (Ni et al., 2012)]. From all these results, it can be concluded no adverse health effects (carcinogenic or non-carcinogenic) are associated with the consumption of eel from the Bizerte lagoon.

CONCLUSIONS

This work is one of few studies including the occurrence of two families of FRs, halogenated and organophosphates, in sediment and eel samples (*Anguilla anguilla*) collected from Bizerte Lagoon. In sediment samples, PBDEs were the main contributors to HFRs of contamination. While, in eel the levels of HNs were higher than PBDEs. As regards OPFRs, they were found in all samples with higher levels than HFRs in both, sediment and eel samples. Our statistically significant correlation between TOC content and OPFRs demonstrated that sediment has the capacity to accumulate more OPFRs than HFRs. The higher ratio between OPFR and HFR levels observed in eel samples could be a result of an additional OPFR source of exposure, probably due to their use also as plasticizers and the large amount of microplastics both in continental and marine waters. Our risk evaluation of eel consumption suggests that there is not significant human health risk. Dietary exposures to PBDEs and OPFRs were much lower than the respective health based guidance values.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SM conceived the study and wrote the manuscript with inputs from BB, ST, MD, and EE. EE provided the methodology, validation, resources,

supervision, and funding. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00067/full#supplementary-material

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Heavy Metals and PAHs in Meat, Milk, and Seafood From Augusta Area (Southern Italy): Contamination Levels, Dietary Intake, and Human Exposure Assessment

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Heavy metals and PAHs were measured in animal foodstuffs from Augusta-Melilli-Priolo area in order to evaluate the potential human health risk associated to their consumption. All heavy metals were detected in seafood products while most of them were <LOD in beef, pork and milks samples. Particularly, seafood products registered higher values of total arsenic (As), mercury (Hg) and lead (Pb) than other food categories, while beef and pork showed higher content of zinc (Zn). Cadmium (Cd) and Pb were below the tolerable limits reported by the European Union in foodstuffs (1) while mercury exceed the threshold value in seafood products. Among the PAHs, chrysene (Chr) was detected in all the terrestrial foodstuffs with higher concentrations found in raw milks. Small quantity of benz(a)anthracene (BaA) were also found in this food. The health risk for consumers was assessed for five age categories of consumers calculating the estimated weekly intake (EWI), the target hazard quotient (THQ) and the cancer risk (CR) for each contaminant. Moreover, the margin of exposure (MOE) was estimated for PAHs. The EWI_{Hg} related to seafood products intake exceeded the Provisional Tolerable Weekly Intake (PTWI) recommended by the European Food Safety Authority. The THQ_{Hg} was >1 for baby, children and teenagers, indicating a non-carcinogenic risk for these age categories by seafood ingestion. The CR_{As} overcame $1*10^{-5}$ for almost age categories (except "baby") and for elderly, by seafood and beef ingestions respectively. Moreover, the MOE for PAHs showed a certain cancer risk for "baby" related to cow milk ingestion.

Keywords: heavy metal, PAH, foodstuff, risk assessment, estimated weekly intake (EWI), target hazard quotient (THQ), cancer risk (CR), margin of exposure (MOE)

INTRODUCTION

Industrial activities release into the environment different wastes (gases, particles, sludge, liquid effluent) containing significant amounts of pollutants, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins (PCDDs) polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Some of them are highly toxic and persistent in the environment, representing a serious threat to human and ecosystem health (2–10).

Heavy metals such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg) lead (Pb), nickel (Ni), and zinc (Zn) occur in the environment both by natural (i.e., soil erosion and weathering of the earth's crust) and anthropogenic sources (i.e., mining, industrial effluents, urban runoff, sewage discharge, insect or disease control agents applied to crops, and many others). Although some heavy metals (Co, Cu, Cr, Ni, and Zn) play key biological functions in the organisms, they can be potentially toxic if present at higher concentrations. The so called non-essential heavy metals (Cd, Pb, and Hg) are toxic even at very low concentration (11–13). Due to their chemical properties, they can escape cellular control mechanisms, bind to native protein, DNA and nuclear proteins, inhibiting their biological activity resulting in toxicity, oxidative deterioration of biological macromolecules (14–16).

The polycyclic aromatic hydrocarbons (PAHs) are generated from the pyrolysis and incomplete combustion of organic matter (17, 18). They have been classified as genotoxic and possibly/probably carcinogenic to humans (19). Among them the benzo(a)pyrene is the most studied and is classified as human carcinogenic in the Group 1 (19). According to the EU Scientific Committee on Food, the benzo(a)pyrene (BaP) and Σ4PAHs (benzo(a)pyrene + benzo(a)anthracene + chrysene + benzo(b)fluoranthene) can be used as a marker for carcinogenic PAHs in food [(1); subsequent amendments and additions]. The area delimited by the municipalities of Augusta, Melilli and Priolo (SE Italy) is a so-called Site of National Interest (SNI) included in the National Remediation Plan by the Italian Environmental Protection Ministry in 2002 (20). The petrochemical industry of Augusta is considered one of the largest and most complex plants in Europe, located in a bay extending about 20 km along the eastern coastline of Sicily (South Italy). Since 1950, different industrial installations have been allocated, in particular chloralkali plant (1958–2005) and oil refineries, petrochemical and chemical industries, cement plants and electric power stations (20–22), generating an uncontrolled discharge of chemical pollutant in the environment (23). Due to the significant level of environmental degradation, this area is considered a site of high environmental risk, both at Italian (24) and international level (25). Local populations are constantly exposed to different contaminant from several pathways (such as air, water and food). Different studies have shown an alarming increase of congenital malformations, abortions, mortality rates, cancer diseases and nervous system malformations affecting the local populations (25–33). Other authors found high level of Hg and PAHs in sediment from the Augusta Bay (20, 21, 34), exceeding the standard limit reported by national and international sediment quality guidelines (SQGs) (35, 36). Moreover, the contamination effects on marine ecosystems and human population exposed to pollutants through the local fish consumption were studied (20, 21, 23, 34, 37–48). Food ingestion is the most important pathway of contaminant exposure for human and actually seafood and terrestrial animal products are the main route of exposure to heavy metals and persistent organic pollutants to human (49–51). Fish and seafood products are important sources of human diet and have been considered good bioindicators of environmental contamination, because of their ability to accumulate contaminants both by absorption from the environment and food ingestion (52). Similarly, food of terrestrial origin (meat and milk) represents an important source of lipophilic contaminants for human consumers. Different studies showed how these matrices can accumulate significant level of pollutants (such as PAHs, PCBs and heavy metals) into hydrophobic compartments through breathing and ingestion of contaminated water, fodder and soil during the grazing (3, 6, 7, 9, 10, 17, 53–61).

Few data are available on contaminant concentrations in foodstuffs from the area of Augusta-Melilli-Priolo, as well as studies aimed to estimate the potential public health risks for local consumers. Therefore, in this study we evaluate heavy metals and PAHs concentrations in seafood products, meats and milks samples collected from the SNI area, in order to assess the human health risk for resident population due to the consumption of local animal foodstuffs.

MATERIALS AND METHODS

Samples Collection

A total of 96 samples of different fish species (*Pagellus bagaraveo, Mullus barbatus, Trigla lucerna, Pagellus erythrinus, Sphyrena sphyrena, Diplodus annularis, Diplodus sargus, Pagellus acarne)* molluscs (*Sepia officinalis*), and crustaceans (*Parapaeneus kerathurus*) were collected during 2018 from local markets and during capture. After collection, they were packed, frozen at −20°C and stored until delivery to the laboratories for further analysis. Specimens with similar size were pooled as single sample and dissected by means of stainless steel scissors in order to avoid contamination (**Table 1**). The edible part of pooled samples was removed, homogenized, and freeze-dried for chemical analysis. Chemical analyses seafood products were conducted at the CNR's laboratories.

From May to August 2018, a total of 30 samples of bulk milks and meats (5 bovine milk, 11 sheep and goat milk, 11 beef and 3 pork meats samples) were collected from 26 different farms located in the SNI area. Particularly, the milks were taken directly in the farms, while the meats in the slaughterhouses. Different criteria were adopted to select the farms from the Italian veterinary data bank after a preliminary study on the livestock productions realized in this area. The following conditions were considered: adoption of extensive management systems, number of animals/farm > 5 (farms with higher number of animals were preferred) and geographical characteristics of their location (morphology and soil use), considering semi-circular

TABLE 1 | Species, number of individuals and pool of fish, mollusc, and crustaceans analyzed for the category "Seafood products."

Specie	Common name	Total individuals	Pool
Fish			
Pagellus erythrinus	Common pandora	28	5
Pagellus acarne	Axillary seabream	8	2
Pagellus bogaraveo	Blackspot seabream	1	1
Mullus barbatus	Red mullet	6	2
Diplodus annularis	Annular seabream	2	1
Diplodus sargus	White seabream	2	1
Trigla lucerna	Tub gurnard	2	1
Sphyraena sphyraena	European barracuda	1	1
Molluscs			
Sepia officinalis	Common cuttlefish	7	6
Crustaceans			
Penaeus kerathurus	Caramote prawn	39	4

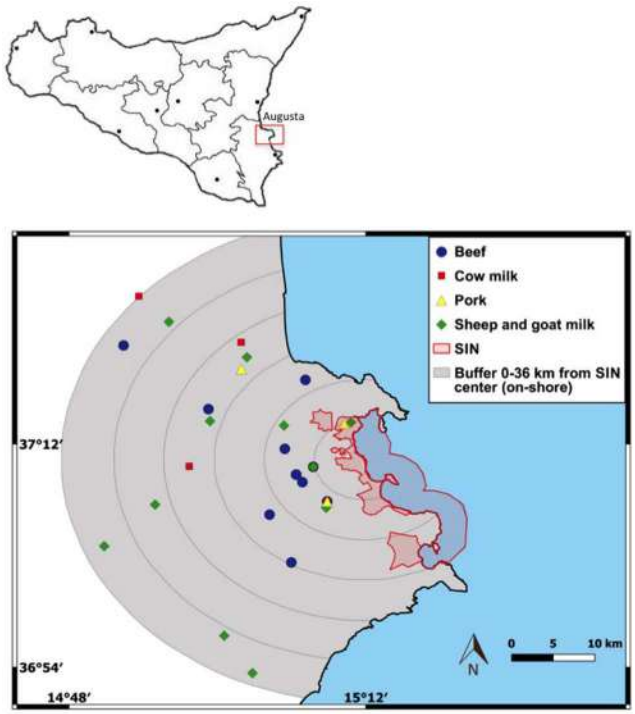

FIGURE 1 | Mapping of terrestrial foodstuff samples and the SNI area.

buffers of 6 km (from 0 to 36 km from the center of the SNI - **Figure 1**). After collection, samples were immediately frozen at −20°C (Thermo scientific: Thermo GPS Series) and storedand analyzed [in accordance with (62)] at the laboratories of Istituto Zooprofilattico Sperimentale della Sicilia (IZSSi).

Chemical Analysis

Seafood Samples

About 0.25 gr of the dried and powdered tissue of seafood products were digested using concentrated nitric acid in microwave (CEM EXPLORER SPD). The digested samples were diluted to 50 ml with deionized water and contents of As, Cd, Cr, Ni, Pb, and Zn were determined by ICP-MS (Icap Q- Thermo-Icap). The accuracy of the method was validated by the analysis of Certified Reference Materials for lobster hepatopancreas (TORT-2, National Research Council Canada). Standards were analyzed every 10 samples and all runs were carried out in triplicated. The accuracy was between 0.4 and 15%. The analytical precision, based on triplicated runs (RSD%, $n = 3$) was <10% and the reproducibility was better than 7% (LODs μg/g: As 0.001; Cd 0.001; Cr 0.002; Ni 0.004; Pb 0.004; Zn 0.06). The concentration of Hg was determined by a direct mercury analyzer atomic absorption spectrophotometer (milestone-DMA-80®), according to analytical procedures reported in EPA 7473. About 0.05 g of dried tissue was loaded in nickel boats and transferred to the DMA-80® system. Acid-cleaned laboratory materials were used in order to minimize contamination risks, during sample preparation and analyses procedures. The Certified Reference Materials-TORT-2 was used to assess analytical accuracy (estimated to be 3%) and precision (routinely better than 4%; RSD%, $n = 3$). Finally, about 20% of the total number of samples were duplicated to estimate reproducibility (which resulted in better than 7%) (LOD 0.2 μg/kg).

For PAHs determination, 1 g of freeze-dried of muscle spiked with a 45 ng of deuterated standard of PAHs, was extracted by Accelerated Solvent Extraction (ASE 200, DIONEX, Thermo Scientific) using a hexane/acetone (80:20 v/v) mixture. The extract was subjected to a saponification reaction by adding sodium hydroxide 6M, concentrated and re-dissolved with 1 ml of cyclohexane. Subsequently it was purified by SPE cartridges containing 6 g silica and cyclohexane:acetone (70:30) mixture. The final extracts were analyzed by Gas Chromatography (GC-MS ISQ; Thermo Finningan) with Mass Spectrometric detection in Selective Ion Monitoring (SIM) mode. Laboratory quality control procedures included analyses of blanks, reference material and spiked samples. The reference material used for quality control was SRM 2974a NIST (recoveries for each analyte ranged between 54 and 111%). The accuracy estimated on multiple analysis of the reference material was estimated at more than ± 10% for each single analyte. The precision estimated on triplicate samples was >90% and the reproducibility was better than 10%. The limit of detection of the method was estimated as 0.8 ng/g for each PAH. All results were converted from dried to wet-weight (μg g^{-1}) applying a conversion factor previously calculated using the following formula: $C_w = C_d$ x (100-%H_2O/100) were C_d and C_w are the concentration expressed relatively to dry and wet mass, respectively. %H_2O is the percentage of humidity in wet tissues calculated after the freeze-drying process [ranging around 80% for almost species—(63–65)].

Milk and Meat Sample

Heavy metals (As, Cd, Cr, Ni, Pb, and Zn) in milk and meat samples were determined by ICP-MS (7700x series, Agilent Technologies, Santa Monica CA, USA) following the method reported by Lo Dico et al. (66), validated in according to Lo Dico et al. (67). About 1 g of sample was transferred into previously

decontaminated PTFE vessels with 3 ml of 65% ultrapure nitric acid (V/V) and 5 ml of deionized water, and subsequently mineralized by microwave digestion (Multiwave 3000 Anton-Paar, Graz, Austria). The extracts were diluted to 50 ml, filtered and analyzed by ICP-MS. A pool of digested samples was used for the test and a calibration curve was made to evaluate the linearity with 8 standard points (BlankCal - 0.01 – 0.05 – 0.1 – 0.5 – 1 – 5 – 10 – 50 μg/l).

Mercury (Hg) concentrations were measured using a direct mercury analyser atomic absorption spectrophotometer (DMA-80®). Aliquots of 0.09 ± 0.01 g (w/w) of each thawed sample was homogenized and added into nickel boats and introduced in the DMA-80® direct analyser (Milestone, Bergamo, Italy). The amount of mercury present was detected and quantified according to calibration curves of 5 concentration points (0.050–2 mg/Kg). Standards for the instrument calibration were prepared on the basis of mono-element certified reference solution ICP standard (VWR, Milan, Italy). The limits of detection and quantification (LOD and LOQ), the repeatability and recovery of the method were calculated as described by Lo Dico et al. (66) (**Table 2**).

Extraction of PAHs, was carried out as follow: an aliquot of 5.00 ± 0.10 g of homogenized sample was taken and weighed in a 50 ml disposable tube by an analytical balance. Into the same tube, 50 μl of Mix SI PAHs (BaA-d12 and BaP-d12) were added to 100 μg/l (prepared at the time of use) of a buffer salts mixture used for the extraction phase (SUPELCO Acetate Extraction Tube cat. 55234-U or equivalent). The sample was centrifuged for 5 min at a speed of 4,000 rpm. The supernatant was transferred into a 15 ml centrifuge tube containing the purification step (SUPELCO QuE Z-Sep/C18 Cleanup Tube cat. 55401-U or equivalent) for purification and subsequently stirred for a minute and centrifuged for 5 min at a speed of 4,000 rpm. After removing the supernatant, it was transferred to a 10 ml glass tube and evaporation was made in a nitrogen stream at a temperature of 30–35°C. The purified formed residue was then recovered with 500 μl of Chrysene-d12 syringe standard at 10 μg/l, prepared at the time of use and transferred into 2 ml amber vials with conical insert for GC autosampler. An automatic GC/MSMS analyzer (Thermo Scientific TSQ Quantum XLS Triple Quadrupole GC/MSMS) was used to perform the detection. An aliquot of 2 μl and a control standard of 10 μg/l were inserted (calibration standard: 1–2–5–10–20–50–100 μg/l) in the instrument. For the calculation of the concentration of analyte in the matrix a conversion factor equal to 0.5/Sample weight was calculated. The limits of detection and quantification (LOD and LOQ), the repeatability and recovery of the method were calculated as described by Lo Dico et al. (66) and reported in **Table 2**. The values obtained were the same for each PAH (benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, and chrysene).

The quality of analytical procedures is in according to (62) and the ISO ENI 17025: 2018. The validity of the method was performed by proficiency test samples (Zscore <2) (Fapas®: Food Chemistry Proficency Test Report 07318, 2018, for Heavy Metals in milk powder; Fapas®: Food Chemistry Proficency Test Report 0677, 2018, for PAHs in smoked fish). In addition, Certified Reference Material DORM-4 (fish protein) was analyzed for analytical batch (**Table 3**). The repeatability limit was lower than the value obtained in the validation process through the analysis of double samples (C_1-C_2 <r; where C are the concentrations of the samples analyzed in duplicate and r is the limit of repeatability at that level). Finally, the accuracy, precision and reproducibility were lower than the limits calculated in the validation process (66–68) (**Table 3**).

Statistical Analysis

Results of heavy metals in terrestrial foodstuffs were integrated with those of the National Residual Plan (2012–2015) for the municipalities of Siracusa, in order to obtain a more representative dataset for the SNI area. Therefore, a statistical analysis was performed by SAS (9.1 version; non-parametric Mann-Whytney U-test by NPAR1WAY, $p \leq 0.05$) to compare the heavy metal concentrations of terrestrial foodstuffs of the SNI area with those detected in the same matrices from other districts of Sicily(National Residual Plan 2012–2015-IZSSi database). Values <LOD were considered as ½ LOD (69).

ESTIMATION OF POTENTIAL PUBLIC HEALTH RISKS

A human health risk assessment was conducted according to the United States Environmental Protection Agency methods (70), considering the ingestion rates of five different age-categories of

TABLE 2 | Validation parameters for terrestrial foodstuffs.

Metals	LOD		LOQ		Repeatability		Recovery %	
	Meats (mg/kg)	Milk (mg/kg)	Meats (mg/kg)	Milk (mg/kg)	Meats (mg/kg)	Milk (mg/kg)	Meats	Milk
Arsenic (As)	0.003	0.003	0.004	0.003	0.0016	0.0015	100.3	99.7
Cadmium (Cd)	0.003	0.001	0.004	0.001	0.0002	0.0013	102.0	100.3
Chromium (Cr)	0.070	0.070	0.090	0.090	0.0200	0.0200	106.3	106.3
Mercury (Hg)	0.041	0.041	0.050	0.050	0.0210	0.0210	103	103
Nickel (Ni)	0.050	0.050	0.060	0.060	0.1330	0.1330	92.0	92.0
Lead (Pb)	0.003	0.003	0.004	0.003	0.0016	0.0013	100.3	100.3
Zinc (Zn)	0.067	0.067	0.079	0.079	0.6820	0.0900	94.0	94.0
PAHs	200.0	200.0	500.0	500.0	60.00	60.000	75.0	75.0

TABLE 3 | Quality parameters for chemical analysis of terrestrial foodstuffs.

Heavy metals	DORM-4 certified values (μg/g)	DORM-4 obtained values (μg/g)	Proficiency test 07318 certified values for milk (μg/kg)	Proficiency test 07318 obtained values for milk (μg/kg)	Z-score (μg/kg)
Arsenic (As)	6.87 ± 0.44	6.22 ± 0.51	76.4	65.00	−0.7
Cadmium (Cd)	0.299 ± 0.018	0.301 ± 0.020	24.9	21.00	−0.7
Lead (Pb)	0.404 ± 0.062	0.411 ± 0.048	70.4	70.00	0.0
Mercury (Hg)	–	–	34.9	42.00	0.9
PAHs			**Proficiency test 0677 certified values for smoked fish (μg/kg)**	**Proficiency test 0677 obtained values for smoked fish (μg/kg)**	**Z-score (μg/kg)**
benzo(a)pyrene			3.20	2.36	−1.2
benzo(a)anthracene			11.5	10.37	−0.5
chrysene			13.6	17.96	1.5
benzo(b)fluoranthene			5.29	5.26	0.0

TABLE 4 | Ingestion rates of animal foodstuffs, considering five age-categories of Sicilian consumers (71).

Population group (men and female) SICILY (Italy)	BW	Beef	Pork	Milk	Seafood*
Age-categories	**(kg)** Mean	**(g/kg BW/die) Mean ± SD**			
Baby (0–2 years old)	11.3	3.14 ± 5.18	0.13 ± 0.28	49.72 ± 75.39	1.82 ± 2.49
Children (3–9 years old)	26.1	1.04 ± 1.01	0.62 ± 0.95	9.29 ± 7.23	1.70 ± 1.96
Teenagers (10–17 years old)	52.6	0.81 ± 0.78	0.32 ± 0.58	3.36 ± 2.20	1.28 ± 1.41
Adult (18–64 years old)	69.7	0.50 ± 0.54	0.24 ± 0.40	1.46 ± 1.46	0.71 ± 0.80
Elderly (65–97 years old)	70.1	0.54 ± 0.47	0.17 ± 0.37	1.82 ± 1.62	0.54 ± 0.65

**Unprocessed and frozen.*

consumers (71), namely children 0–2 years (11.3 kg), children 3–9 years (26.1 kg), adolescent 10–17 years (52.6 kg), adult 18–64 years (69.7 kg), seniors 65–97 years (70.1 kg) (**Table 4**).

The sheep and goat milks are not consumed unprocessed but used for cheese making. Heavy metals and PAH concentrations were detected on fresh milk. It was not possible to estimate their concentration in processed products due to the lack of the conversion factors from milk to cheese. Therefore, sheep and goat milks were excluded in the estimation of potential human health risk. Concerning the risk exposure due to the seafood consumption, we considered the average values of each pollutant, independently from the specie (as indicative of "Seafood products") in according to the foodstuffs categories reported by INRAN (71).

The human exposure of heavy metals and PAHs due to animal products ingestion was assessed calculating the Estimate Weekly Intake (EWI), the Target Hazard Quotient (THQ) and the Lifetime Cancer Risk (CR). According to previous studies, we assumed that the ingestion dose is equal to the adsorbed contaminant dose and that cooking has no effect on contaminant concentrations (72). The EWI, the THQ and the CR for each contaminant (inorganic and organic) were calculated on mean concentrations, considering ½ LOD where data were <LOD (69). The EWI for each metal was compared with the Provisional Tolerable Weekly Intake (PTWI) recommended by the European Food Safety Authority and WHO (Cd = 25 μg/kg bw; Cr = 700 μg/kg bw; Ni = 35 μg/kg bw; Pb = 25 μg/kg bw; Zn = 7,000 μg/kg bw).

Concerning the Hg exposure, we considered the PTWI for methylmercury (MeHg), the most toxic organic form of Hg (73–75) present in fish as more than 70% of total Hg (76–78).

The inorganic form of As (iAs) is considered more toxic than organic arsenic compounds and predominant in terrestrial animal foodstuffs, together with single methylated arsenic species. In this survey, iAs contents in seafood products was estimated applying 2% of the total As (79–86). Actually, the organic As species are commonly present in seafood (i.e., arsenobetaine and different arsenosugars) and considered less

dangerous for human health (87, 88). Otherwise, the iAs in terrestrial foodstuffs was calculated as 70% of the total As according to EFSA (89). Therefore, we estimated the iAs human exposure comparing the dietary intake with the lower limit on the benchmark dose for a 0.5% (BMDL0.5) (range: 2–7 μg/kg bw per day) calculated by JECFA (90) for cancer incidence.

Estimated Weekly Intake

The metals human exposure was assessed according to the following equation:

$$EWI = (Cm \times IR_w)/BW \quad (1)$$

where Cm represents the average concentrations of contaminant for each category of considered food (μg g^{-1}); IR_w is the weekly ingestion rate (g $week^{-1}$) derived from the INRAN database (2010) for seafood products, cow milk and meats in Sicily; BW is the body weight (kg) reported by INRAN database (2010) for 5 different age-category of consumers.

The total EWI for each metal derived from meat (beef and pork), cow milk and seafood products ingestion was calculated as follow:

$$\text{Total } EWI_c = EWI_{(c-beef)} + EWI_{(c-pork)} + EWI_{(c-milk)} + EWI_{(c-seafood)} \quad (2)$$

To assess the risk due to PAHs exposure, the total BaP equivalent concentration (BEC) was estimated in each foodstuff as follow:

$$BEC = \sum_{i=1}^{n} c_i \; x \; TEF_i \quad (3)$$

Where c_i is the concentration of PAH congener *i* in the foodstuff and TEF is the toxic equivalency factor used to quantify the carcinogenicity of BaA (0.1), BbF (0.1), and Chr (0.01) respect to BaP (91).

The BEC was used to estimate the EWI of all congeners (∑4PAH):

$$EWI = (BEC^{*}IR_w)/BW \quad (4)$$

Similarly to metals, the total EWI was calculated according to the Equation 2.

Non-carcinogenic Health Hazard

The risk of non-carcinogenic effects was estimated using the Target Hazard Quotient (THQ), that is the ratio between the exposure and the reference dose (RfD). THQ was calculated according to the USEPA (92) method using the following equation:

$$THQ = [(EF \times ED \times FIR \times C/RfD \times BW \times AT)] \times 10^{-3} \quad (5)$$

where EF is the exposure frequency (365 days $year^{-1}$ for people who eat each categories of food every day); ED is the exposure duration (years); FIR is the food ingestion rate for each categories of food (g day^{-1}) (71); C is the metal concentration in foodstuff (μg g^{-1}); RfD is the oral reference dose in μg $g^{-1}day^{-1}$: As = 3.0×10^{-4}, Cd = 1.0×10^{-3}, Cr = 3×10^{-3}; Hg = 1.0×10^{-4}, Ni = 2×10^{-2}; Pb = 4.0×10^{-3}; Zn = $3.0 \times 10^{-1;}$ BaP = 0.0003 (92); AT is the average time for non-carcinogens (it is equal to 365 days $year^{-1}$ x ED). The ED used in this study were 1 year (children 0–2 years), 6 years (children 3–9 years), 14 years (adolescent 10–17 years), 41 years (adult 18–64 years), 81 years (seniors 65–97 years).

The THQ for ∑4PAH was calculated by replacing the concentration of metal "C" with the BEC value.

A THQ value <1 indicates negligible non-carcinogenic risks for consumers (72). Higher THQ values indicate significant risks for long-term non-carcinogenic effects (93, 94). Additionally, the total target hazard quotient (TTHQ) for each contaminant (Equation 6) end for all considered toxicants (Equation 7) were also calculated in order to evaluate the non-carcinogenic risk for human resulting from consumption of different foodstuffs:

$$TTHQ_c = THQ_{(c-beef)} + THQ_{(c-pork)} + THQ_{(c-milk)} + THQ_{(c-seafood)} \quad (6)$$

$$TTHQ_i = \sum THQi \quad (7)$$

Carcinogenic Risk Assessment of Metals and PAHs

The cancer risk (CR) associated with consumption of selected foodstuff was assessed following the equation:

$$CR = [(EF \times ED \times FIR \times C \times C_SF)/(BW \times AT)] \times 10^{-3} \quad (8)$$

Were CsF is the cancer slope factor derived by response-dose curve for toxicant ingestion: As = 1.5 kg-day/mg; Cd = 6.3 kg-day/mg; BaP = 1 kg-day/mg (70); Cr = 5×10^{-1} kg-day/mg; Pb = 8.5×10^{-3} kg-day/mg) (95, 96); Ni = 0.91 kg-day/mg (95).

The CR for ∑4PAH was calculated by replacing the concentration of metal "C" with the BEC value.

Usually, the CR between 10^{-6} (risk of developing cancer over a human lifetime is 1 in 1,000,000) and 10^{-4} (risk of developing cancer over a human lifetime is 1 in 10,000) indicate a low health risk for carcinogens, while a value more than 10^{-4} involves a serious potential health risk (97, 98). In this study, we consider 10^{-5} as cancer benchmark.

Furthermore, the margin-of-exposure approach (MOE) was used according to the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) (99). The MOE is a useful tool for health risk characterization for a given population exposed to genotoxic and carcinogenic substances as PAHs (99) and is defined as:

$$MOE = BMDL_{10}/EDI \quad (9)$$

where $BMDL_{10}$ represents the lower bound of the 95% confidence interval on the benchmark dose corresponding to a 10% to more incidence in experimental animals (BaP = 0.1 mg /kg BW/day) (99), while EDI stands for the chronic daily dietary PAHs exposure (mg/kg BW/day). Similarly to the other risks indices, the total MOE for Σ4PAH was calculated considering the BEC value. MOEs <10,000 represent a potential concern for human health (99).

RESULTS

Heavy Metals and PAHs Content

Heavy metal shows wide range of concentrations among the analyzed samples (**Table 5**). In general, all heavy metals were detected in seafood while most of them were <LOD in beef, pork and milks (**Table 5**). Particularly, higher values of total As (mean 7.06 μg/g), Hg (mean 0.99 μg/g) and Pb (mean 0.09 μg/g) were found in seafood products respect to the other categories of foodstuff, while beef and pork showed higher content of Zn (mean 48.94 μg/g and 44.91 μg/g, respectively).

Focusing on individual marine organisms, Hg concentrations exceed the threshold limits imposed by European Community (1) for seafood in almost the fish species (*P. erythrinus:* 1.13 μg/g; *D. annularis:* 3.51 μg/g; *M. barbatus*: 1.91 μg/g; *T. lucerna:* 0.66 μg/g and *S. sphyraena*: 0.78 μg/g) and in the crustacean *P. kerathurus* (0.59 μg/g), while Pb content was above the threshold limit for fish [0.3 μg/g; (1)] only in the specie *P. acarne* (0.57 μg/g). Cd concentrations remain within the normative limits in all species (**Table 5**).

The highest values of Pb were found in meats (beef: 0.098 μg/g; pork: 0.064 μg/g) sampled in the farm located at about 6 km from the SNI.

As regard PAHs concentrations, the BaA was detected in cow (mean 0.30 ng/g), sheep and goat milks (mean 0.13 ng/g), while Chr was found in beef (men 0.34 ng/g), cow (mean 12.56 ng/g) and in sheep and goat milks (9.25 ng/g). The BaP and the BbF were <LOD in all foodstuff.

In particular, highest values of chrysene (43.96 ng/g and 95.12 ng/g in cow and sheep and goat milks, respectively) were found in milks collected in the farms located very closed to the SNI.

Dietary Exposure and Health Risk

The total EWI for all metals were below the PTWI except for Hg, mainly related to seafood ingestion (**Table 6**). Similarly, values of $TTHQ_{Hg}$ >1 were obtained in all age classes of consumers primarily due to the intake of milk and seafood products. In particular, THQ_{Hg} >1 were recorded in 2 age classes due to milk consumption (10.19 and 1.90 for baby and children, respectively) and in 3 age classes due to seafood consumption (2.38, 2.23, and 1.68 for baby, children and teenagers, respectively). Moreover, As and Zn showed a TTHQ>1 in the age class "baby."

The CR_{As}, $CR_{Cr,}$ and CR_{Ni} exceed the acceptable lifetime risk of 10^{-5} in beef, milk and seafood products. In particular, beef ingestion determined a certain risk for "elderly," while cow milk consumption showed critical values of CR_{Cr} and CR_{Ni} for the all age classes. Furthermore, the CR_{As} related to seafood ingestion indicated a risk for all age classes, excepted for "baby" (**Table 6**).

The cow milk mainly contributed to the EWI of PAHs, determining a MOE value < 10,000 in the "baby" age class (7561; **Table 7**). The THQ and CR values due to PAHs ingestion did not show relevant risk for any foodstuff and age class.

DISCUSSION

The foodstuffs of animal origin (terrestrial and aquatic) play a key-role in the human diet as they provide proteins, vitamins, and other important nutrients (59, 100) with potential health benefits. Otherwise, their consumption represents the principal pathway of exposure to potentially deleterious compounds, such as heavy metals and persistent organic pollutants, constituting therefore an important public health issue (50, 59). Indeed, animals are constantly exposed to contaminants present in the environment and are able to accumulate pollutants in elevated concentration in their tissues, in particular in the fat (53, 101). Therefore, the EU Scientific Committee on Food has set maximum level for certain contaminants in foodstuffs, periodically monitoring by the European Food Safety Authority (EFSA).

In this preliminary study, we evaluated the content of heavy metals and PAHs in local products from a contaminated site (SNI of Augusta), in order to assess the risk for resident population derived from their consumption.

Occurrence of Heavy Metals in the Study Area

This investigation shows that heavy metal concentrations were not relevant in meat and milks and that seafood products give the main contribution to the total dietary uptake.

Cd and Pb, concentrations were below the regulation limit in all matrices analyzed (1) and along with Cr, Ni, and Zn were generally consistent with those reported by EFSA (102–106). Differently, the pork meat showed a higher concentration of Pb (mean 0.024 μg/g) respect EFSA value [0.011 μg/g; (103)] due to the highest concentration (0.064 μg/g) found in one sample collected in a farm very close to the SNI center. Moreover, Cr and Ni concentrations were lower than those found in meats collected in Europe (104, 105). Data on contaminant concentrations in terrestrial foodstuffs were also compared with those collected (IZSSi database) during the National Residual Plan carried out in Sicily from 2012 to 2015. Ni and As contents in dairy products collected in the SNI area (municipalities of Siracusa) were lower than those found in the same food category in other districts of Sicily ($P < 0.05$; **Table 8**), while heavy metals in meats did not show significant difference.

Hg concentrations here found in seafood products are of great concern, exceeding the limits set for fishery products by the European Regulation (1). The Hg content in meats and milks was < LOD, according to EFSA (73) that considers only Hg content in fish and other seafood for the evaluation of human exposure.

The higher Hg concentrations measured in seafood samples is probably related to ecological habits of species and are consistent with results reported by previous studies in the same area (23, 39, 43, 107). Specifically, Bonsignore et al. (43) have found elevated Hg concentrations in fish caught from inside and outside the Augusta Bay, suggesting an active release mechanism of Hg from sediments to the environment and thus a potential risk, both for marine organisms and consumer, due to seafood ingestion.

Arsenic, particularly in the inorganic form, is classified by IARC as carcinogenic to humans [Group 1, (108, 109)] and thus is often considered for human health risk assessment, although there is no legislation defining As limit in food.

TABLE 5 | Mean, standard deviation, and range values of heavy metals and PAHs in the animal foodstuffs.

Food	Heavy metals Means ± SD (range) μg/g							PAHs Means ± SD (range) ng/g			
	As	Cd	Cr	Hg	Ni	Pb	Zn	BaA	BaP	BbF	Chr
Beef	0.012 ± 0.008 (0.002–0.031)	<LOD	<LOD	<LOD	<LOD	0.019 ± 0.027 (0.002–0.098)	48.94 ± 11.94 (32.11–70.15)	<LOD	<LOD	<LOD	0.34 ± 0.65 (0.10–2.26)
Pork	0.015 ± 0.012 (0.002–0.022)	<LOD	<LOD	<LOD	<LOD	0.024 ± 0.034 (0.002–0.064)	44.91 ± 13.43 (34.17–59.96)	<LOD	<LOD	<LOD	<LOD
Cow milk	<LOD	<LOD	<LOD	<LOD	<LOD	0.002 ± 0.001 (0.001–0.004)	2.92 ± 0.96 (2.06–4.44)	0.30 ± 0.46 (0.10–1.12)	<LOD	<LOD	12.56 ± 19.17 (0.10–43.96)
Sheep and goat milk	<LOD	<LOD	<LOD	<LOD	<LOD	0.002 ± 0.002 (0.001–0.007)	2.77 ± 1.18 (1.69–5.18)	0.13 ± 0.09 (0.10–0.39)	<LOD	<LOD	9.25 ± 28.49 (0.10–95.12)
Fish											
Pagellus erythrinus	3.62 ± 0.98 (2.73–5.02)	0.001 ± 0.0002 (0.0003–0.001)	0.019 ± 0.007 (0.01–0.03)	1.13 ± 0.22 (0.94–1.45)	0.002 ± 0.001 (0.0003–0.003)	0.007 ± 0.003 (0.003–0.01)	3.40 ± 0.76 (2.84–4.66)	<LOD	<LOD	<LOD	<LOD
Pagellus acarne	4.72 ± 0.52 (4.35–5.09)	<LOD	0.02 ± 0.02 (0.005–0.04)	0.59 ± 0.11 (0.52–0.67)	0.01 ± 0.006 (0.009–0.018)	0.57 ± 0.079 (0.52–0.63)	2.46 ± 0.26 (2.28–2.64)	<LOD	<LOD	<LOD	<LOD
Pagellus bogaraveo	2.47	0.0005	0.01	0.06	0.016	0.02	3.72	<LOD	<LOD	<LOD	<LOD
Mullus barbatus	9.94 ± 2.65 (8.1–11.8)	0.001 ± 0.00002 0.001	0.02 ± 0.01 (0.02–0.03)	1.91 ± 0.28 (1.71–2.11)	0.003 ± 0.001 (0.002–0.004)	0.03 ± 0.001 0.03	4.14 ± 1.55 (3.05–5.24)	<LOD	<LOD	<LOD	<LOD
Diplodus annularis	4.52	0.001	0.04	3.51	0.001	0.01	6.31	<LOD	<LOD	<LOD	<LOD
Diplodus sargus	7.42	0.001	0.01	0.48	0.003	0.17	3.00	<LOD	<LOD	<LOD	<LOD
Trigla lucerna	4.41	0.0004	0.02	0.66	0.002	0.01	4.22	<LOD	<LOD	<LOD	<LOD
Sphyraena sphyraena	1.37	0.006	0.01	0.78	0.003	0.001	4.40	<LOD	<LOD	<LOD	<LOD
Molluscs											
Sepia officinalis	22.68 ± 14.88 (8.73–44.60)	0.003 ± 0.002 (0.001–0.006)	0.01 ± 0.01 (0.002–0.02)	0.21 ± 0.08 (0.07–0.78)	0.05 ± 0.03 (0.003–0.09)	0.09 ± 0.11 (0.001–0.29)	13.94 ± 1.88 (4.40–16.22)	<LOD	<LOD	<LOD	<LOD
Crustaceans								<LOD	<LOD	<LOD	<LOD
Penaeus kerathurus	9.45 ± 2.67 (7.90–13.45)	0.003 ± 0.00 (0.002–0.003)	0.02 ± 0.01 (0.02–0.03)	0.59 ± 0.02 (0.56–0.62)	0.01 ± 0.00 (0.004–0.01)	0.01 ± 0.01 (0.01–0–02)	18.05 ± 4.00 (15.67–24.03)	<LOD	<LOD	<LOD	<LOD
Total seafood products	7.06 ± 6.17 (1.37–44.60)	0.002 ± 0.002 (0.0003–0.006)	0.019 ± 0.011 (0.002–0.044)	0.99 ± 1.02 (0.057–3.51)	0.010 ± 0.014 (0.0004–0.085)	0.09 ± 0.18 (0.001–0.63)	6.36 ± 5.27 (2.28–24.03)	<LOD	<LOD	<LOD	<LOD

TABLE 6 | Estimated weekly intake (EWI; μg/kg b.w.), Target hazard quotient (THQ), Total Target hazard quotient (TTHQ) and Target carcinogenic risk (CR) for each analyzed metals in the five age-categories considering an exposure time of 365 days year^{-1}.

Food		EWI (μg/kg BW)							THQ							CR				
		As	Cd	Cr	Hg	Ni	Pb	Zn	As	Cd	Cr	Hg	Ni	Pb	Zn	As	Cd	Cr	Ni	Pb
Beef	Baby (0–2 yr)	0.35	0.03	0.77	0.45	0.55	2.16	262.40	0.16	0.00	0.04	0.64	0.00	0.02	0.51	1.1E-06	4.2E-07	7.9E-07	1.0E-06	7.2E-09
	Children (3–9 yr)	0.11	0.01	0.25	0.15	0.18	0.71	86.91	0.05	0.00	0.01	0.21	0.00	0.01	0.17	2.1E-06	8.4E-07	1.6E-06	2.0E-06	1.4E-08
	Teenagers (10–17 yr)	0.09	0.01	0.20	0.12	0.14	0.56	67.69	0.04	0.00	0.01	0.17	0.00	0.01	0.13	3.8E-06	1.5E-06	2.8E-06	3.7E-06	2.6E-08
	Adult (18–64 yr)	0.06	0.01	0.12	0.07	0.09	0.34	41.78	0.03	0.00	0.01	0.10	0.00	0.00	0.08	6.9E-06	2.8E-06	5.1E-06	6.7E-06	4.7E-08
	Elderly (65–97 yr)	0.06	0.01	0.13	0.08	0.09	0.37	45.13	0.03	0.00	0.01	0.11	0.00	0.00	0.09	**1.5E-05**	5.9E-06	**1.1E-05**	**1.4E-05**	1.0E-07
Pork	Baby (0–2 yr)	0.01	0.00	0.03	0.02	0.02	0.06	40.87	0.00	0.00	0.00	0.03	0.00	0.00	0.02	3.0E-08	1.8E-08	3.3E-08	4.2E-08	3.8E-10
	Children (3–9 yr)	0.05	0.01	0.15	0.09	0.11	0.28	194.90	0.02	0.00	0.01	0.13	0.00	0.00	0.09	8.4E-07	5.0E-07	9.3E-07	1.2E-06	1.1E-08
	Teenagers (10–17 yr)	0.02	0.00	0.08	0.05	0.06	0.14	100.59	0.01	0.00	0.00	0.07	0.00	0.00	0.05	1.0E-06	6.1E-07	1.1E-06	1.5E-06	1.3E-08
	Adult (18–64 yr)	0.02	0.00	0.06	0.03	0.04	0.11	75.44	0.01	0.00	0.00	0.05	0.00	0.00	0.04	2.2E-06	1.3E-06	2.5E-06	3.2E-06	2.9E-08
	Elderly (65–97 yr)	0.01	0.00	0.04	0.02	0.03	0.08	53.44	0.01	0.00	0.00	0.03	0.00	0.00	0.03	3.1E-06	1.9E-06	3.4E-06	4.5E-06	4.0E-08
Cow milk	Baby (0–2 yr)	0.34	0.19	12.18	**7.13**	8.70	1.44	1014.76	0.16	0.01	0.58	**10.19**	0.06	0.03	0.48	1.0E-06	2.5E-06	**1.2E-05**	**1.6E-05**	1.1E-08
	Children (3–9 yr)	0.06	0.04	2.28	1.33	1.63	0.27	189.60	0.03	0.00	0.11	**1.90**	0.01	0.01	0.09	1.2E-06	2.8E-06	**1.4E-05**	**1.8E-05**	1.3E-08
	Teenagers (10–17 yr)	0.02	0.01	0.82	0.48	0.59	0.10	68.58	0.01	0.00	0.04	0.69	0.00	0.00	0.03	9.9E-07	2.3E-06	**1.2E-05**	**1.5E-05**	1.1E-08
	Adult (18–64 yr)	0.01	0.01	0.36	0.21	0.26	0.04	29.80	0.00	0.00	0.02	0.30	0.00	0.00	0.01	1.3E-06	3.0E-06	**1.5E-05**	**2.0E-05**	1.4E-08
	Elderly (65–97 yr)	0.01	0.01	0.45	0.26	0.32	0.05	37.15	0.01	0.00	0.02	0.37	0.00	0.00	0.02	3.1E-06	7.3E-06	**3.7E-05**	**4.8E-05**	3.4E-08
Seafood products	Baby (0–2 yr)	1.80	0.02	0.24	**12.60**	0.13	1.18	81.09	0.86	0.00	0.01	**2.38**	0.00	0.06	0.04	5.5E-06	2.7E-07	2.5E-07	2.3E-07	2.1E-08
	Children (3–9 yr)	1.68	0.02	0.22	**11.77**	0.12	1.10	75.74	0.80	0.00	0.01	**2.23**	0.00	0.05	0.04	**3.1E-05**	1.5E-06	1.4E-06	1.3E-06	1.2E-07
	Teenagers (10–17 yr)	1.27	0.01	0.17	**8.86**	0.09	0.83	57.03	0.60	0.00	0.01	**1.68**	0.00	0.04	0.03	**5.4E-05**	2.7E-06	2.4E-06	2.35E-06	2.0E-07
	Adult (18–64 yr)	0.70	0.01	0.09	**4.92**	0.05	0.46	31.63	0.33	0.00	0.00	0.93	0.00	0.02	0.02	**8.8E-05**	4.4E-06	3.9E-06	3.8E-06	3.3E-07
	Elderly (65–97 yr)	0.53	0.01	0.07	**3.74**	0.04	0.35	24.06	0.25	0.00	0.00	0.71	0.00	0.02	0.01	**1.3E-04**	6.5E-06	5.9E-06	5.6E-06	5.0E-07
Total	Baby (0–2 yr)	2.50	0.25	13.22	**20.21**	9.40	2.27	1399.12	**1.19**	0.01	0.63	**13.25**	0.07	0.11	**1.05**					
	Children (3–9 yr)	1.91	0.07	2.91	**13.34**	2.03	1.47	547.16	0.91	0.00	0.14	**4.47**	0.02	0.07	0.39					
	Teenagers (10–17 yr)	1.40	0.04	1.27	**9.51**	0.87	1.04	293.89	0.67	0.00	0.06	**2.60**	0.01	0.05	0.24					
	Adult (18–64 yr)	0.78	0.02	0.63	**5.23**	0.43	0.59	178.66	0.37	0.00	0.03	**1.38**	0.00	0.03	0.15					
	Elderly (65–97 yr)	0.62	0.02	0.69	**4.10**	0.48	0.47	159.77	0.29	0.00	0.03	**1.23**	0.00	0.02	0.14					

Significant values are indicated in bold.

TABLE 7 | Estimated weekly intake (EWI; μg/kg b.w.), Target hazard quotient (THQ), Total Target hazard quotient (TTHQ), Target carcinogenic risk (CR) and Margin-of-exposure (MOE) for each PAH and their sum in the five age-categories.

Food		EWI (ng/kg BW)					THQ	CR	MOE
		BaA	BaP	BbF	Chr	Σ4 PAH (BEC)	Σ4 PAH (BEC)	Σ4 PAH (BEC)	Σ4 PAH (BEC)
Beef	Baby (0–2 yr)	2.20	2.20	2.20	7.39	2.71	0.00	5.5E-09	258157
	Children (3–9 yr)	0.73	0.73	0.73	2.45	0.90	0.00	1.1E-08	780009
	Teenagers (10–17 yr)	0.57	0.57	0.57	1.91	0.70	0.00	2.0E-08	1001493
	Adult (18–64 yr)	0.35	0.35	0.35	1.18	0.43	0.00	3.6E-08	1622419
	Elderly (65–97 yr)	0.38	0.38	0.38	1.27	0.47	0.00	7.7E-08	1502240
Pork	Baby (0–2 yr)	0.09	0.09	0.09	0.09	0.11	0.00	2.3E-10	6357279
	Children (3–9 yr)	0.43	0.43	0.43	0.43	0.53	0.00	6.4E-09	1331877
	Teenagers (10–17 yr)	0.22	0.22	0.22	0.22	0.27	0.00	7.8E-09	2580512
	Adult (18–64 yr)	0.17	0.17	0.17	0.17	0.20	0.00	1.7E-08	3440683
	Elderly (65–97 yr)	0.12	0.12	0.12	0.12	0.14	0.00	2.4E-08	4857434
Cow milk	Baby (0–2 yr)	105.80	34.80	34.80	4371.38	92.58	0.04	1.9E-07	**7561**
	Children (3–9 yr)	19.77	6.50	6.50	816.78	17.31	0.01	1.1E-07	40437
	Teenagers (10–17 yr)	7.15	2.35	2.35	295.41	6.26	0.00	1.8E-07	111803
	Adult (18–64 yr)	3.11	1.02	1.02	128.36	2.72	0.00	2.3E-07	257300
	Elderly (65–97 yr)	3.87	1.27	1.27	160.01	3.39	0.00	5.6E-07	206405
Seafood	Baby (0–2 yr)	5.10	5.10	5.10	5.10	6.17	0.00	1.3E-08	113523
	Children (3–9 yr)	4.76	4.76	4.76	4.76	5.76	0.00	7.0E-08	121536
	Teenagers (10–17 yr)	3.58	3.58	3.58	3.58	4.34	0.00	1.2E-07	161415
	Adult (18–64 yr)	1.99	1.99	1.99	1.99	2.41	0.00	2.0–07	291002
	Elderly (65–97 yr)	1.51	1.51	1.51	1.51	1.83	0.00	3.0E-07	382614
Total	Baby (0–2 yr)	113.19	42.19	42.19	4383.96	101.57	0.05		
	Children (3–9 yr)	25.69	12.43	12.43	824.42	24.49	0.01		
	Teenagers (10–17 yr)	11.53	6.73	6.73	301.13	11.57	0.01		
	Adult (18–64 yr)	5.61	3.53	3.53	131.70	5.76	0.00		
	Elderly (65–97 yr)	5.88	3.28	3.28	162.92	5.83	0.00		

The total BaP equivalent concentration (BEC) and an exposure time of 365 days year^{-1} were considered. The Significant values are indicated in bold.

TABLE 8 | Significative difference by Mann-Whytney's test (*P*-value < 0.05).

Metal	Samples from the SNI area *n*	Samples from other districts *n*	SNI area mean score	Other districts mean score	*P*-value
Arsenic (As)	18	7	9.50	22.00	<0.0001
Nickel (Ni)	18	6	9.88	20.33	0.0002

Polluted water and soil are the principal pathways carrying As to the food chain. Thus, intake of As contaminated food is a growing issue of public concern (60, 89, 110–113). In this study As was detected in most of foodstuffs samples, with a variable range of concentration. The higher values were recorded in seafood products. These concentrations fall within the range found in seafood products in other Mediterranean areas (114–116), while the estimated iAs concentration (0.14 μg/g) was higher than value recorded by EFSA [0.025 μg/g; (89)] for seafood products, probably due to the specific environmental condition. Beef and pork showed an estimated iAs average contents (0.008 and 0.010 μg/g, respectively) similar to those found by EFSA (89) in livestock meats (mean 0.006 μg/g).

Occurrence of PAHs in the Study Area

In this study, detectable Chr concentrations were found in all terrestrial foodstuffs samples, with higher concentrations detected in raw milks. These results were compared to the European limits (1, 117) that are set on the maximum levels for benzo(a)pyrene and on the sum of the four PAHs (benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, and chrysene). This approach was introduced by European Community (117) to guaranty not entering in the market products in which BaP is not detectable, but where others PAH are present. Although there aren't PAHs limits referred specifically to raw milk, Chr showed higher values both in cow (mean 12.56 ng/g), sheep and goat milks (mean 9.25 ng/g)

compared to the lowest maximum food concentrations set for infants and young children [1 ng/g; (117)]. Chr and BaA are classified as IARC group 2B, being considered an agent (mixture) possibly carcinogenic to humans. Chr concentrations here found in milk samples are similar to those found in cow milk collected in polluted Kuwait area (54) and in smoked dairy products (99), but much higher than those found in unprocessed and heat-treated cow milk in south Italy and Europe (17, 54, 118), breast milk and infant formula (18) and dairy products (58). Otherwise, BaA concentrations here found in milk samples (<LOD) are similar to those found in other investigations (54, 58) and lower than those found in milk from European industrial areas, where values until 1.5 ng/g were registered (54).

Since the food contamination by PAHs is related also to industrial food processing and some cooking treatment, the presence of PAHs in raw matrices nearest the SNI, reflects a certain level of environmental contamination.

The limits for PAHs concentrations in meats and seafood are set only for smoked products [2 ng/g and 12 ng/g for BaP and $\sum$4PAH, respectively; (117)]. Concentrations here found in unprocessed products were much lower than the maximum level set for smoked products and consistent with those observed in other studies carried out in Europe (54, 58, 99).

Human Health Risk Assessment

The human health risk assessment based on the evaluation of EWI, THQ, and CR showed a certain degree of risk, principally related to Hg exposure. Particularly, the elevated values of the indices EWI_{Hg} (for all the age categories) and THQ_{Hg} (for baby, children, and teenagers) estimated for seafood category suggested that consumption of these products from the Augusta bay is not recommended, especially for more susceptible categories.

The CR_{As} related to seafood ingestion exceed the threshold limit (1×10^{-5}) for all age categories, (except for the "baby") indicating a risk for consumers. As previously mentioned, we estimated the iAs content applying a specific percent to the total As but, until now, it is not possible to certainly predict the inorganic content of iAs in seafood (89, 119). Further investigations are recommended to clarify this issue.

We used a conservative approach for human exposure considering a value equal to ½ LOD for contaminants with concentration <LOD. Therefore, the significant value obtained for EWI_{Hg}, THQ_{Hg}, CR_{Ni}, and CR_{Cr} by cow milk and beef ingestion (**Table 5**) could be overestimated and need to be carefully considered.

Among the risk indices calculated for PAHs, the margin-of-exposure approach (MOE) was significant (7561) for "baby" by cow milk ingestion, probably related to the high Chr content found in this food and the elevated ingestion rate from this age category (**Table 7**).

CONCLUSIONS

This study represents a first investigation of heavy metals and PAHs concentrations in different foodstuffs from the SNI of Augusta-Melilli-Priolo. Results indicated that the seafood exceeded the mercury limits established by the European legislation and contributed more respect the other foodstuffs to the heavy metals dietary intake. Otherwise, the terrestrial matrices, in particular milks, presented significant contents of chrysene reaching higher values than those set for food for infants and young children by European legislation. The high heavy metals concentration in seafood as well as the high PAHs concentrations in raw foodstuff sampled near the SNI suggest an environmental contamination of the Augusta Bay due to anthropogenic activities. The evaluation of human health risk related to seafood products consumption evidenced the overcoming of Provisional Tolerable Weekly Intake (PTWI) for Hg recommended by the European Food Safety Authority and WHO, and a non-carcinogenic risk (THQ) for Hg intakes occurs in baby, children and teenagers. The arsenic cancer risk (CR_{As}) exceeded the threshold limit for almost age categories (except "baby") and for elderly, due to seafood products and beef ingestions, respectively. Finally, the margin-of-exposure calculated for "baby" showed a certain cancer risk due to cow milk ingestion, probably related to the high chrysene content found in this food and the elevated ingestion rate from this age category.

The consumption of local animal foodstuffs, in particular seafood, should represent a risk for local population health, and further studies are recommended to evaluate the contaminants' exposure, especially for certain vulnerable categories of consumers.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they are part of an ongoing project. Requests to access the datasets should be directed to www.cisas.cnr.it.

AUTHOR CONTRIBUTIONS

CD, AT, and CG made substantial contributions to conception and design of the study, as well as the monitoring plan. They drafted and critically revised the manuscript for its intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work. VF, DC, GL, AB, MD, FF, SG, and MU made substantial contributions to foodstuffs analysis and data acquisition. Each of the authors read and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Persistent and Emerging Organic Pollutants in the Marine Coastal Environment of the Gulf of Milazzo (Southern Italy): Human Health Risk Assessment

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The Gulf of Milazzo (north-eastern Sicily) has been recognized as Italian Site of National Interest (SNI; areas characterized by high level of contamination with potential effects on human health) in 2005 because of its high level of pollution. In this study we measured the concentration of polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), and polyBrominated diphenyl ether (PBDE) in seawater and sediments sampled from the Gulf of Milazzo in order to assess (i) the environmental status of contamination, and (ii) cancer and non-cancer human health risk potentially due to dermal absorption from contaminated seawater and/or ingestion of contaminated fish. Particularly, POPs content in pelagic and demersal fish of different size classes (small, medium, and large) were estimated, starting from the measured seawater and sediments concentrations, using the KABAM model. In particular, Monte Carlo simulation techniques were applied to address uncertainty in assessment of the risk and to provide quantitative estimates of probability of exposition. Ingestion of contaminated pelagic and demersal fish was the dominant pathway of exposition with high probability of significant cancer risk (Ingestion Cancer Risk $>10^{-4}$) and significant non-cancer risk (Hazard Index >1). No human health risks emerged to be associated to dermal adsorption from contaminated seawater. Benzo(a)pyrene show the highest Ingestion Cancer Risk with respect to the other PAHs, while the highest Hazard Index for non-cancerogenic molecules was estimated for the PBDE47 congener.

Keywords: human health risk, KABAM, polycyclic aromatic hydrocarbons, organochlorine pesticides, polybrominated diphenyl ether, bio-accumulation factor, risk assessment

INTRODUCTION

Persistent organic pollutants (POPs) are organic compounds with a worldwide occurrence in the marine environment because of their resistance to environmental degradation. The high lipophilicity makes them capable to bioaccumulate in large quantities in tissues of marine organisms. The polycyclic aromatics hydrocarbons (PAHs) are a mixture of aromatic compounds of great environmental concern released both by natural sources (pyrolysis, incomplete combustion

of organic matter) and anthropogenic activities (industrial processes, combustion of wood and fossil fuels, motor vehicles, incinerators, oil plants and oil spills). Several researches indicate that high environmental PAHs concentrations are related to increasing incidence of cancer and mutagenic events for exposed organisms (Ramesh et al., 2012). As a consequence, several high molecular weight PAHs have been classified as recognized (class 1), probable (class 2A), or possible (class 2B) human carcinogens (International Agency for Research on Cancer [IARC], 1987). On the other side, the intensifications of agricultural activities has led to increased concentration in the environment of pesticides, a group of POPs belonging to organochlorines compounds (OC). Organochlorines are commonly classified as endocrine disrupting chemicals (EDCs) and are reported to increase the risk of hormone-related cancers by interfering with the endocrine metabolism (Wolff et al., 1993; Sohail et al., 2004). Moreover, epidemiological studies demonstrated a positive correlation between high levels of OCs and incidence of hypertension, cardiovascular diseases and other health-related issues in humans (Subramaniam and Solomon, 2006; Jayaraj et al., 2016). Finally, Polybrominated diphenyl ethers (PBDEs) are a group of emerging ubiquitous POPs used as flame-retardants during the production of textiles, paints, furnitures, electronic circuit boards and plastics. Toxicological studies on laboratory animals demonstrated that PBDEs exposure is positively correlated with thyroid homeostasis disruption, neurotoxic effects, reproductive disorders and cancer (Linares et al., 2015). As recommended by the Marine Strategy Framework Directive (MSFD) (2008/105/CE; Descriptor D8), monitoring studies are needed to assess the environmental risk due to traditional and emerging contaminants and their possible adverse effects on wildlife and human health. The Gulf of Milazzo, located in northern Sicily (southern Italy), has been recognized as Site of National Interest (SNI) in 2005 because of its high level of pollution due to the intensive industrial activities started in the 1950. High arsenic concentrations were recorded in seawater (La Pera et al., 2008) while high concentrations of zinc, lead, cobalt, and copper were recorded in sediments (Pepe et al., 2010). In this paper, we report POPs concentration in seawater and sediments of the Gulf of Milazzo in order to assess the human health risk due to the presence of these contaminants. In particular, we evaluate human cancer and non-cancer risk due to POPs in seawater through two ways of exposure: direct contact by skin during summer activities and by ingestion of contaminated fish. Monte Carlo simulation techniques were run to verify the probability distribution of risk indices, useful for a better assessment of pathways of exposition.

MATERIALS AND METHODS

Sampling Strategy

Seawater stations (n = 16) were sampled in the Gulf of Milazzo, during two oceanographic cruises: Cisas I Milazzo (July–August, 2017) and Cisas II Milazzo (April, 2018) aboard R/V Luigi Sanzo, at three different depths of the water column (surface, intermediate, and bottom) with 10 L "Niskin" bottles. Sediment samples (n = 8) were collected using a box-corer. The sampling stations are shown in **Figure 1**. Analyses on sediment samples were carried out on samples collected from the superficial 5 cm depth. Seawater samples were preserved in dark glass bottles, previously cleaned with acetone and rinsed with Milli-Q. All the samples were stored at −20°C, until the analysis. During each survey, hydrological parameters (temperature, salinity, dissolved oxygen, fluorescence, and pH) were measured along the water column (more details are reported in the **Supplementary Material**). The hydrology study showed evidence of a stratified system with a mixed layer depth (MLD) of about 15 m and an evident thermocline, at about 20 m of depth (**Supplementary Material**). We measured the contaminants only in surface waters.

Chemical Materials

All reagents used, methanol (CH_3OH), methylene chloride (CH_2Cl_2), n-hexane (n-C_6H_{14}), ethyl acetate ($C_4H_8O_2$), purchased by VWR were pure and pesticides free. The solid phase disk C18 was Empore disk C18 of 50 mm. The PAHs mix standard containing naphthalene (Nap), acenaphthylene (Acn), acenaphthene (Ace), fluorene (Flo), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flu), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaPy), indeno(1,2,3-cd)pyrene (InP), dibenzo(a,h)anthracene (DahA), and Benzo(g,h,i)perylene (BghiP), with a concentration of 100 μg/ml per each congeners, get by Dr. Ehrenstorfer GmbH (PAH-MIX9), was used to calibrate the instrument. The internal standard was a mix of four deuterated PAHs (PAH Mix 25), and a mix of three more deuterated PAHs (PAH Mix77 get by Dr. Ehrenstorfer GmbH) was used to check the recovery. A mix of PBDE (28, 47, 99, 100, 153, 154, and 209) was prepared *ad hoc* by LabStandard (Italy). Two mixes of OCPs, one containing Alachlor, Aldrin, Dieldrin, Endrin, Isodrin, DDE, DDD, DDT (Σ isomers), Endosulfan (Σ isomers), with a concentration of 100 mg/l each one; and the other containing Hexachlorobenzene, Hexachlorobutadiene, Hexachlorocyclohexane (Σ isomers), Pentachlorobenzene with

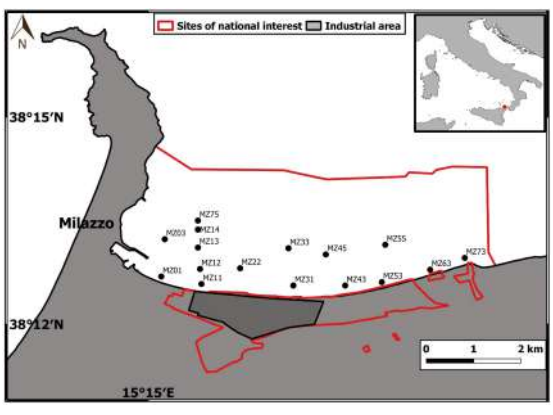

FIGURE 1 | Sampling stations in the study area.

a concentration of 100 mg/l each-one, were prepared *ad hoc* by LabStandard (Italy).

Chemical Analysis

Seawater Analysis

USEPA Method 525.1, EPA 8270D e EPA 8081 was run to extract and analyze POPs from seawater. Particularly, the extraction of POPs from seawater was carried out, according to US EPA Method 525.1, using a solid phase disk bakerbond speedisk C18 of 50 mm and an apparatus of six extractors coupled with a vacuum system. 1 L of seawater sample was poured onto the disk previously cleaned with 10 ml of CH_2Cl_2, conditioned with 20 ml of CH_3OH and 20 ml of distilled water (with 0.5% of CH_3OH). The elution was run by (i) drying the disk, venting air for at least 10 min with a vacuum pump; (ii) pouring 10 ml of $C_4H_8O_2$ (ethyl acetate) onto the disk (this step was necessary to eliminate any remaining water from the disk and to increase the recovery of the contaminants); (iii) pouring 10 ml of a mix 50:50 of $C_4H_8O_2/CH_2Cl_2$ and at last 20 ml of CH_2Cl_2. All eluted solvents were collected in the same test tube and dried using a multivapor (got by BÜCHI). Finally, 1 ml of n-Hexane (containing internals standard) was spilled inside the test tube and recovered for GC/MS. Analysis was carried out using a GC/MS Triple Quadrupole (by Thermo Fisher GC Trace 1310 coupled with a TSQ8000 mass spectrometry and Triplus RSH autosampler). The GC was equipped with a DB-5 ms capillary column (30 m × 0.25 mm, 0.25 μm) and with a PTV injector set in large volume mode. PAHs, OCPs and PBDE were determined in SRM Mode as reported in the Thermo Scientific application note 52389.

Sediment Analysis

USEPA Method 3545, EPA 8270D, EPA 8081 was used to extract and analyze POPs from sediments. Synthetically, 2 g of dry, sieved and homogenized sediment, spiked with surrogate standards, was extracted by Accelerated Solvent Extraction (ASE 200, DIONEX, Thermo Scientific) using a hexane/acetone (80:20 v/v) mixture. The clean up was run using SPE Silica Gel and SPE Florisil to PAH and OCPs, respectively. The final extracts were analyzed by Gas Chromatography (GC-MS ISQ; Thermo Finnigan) with Mass Spectrometric detection in Selective Ion Monitoring (SIM) mode for PAH with a limit of detection estimated as 1 μg/kg for each PAH and by Gas Chromatography with mass spectrometric Ion-Trap with tandem mode (GC-Ion Trap Polaris; Thermo Finnigan) in MSN mode for OCPs with a limit of detection estimated as 0,25 μg/kg.

Total Organic Carbon in Sediments

Total organic carbon was determined by a Thermo Electron Flash EA 1112 coupled to a Thermo Electron Delta V Advantage mass spectrometer. Analysis was carried out after elimination of all of the carbonate present in the samples (ca. 10–15 mg of bulk sediment samples were de-carbonated using HCl 1 M in silver cups for 24 h at ambient temperature and then dried in an oven at 60°C). An internal standard (urea with C = 20%, N = 46%) was run every six samples. The detection limit for TOC measurements was established at 0.05%, considering chromatogram peak > 500 mV and correspondingly determining the sample weight.

Quality Control

Laboratory quality control procedures for PAHs, OCPs and PBDEs both in seawater and sediments included analyses of blanks, spiked samples and reference materials for sediments. The recovery measured on artificial seawater spiked with a final concentration of 100 ng/l per each contaminant was between 75 and 115%. A mix of deuterated PAH with a final concentration of 10 ng/l was added before the extraction to monitor the recovery percentage (PAH deuterated MIX) in each seawater sample. The reproducibility measured by three consecutive extractions was less than 20%.

The reference material used for quality control, on sediment analyses, was SRM 1941b-NIST.

The recoveries for each analyte of PAHs ranged between 94 and 107%, the reproducibility was about 10% for all substances, and the accuracy was estimated greater than 10% for each single analysis. The SRM 1941b-NIST.reference material was also used to measure the accuracy of total organic carbon percentage (TOC%) in sediment. The accuracy and reproducibility were about 5 and 0,4%, respectively.

Human Health Risk Assessment

The human health risk for resident population was assessed hypothesizing two routes of contaminant exposure:

(1) Dermal adsorption during summer activities (e.g., swimming).
(2) Ingestion of contaminated fishes.

Dermal Cancer and Hazard Risk

Dermal Hazard Risk (DHR) and Dermal Cancer Risk (DCR) were assessed according to US EPA protocol, Risk Assessment Guidance for Superfund: part E (RAGS, E), following the equations 1–5:

$$\text{Dose Adsorbed (DA)}_{\text{event}-\text{water contact}} = 2 \text{ x FA x Kp x Cw x } \sqrt{\frac{6 \text{ x } \tau\_\text{event} \times \text{ t\_event}}{\pi}} \quad (1)$$

$$\text{Dermal Adsorbed Dose (DAD)} = \frac{\text{DA}_{event} \text{ x EV x ED x EF x SA}}{\text{BW x AT}} \quad (2)$$

$$\text{Dermal Cancer Risk (DCR)} = \text{DAD} \times \text{SFo}/\text{ABS}_{\text{GI}} \quad (3)$$

$$\text{Hazard Quotient Dermal (HQ)} = \text{DAD}/(\text{RfDo} \times \text{ABS}_{\text{GI}}) \quad (4)$$

$$\text{Hazard Index (HI)} = \Sigma\text{HQD} \quad (5)$$

where FA is the fraction of the adsorbed water, K_p is dermal permeability coefficient calculated per each compound (cm/h) as function of K_{ow} and molecular weight, C_w is pollutant

concentration in surface water (ng/l), τ event is lag time per event (h/event), τ_event is the duration of the event (h/event), EV is the frequency of the event (events/day), ED is the exposure duration (years), EF is the frequency of exposure (days/year), SA is the skin surface area (cm^2), BW is the body weight (kg), AT is the average lifespan (years, AT = ED for not cancer risk), SFo is Cancer Slope Factor oral (mg/kg/day), RfDo is oral Reference Dose (mg/kg/day), and ABS_{GI} is the fraction of contaminant absorbed in the gastro-intestinal tract. The values of these parameters are shown in **Table 1**. The combined DCR associated to all congeners of PAH was calculated as Benzo(a)pyreneTEQ (Eq. 6)

$$\text{BaPy\_TEQ} = \Sigma \text{Ci} \cdot \text{TEFi} \quad (6)$$

where TEFi is Toxicity Effect Factor and Ci is the concentration of each congener in seawater (ng/l). The combined DCR associated to all POPs was calculated as the sum of each carcinogenic compound that can injury the same target organ.

Contaminant Concentrations in Fish Tissues

Contaminant concentrations in fish were simulated using the model KABAM (K_{ow} based Aquatic Bioaccumulation Model) following Arnot and Gobas (2004), who parameterized a bioaccumulation model in aquatic ecosystems using the octanol-water partition coefficient (K_{ow}) to estimate uptake and elimination constants through respiration and diet of organisms in different trophic levels. We used the model to estimate the concentration of contaminants in edible tissues of pelagic and demersal fish of 3 size classes, small fish (SF, 10–100 g), medium fish (MF, 100–1,000 g), and large fish (LF, −1,000 g), starting from the contaminant concentrations in seawater column and in pore waters, respectively. Contaminant concentrations in seawater and sediments were calculated as the average of concentrations measured in all samples during the two surveys. We used values corresponding at half of detection limit for not quantifiable measures. We used the sediment/liquid partition coefficients (Kd = Koc· foc = $C_{sediment}/C_{pore\ water}$) to estimate contaminant concentrations in pore waters starting from concentrations measured in sediment samples. Time to steady-state (Tss) for each compound was calculated using Kow as follows:

$$\text{Tss(days)} = (0.00654 \cdot \text{Kow} + 55,31)/24 \quad (7)$$

Contaminant concentrations in fish were normalized at 2-year life for each compound with Tss greater than 730 days (2 years). Furthermore, we set the KABAM model (ecosystem input paragraph) according to the diet and habitat of the fish.

Ingestion Cancer and Hazard Risk

The Average Daily potential Dose (ADD) was calculated as follows:

$$\text{ADD} = C_{fish} \times \text{IR} \times \text{EF} \times \text{ED}/(\text{BW} \times \text{AT}) \quad (8)$$

where C_{fish} is the estimated concentration of contaminant in fish tissue, IR is the Ingestion Rate (gr/days); EF is the Exposure Frequency (days/years); ED is the Exposure Duration (years); BW is the Body Weight (kg) and AT is the average lifespan (years, AT = ED for not cancer risk). The values of these parameters are shown in **Table 1**. The Ingestion Cancer Risk (ICR) was calculated multiplying the ADD by the Cancer Slope Factor oral (CSF) of each contaminant:

$$\text{ICR} = \text{ADD} \times \text{CSFo}/\text{ABS}_{GI} \quad (9)$$

The combined ICR associated to all POPs was calculated as the sum of each compound that can injury the same organ. The Hazard Quotient risk (HQ) regarding non-cancerogenic health issues was calculated dividing the ADD by the Reference Dose oral (RfDo) of each contaminant:

$$\text{HQ} = \text{ADD}/(\text{RfDo} \times \text{ABS}_{GI)} \quad (10)$$

$$\text{HI} = \Sigma \text{HQ} \quad (11)$$

Statistical Analysis

Monte Carlo simulation techniques with 5,000 iterations were applied to estimate variability and uncertainty in risk assessment. The repeated sampling was based on probability distributions of contaminant concentrations in water and fish. The procedure was performed by R 3.6.1 (R Core Team, 2019). Differences of contaminant contribution for the ingestion cancer risk were tested by a non-parametric test (Kruskal–Wallis test) and by pairwise comparison. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Occurrence of Contaminants in the Study Area

The concentrations of PAHs, OCPs, and PBDEs in seawater and superficial sediments of the Gulf of Milazzo are shown in **Table 2**. Higher concentrations of low molecular weight PAHs (sum of congeners with 2 and 3 rings) were found in the water column because of their greater solubility, while high molecular weight PAHs (sum of congeners 4, 5 and 6 rings) were more abundant in sediments. Alachlor is the most concentrated OCPs in seawater (average 2.84 ng/l) followed by DDE (average 1.06 ng/l), while PBDE47 is the most concentrated PBDEs (3.58 ng/l). Contaminant concentrations in seawater samples are lower than the maximum admissible concentrations (MAC) set by the Italian Regulations n.172/2015 (application of the European Directive 2013/39/EU, 2008/105/CE, and 2000/60/CE) except for Benzo(ghi)Perylene and ΣPBDE that exceeds the threshold limits in 13 and 5 sampling stations, respectively. Depending on PAHs contamination, seawater could be classified as: micro-polluted, ΣPAHs 10–50 ng/l; light-polluted, ΣPAHs 50–250 ng/l; moderately polluted, ΣPAHs 250–1,000 ng/l and heavily polluted, ΣPAHs >1,000 ng/l according to Chen (2008). The concentrations of ΣPAHs in seawater

TABLE 1 | Variables used for risks determination.

Variable (symbol)	Unit	Value (dermal absorption)	Value (ingestion rate)	References
Fraction absorbed water (FA)	Unitless	Chemical specific		Environmental Protection Agency [EPA], 2004
Dermal permeability coefficient (Kp)	Cm/h	Chemical specific		Environmental Protection Agency [EPA], 2004
Lag time per event (tau event)	h/event	Chemical specific		Environmental Protection Agency [EPA], 2004
Event duration (tevent)	h/event	0,33		
Event frequency (EV)	Event/day	3	1	
Exposure duration (ED)	Year	30	30	Environmental Protection Agency [EPA], 2004
Exposure frequency (EF)	Days/year	90	104	
Surface area (SA)	cm^2	18.000		Environmental Protection Agency [EPA], 2004
Body weight (BW)	kg	70	70	Environmental Protection Agency [EPA], 2004
Averaging time (AT)	Year	70/30	70/30	Environmental Protection Agency [EPA], 2004
Cancer Oral slope factor (CSfo)	(mg/kg-day)−1	Chemical specific		Health Canada [HC] (2007a,b); IRIS (2007)
Absorption fraction (ABSgi)	Unitless	Chemical specific		Environmental Protection Agency [EPA], 2004
Oral reference dose (RfDo)	mg/kg-day	Chemical specific		IRIS database
IR	Portion_gr/day		170	INRAN

ranged from 1.6 to 70.3 ng/l, showing that the site could be classified as micro-polluted/light-polluted by PAHs. Particularly, light-polluted seawaters were found in the stations MZ13 (ΣPAHs = 59.2 ng/l) and MZ43 (ΣPAHs = 70.3 ng/l). PAHs concentrations in sediments (26.94–862.0 μg/kg), are much higher than organochlorine pesticides (2.50–3.72 μg/kg) and PBDE concentrations (0.93–3.72 μg/kg). Hexachlorobenzene (HCB) is the most concentrated organochlorine pesticide in sediments followed by DDD and DDT, while the same concentrations were found for PBDE congeners. Contaminant concentrations in superficial sediments are lower than the maximum admissible concentrations (MAC) set by the European Directive 2013/39/EU, except for Fluoranthene concentrations in 2 sampling stations (MZ13 and MZ33), Benzo(b)fluoranthene in 1 sampling station (MZ13), Benzo(k)fluoranthene in 1 sampling station (MZ13) and Benzo(a)pyrene in 1 sampling station (MZ13). Total PAH concentrations, sediments may be classified according to Soclo et al. (2000) as highly contaminated with PAHs > 500 μg/kg, moderately contaminated with PAHs > 250, and slightly contaminated with PAHs < 250 μg/kg. Our data demonstrate that the superficial sediment samples of the Gulf of Milazzo cover the entire range of contamination. Particularly, high PAHs contamination was found in the stations MZ13 (ΣPAHs = 861 μg/kg) and MZ33 (ΣPAHs = 544 μg/kg). Relatively low values of organic chlorinated pesticides (OCPs) concentrations were found in seawaters and superficial sediment samples suggesting a reduced level of this class of contaminant contribution from agricultural activities in the study area. A comparison of PAHs concentrations in sediments from other industrialized marine sites is shown in **Table 3**. No sufficient data on OCP and PBDE concentrations in the Mediterranean Sea are available in literature for robust and statistically significant comparisons. This work offers one of the first complete datasets of these classes of contaminants in seawater and sediments from highly contaminated industrial areas.

PAHs Source Identification

The identification of the PAHs source was assessed calculating Fluo/Fluo + Pyr and Ant/Ant + Phe ratios per each sample. Fluo/Fluo + Pyr <0.40 show a petroleum contamination, between 0.40 than 0.50 a liquid fossil fuel (vehicle and crude oil) combustion and >0.50 are characteristic of grass, wood, or coal combustion; Ant/Ant + Pyr <0.1 petroleum and >0.1 pyrogenic sources (Yunker et al., 2002; He et al., 2014; Soliman et al., 2014; Ya et al., 2014). Fluo/Fluo + Pyr and Ant/Ant + Pyr show mean value of 0.41(±0.14) and 0.28(±0.14) in seawater and 0.54(±0.07) and 0.21(±0.2) in sediment. These results suggest both petroleum contamination and pyrogenic source due to refinery activities/marine traffic and thermopower plant.

Health Risk Assessment

Cancer Risk Assessment

The Dermal Cancer Risk (DCR) due to dermal absorption of all contaminants in surface seawater (ΣDCR) for each sampling station ranged from $4.8 \cdot 10^{-7}$ to $6.0 \cdot 10^{-6}$. These values are shown in the distribution map (**Figure 2**), using inverse distance weighting (IDW) and setting a buffer with a fixed distance of 1 km from sampling station, in order to identify the most dangerous area. The average values of DCR for each contaminant ranged from 10^{-13} to 10^{-7} (**Table 4**). The cancer risk due to fish ingestion ranged from 10^{-13} to $5.0 \cdot 10^{-4}$ (**Table 4**). We estimated the combined cancer risk for different organs supposing additive carcinogenic effects of contaminants with the same target. The information about the target organs of each contaminant was checked using IRIS database. The combined DCR per target organs ranged from $7.0 \cdot 10^{-9}$ for breast cancer to $9.4 \cdot 10^{-7}$ for lung cancer, while the combined ICR per target organs ranged from $1.7 \cdot 10^{-7}$ for breast cancer to $5 \cdot 10^{-4}$ for gastro-intestinal cancer (**Table 5**). The target organ with the highest potential to develop a carcinogenic event is the gastro-intestinal tract (followed by liver, skin, lung, and breast) because of the higher ICR associated with BaPy ($2.7 \cdot 10^{-4}$, **Table 4**). The risks here analyzed are compared with the acceptable risk levels proposed by the international agencies. The US EPA assumed acceptable risk level in a range from 10^{-6} to 10^{-4} (Environmental Protection Agency [EPA], 1991). No remediation measures are required when the cancer risk falls

TABLE 2 | Range and mean contaminant concentrations in seawater and sediment.

	Contaminant	Water				Sediment			
		Average (ng/l)	Range (ng/l)	MAC (ng/l)	d.l. (ng/l)	Average (μg/kg)	Range (μg/kg)	MAC (μg/kg)	d.l. (μg/kg)
PAH	Naphthalene	6.43	0.10–37.72	130000.00	0.20	1.28	0.50–2.88	35.00	1.00
	Acenaphtylene	0.12	0.10–0.17		0.20	1.39	0.50–4.63		1.00
	Acenaphtene	0.47	0.10–1.20		0.20	4.49	0.50–13.80		1.00
	Fluorene	1.52	0.10–3.46		0.20	3.41	0.50–17.40		1.00
	Phenanthrene	5.13	0.10–12.90		0.20	20.50	1.78–73.60		1.00
	Antracene	0.74	0.10–5.65	100.00	0.20	8.52	0.50–19.8.	24.00	1.00
	Fluoranthene	1.18	0.10–4.82	120.00	0.20	45.34	4.63–177.00	110.00	1.00
	Pyrene	2.75	0.10–14.10		0.20	38.21	4.28–149.00		1.00
	Benzo(a)anthracene	0.63	0.10–3.18		0.20	20.22	0.50 –84.10		1.00
	Chrysene	0.66	0.10–3.35		0.20	22.09	0.47–99.70		1.00
	Benzo(b)fluoranthene	0.15	0.10–1.53	17.00	0.20	14.10	0.50 –79.10	40.00	1.00
	Benzo(k)fluoranthene	0.16	0.10–1.59	17.00	0.20	5.80	0.50 –33.70	20.00	1.00
	Benzo(a)pyrene	0.16	0.10–1.82	27.00	0.20	11.18	0.50 –60.40	30.00	1.00
	Indeno (123) pyrene	0.21	0.10–3.07		0.20	12.79	0.88–50.90	70.00	1.00
	Dibenzo(ah)anthracene	0.21	0.10–3.03		0.20	2.59	0.50 –10.30	55.00	1.00
	Benzo(ghi)perylene	1.18	0.10–4.50	0.82	0.20	12.34	1.03–41.30		1.00
	ΣPAH	21.69	1.60–70.36			224.27	26.94–862.0		
OCP	Alachlor	2.84	0.25–13.00	700.00	0.50	0.13	0.13–0.13		0.25
	Aldrin	0.09	0.02–0.50	Σ = 5.00	0.05	0.13	0.13–0.13	0.20	0.25
	Isodrin	0.03	0.03–0.03		0.05	0.13	0.13–0.13		0.25
	Dieldrin	0.06	0.03–0.85		0.05	0.13	0.13–0.13	0.20	0.25
	Endrin	0.03	0.03–0.03		0.05	0.13	0.13–0.13		0.25
	Endosulfan	0.03	0.03–0.03	4	0.05	0.13	0.13–0.13		0.25
	Hexachlorobutadiene	0.01	0.01–0.10	600.00	0.02	nd	nd		
	HexachlorocycloHexane	0.06	0.03–0.13	20.00	0.05	0.13	0.13–0.13		0.25
	Hexachlorobenzene	0.14	0.01–0.62	50.00	0.02	0.35	0.10–1.50		0.25
	DDE	1.06	0.15–4.86	Σ = 25.00	0.30	0.16	0.13–0.34	1.80	0.30
	DDD	0.19	0.15–0.59		0.30	0.25	0.25–0.25	0.80	0.50
	DDTop	0.19	0.15–0.89		0.30	0.25	0.25–0.26	1.00	0.50
	DDTpp′	0.16	0.15–0.55	10.00	0.30	0.25	0.25–0.27	1.00	0.50
	ΣOCPs	4.52	0.24–20.82			2.71	2.5–3.72		
PBDE	PBDE28	0.03	0.03–0.03	Σ = 14.00	0.05	0.10	0.10–0.10		0.20
	PBDE47	3.58	0.03–21.8		0.05	0.11	0.10–0.20		0.20
	PBDE99	0.03	0.03–0.03		0.05	0.10	0.10–0.10		0.20
	PBDE100	0.03	0.03–0.03		0.05	0.10	0.10–0.10		0.20
	PBDE153	0.03	0.03–0.03		0.05	0.10	0.10–0.10		0.20
	PBDE154	0.03	0.03–0.03		0.05	0.10	0.10–0.10		0.20
	PBDE209	0.05	0.05–0.05		0.10	0.41	0.18–0.79		0.50
	ΣPDBEs	3.70	0.15–21.8			1.02	0.78–1.39		
	TOC (%)					0.80	0.11–3.73		

MAC, Maximum Admissible Concentrations set by European Directive 2013/39. d.l., detection limit; nd, not determined; the minimum value was set, arbitrarily, for the risk assessment study half of d.l.

within this range. The US EPA assumes the unconditionally acceptable risk level below 10^{-6} (Environmental Protection Agency [EPA], 2004) while a risk greater than 10^{-4} requires protective measures to mitigate the risk. All the DCRs here found were under the USEPA acceptable range (10^{-6}–10^{-4}). We recorded ICRs above the upper bound of the USEPA acceptable range (10^{-4}) due to the ingestion of demersal medium and large fishes and pelagic large fishes (**Tables 4**, **5**), suggesting a significant risk to develop cancer for the resident population. The probabilistic distributions of Dermal Cancer Risk (DCR) and Ingestion Cancer Risks (ICR) for small (SF), medium (MF), large (LF) pelagic, and demersal fish were obtained from Monte Carlo simulations (Figures in **Supplementary Material**). The descriptive statistical values (including 5th percentile, 25th

TABLE 3 | Contaminant concentration in sediments from others industrialized bay.

ΣPAH (ng/g)			
France	Arcachon Bay	32–4120	Baumard et al., 1998
	Marseille Bay	43–1,984	Asia et al., 2009
	Santander Bay	<d.l.– 4900	Antizar-Ladislao, 2009
Italy	Augusta Bay-Sicily	50–37000	ICRAM, 2006
	Chioggia and Ancona	24.1–507	Magi et al., 2002
	Porto Torres, Sardinia	70–1210	De Luca et al., 2004
Croazia	Rijeka Bay area	32–13681	Alebic-Juretic, 2011
Egypt	Abu Qir Bay, Alexandria	<dl. – 2660	Khairy et al., 2009
Italy	Milazzo Bay	26.9–862	This study
ΣPBDE (ng/g)			
China	Shenzhen Bay	0.07–4.85	Qiu et al., 2009
	Jiaozhou Bay	2.18–10.59	Ju et al., 2016
	Hong Kong	1.7–53.6	Liu et al., 2005
Korea	Ulsan Bay	3.97–290	Moon et al., 2007
	Busan Bay	14.4–2253	Moon et al., 2007
	Jinhae Bay	2.03–151	Moon et al., 2007
Italy	Milazzo Bay	0.78–1.39	This study

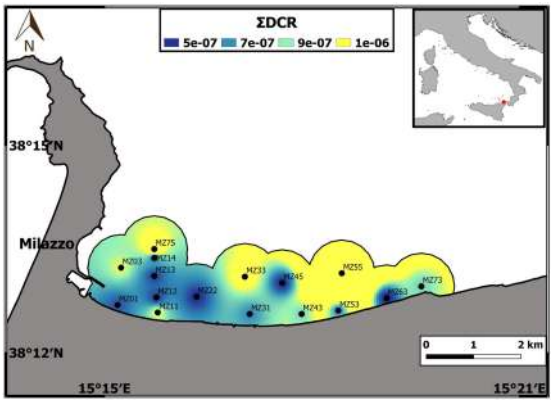

FIGURE 2 | Dermal Cancer Risk (ΣDCR) distribution in the study area.

percentile, median, 75th percentile and 95th percentile) are reported in **Table 7**. Integration of variability can lead to a more realistic evaluation of risk estimation, that could be affected by data scarcity, parameter variability and model limitations (Chen et al., 2015; Qu et al., 2015). According to the guideline established by United States Environmental Protection Agency [US EPA] (1991), the unconditionally acceptable risk level is below 10^{-6} and the acceptable risk levels range from 10^{-6} to 10^{-4}. All the simulated statistical values of DCR and ICR of PBDE of pelagic and demersal fish were lower than 10^{-6}. On the contrary, the density distributions of ICR due to PAHs exposure from ingestion of pelagic and demersal fish estimated the unacceptable risk level (10^{-4}) in correspondence with the 93th percentile for medium pelagic fishes, the 80th percentile for large pelagic fishes, the 85th percentile for small demersal fishes, the 65th percentile for medium demersal fishes, and 38th percentile for large demersal fishes. This means that the probability to exceed the unacceptable threshold is 7, 20, 15, 35, and 62%, respectively. About 5% of ICR due to exposure to OCPs for large pelagic fish, 8% for medium demersal fish and 22% for large demersal fish is above the unacceptable risk level (10^{-4}).

Non-cancer Risk Assessment

Hazard Quotients (HQ) were applied for the evaluation of non-carcinogenic health risk through ingestion and dermal adsorption of single contaminant. It is well known that POPs exert hemato-, cardio-, renal-, neuro-, immuno-, reproductive toxic effects in laboratory animals and humans (Ramesh et al., 2004; WHO, 2010). Hazard Index (HI) were applied to evaluate the non-cancerogenic health risk through ingestion and dermal adsorption of the sum of contaminants found in the study area. The hazard quotients calculated for the dermal adsorption (DHQ) and the ingestion of contaminated fishes (IHQ) and hazard index (HI) for the sum of all POPs are given in **Table 6**.

We found HQ values less than 1 for all the measured POPs due to ingestion and dermal adsorption, which indicated little or no potential adverse effects on local residents' health through ingestion and/or dermal absorption of a single contaminant. The exception is represented by the HQ of PBDE47 due to the ingestion of pelagic and demersal large fishes (IHQ = 2.47 and 2.49, respectively; **Table 6**). According to **Table 6**, the HIs due to the ingestion of pelagic and demersal medium-large fishes are above the unity, indicating potential adverse effects on local resident's health due to the ingestion of a mix of pollutants from contaminated commercial fishes. Thus, it can be concluded that the mix of pollutants can cause harmful non-carcinogenic health effects in the resident population of Milazzo. The HQs and HI due to dermal absorption of all contaminants are negligible. The HI values are shown in a distribution map (**Figure 3**) using inverse distance weighting (IDW). The probabilistic distributions of Dermal Hazard Risk (DHR) and the Ingestion Hazard Risks (IHR) for small (SF), medium (MF), large (LF) pelagic and demersal fishes were obtained from Monte Carlo simulations (Figures in **Supplementary Material**). The descriptive statistical values (5th percentile, 25th percentile, median, 75th percentile and 95th percentile) are reported in **Table 8**. According to the guideline established by United States Environmental Protection Agency [US EPA], 1991, the acceptable risk levels is below the unit. All the simulated statistical values of DHR and IHR were lower than 1, except for IHR from OCPs exposition of medium and large pelagic fish and of all demersal fish and PBDEs exposition of all fishes. Specifically, the estimated probability to exceed the unacceptable threshold (1) is 5% for IHR due to exposure to OCPs from ingestion of medium pelagic fishes, 32% from large pelagic fishes, 25% from small demersal fishes, 84% from medium demersal fishes and 55% from large demersal fishes. Moreover, the exposition to PBDEs determinate a percentage of 9% of IHR higher than the unit for small pelagic fishes, 25% for medium pelagic fishes, 71% for large pelagic fishes, 8% of small demersal fishes, 25% for medium demersal fishes and 70% for large demersal fishes.

TABLE 4 | Mean Ingestion Cancer Risk (ICR) for small (SF), medium (MF), large (LF) pelagic, and demersal fish and mean Dermal Cancer Risk (DCR).

	Contaminant	Target organ	DCR	ICR SF		ICR MF		ICR LF	
				Pelagic	Demersal	Pelagic	Demersal	Pelagic	Demersal
PAHs	Naphthalene	Generic	3.72E-09	2.2E-08	2.3E-08	2.2E-08	2.3E-08	2.2E-08	2.3E-08
	Phenanthrene	Generic	2.6E-10	4.4E-09	1.2E-08	4.9E-09	1.4E-08	5.8E-09	1.7E-08
	Antracene	Generic	2.97E-09	5.2E-08	3.3E-07	5.7E-08	3.7E-07	6.4E-08	4.3E-07
	Fluoranthene	Generic	1.46E-10	3.5E-09	3.5E-08	5.0E-09	5.8E-08	8.7E-09	9.9E-08
	Benzo(a)anthracene	Skin	3.41E-08	1.4E-06	8.8E-06	2.9E-06	2.1E-05	7.2E-06	4.9E-05
	Chrysene	Skin	4.49E-10	1.5E-08	9.3E-08	3.1E-08	2.2E-07	7.6E-08	5.2E-07
	Benzo(b)fluoranthene	Generic	1.34E-08	3.4E-07	2.6E-06	7.0E-07	6.3E-06	1.7E-06	1.5E-05
	Benzo(k)fluoranthene	Generic	1.39E-10	3.5E-09	1.3E-08	7.3E-09	3.0E-08	1.8E-08	7.1E-08
	Benzo(a)pyrene	Generic	2.33E-07	4.9E-06	4.0E-05	1.1E-05	**1.1E-04**	3.0E-05	**2.7E-04**
	Indeno (123) pyrene	Skin, Lung	7.54E-08	3.3E-07	2.8E-06	7.8E-07	7.4E-06	2.0E-06	1.9E-05
	Dibenz[a,h]anthracene	Lung	8.69E-07	2.3E-06	6.2E-06	5.2E-06	1.6E-05	1.3E-05	3.8E-05
	Benzo(ghi)perylene	Gastrointestinal tract	7.9E-09	8.3E-08	1.7E-07	2.0E-07	4.5E-07	5.2E-07	1.2E-06
	Benzo(a)pyreneTEQ	Gastrointestinal tract	7.33E-07	1.6E-05	7.4E-05	3.6E-05	**1.9E-04**	9.7E-05	**5.0E-04**
	Σ PAH		1.2E-06	9.4E-06	6.1E-05	2.1E-05	**1.6E-04**	5.5E-05	**3.9E-04**
OCPs	HexaClCycloHexane(a + b)	Liver	5.76E-08	3.3E-08	1.9E-07	3.4E-08	2.0E-07	3.5E-08	2.1E-07
	HexaClBenzene	Liver	6.1E-09	6.0E-07	1.0E-06	1.2E-06	2.1E-06	2.7E-06	4.9E-06
	Aldrin	Liver	2.12E-07	6.2E-06	1.1E-05	1.5E-05	2.9E-05	4.0E-05	7.7E-05
	Dieldrin	Liver	8.65E-10	7.0E-08	1.1E-07	7.1E-08	1.1E-07	7.3E-08	1.2E-07
	Alachlor	Gastrointestinal tract	3.78E-10	6.6E-10	1.3E-09	6.7E-10	1.3E-09	6.9E-10	1.3E-09
	DDE	Liver	5.46E-08	1.5E-06	1.6E-06	3.5E-06	3.8E-06	9.6E-06	1.0E-05
	DDD	Liver	3.19E-09	1.8E-07	2.4E-07	4.0E-07	5.5E-07	1.0E-06	1.4E-06
	DDTop	Liver	1.33E-08	6.4E-08	1.2E-07	1.4E-07	2.9E-07	3.4E-07	6.9E-07
	DDTpp′	Liver	1.31E-08	5.7E-08	1.2E-07	1.2E-07	2.7E-07	3.0E-07	6.6E-07
	Σ OCPs		3.61E-07	8.68E-06	1.46E-05	2.04E-05	3.60E-05	5.45E-05	9.50E-05
PBDEs	PBDE28	Liver	3.77E-13	3.6E-11	6.5E-11	6.2E-11	1.2E-10	1.3E-11	2.5E-10
	PBDE47	Liver	7.82E-11	1.2E-08	1.2E-08	2.7E-08	2.8E-08	7.4E-08	7.5E-08
	PBDE99	Liver	1.16E-12	1.8E-11	6.3E-11	3.9E-11	1.5E-10	9.5E-11	3.6E-10
	PBDE100	Liver	1.16E-12	1.8E-11	6.3E-11	3.9E-11	1.5E-10	9.5E-11	3.6E-10
	PBDE153	Liver	2.03E-12	4.5E-13	8.2E-12	5.5E-13	1.3E-11	8.3E-13	1.9E-11
	PBDE154	Liver	2.03E-12	4.5E-13	8.2E-12	5.5E-13	1.3E-11	8.3E-13	1.9E-11
	PBDE209	Liver	4.06E-12	9.0E-13	1.6E-11	1.1E-12	2.6E-11	1.6E-12	3.8E-10
	Σ PBDE		8.9E-11	1.21E-08	1.22E-08	2.71E-08	2.84E-08	7.42E-08	7.6E-08

In bold: significant cancer risks.

TABLE 5 | Combined Ingestion Cancer Risk for small (SF), medium (MF), large (LF) demersal-pelagic fishes, and Combined Dermal Cancer Risk calculated for the target organ of each carcinogenic contaminant.

Target organ	ICR (demersal fish)			ICR (pelagic fish)			DCR
	SF	MF	LF	SF	MF	LF	
Breast	1.7E-07	4.5E-07	1.2E-06	8.7E-06	2.0E-05	5.5E-05	7.9E-09
Gastro intestinal	7.4E-05	**1.9E-04**	**5.0E-04**	1.7E-07	3.2E-07	7.3E-07	7.3E-07
Generic	3.0E-06	6.8E-06	1.5E-05	2.1E-05	4.8E-05	**1.3E-04**	2.1E-08
Liver	1.5E-05	3.6E-05	9.5E-05	4.3E-07	8.0E-07	1.8E-06	3.6E-07
Lung	9.0E-06	2.3E-05	5.7E-05	1.8E-06	3.8E-06	9.3E-06	9.5E-07
Skin	1.2E-05	2.8E-05	6.8E-05	2.7E-06	6.0E-06	1.5E-05	1.1E-07

In bold: significant cancer risks.

Contribution of Different Pathways

It is noteworthy that food ingestion is the main pathway of contaminants exposure for humans when compared with other routes such as inhalation, dermal contact and drinking water (Lioy et al., 1988; Butler et al., 1993). Studies conducted on human exposure to BaPy revealed that the range and magnitude of dietary exposures (2–500 ng/day) were larger for inhalation and dermal contact (Lioy et al., 1988). Diet makes a substantial

TABLE 6 | Ingestion Hazard Quotient (IHQ) for small (SF), medium (MF), large (LF) pelagic and demersal fishes, Dermal Hazard Quotient (DHQ) and Hazard Index (HI) as the sum of the HQs.

	Contaminant		IHQ SF		IHQ MF		IHQ LF	
		DHQ	Pelagic	Demersal	Pelagic	Demersal	Pelagic	Demersal
PAHs	Naphthalene	3.62E-06	2.11E-05	2.19E-05	2.13E-05	2.21E-05	2.16E-05	2.24E-05
	Acenaphtene	1.71E-07	1.96E-06	4.37E-06	2.01E-06	4.54E-06	2.12E-06	4.76E-06
	Fluorene	1.30E-06	1.81E-05	2.04E-05	1.90E-05	2.16E-05	2.09E-05	2.37E-05
	Antracene	1.01E-07	1.77E-06	1.11E-05	1.93E-06	1.26E-05	2.17E-06	1.44E-05
	Fluoranthene	3.72E-06	8.77E-05	8.95E-04	1.27E-04	1.48E-03	2.21E-04	2.50E-03
	Pyrene	8.90E-06	1.67E-04	5.94E-04	2.08E-04	8.09E-04	2.99E-04	1.14E-03
	Benzo(a)pyrene	2.49E-04	5.26E-03	4.23E-02	1.22E-02	1.12E-01	3.24E-02	2.89E-01
	Benzo(a)pyreneTEQ	7.82E-04	3.20E-02	7.93E-02	1.80E-02	2.08E-01	1.30E-01	5.36E-01
OCPs	HexaClCycloHexane(a + b)	2.67E-06	1.54E-06	8.86E-06	1.57E-06	9.17E-06	1.63E-06	9.53E-06
	HexaClBenzene	1.11E-05	1.10E-03	1.85E-03	2.14E-03	3.84E-03	5.00E-03	8.91E-03
	Aldrin	9.70E-04	3.36E-02	6.08E-02	8.09E-02	1.55E-01	2.19E-01	4.16E-01
	Dieldrin	2.52E-06	2.03E-04	3.25E-04	2.07E-04	3.32E-04	2.14E-04	3.42E-04
	Endrin	3.16E-07	1.34E-05	3.47E-05	1.37E-05	3.56E-05	1.41E-05	3.67E-05
	Endosulfan	1.56E-08	8.39E-07	4.87E-06	8.57E-07	5.04E-06	8.92E-07	5.23E-06
	Alachlor	1.58E-06	2.76E-06	5.31E-06	2.80E-06	5.39E-06	2.86E-06	5.50E-06
	DDE	1.25E-03	3.97E-02	4.24E-02	9.55E-02	1.03E-01	2.59E-01	2.78E-01
	DDD	1.04E-03	5.73E-02	7.72E-02	1.28E-01	1.80E-01	3.35E-01	4.66E-01
	DDTop	1.83E-04	2.69E-03	5.17E-03	5.78E-03	1.20E-02	1.41E-02	2.90E-02
	DDTpp′	1.81E-04	2.31E-03	4.74E-03	4.99E-03	1.11E-02	1.22E-02	2.67E-02
PBDEs	PBDE28	1.26E-05	8.50E-03	2.18E-03	9.80E-03	4.00E-03	9.90E-04	8.33E-03
	PBDE47	2.61E-03	3.87E-01	3.89E-01	9.14E-01	9.19E-01	**2.47E+00**	**2.49E+00**
	PBDE99	3.87E-05	1.78E-03	6.28E-03	3.85E-03	1.50E-02	9.42E-03	3.60E-02
	PBDE100	3.87E-05	1.78E-03	6.28E-03	3.85E-03	1.50E-02	9.42E-03	3.60E-02
	PBDE153	3.39E-05	1.11E-04	2.04E-03	1.37E-04	3.30E-03	2.05E-04	4.68E-03
	PBDE154	3.39E-05	1.11E-04	2.04E-03	1.37E-04	3.30E-03	2.05E-04	4.68E-03
	HI	7.45E-03	5.65E-01	7.23E-01	**1.27E+00**	**1.75E+00**	**3.50E+00**	**4.63E+00**

In bold: significant risks.

contribution (more than 70%) to the exposure to PAHs (Beckman et al., 1998; Phillips, 1999). We found significantly higher ICRs with respect to DCRs that could be affecting the resident population, confirming these assumptions. As expected, higher ICRs were recorded for large pelagic/demersal fish due to biomagnification process that enhances the bioaccumulation factor of contaminants in the large fishes that live on the top of the food chains. The affinity of POPs for organic materials in sediments are high enough to cause accumulation in this matrix with long-lasting consequences for environmental health. Thus, sediment-dwelling, filtering organisms and demersal fishes are most susceptible to POPs contamination. Our results reported higher ICRs for demersal fishes with respect to pelagic fishes due to their feeding and breathing in the pore water in contact with contaminated sediments.

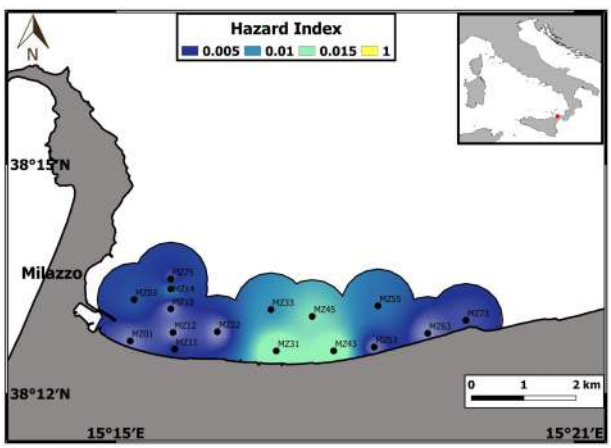

FIGURE 3 | Dermal Hazard Index distribution in the study area.

Contribution of Different Contaminants

Statistically significant differences (KW test, p = 0,002) between contaminant contribution for the ICRs were evidenced. Particularly, higher PAHs contribution was recorded concerning OCPs and PBDEs because of their higher concentrations in seawater and sediments of the study area. BaPy has the highest ICR with respect to the other PAH congeners that exceed the upper bound of EPA acceptable range ($2.7 \cdot 10^{-4}$ for the ingestion of large demersal fishes, **Table 3**). BaPy is the most known and studied member of PAHs because of its highest carcinogenic

TABLE 7 | Monte Carlo simulation, probabilistic distribution of DCR and ICR, statistical values (percentiles).

		5%	25%	50%	75%	95%
DCR						
	PAHs	8.9E-9	8.5E-8	1.7E-7	7.2E-7	2.7E-6
	OCPs	8.6E-9	8.3E-8	2.6E-7	6.2E-7	1.5E-6
ICR pelagic fishes						
SF	PAHs	4.3E-7	1.7E-6	4.9E-6	1.4E-5	6.2E-5
	OCPs	1.0E-6	2.5E-6	4.9E-6	9.6E-6	2.4E-5
	PBDEs	8.0E-10	2.5E-9	5.7E-9	1.3E-8	3.9E-8
MF	PAHs	9.4E-7	4.2E-6	1.2E-5	3.2E-5	**1.4E-4**
	OCPs	2.6E-6	6.2E-6	1.2E-5	2.3E-5	5.6E-5
	PBDEs	1.9E-9	6.4E-9	1.4E-8	3.1E-8	9.6E-8
LF	PAHs	2.0E-6	9.6E-6	2.6E-5	7.5E-5	**3.7E-4**
	OCPs	1.8E-5	3.1E-5	4.3E-5	6.1E-5	**1.0E-4**
	PBDEs	1.2E-8	2.8E-8	5.1E-8	9.1E-8	2.2E-7
ICR demersal fishes						
SF	PAHs	1.9E-6	8.5E-6	2.3E-5	6.6E-5	**2.9E-4**
	OCPs	1.2E-6	3.3E-6	6.2E-6	1.2E-5	2.9E-5
	PBDEs	8.0E-10	2.6E-9	5.7E-9	1.3E-8	4.3E-8
MF	PAHs	4.8E-6	2.2E-5	6.3E-5	**1.7E-4**	**7.4E-4**
	OCPs	5.5E-6	1.3E-5	2.6E-5	4.9E-5	**1.2E-4**
	PBDEs	1.9E-9	6.3E-9	1.4E-8	3.0E-8	9.7E-8
LF	PAHs	1.4E-5	6.1E-5	**1.7E-4**	**4.6E-4**	**2.1E-3**
	OCPs	1.0E-5	2.5E-5	4.8E-5	9.0E-5	**2.7E-4**
	PBDEs	1.2E-8	2.8E-8	5.1E-8	9.1E-8	2.2E-7

In bold: significant risks.

potential (Howard and Fazio, 1980). A lot of epidemiological studies confirm the strong relationship between the ingestion of highly contaminated food with BaPy and risk for gastro-intestinal cancer, particularly stomach, esophagus (Ward et al., 1997) and colorectal cancer (Schiffman and Felton, 1990; Muscat and Wynder, 1994; Sinha et al., 1999). BaPy concentration higher than the MAC set by the Directive 2013/39/EU here found in sediment of the sampling station MZ13 located near the industrial pole (where both thermopower plant and refinery plant are present), suggest a significant anthropogenic input of this contaminant in the study area with a relevant health risk for local populations. Although a not statistically significant level, the higher mean contribution of OCPs was found for non-cancerogenic health issues with respect to PAHs and PBDEs contributions (**Table 5**). OCPs, such as Endosulphan and Lindane (γ-hexachlorocyclohexane), are well known to exert neurotoxic effects inhibiting the calcium ion influx and Ca- and Mg-ATPase and causing release of neurotransmitters (Mathew, 2012). Moreover, epidemiological studies have shown that exposure to OCPs is strongly associated with type 2 diabetes (Lee et al., 2006) and Parkinson's disease (Steenland et al., 2014). As shown in **Table 5**, the HI associated to OCPs and due to the ingestion of large demersal fishes is higher than 1 suggest that local population could experience these pathologies after a long-time exposure of OCPs. As shown in **Table 2**, all the OCPs concentrations in seawater and sediment samples are lower than the MAC set by the Directive 2013/39/EU. This study evidence that the threshold limits imposed by legislation could not be sufficiently protective against a long-time exposure of a mix of highly toxic contaminants. Although the OCPs has the highest mean contribution, the highest HI was reported for PBDE47 (HI = 2,5; **Table 5**). PBDE-47 is an emerging contaminant diffuse worldwide in the marine environment at constant increasing concentrations (Hites et al., 2004; Schecter et al., 2007) although its use has been banned in many countries. In spite of these concern, limited information on the PBDE47 toxicity is available. Exposure of neonatal mice to PBDE47 is reported to exert neurodevelopment toxicity causing behavioral alterations, learning and memory deficits and dysfunctions in the cholinergic system in adult stage (Eriksson et al., 2001, 2002; Branchi et al., 2003). PBDE47 exceed the MAC set by the Directive 2013/39/EU in seawater column of 5 sampling stations, suggesting relevant contamination of this pollutant in the study area.

Uncertainty

As reported in the Basic Information about the Integrated Risk Information System (IRIS), Cancer Oral slop factor (CSFo), and Reference Dose (RfDo) are parameters estimated with uncertainty due to limitations of the data used and could represent an uncertainty factor for risk assessment. Fixed exposure factors, commonly used in risk assessment, may not be adequately accurate in reproducing reality because of their variability due to different life stages and/or different environmental condition. Finally, the cancer risks per target organs here calculated are probably underestimated for the lack of information about carcinogenic action of every single PAH on human. For example, we established a specific carcinogenic

TABLE 8 | Monte Carlo simulation, probabilistic distribution of DHR and IHR, statistical values (percentiles).

		5%	25%	50%	75%	95%
DHR						
	PAHs	2.7E-7	2.7E-5	2.4E-4	9.8E-4	3.7E-3
	OCPs	6.6E-5	6.4E-4	2.0E-3	4.7E-3	1.2E-2
IHQ pelagic fish						
SF	PAHs	4.5E-4	1.8E-3	5.2E-3	1.4E-2	6.6E-2
	OCPs	1.8E-2	4.5E-2	8.8E-2	1.7E-1	4.3E-1
	PBDEs	2.7E-2	8.2E-2	1.9E-1	4.3E-1	**1.3**
MF	PAHs	1.0E-3	4.4E-3	1.2E-2	3.4E-2	1.5E-1
	OCPs	4.6E-2	1.1E-1	2.2E-1	4.1E-1	**1.0**
	PBDEs	6.4E-2	2.1E-1	4.6E-1	**1.0**	**3.2**
LF	PAHs	2.2E-3	1.0E-2	2.7E-2	7.9E-2	3.9E-1
	OCPs	3.3E-1	5.5E-1	7.7E-1	**1.1**	**1.8**
	PBDEs	3.9E-1	9.3E-1	**1.7**	**3.0**	**7.2**
IHQ demersal fish						
SF	PAHs	2.1E-3	9.0E-3	2.4E-2	6.9E-2	3.1E-1
	OCPs	1.1E-1	2.9E-1	5.6E-1	**1.1**	**2.6**
	PBDEs	2.7E-2	8.8E-2	1.9E-1	4.3E-1	**1.4**
MF	PAHs	5.1E-3	2.3E-2	6.7E-2	1.8E-1	7.9E-1
	OCPs	5.5E-1	**1.4**	**2.6**	**5.0**	**12.6**
	PBDEs	6.6E-2	2.1E-1	4.6E-1	**1.0**	**3.2**
LF	PAHs	1.5E-2	6.4E-2	1.8E-1	4.9E-1	2.2
	OCPs	2.3E-1	5.8E-1	**1.1**	**2.1**	**5.3**
	PBDEs	4.0E-1	9.3E-1	**1.7**	**3.0**	**7.3**

In bold: significant risks.

action of BaPy in the gastro-intestinal tract based on IRIS database, excluding other target organs for the carcinogenic action of this compound for lack of evidence in humans. However, several studies with experimental animals suggest that oral, intratracheal and subcutaneous injection of BaPy led to carcinogenic events in multiple sites (IARC, 2010). Furthermore, BaPy has been proven to induce breast tumors in animal through genotoxic activities implied in p53 mutations (Morris and Seifter, 1992). This information suggests that the carcinogenic action of BaPy could affect other organs and not exclusively the gastro-intestinal tract. Moreover, uncertainty is not considered in the models applied to mixtures of compounds thus possibly affecting their mobility, partitions and health impacts.

CONCLUSION

The study investigated, for the first time, persistent and emerging pollutants in seawater and sediments of the Gulf of Milazzo. The concentration of BghiP, PBDE in seawater samples and BbF, BkF, BaPy in sediment samples higher than MAC show a low quality of this marine environment.

The human health risk was calculated through cancer and non-cancer risk indices. These indices provide only point estimates giving little information about uncertainty and variability surrounding the risk impact. Therefore, Monte Carlo simulation was necessary to provide complete information on the likelihood of the various risk levels. The cancer risk assessment shows higher risks for resident population through the ingestion of large demersal fish, especially gastro-intestinal cancer caused by BaPy. Negligible cancer risks were associated with dermal adsorption of contaminants from seawater. The non-cancer risk assessment (HI) shows significant risks for resident population to develop health issues due to the ingestion of large demersal fish contaminated by mixture of the analyzed pollutants. OCPs and especially PBDE47 represent the major contribution for non-cancer risk. The present study might provide, assuming permanent environmental conditions, useful information on human exposure to POPs in Milazzo bay and will be useful for strategies focused on risks mitigation. Particular attention should be done to reduce BaPy emission from refinery plant, thermopower plant and maritime transport. The use of other less toxic flame retardants than PBDE47 for the production of plastic and fiber materials is also recommended.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FD'A led the conception and design of the manuscript. AB led the conception and writing of the manuscript. MD led the sampling

strategy. EQ carried out the statistical analysis and developed the risk map. SG carried out the chemical analysis on sediment samples. FP carried out the interpretation of oceanographic data. NS carried out the total organic carbon analysis on sediment samples. GB led the oceanographic survey. GA and VD contributed to carry out the chemical analysis on seawater samples and to review the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Environmental Science

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Linking Bioeconomy to Redevelopment in Contaminated Sites: Potentials and Enabling Factors

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This article proposes a bioeconomy approach to the management of contaminated sites with the aim to identify actions for the development of a common policy framework for environmental protection and sustainable development. Among the policies addressing pollution on land and at sea, we identify four main gaps that hamper the implementation of measures for the prevention and management of contaminated sites from local to systemic scales. We introduce three concepts from bioeconomy—(i) value-chain, (ii) regional perspective, and (iii) multi-sector approach—that are potentially conducive to socio-economic and environmental improvement of degraded areas in Europe.

Keywords: bioeconomy, circular economy, bioremediation, pollution, nature-based solution, smart specialization

INTRODUCTION

The last 70 years of super-exponential growth of the world economy led to the exploitation of not-renewable resources and the increasing production of mismanaged waste (Steffen et al., 2015). In this context, land and coastal regions worldwide have been extremely under pressure coming from urbanization and the related growth of infrastructures and industries. The Mediterranean coastal areas, for example, witness the legacy of this economic growth with numerous refineries, chemical and steel plants, often decommissioned or in a phase of recasting, accompanied by high volumes of dangerous materials in extensive landfills and near-shore dumps. As a consequence, contamination caused by anthropic activities represents a major threat affecting terrestrial and marine ecosystems, and for this reason, such areas are monitored and occasionally recovered at the European, national, and local levels (Payá Pérez and Rodríguez Eugenio, 2017; Eea Report, 2019). However, the restoration and reintroduction of such areas into productive, and hopefully sustainable, value chains are difficult to achieve while management measures are fragmented and lack an integrated plan for remediation and development, from a local to a systemic level. In the context of a growing population, facing also global challenges like climate change, sea level rise and land/coastal ecosystem degradation, the interest in the use of renewable resources is growing, with the aim to catalyze the economic transformation and to implement more sustainable consuming strategies.

With this aim, we propose a new approach to the management of contaminated sites, i.e., areas of environmental and ecosystem degradation and resource depletion with consequent socio-economic loss, by applying bioeconomy principles to help overcome major limits and gaps that prevent the reintegration of these areas into productive chains. We discuss how this approach could drive the choice of restoration measures in contaminated areas, while producing value and jobs. The study (i) presents an analysis of policy and management measures undertaken at the local, regional, and country levels and of main existing gaps; (ii) discusses the added value of bioeconomy approaches to recover contaminated areas from an environmental, economic, and social point of view; and (iii) proposes an integrated concept of management of contaminated areas in a circular bioeconomy perspective for business operators and decision makers.

OVERVIEW OF POLICY FRAMEWORK FOR PREVENTING AND MANAGING SOIL/COASTAL POLLUTION: MAJOR GAPS AND LIMITS

The urgent need to reduce pollution across Europe and to maintain healthy soils and seas (water column and sea floor) is widely recognized, although this is a goal difficult to achieve because chemical substances, including heavy metals, persistent organic pollutants, and also emerging contaminants tend to persist in all environmental matrixes. Indeed, several classes of contaminants are widespread and still above the legal threshold levels in extensive portions of Europe's terrestrial and marine environments (Payá Pérez and Rodríguez Eugenio, 2017; Eea Report, 2019). Presently, contamination is mainly driven by increasing economic activity together with mismanaged waste storage and disposal practices, all leading to the dispersal of contaminants into soil, groundwater, and marine environments, from coastal to offshore regions (Payá Pérez and Rodríguez Eugenio, 2017; Eea Report, 2019). The main sources of these contaminants include inadequate and unsustainable agricultural and forestry practices, industrial activities, treated and untreated waste water, tourism, urban and industrial sprawl, shipping, port activities, aquaculture, fisheries, offshore oil exploitation, and consumption of fossil fuels (Payá Pérez and Rodríguez Eugenio, 2017; Eea Report, 2019). Based on the analysis of the JRC report on progress in management of contaminated sites (Payá Pérez and Rodríguez Eugenio, 2017) and the EEA report on contaminants in Europe's seas (Eea Report, 2019), we focus on existing policies and cross-policy approaches, identifying *four* potential gaps that should be considered for implementing a common framework and for progress in environmental protection and restoration actions.

Spatial 'Separation' (Gap 1)

Across European countries, the assessment and management of contaminated areas deal with policies that separately address marine and terrestrial environments. This existing approach is probably due to the specificity of features (from chemical and physical properties to the ecosystems structure and functioning) that distinguish each environmental compartment and consequently influence and drive management and remediation practices. Land and sea also differ in terms of anthropic uses, multiplicity of sectors, and activities with distinctive environmental and socioeconomic impacts. The "land or sea" approach intrinsically fails in linking causes to effects in an integrated framework that would rather lead to measures preventing further pollution through long-term-oriented remediation strategies. In particular, transitional coastal areas, with their relevant anthropic impacts and specific and fragile ecosystems, suffer from the lack of integrated approaches to environment restoration and recovery.

'Fragmented' Policies (Gap 2)

The policy framework in use lacks a coordinated approach aimed at preventing and managing the contamination on land and sea. On land, the protection and sustainable use of soil is regulated by indirect measures (Com/2012/046, 2012) and includes policies addressing contamination sources (targeting industrial policies or chemicals directives) and specific actions dedicated to soil conservation or land use regulation. The existing framework of policy regulation primarily encompasses major compartments: waste management (Directive 2008/98/EC, 2008); landfill (Council Directive 1999/31/EC, 1999); wastewater and water resource protection (Directive 2000/60/EC, 2000); evaluation, authorization, and restriction of chemical industrial activities (Directive 2004/35/CE, 2004; Regulation (EC) No 1907/2006, 2006); nature protection and biodiversity conservation (Directive 2009/147/EC, 2009; COM/2011/244, 2011); nitrates and pesticides (Council Directive 91/676/EEC, 1991; Directive 2009/128/EC, 2009); sewage sludge (Council Directive 86/278/EEC, 1986); forestry strategy (COM 2013/659, 2013); climate change adaptation and mitigation (COM/2013/216, 2013); and energy (Directive 2009/28/EC, 2009). Remarkably, none of these regulations includes guidelines to systematically manage and specifically regulate soil contamination (Frelih-Larsen et al., 2016). Moreover, the effectiveness of all those policy instruments depends on the implementation actions at the national/regional level with a potential consequent fragmentation in terms of measures/regulations adopted among—and within—member states. In the marine compartment, instead, the need to substantially reduce pollution (both in coastal marine areas and offshore) is widely recognized, but specific remediation actions are difficult to achieve due to the heterogeneity of the environmental matrices and the lack of available technologies. Differently from soil, the comprehensive European Integrated Maritime Policy (COM 2007/575, 2007) aims to achieve coherence across the range of economic activities in the marine environment carried out by different marine sectors. The uses of marine resources should be constrained within sustainable limits, as stated by the 'Blue Growth Directive' (SWD 2017/128, 2017) and need to be harmonized as outlined by the Maritime Spatial Planning Directive (Directive 2014/89/EU, 2014). In this context, the Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC, 2008) sets the standards conducive to the achievement, or preservation, of the good environmental

status through a sustainable use of marine ecosystems and provides the overarching compliance criteria to adopt in conducting economic activities (e.g., Borja et al., 2017). The main pressures affecting European seas have been identified by different reports (UNEP, 2018; Eea Report, 2019) and are considered as central in the MSFD (Directive 92/43/EEC, 1992; Directive 2000/60/EC, 2000).

Lack of a Common Framework for Local and Regional Development and Funding (Gap 3)

Financing is key among the indirect measures aimed at addressing the problem of remediation of polluted sites. More than 42% of the total costs for remediation come from public budgets (only referring to soil remediation; Payá Pérez and Rodríguez Eugenio, 2017) with an urgent need to include local and private stakeholders in the management of contaminated sites to find productive and inclusive solutions. Financing instruments and thematic strategies useful for supporting remediation and risk reduction are identified in the JRC report (Payá Pérez and Rodríguez Eugenio, 2017) and include instruments, such as H2020 and Life Programme, that may contribute to advancing environmental protection on land and at sea through the promotion and financing of relevant research and innovation. However, a cross-policy analysis reveals the lack of a dedicated strategy for attracting private funding as well as a robust framework for their implementation. Even if the European Regional Development Funds (ERFD) and Cohesion Fund (CF) are mentioned among the relevant indirect measures (Payá Pérez and Rodríguez Eugenio, 2017) no further action defining a medium- to long-term plan for development is clearly defined both at regional or European level, thus reducing the effective adoption of specific and concrete measures. This weakness reflects the lack of a strategic economic plan of development based on a new use of contaminated areas, thus limiting the interest of private stakeholders and investors.

Uncertain Potential for Economic Outlooks (Gap 4)

A productive use of contaminated areas mentioned by the cross-report analysis suggests further uses of biomass produced on contaminated soils as biofuel (Directive 2009/28/EC, 2009) for its positive impact in soil protection. No other reference is, however, made to additional products or services provided, among which the ecosystem services, even though their value is not easily quantifiable. Furthermore, the policies that cover aspects related to industrial production are described for their role in preventing pollution arising from industrial activities (Directive 2010/75/EU, 2010) while strategies for remediation purposes or sustainable development of industrial processes are not indicated. Strategies of circular economy are mentioned in the EEA report (Eea Report, 2019) but only in relation to the prevention of plastic pollution at sea and reduction of soil contamination (Frelih-Larsen et al., 2016). This analysis reveals that the approaches commonly undertaken at the regional, national, and European scale focus on risk assessment, mitigation, and recovery from pollutants but lack a broader strategic plan for the long-term reincorporation of contaminated areas into productive and sustainable economic activities.

A NEW APPROACH TO THE MANAGEMENT OF CONTAMINATED AREAS THROUGH BIOECONOMY

The goal of bioeconomy is to improve sustainably the productivity and quality of products of economic sectors by creating longer and more locally routed value chains, where the actions of public and private stakeholders integrate across all major sectors (BIT II, 2019). A key aspect in bioeconomy is exploiting terrestrial/marine biodiversity, ecosystem services, and circularity to regenerate abandoned marginal lands and former industrial sites.

We here propose a new approach to the management of contaminated areas to fill the gaps identified in the previous section. To this end, we moved from a sectorial solution-oriented approach to a (sustainable) development-oriented approach primary considering the modern European Economic strategies. Among these strategies, bioeconomy offers key principles to advance in this direction. Bioeconomy, often referred also as bio-based economy, involves the sustainable use of renewable biological resources, biological waste and residual material to produce food, energy, and industrial goods. Bioeconomy is driving the transition toward a more sustainable economy by addressing major global challenges (including food security, climate change, resource scarcity) and ultimately reconciling economic activity within the planetary boundaries (Lewandowski, 2018). Bioeconomy exploits biological (bio-based) resources through efficient production and conversion technologies providing environmental and economic services and promoting the transition to a more sustainable society. We thus propose as a new model the *Integrated Bioeconomy Approach to the Management of Contaminated Areas* (hereinafter Integrated Bioeconomy Approach) that combines (i) the environmental restoration through nature-based solution and (ii) the establishment of productive bio-based value chains (described in section "Bioeconomy as a successful 'horizontal strategy' to build integrated policies for contaminated site management"), specifically focused on socio-economic development of contaminated areas. This approach is expected to break through some major blocks in the application of remediation solutions and to overcome the current limits in the management of contaminated areas.

In **Table 1** we report the four gaps described in the previous section, together with proposed specific activities that result from the application of the Integrated Bioeconomy Approach. These activities are particularly oriented to implement specific EU policy trajectories and to achieve integrated actions for preventing further pollution and managing contaminated areas by using modern approaches of environment restoration and socioeconomic valorization.

Synthetically, the spatial gap shows the limits in addressing the management and remediation of contaminated sites by referring

TABLE 1 | Gaps in EU Policy framework and suggested actions.

GAP 1	**Description**
Spatial separation	Policies related to the prevention and management of contaminated sites are commonly overseen separately with respect to specific areas of interest on land and offshore. The lack of an integrated approach mainly affects the coastal area that, for its hybrid nature, should be managed by taking into account both land and offshore related features. The spatial approach prevents from considering the multiplicity of activities, factors, and sectors that are recognized to be responsible for contamination.
Suggested Action 1	**Expected outcome**
Integration of policies considering the land-sea continuum	An integrated approach to land and sea related policies will result in: better addressing land/sea interaction related features; improvement of coastal management; consequent broadening of the analysis of economic sectors responsible for contamination; and development of a comprehensive framework of policy references.
GAP 2	**Description**
Fragmented policies	The fragmentation in the measures adopted among—and within—member states influences their effectiveness.
Suggested Action 2	**Expected outcome**
Building an integrated and common policy framework	Allowing crosscutting policy building; facilitating the dissemination and increase the adoption of management measures for contaminated sites from local to systemic level
GAP 3	**Description**
Lack of a common framework for basin or regional development strategies	Despite the link with the Cohesion Fund (CF) or European Regional Development Fund (ERDF) suggested in the case of soil management (JRC), the lack of a framework supporting regional or macro-regional development opportunities limits the implementation plans for the management of contaminated sites and hinders the development of potentially productive and inclusive solutions.
Suggested Action 3	**Expected outcome**
Prioritization of domains, areas and economic activities	Prioritization of specific assets and resources related to the prevention and management of contaminated sites, with unique opportunities for development and growth; facilitating the exchange of good practices and addressing measures for managing contaminated site; promoting multi-stakeholder engagement for contaminated site's solutions; favoring the integration of policies from local to regional and macro-regional dimensions; support effective measures to favor private Research and Innovation investments
GAP 4	**Description**
Uncertain potential economic outlooks	Value chains fully implemented and specifically linked to economic sectors and market of reference; lack of a pilot or good practice in business models construction
Suggested Action 4	**Expected outcome**
Identification of development scenarios promoting business and investments	Developing bio-based sustainable value chains and services in degraded areas; bringing private and public investments in establishing value chains; fostering new integrated business models in the bioeconomy; opening multiple possibilities among different sectors and targets

to the land or marine environment, separately. Integration of policy across diverse environmental compartments is proposed as a tool to better address cross-cutting issues and by building on the specificity of each compartment in terms of technical features and socio-economical attributes (suggested action 1). Building an integrated policy framework (suggested action 2) would overcome the fragmentation of policies (gap 2) by setting the framework for the future development of mitigation measures based on cross-sectorial policy and multi-stakeholder analysis. This approach looks at incorporating in the remediation and management policies those aspects related to the productive use of such areas and other direct/indirect policies relevant for their implementation from local to systemic level. "Prioritization of domains, areas and economic activities related to management of contaminated sites" (suggested action 3) would address the lack of a common framework for local or regional development strategies (gap 3). To reach this goal, a broad stakeholder analysis can influence policymakers to design suitable strategies accordingly. Strategies for regional development and European economic policies are also crucial to push efficiently integrated policies (for remediation) from local to systemic level. In this regard, targeted pilot actions—ranging from the technical features to the integrated policy design addressing the socioeconomic and environmental sustainability as a whole—represent the key to unlock the local to systemic transition by providing their feasibility in terms of replication capacity, and transferability potential. Lastly, the lack of a well-defined economic potential (gap 4) can be overcome by identifying strategic areas for business development and investments (expected outcome/suggested action 4). The use of regenerative, sustainable, and bio-based value chains or services applied to the management of contaminated sites would set up new integrated business models, thus opening possibilities for multiple market sectors and job creation at the local level. Pilot actions are

necessary to demonstrate the economical sustainability of the models but also to demonstrate that the model is transferable to other Areas/Regions/Countries characterized by a diverse set of assets. The business model has to be adapted to local peculiarities in terms of socio economical aspects, environmental features, level of contamination, but also to the maturity of those enablers that are responsible for the implementation of the identified business models (taking into account technological, infrastructure, and logistic capabilities).

The proposed actions demand a highly innovative 'socio-economic ecosystem' and a policy framework centered on the key concept of sustainability through the adoption of adaptable, scalable and transferable business models. Notably, the suggested actions also reflect an approach focused on the complexity of the management of contaminated areas as an *integrated* system, linking causes and effects, as well as connecting different sectors and application fields. In the next paragraph, we discuss three specific aspects of the bioeconomy (hereinafter indicated *lines*) that are responsible for the Integrated Bioeconomy Approach effectiveness.

Bioeconomy as a Successful 'Horizontal Strategy' to Build Integrated Policies for Contaminated Site Management

The European Bioeconomy Strategy, launched and adopted in 2012, addresses the production of renewable biological resources and their conversion into value-added products, including food, feed, bio-based products, and bioenergy. By definition, "bioeconomy covers all sectors and systems that rely on biological resources (animals, plants, micro-organisms and derived biomass, including organic waste), their functions, and principles. It includes and interlinks: land and marine ecosystems and the services they provide; all primary production sectors that use and produce biological resources (agriculture, forestry, fisheries, and aquaculture); and all economic and industrial sectors that use biological resources and processes to produce food, feed, bio-based products, energy and services" (Innovating for Sustainable Growth: A Bioeconomy for Europe, 2012). From its first release in 2012, the Strategy has been updated in 2018 in line with European priorities (State of the Union, 2018) with the aim to strengthen the connections among economy, society, and the environment. Since the first release, the bioeconomy strategy proposed a comprehensive approach to address specific deteriorations that our planet is facing (e.g., loss in biodiversity and environmental resources, energy, food supply). The updated European Bioeconomy Strategy (Updated Bioeconomy Strategy, 2018) better focuses on actions to accelerate the adoption of a sustainable European bioeconomy plan and to maximize the impact on the 2030 Agenda, its Sustainable Development Goals (SDGs), and the Paris Agreement. Now the strategy looks at circularity as an economy process that provides multiple value-creation. This approach would allow a substantial change from the consumption of finite resources (Growth within, 2015) and accelerate the transition toward a circular, carbon-neutral economy in the framework of the three axes of renewed Industrial Policy Strategy (COM/2017/0479, 2017, final), Circular Economy Action Plan (COM/2019/190, 2019), and Accelerating Clean Energy Innovation (COM 2016/0763, 2016). This updated strategy cuts across several sectors and allows synergies that favor industrial symbiosis. The strategy refers, among others, to innovative policies impacting production routes (supply chains), use of bioresources (such as biomass), ecosystem protection, and smart specialization as reported in **Figure 1**. No specific EU bioeconomy legislation exists but rather an ensemble of sectorial legislation developed in a common framework.

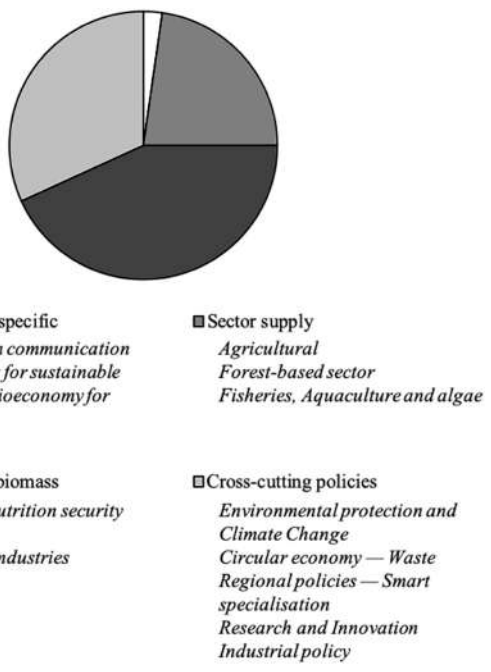

FIGURE 1 | Bioeconomy-related policies and strategies. Description of relevant policies and strategies for the bioeconomy. Based on A sustainable bioeconomy for Europe: strengthening the connection between economy, society and the environment (Updated Bioeconomy Strategy, 2018).

With this in mind, we propose three specific lines of actions for the remediation/recovery and development of contaminated sites. These actions are expected to provide specific instruments to overcome the above-identified gaps and to support the development of a new integrated management plan of contaminated areas.

Line 1: Generating Value Chains and Engaging Stakeholders: Potentials and Main Obstacles

Bioeconomy has a positive potential impact on the recovery of contaminated sites as indicated by two specific actions in the updated EU Bioeconomy Strategy (namely Action 1.6 and Action 2.2), hereinafter described (Updated Bioeconomy Strategy, 2018). Applying circular-bioeconomy principles to soft biological remediation techniques (bioremediation) provides a cost-effective solution for the rehabilitation of degraded areas and would potentially create an economy that is restorative and

regenerative. This approach is based on the establishment of *bio-based value chains,* a set of interlinked activities performed to deliver products/services for the market by adding value to biological bulk material (feedstock) (Lokesh et al., 2018). The H2020 Programme (For a better innovation support to SMEs, 2019) defines an industrial value chain as "the stages of value creation by enterprises and other organizations as part of the process of designing and delivering goods and services for their users." The bioeconomy represents a large potential for the development of new value chains from renewable biological materials such as bio-based products from lignocellulose, microalgae for the production of food and feed, including the conversion of organic waste into valuable products. Innovation may result from new combinations along and across existing value chains or by an innovative technology or process brought from one sector into another resulting in a disruptive effect (SuperBIO, 2016–2019). This valorization process incorporates a large number of different actors and can positively contribute to socio-economic, environmental, and technological advances (Lokesh et al., 2018), through the addition of value and building new cross-border and cross-sectorial collaboration, innovation, and entrepreneurship. A schematic description of the key elements involved in the establishment of a bio-based value chain is shown in **Figure 2** as a reference example.

However, estimates of jobs and growth created by the bioremediation actions in bioeconomy are still lacking and mirror the lack of a value chain on contaminated areas because of unclear market reference and applications. The potential intrinsic value of bioresources impacts on the production of goods or services also in contaminated areas. The development of sustainable products and processes from contaminated areas requires an interdisciplinary systemic analysis of entire value chains from feedstock, processing, and conversion, up to the levels of manufacture and marketing of products. The main bottlenecks for process implementation and value chain creation include the technological issues related to scientific knowledge on biomass exploitation and industrial scale-up and the need of a clear definition of the proper policy landscape building. For this reason, the engagement of key stakeholders across all elementary value chain fragments would represent a priority action to unlock the potential and thus implement the entire process combining the effects of research knowledge, industrial interest, social acceptance, and policy orientation. A number of research and pilot industrial projects already demonstrated that nature-based solutions are effective in restoring ecosystems from complex soil and water pollution (Fiorentino et al., 2010; Bianconi et al., 2011; Dubois and Gomez San Juan, 2016; Pietrini et al., 2018); among these, phytotechnologies and bioremediation rely on the ability of specific plants, fungi, or bacteria to degrade, stabilize, or remove pollutants in specific environmental compartments. Nonetheless, the entire value chain has not yet been designed on a large scale and reference markets or economically viable scenarios have still to be set up. Indeed, so far the biomass produced as a result of the phyto- and bioremediation interventions is generally treated as a waste. This approach represents a loss in terms of exploitable feedstock, costs for waste disposal, and sustainability of the entire process with a consequent negative effect on the application of the technology on a larger scale. The Action 1.6 of the Bioeconomy Strategy "Strengthen and scale-up the bio-based sectors, unlock investments and markets" is expected to contribute to the development of further bioremediation methods through their integration with dedicated markets. There is a wide variety of knowledge and technological expertise needed for bioeconomy-related activities, and the challenge to apply bioeconomy principles to contaminated sites includes research- and industry-driven aspects and demands innovation in bioeconomy value chains. As an example, there are still relevant gaps of knowledge on the effects of the presence of contaminants on biomass quality and conversion/valorization potential (Bianconi et al., 2011; Pietrini et al., 2019). Preliminary studies on processes for biomass treatment coming from phytoremediation activities and consequent valorization into a biorefinery perspective also using microorganism (Sotenko et al., 2017) have been undertaken with the aim to pave the way for successful commercialization of bio-based products and services from contaminated areas. On the other hand, in the marine compartment, some solutions have been explored to convert bioresources such as microalgae into valuable products (e.g., fertilizers) from waste (SABANA Project, 2016–2021) as well as promoting the entry of new technologies to use bioremediator organism for the restoration of polluted environments (REMEDIA Project, 2017–2021). Nonetheless, despite the specific context of bioeconomy application, the capacity to create value chains in a framework of contaminated sites varies according to the local assets, to the availability of specific bioresources, and to the presence of research infrastructure or biorefineries (Spatial Foresight, 2017). Among the key factors conducive to the development of a bioeconomy plan, the capacity to engage the actors from multiple domains is the primary enabling factor to set up new bio-based value chains (BioSTEP Consortium, 2017) and scale up processes. In this direction, a number of specific H2020 projects have been funded to promote multi-actor dialogue and multi-stakeholder co-creation of research, innovation, development, and political context in the bio-based economy (BIOVOICES Project, 2018–2020). Therefore, the dissemination of good practices for multi-stakeholder and cross-sectorial collaboration appears crucial, and examples of multi-stakeholder collaboration in the frame of European projects are numerous (Hasenheit et al., 2016). Also, the engagement of industrial actors is

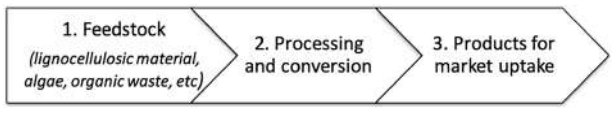

FIGURE 2 | Simplified scheme of a bio-based value chain. The figure represents a non-exhaustive description of the elements that are involved in the establishment of a bio-based value chain. Blocks indicate, respectively: sustainable biomass production and collection (block 1), breakthrough technology of processes and their conversion routes (block 2), and production of innovative added value products to the market (block 3).

necessary for reaching the technological maturity across the value chain segments in the bio-based sector and to bring the value chain closer to the market. At European level, the Bio-Based Industries Joint Undertaking (BBI JU) Public-Private Partnership promotes a strong European bio-based industrial sector developing new biorefining technologies to sustainably transform renewable natural resources into bio-based products, materials, and fuels. As an example, the GRACE project demonstrated large-scale Miscanthus and hemp production on contaminated/degraded soil with the aim to secure the supply of sustainably produced raw materials for the growing European Bioeconomy (GRACE Project, 2017–2022).

Line 2: Stimulating a Regional Perspective Toward Smart Specialization

The bioeconomy strategy (Updated Bioeconomy Strategy, 2018) calls Member States and regions to boost sustainable bioeconomies through their Research and Innovation Strategies for Smart Specialization (RIS3). Pilot actions to support local bioeconomy development (at rural, coastal, and urban level) via Commission instruments and programs are available, and a number of European regions have already included bioeconomy-related priorities (Spatial Foresight, 2017) in their mid-term strategy plans. This reflects the overarching goal of the European Bioeconomy Strategy to deploy bioeconomy across Europe acting as a vehicle for inclusive and sustainable growth at the local level. The targets for local development are the member state territories, such as regions, rural areas, cities, and coastal areas. The main goal of this bioeconomy framework is fostering local developments in the EU regions and cities while addressing sustainability targets. Once associated to the bioeconomy development of areas of environmental risk, tailored focused actions could reasonably boost the potential of innovation associated to supporting emerging sustainable value-chains and ecosystem services. This would in turn result in promoting sustainable and regenerative economies preventing further pollution associated to non-circular industrial activities and realizing remediation solutions. As an example, in Italy, a ministerial decree (D.P.C.M. May 19, 2005) declared the state of socio-economic and environmental emergency in the catchment of river Sacco after the detection of concentrations of betahexachlorocyclohexane (β-HCH) above the limit level of 0.003 mg/kg in a sample of milk from a farm located in the municipality of Gavignano (RM). The perimeter of the contaminated area (Site of National Interest – SIN) was later enlarged to cover over 8,000 hectares included in 19 municipalities between the Provinces of Rome and Frosinone, where several industrial clusters are located. Since 2012 bioremediation approaches have been tested both in lab and pilot scale (Bianconi et al., 2011; Pietrini et al., 2019) with the double aim to remediate from contamination and turn the system into a sustainable value chain. Despite this serious situation, no political frameworks have been developed in the long term in the Region. This reflects the limits previously discussed (mostly technological showing the low maturity of funding for the bio-based sector, at that time) and the fragmentation of actions at the policy level that lacked measures to sustain the implementation of the actions in the medium to long term. Recently, on 7 March 2019, Lazio Region and the Ministry of the Environment and Land and Sea Protection signed a memorandum of understanding anticipating major financing for the SIN of the Sacco Valley that mainly looks at the characterization of the whole territory and at securing most critical areas. This action is key in sustaining the process of remediation of contaminated areas but needs to be implemented with measures able to build frameworks (from pilot building, value chain creation, etc.) to assure the long-term stability of the process. In the last few years, a number of actions emerged with the aim to test economic value chains at the local level from enterprises, associations, and research bodies supported by municipalities or by generic funding tools (as for example the Rural Development Program of Lazio Region, REG UE N. 1305/2013, 2013) without coordination or strategic alignment. So far, the increasing interest at the stakeholder level in the region has not been supported by specific funding at local scale, capable to demonstrate the full value chain within a general policy framework, targeting the economic and environmental rehabilitation of the territory in a whole bioeconomy perspective. Indeed, the updated strategy has recognized the remediation of contaminated sites in relation to the development of local bioeconomies within the 2.2 Action "Pilot actions to support local bioeconomy development (rural, coastal, urban) Point iii: 'Develop urban bioeconomies through piloting circular bioeconomy cities through Horizon Europe.'" Thus, the management of contaminated sites can be seen as a potential effective driver for local bioeconomy development. The Lazio Region included among the drivers in its RIS 3 the bioeconomy challenges and specifically referred to the role of sustainable and competitive bio-based industries. This alignment would help, once the barriers for implementation at local scale are identified, the setting of a framework between (i) regional development strategies, (ii) bioeconomy strategies, and (iii) management policies for contaminated sites with the result to fully unlock its potential in a perspective of regional development of a new smart specialization field in synergy with environment and society.

Line 3: Multi-Sectoral Approach Across the Land-Sea Transition

The ocean, including coastal regions, represents a new economic frontier, covering more than two-thirds of the Earth's surface, spanning an increasingly diverse range of activities directed to exploit biological and abiotic resources and space (for traffic, tourism, cable/pipeline connections, energy or aquaculture platforms and waste disposal). The ocean will play a key role in the next decade, particularly in scenarios of accelerated exploitation to fuel a blue great acceleration (Jouffray et al., 2020). Coastal areas present diverse criticalities, ranging from those derived from the extreme effects of climate change to those induced by rapidly increasing anthropic sprawling

witnessed in Europe by the presence of half the population within 50 km from the coast and substantially increasing during touristic seasons (Collet and Engelbert, 2017). The coastal zone marks the border and may potentially represent a link, between land-based economies and the oceans but also the springboard to the offshore economy. Particular attention is dedicated to coastal development, boosted through the use of sea basin strategies and through dedicated strategic research and innovation agenda such as the ones for the Mediterranean (BLUEMED Initiative), the Black Sea, the Atlantic, and the Baltic. The aim to unlock the potential of the Blue Bioeconomy is a common objective of such strategies and agenda, in line with the "food security, sustainable agriculture and forestry, marine and maritime, and inland water research and the bioeconomy societal challenge" (Horizon 2020 Work Programme 2018-2020, 2020). The bioeconomy strategy specifically promotes local blue bioeconomies for the expected effect in generating actions addressing, among others, the issue of pollution at sea (Updated Bioeconomy Strategy, 2018). Some EU Member States and regions have R&I priorities for their smart specialization in the field of blue bioeconomy that mainly refer to water bio-resources across the coastal zone. Even if the blue bioeconomy specialization pattern is present across European regions and countries, the bioeconomy potential is not fully applied and exploited to marine/coastal resources. The existing policy, if properly implemented, may help the management and remediation of complex contaminated sites that appear unfortunately widespread in the coastal area, in a policy framework that would possibly overcome the spatial separation (gap 1) and maximize the valorization of the overall environment by connecting multiple sectors (gap 2). Bioeconomy is leading European economies and Member States are developing strategies accordingly to valorize their resources (Spatial Foresight, 2017; Ronzon and M'Barek, 2018). At the regional level, the actions are mainly driven by the availability of natural resources and cultural heritage, accompanied by a motivated research environment, an established primary value chain, and a developed industrial biotech sector (Spatial Foresight, 2017). Most strategies combine several thematic focus areas and develop interconnections according to their internal or external drivers (Spatial Foresight, 2017). This aspect would favor the adaptability of business models and management approaches across multiple economic sectors and regional policy contexts. Therefore, rather than just planning the safety and recovery of a contaminated area, a targeted plan for management and valorization should include the analysis of all possible uses of that area. Business solution should take into account both risks and benefits in a whole environmental and socio-economic perspective of a region and should look at each segment of the bio-based value chain as a possibility to valorize cross-sectorial synergies. As demonstrated by the project SABANA (SABANA Project, 2016–2021), an interlink between multiple sectors as agriculture, aquaculture, and waste management is possible within a bioeconomy framework that targets the sustainable management of both marine and terrestrial environments. This approach is based on the development of an integrated microalgae-based biorefinery for the production of biostimulants, biopesticides, and feed additives, together with biofertilizers and aquafeed, using marine water and nutrients from wastewaters. The adoption of a bioeconomy-based approach would lead to solutions able to catch the peculiarity of each scenario and to valorize the drivers present in such a region even if apparently far from each other, to promote a new value chain that would potentially open new and multiple markets. This approach may rapidly lead to an enrichment of the portfolio of activities in a given coastal region, adding, for instance, advanced industrial activities to tourism or fisheries. Bioeconomy should support nature-based solutions in coastal areas offering new opportunities to local economies and also supporting the definition of new solutions for environmental recovery in a circular economy perspective.

In line with actions aimed at preventing pollution and/or managing contaminated areas (analysis of directives/regulations/strategies as shown in sections "Overview of policy framework for preventing and managing soil/coastal pollution: major gaps and limits" and "Bioeconomy as a successful 'horizontal strategy' to build integrated policies for contaminated site management"), the bioeconomy strategy provides significant added value in safeguarding resources by (i) promoting sustainable business models (value chains and stakeholders engagement), (ii) fostering principles of sustainability and circularity (multisectoral approaches of the bioeconomy strategy), and (iii) designing a strategic integrated perspective (defining a regional approach to development). Bastioli (2019) highlighted how the bioeconomy transition should be played on interdisciplinary and interconnected local projects and on our capacity of inclusion. It is therefore urgent to develop guidelines for a bioeconomy-based, integrated policy framework, from a local to a systemic level, to exploit the full bioeconomy potential also in environmentally degraded and economically depressed areas, founded on a sustainable use of renewable biological resources. Like this, the application of the Integrated Bioeconomy Approach will ultimately result in a new inclusive management of polluted terrestrial and aquatic ecosystems through a harmonized framework of policies with high socio-economic and environmental impact.

DESIGN OF AN INTEGRATED CONCEPT FOR MANAGEMENT: TRANSITION TO ECONOMICALLY VIABLE SUSTAINABILITY FROM A LOCAL TO SYSTEMIC SCALE

As already mentioned, innovative technologies and approaches to the management of contaminated environments are now available (Payá Pérez and Rodríguez Eugenio, 2017; Eea Report, 2019) with opportunities for remediation through biological-based technologies (natural based solutions, phytotechnologies, etc.), which guarantee sustainable and smart solutions (Lord et al., 2008; Regional Biotechnology, 2011; COM/2017/0479,

2017). Nevertheless, the policy framework at the European, national, and regional levels on the management of risk areas and soil and water/sea protection never refers to circular bioeconomy as an instrument of direct or indirect prevention, mitigation, or remediation. Also, bioeconomy strategies do not mention the existing regulatory obstacles and seldom refer to the drivers associated to contaminated biomass exploitation. This regulatory gap represents a limit to the use of renewable bioresources both for remediation and for an economic perspective of contaminated areas. This gap reflects non-exhaustive scientific and methodological knowledge as well as the lack of potential actions supporting the implementation and spreading of good practices. The inclusion of the three above-described lines of actions oriented to environmental recovery would result in a crosscutting approach able to favor the policy evolution at:

- multiple complementary scales (local, regional, national, European).
- cross-sectorial levels (agriculture production, waste management, industrial manufacture, etc.).
- integrated spatial levels on land (rural, urban, transitional zone) and their impact on marine coastal and offshore areas.

Figure 3 summarizes how to design a comprehensive strategy and promote sustainable and inclusive roadmaps for sustainable solutions based on research and innovation driving business model development. The overall idea, reflecting also what was discussed in the final report of the High-Level Panel of the European Decarbonisation Pathways Initiative (Final Report, 2019), is that the new trend for implementation challenges of this general transition should be based on the combination of credible policies and their integration with solid markets. Thus, to overarch strong policy guidance, quantitative models should support the selection of policy targets for restoration activities. Consequently, the environmental benefits and economic potentials derived from the application of bioeconomy principles to contaminated areas, described in this paper, should be incorporated and integrated in appropriate models to drive the policy process in the medium and long term and at multiple space-scale. Costanza et al. (2017) highlights

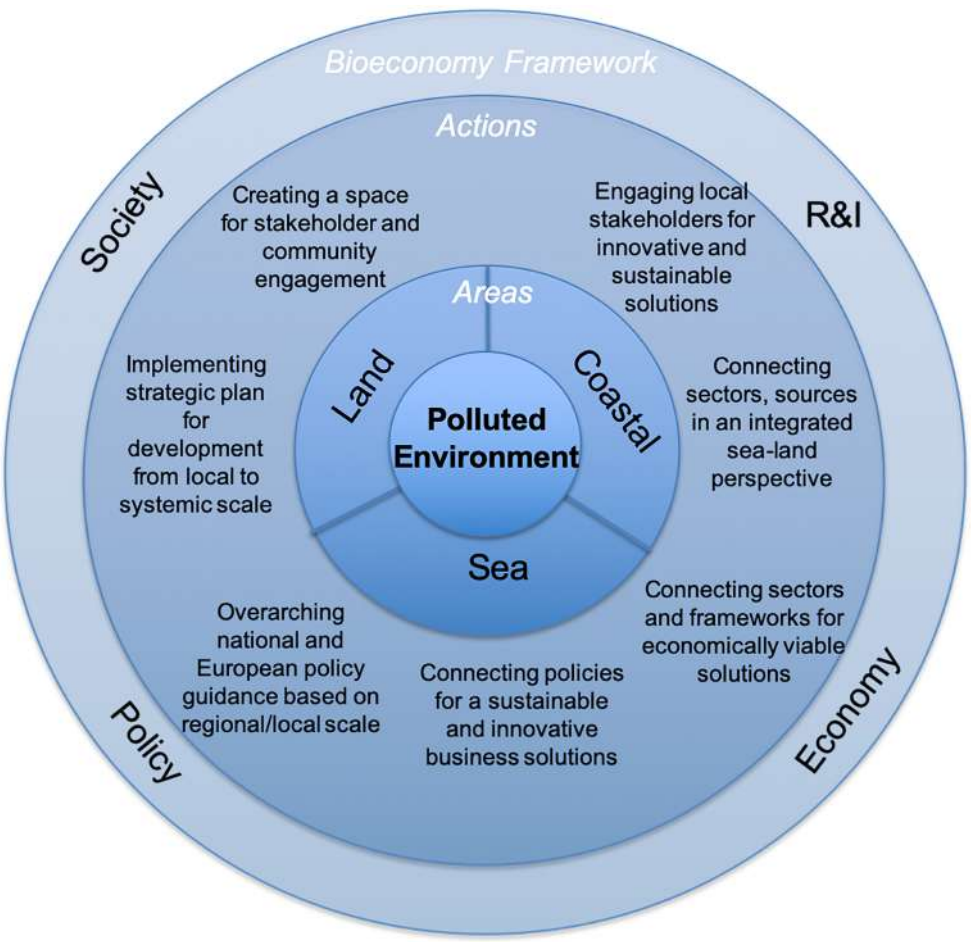

FIGURE 3 | Integrated concept for the management of contaminated areas in a circular bioeconomy perspective. The proposed conceptual model highlights areas, actions, and framework that would potentially contribute to the design of a comprehensive strategy of the polluted environment.

the crucial importance, in the process of integrating ecosystem services and natural capital into mainstream economic policy, that the process be orchestrated to allow the wider dialogue and participation of actors. Building a comprehensive model including the bioeconomy stakeholder ecosystem would elicit and successfully exploit such process by taking into account the complexity of technological, economic, environmental, and social aspects. Indeed the integration of environmental and economic models for development is in line with the European Green Deal strategy's aim that looks at the transition to a prosperous society as beneficial for the EU economy, society, and natural environment. This includes the up-scaling restoration efforts for damaged ecosystems at sea and on land with the final aim to positively impact biodiversity and deliver a wide range of ecosystem services (COM 2019/640, 2019).

Integration from local to systemic approach should be encouraged with the perspective also to align policies. As suggested in the report on 'Regional Biotechnology—Establishing methodology and performance indicators for assessing bio-cluster and bio-regions relevant to the KBBE area' (Regional Biotechnology, 2011), among the success factors in bio-regions, the high level of awareness was instrumental for bringing bioeconomy on national and EU agendas. Locally, building multiple value chains based on remediation of contaminated areas would positively influence the allocation of resources and the attention of regional policy makers and politicians from local to systemic scale. If the design of national/regional research and innovation strategies for smart specialization would clearly take into account bioeconomy drivers associated to the management of contaminated areas, an integrated approach would become possible across all regions. Consequently, the European Structural and Investment Funds (ESIF), supporting the economic development across all EU countries, would unlock the potential of contaminated areas and allow a better harmonization of the implementation of diverse interventions. If the adoption of the bioeconomy takes place at multiple levels, taking into account existing productions, industrial ecosystems and policy-support systems related to both soil/water/sea protection and management of risk areas, then the political framework would evolve in line with the changing demands of a centralized, circular and regenerative model of production. In this view, a recent measure of the Italian Ministry of Environment (Decree 46/19, 2019) strongly directs the reclamation of agricultural areas toward the bio/phytoremediation technology by also envisaging the exploitation toward an economic perspective. This is a positive and promising policy action that goes in the direction of coupling the management of contaminated areas to a sustainable economic framework. This action should be integrated and sustained by adequate business models tools and frameworks for their implementation, including targeted policies addressing the controversial issue of the exploitation of resulting biomass/waste, together with dedicated financial measures and regional support from pilot to industrial scale, to be effective and to act as a model for similar actions across Europe.

The process of implementation of SDGs at the regional level may represent an enabling condition to test and demonstrate the integrated policy approach to contaminated area management toward a bioeconomic perspective. Local and regional authorities, together with local civil society, play an important role in implementing the Agenda 2030 by catalyzing EU financial and policy instruments to foster innovation and boost investments in transformative community-based services supporting the achievement of the SDGs (United Nations, 2015). In this context, the European Commission should consider the territorial dimension of relevant policies in particular when they bring, and this is the case, socio-economic value. In 2019, The Ministry of the Environment and Land and Sea Protection of Italy launched a National call for proposals (Bando SNSVS 2, 2019) to promote research projects supporting the implementation of the National Strategy for Sustainable Development. In particular, and in line with the sustainable development strategy, specific actions are expected to define and evaluate policies, plans, programs, and projects for a full integration of sustainability targets. The Ministry recognized the importance of bioeconomy applied to the remediation of contaminated sites in the achievement of SDGs by funding the project *BioGoal* (Contaminated areas and circular bioeconomy: how to build regional strategies starting from sustainable development goals, *personal communication*) that looks at the Sicilian environment, economy, and society as a relevant study case for future replication.

CONCLUSION

Human activities are responsible for dramatic, extensive, and pervasive pollution worldwide as one of the environmental costs devoted to the unsustainable, great acceleration. While recognizing the prevention of pollution as a key priority, human society needs to make major efforts to prevent pollution and restore increasing portions of the territory through strategic and integrated policy approaches. At the global level, the importance of soil, land, and coastal zone management combined with human activities is increasingly stimulated by international political agenda through dedicated prevention, remediation, and restoration of contaminated sites at the regional, national, and EU levels. A systemic and harmonized implementation action is, however, still missing; to bridge this gap, we propose a cross-cutting approach, based on a comprehensive bioeconomy framework. Coordinated cross-sectorial actions that include wide stakeholder participation could offer, through a bioeconomy approach, a new vision for an inclusive and sustainable growth. This will require the development of business models for the management of contaminated areas based on cutting-edge research and nature-based solutions that are developed in a strategic policy framework able to recognize the economic value of the reintegration of contaminated sites in a frame of regional development. The bioeconomy offers multiple benefits and frameworks for process implementation not only at regional scale but also up to European level. Positive feedback is expected by this approach, by promoting regenerative economies,

with paramount benefits for the environment and human health. This would also make a step forward in the integration and alignment of policies by building favorable conditions based on the framework of the smart specialization strategy.

AUTHOR CONTRIBUTIONS

FF designed the structure and wrote the manuscript. FT checked the manuscript, proposed the comments, and revised the manuscript. MS checked and revised the manuscript. MZ and AB revised the manuscript. All the authors contributed to the article and approved the submitted version.

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