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ECOTOXICOLOGICAL EFFECTS OF CIGARETTE BUTTS IN MARINE ORGANISMS

Academic Tutor:
Prof. Stefania Gorbi

PhD Candidate:
Dr. Giulia Lucia

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Contents

1. Introduction

1.1. Cigarette consumption and global threatening waste: cigarette butts

Cigarette consumption is a global phenomenon related to the characteristics of cigarettes and tobacco in general, including the relative low price and rapid human addiction to nicotine (Ghasemi et al., 2022). According to the Tobacco Atlas, a project of the American Cancer Society and the Vital Strategies, despite cigarette consumption has declined from 22.7% in 2007 to 19.6% in 2019, the total number of smokers remains high due to population growth. Nowadays 1.3 billion people use some tobacco products daily and 1.1 billion of them consume cigarettes (https://tobaccoatlas.org). 5.5 trillion filtered cigarettes are produced annually, and their consumption varies among countries and continents: China is the greatest producer and consumer with 2.1 trillion cigarettes smoked in 2020 and an average consumption of 1970 cigarettes per capita in 2019, compared to 0.9 trillion cigarettes smoked in European Union with Belarus, Czech Republic and Bulgaria in which more than 2000 cigarettes per smoker were consumed in 2019 (Fig. 1.1 and 1.2). In some countries, this habit has been decreasing during the last decade due to more awareness and sensibilization campaigns on the health risk related to smoke. In fact, more than 8 million deaths were correlated to cigarette consumption and were estimated nearly US $ 2 trillion of economic damages (WHO, 2021).

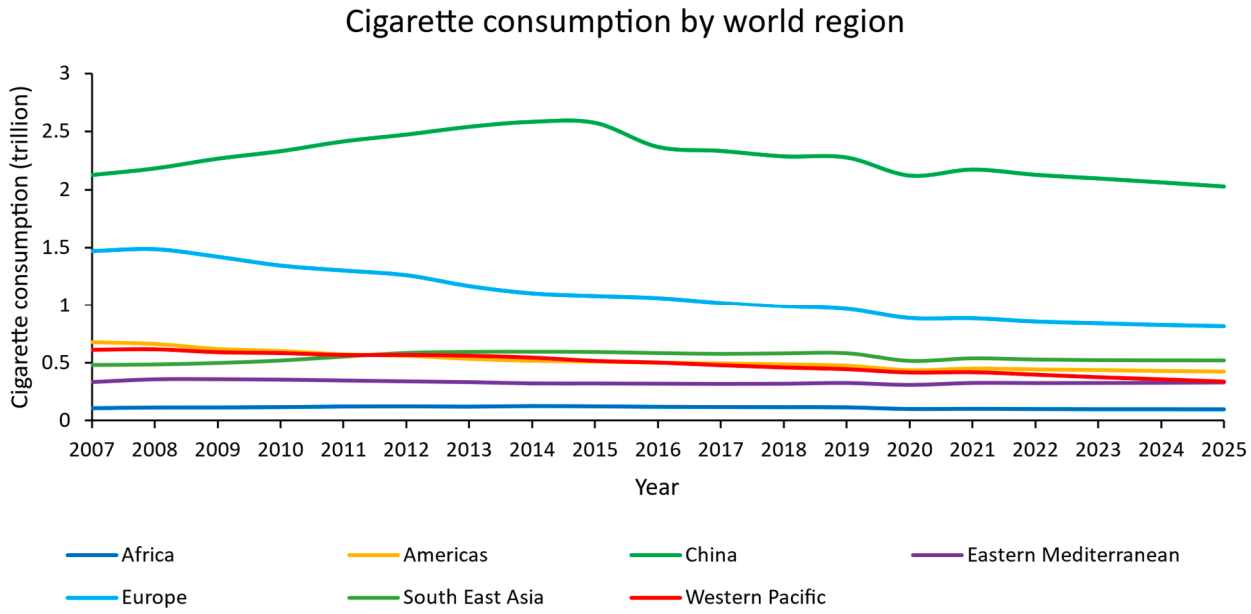

Figure 1.1. *Number of cigarettes consumed by world regions from 2007 to 2025 (2021-2025 are projections). Author elaboration from Euromonitor and WHO, 2021.*

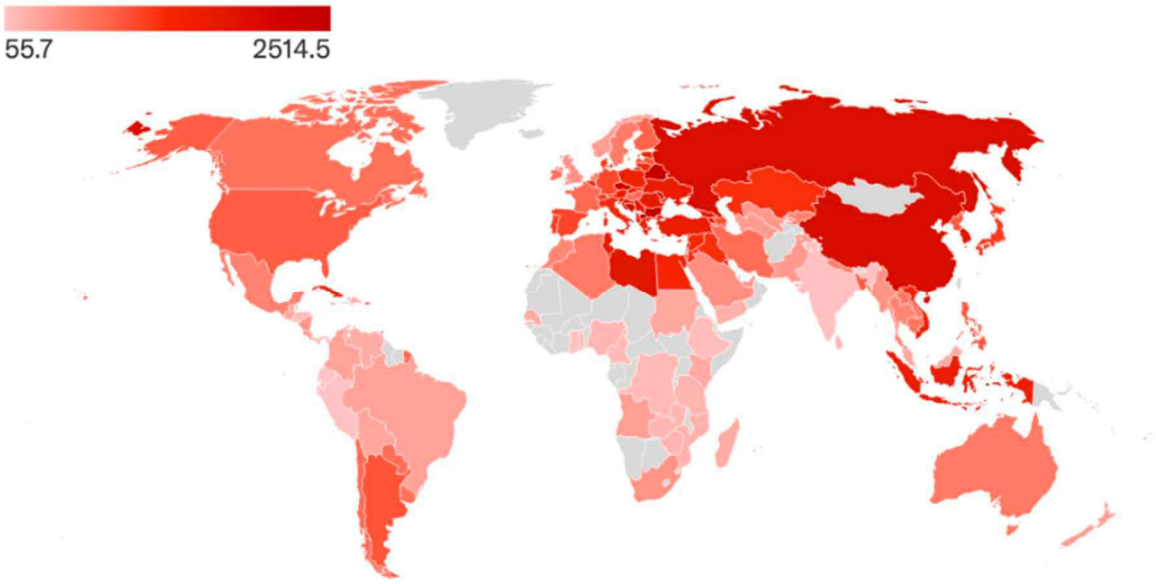

Figure 1.2. *Average number of cigarettes consumed in one year per adult (people with more than 15 years). Data are referred to 2019, source Euromonitor, Tobacco Atlas and WHO, 2021.*

World Health Organization affirmed: "Tobacco threatens many of the Earth's resources" (WHO, 2017). Every year the tobacco industry consumes a huge amount of world natural resources: 22 billion tonnes of water are used for tobacco production, 200000 hectares of land are employed in tobacco agriculture and 600 million trees are cut down for tobacco growing (WHO, 2022). Moreover, tobacco-related waste contributes to the depletion of environmental health: 2 million tonnes of packaging are wasted, more than 80 million tonnes of CO_2 are released contributing to global warming and 4.5 trillion cigarette butts (CBs) are thrown into the environment every year (dos Santos et al., 2017; Zafeiridou et al., 2018; Araújo & Costa, 2019; WHO, 2022), with a total weight of 1.2 million tons of CBs per year present in the environment. Researchers estimated that this quantity would increase of 50% by 2025 considering different parameters such as population growth (Torkashvand et al., 2020). Roder Green et al. (2014) revealed that CBs were the most common form of personal litter found in urban areas and represent 22-46% of the visible wastes in the streets, urban roads, public places and beaches where, according to the Ocean Conservancy report (2020), more than 4 million CBs were daily collected during cleaning activities in more than 100 countries. Their distribution and concentrations aren't also the same in urban areas and depend on population density and the availability of cigarette sales (Araújo & Costa, 2019). Moreover, this litter is more abundant near locations of sales and consumption or in proximity of bars, convenience and liquor stores, gas stations, supermarkets, restaurants, bus stops and train stations (Marah & Novotny, 2011; Roder Green et al., 2014) or in parks and beaches (Moerman & Potts, 2011; Chevalier et al., 2018; Dobaradaran et al., 2018; Drope et al., 2018). Considering this global situation many

researchers have been studying the environmental problem but most of them mentioned only the number of CBs (Torkashvand et al., 2020) and didn't provide a criterion for ranking the degree of environmental pollution (Ghasemi et al., 2022). Torkashvand et al. (2021), developed a cigarette butt pollution index (CBPI), which identified six statuses of environmental pollution ranking from 1 to 10. This index was defined by CBs density, weather conditions, and urban characteristics which included the structure of the walkways and distance from groundwater. The work proposed the city of Qazvin in Iran as an example of CBPI using: 20.5% of the city presents very low pollution, 7.7% low pollution, 53.8% pollution, 10.3% significant pollution and 5.1% sever pollution status.

There are different reasons why CBs are so abundant in the environment. The first is that smokers dispose of CBs improperly or throw CBs on the ground, not recognizing this act as a pollution source. They justify it as a natural and acceptable continuation of smoking and believe that this waste is less impactful than other litter (such as plastic items) because most smokers think that CBs are biodegradable (Araújo & Costa, 2019). The lack of waste management facilities and equipment can also contribute, but in a small extent: as demonstrated by Patel et al. (2013), despite the number of trash bins or ashtrays are sufficient and are arranged at appropriate distances from each other, CBs are present in the urban environment anyway. Public cleaning services can also determine the degree of CBs pollution: poor practices may cause the permanence of this waste in the environment and in any case, regular cleanings by municipal services and episodic events of volunteers cleaning up activities are inefficient in total removal of CBs due to their small size (Araújo & Costa, 2019). The European List of Waste (EC, 2000; EU, 2014) considers CBs as "Municipal waste including separately collected fraction/Separately collected fraction/Other fractions not otherwise specified" (Rebischung et al., 2018). Nowadays most countries collect CBs and bring them, with other garbage, to landfills or incinerators, where continue to pollute due to toxic fumes generated by the incineration process (WHO, 2022; Conradi & Sánchez-Moyano, 2022) or to substances leached by CBs in the groundwater (Masoner et al., 2015; WHO, 2022). Fortunately, some researchers have been trying to find sustainable recycling methodologies which could recover efficiently the materials from CBs, reducing the dispersion of this waste into the environment (Kurmus & Mohajerani,2020; Marinello et al., 2020; Moroz et al., 2021; Yousefi et al., 2021; Ghasemi et al., 2022). Finally, anti-smoking laws are diffused in some countries for indoor environments like restaurants, urban transport stations, stadiums, public offices and buildings for preventing human health, but, as well as smoking prevention laws, a ban for CBs littering is necessary for protecting also environmental health (Ghasemi et al., 2022). The Single-Use Plastic Directive (Directive (EU) 2019/904) was a step

ahead this aim, in fact this directive, entered into force in July 2021, including cigarette butts and other filtered tobacco products in the category of products made with plastic, which are used only one-time. The directive obligates producers to cover the costs of clean-up activities related to this waste. Finally, proper public education and sensitizing campaign about the risk for the environment related to CBs are necessary to increase citizen awareness of the environmental risk of CBs and change the mentality of smokers with wrong habits and incorrect behaviour of littering (Conradi & Sánchez-Moyano, 2022).

1.2. Environmental impact of cigarette butts

The environmental impact of cigarette butts is related to their composition and chemical substances they contain. CBs are made of three main parts: the filter, wrapping paper and unsmoked tobacco and ash (Kurmus & Mohajerani, 2020; Marinello et al., 2020).

Since the 1950s, cigarettes were produced adding a cellulose acetate filter, introduced to capture and retain some toxic chemicals contained in smoke, tar and fine particles preventing their inhalation by smokers during the combustion of tobacco. Even though a recent study demonstrated that the effectiveness of filters was dubious, and they were not considered a health-protective device (Evans-Reeves et al., 2020), most cigarettes and other tobacco products continue to be manufactured with cellulose acetate (CA) filters (Green et al., 2022). A standard filter is made by 15000 microfibers of a typical Y-shape, with a diameter of about 20 to 50 µm and 20 mm of length, treated with titanium dioxide (a delustrant) and bound together with glycerol triacetate (a plasticizer) (Slaughter et al., 2011; Bao, 2015; Marinello et al., 2020). CA is an artificial polymer made of cellulose through a process of two steps: the esterification between acetic acid and cellulose and the partial hydrolysis of the resulting ester groups (Bao et al., 2015). Due to its physic and chemical characteristics, this material is largely used for also producing textiles, plastics and films (Puls et al., 2011). CA is a degradable-resistant material, which determines the persistence of CBs in the environment (Kurmus & Mohajerani 2020). Different studies estimated and evaluated the deterioration time-dependent to degradation processes, such as photodegradation, biodegradation and mechanical degradation or a combination of them which are related to the typology of the environment. Kadir & Sarani (2015) evaluated from 1 to 2 months under anaerobic conditions, from 6 to 9 months in soil, 12 months in fresh water and 36 months in sea water, desert and underground stations. Joly & Coulis (2018) estimated 7.5 and 14 years to disappear in compost and on soil surface,

respectively. Bonanomi et al. (2020) demonstrated a slow degradation process through an experiment of 5 years of CBs decomposition in an organic substrate, showing a reduction of 66% of their initial mass after this time. Niaounakis (2017) estimated from 1 to 30 years in the marine environment in relation to the weather, temperature and light conditions. Moreover, during this time of complete degradation, CBs continue realising 100 microfibers of 200 µm length daily in the aquatic environment (Belzagui et al., 2021) and nanoparticles (Chevalier et al., 2018). Entire CBs filter or their micro/nanofibers can be ingested by organisms and determine some biological effects, like immobilization of crustacean *Daphnia magna* caused by microfibers (Belzagui et al., 2021). Moreover, they go on dispersing some CBs chemicals and/or adsorb toxins present in the environment acting as a vector of pollutants (Chevalier et al., 2018; Acosta-Coley et al., 2019; Santos-Echeandía et al., 2020).

Wrapping paper is another part of the CB that covers the cigarette filter. It contains ventilation holes that, during smoking, create an airflow in the filter, mixing fresh air with the smoke and allowing the dilution of inhaled toxic chemicals. When CBs are discarded into the environment, the degradation of this paper takes place from 1 to 5 months depending on external conditions (Bonanomi et al., 2015, 2020; Kurmus & Mohajerani, 2020).

In addition to the persistence of CBs in the environment due to the slow degradation of CA filters, the presence of chemicals trapped in filters, tobacco and ash is the second aspect which classifies CBs as an environmental hazard. During smoking some components of cigarettes are converted to the gas phase, pass through cigarette filters, are inhaled or finished in the air; other compounds are in the particulate phase, which is the matter captured in the filter (Kurmus & Mohajerani, 2020). More than 7000 chemicals are contained in cigarette smoke, such as nitrogen, oxygen, carbon dioxide, carbon monoxide, water, argon, hydrogen, acetone, nitrogen oxides and volatile sulfuric compounds (Hoffmann & Hoffman, 1997). In tobacco there are thousands of substances that react to form other compounds, which can be trapped in the filter, remain in unsmoked tobacco or are present in ash; hundreds of them are toxic and about 70 were classified as carcinogens (Torkashvand et al. 2020). Some of these compounds include nicotine and cotinine (Roder Green et al., 2014; Rebischung et al., 2018; King et al., 2021; Venugopal et al., 2021), polycyclic aromatic hydrocarbons (Dobaradaran et al., 2020; Venugopal et al., 2021), heavy metals (Moriwaki et al., 2009; Moerman & Potts, 2011; Hernandez, 2018; Venugopal et al., 2021), pesticides, herbicides, insecticides, fungicides (Dane et al., 2006; Slaughter et al., 2011; Dieng et al., 2013) and other organic compounds like aldehydes, alcohols and phenol (Venugopal et al., 2021), benzene, toluene, ethylbenzene and

xylene (BTEX, Dobaradaran et al., 2021), additives, flavours and humectants (Baker et al., 2004; Venugopal et al., 2021). These contaminants can be transferred from CBs to the environment when CBs enter in contact with water derived from external humidity, precipitations, infiltration to soil and sand layers, finish in street manholes, or are thrown directly into aquatic environments such as seas, rivers and lakes (Ghasemi et al., 2022).

Nicotine and cotinine are alkaloids associated specifically with tobacco and/or its metabolism. The mean concentration of nicotine in tobacco is in the range of 1080–16800 μg/g of cigarette and 1018.60 μg/g of CBs (Roder Green et al., 2014; Moriwaki et al., 2009; Venugopal et al., 2021; King et al., 2021), while cotinine has a mean concentration of 32.94 μg/g of CBs (King et al., 2021). Nicotine is eluted from CBs in water readily and after only one day can contaminate 1000 L of water with a concentration of 2.5 mg/L (Roder Green et al., 2014) and after 5 days is accumulated in the sediment at a concentration of about 2.5 μg/g sediment per CB, where it persists for more than 60 days reducing its concentration along time (King et al., 2021). Cotinine, an alkaloid found in tobacco leaves and the main metabolite of nicotine, is also present in large concentrations in the environment: a study on the wastewater of Milan revealed about 500 g/day derived from human urine (Castiglioni et al., 2014) and a maximum concentration of 30400 ng/L of this compound in a study of 22 landfills leachate in the USA (Masoner et al., 2016). Cotinine is also accumulated in marine sediments but in less concentration than nicotine (King et al., 2021). Nicotine is a very toxic compound because alters several biological functions from gene expression, regulation of hormone secretion, and enzyme activities especially related to antioxidant responses and neural signalling (Yildiz, 2004; Novotny et al., 2011; Wright et al., 2015; Nathiga Nambi et al., 2017; Oropesa et al., 2017). This alkaloid is easily absorbed through the oral cavity, skin, lung, urinary bladder, and gastrointestinal tract and its rate absorption, through the biological membranes, is positive correlated to pH (Schievelbein, 1982). Organisms can absorb nicotine, cotinine or other secondary metabolites either directly eluted by CBs in the environment or by urban wastewater, in which nicotine but above all cotinine are present due to their release by human urine (Yildiz, 2004).

Polycyclic aromatic hydrocarbons (PAHs) are present in CBs due to incomplete combustion and pyrolysis of organic materials in cigarettes (Zhang et al., 2016). They are released by CBs in water in the long term, reaching an estimated amount of 4.96 tons of total PAHs dispersed in the environment annually with 4.2 tons of naphthalene and 0.88 tons of acenaphthene (Dobaradaran et al., 2019). Moriwaki et al. (2009) reported that the total amount of PAHs analysed in a CB was 0.39 μg/g of wet CB with a range of concentration of each PAH from 0.0065 μg/g of

dibenzo[a,h]anthracene to 0.091 μg/g of and pyrene. It was also estimated an average potential emission of 0.032 mg/km/month of these compounds in a roadway. PAHs of low molecular weight (e.g. naphthalene, acenaphthylene, acenaphthene and fluorene) have high volatility, are more water soluble and less lipophilic than the high molecular weight PAHs (e.g. benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, 7H-dibenzo[c,g]carbazole, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, indeno[1,2,3-cd]pyrene, benzo[k]fluoranthene, dibenzo[a,e]pyrene, dibenzo[a,l]pyrene and 5-methyl chrysene). So the first group can be released easily from CBs into the environment while the second one can remain in CBs for a long time (Dobaradaran et al., 2019). The toxicity is dependent on the typology of PAHs: PAHs with low molecular weight generate acute toxicity, while high molecular weight PAHs are considered genotoxic. In any case, both are considered as bioavailable compounds for organisms which can be accumulated in their tissues and cause adverse health effects, such as decreasing in immune responses, breathing problems, mutagenicity, teratogenicity and carcinogenicity (Perugini et al., 2007; Moorthy et al., 2015).

Metals are presented in CBs due to the cultivation of tobacco which easily accumulate these elements from the soil. The application of fertilizers, pesticides, insecticides and herbicides also contributes to the availability of metals for tobacco leaves (Frank et., 1987). Farzadkia et al. (2022) estimated 58.37 to 147.56 kg of metals released from CBs in the Iranian environment in only one year, considering the annual consumption of 165 billion cigarettes. Other previous studies investigated the typology, the amount and leaching behaviour of metals from CBs: Moerman and Potts in 2011 evaluated the concentrations of metals eluted from CBs during 34 days, founding that Ba, Fe, Mn, and Sr increased their leached concentration over time; Ti, Pb, Zn, and Ni concentrations remained constant; Cu, Al, Cr, and Cd were eluted readily during first days and then their elution decreased. In the study of Moriwaki et al. (2009), the potential leak of heavy metals from CBs in a roadway was estimated in 0.02-1.7 mg/km/month, with Cd, Cu, Pb, Cr and As reported to be 0.02, 1.7, 0.59, 0.15 and 0.81 mg/km/month, respectively. Dobaradaran et al. (2017 and 2018) revealed that CBs released Cd, Fe, As, Ni, Cu, Zn, Mn, Hg and Pb in marine environment and coastline areas with different concentrations after 1 and 10 days: 0.16 and 0.67 μg/g CBs for Cd, 79.01 and 244.97 μg/g CBs for Fe, 0.12 and 0.48 μg/g CBs for As, 1.13 and 3.27 μg/g CBs for Ni, 4.29 and 12.29 μg/g CBs for Cu, 6.39 and 21.17 μg/g CBs for Zn, 38.29 and 123.1 μg/g CBs for Mn, 0.0495 and 0.0331 μg/g CBs for Hg and 4.79 and 3.63 μg/g CBs for Pb. Metals and especially heavy metals (Pb, Ni, Cd, Cr, As and Hg), once in the environment, are highly persistent because are not degraded by

microorganisms and can be accumulated in soil, sediments and organisms, causing some toxic effects related to oxidative stress, stimulating the production of reactive oxygen species (Woo et al., 2009). Moreover, can affect embryonic development, organisms' behaviour, and are potentially teratogenic and lethal at high concentrations (Lee & Lee, 2015; Parker & Rayburn, 2017). Considering the high quantity of metals released by CBs into the environment annually, they can cause acute and chronic toxicity to terrestrial and aquatic species for their biomagnification along food webs (Soleimani et al., 2022).

Other toxic compounds released by CBs are pesticides, herbicides, insecticides and fungicides which are largely employed in the cultivation and production process of tobacco (Mavrikou et al., 2008; Dieng et al., 2013; Sacchetto, 2012). From 50 to 100 different compounds, belonging to different chemical groups (e.g. organophosphates, carbamates, organochlorine and heterocyclic pesticides, nitro compounds, pyrethroids and amides) are used regularly, such as alachlor, clomazone, metolachlor, napropamide, pebulate, pendimethalin, sethoxydim, sulfentrazone, imidacloprid, chlorpyrifos, 1,3-dichloropropen, aldicarb, and methyl bromide (McDaniel et al., 2005; Bailey, 2013; Mavrikou et al., 2008; Dieng et al., 2013; Sacchetto, 2012). Few studies evaluated the concentrations of these chemicals in cigarette tobacco or smoke (Dane et al., 2006; Asubiojo et al., 2009; López-Dávila et al., 2020). These compounds can determine respiratory, neurological, genotoxic and carcinogenic damages, acting as inhibitors of acetylcholinesterase activity, the fundamental enzyme for nerve impulse transmission (Mavrikou et al., 2008), as endocrine disruptors, producing effects on development, reproduction and hormonal system (Dane et al., 2006), and as tumour promoters (Dich et al., 1997). Finally, they contribute to growing the number of metals dispersed in the environment (Araújo & Costa, 2019), with their biological consequences described before.

Other organic compounds like aldehydes, phenol, alcohols (Venugopal et al., 2021) and BTEX (Dobaradaran et al., 2021) are produced during the combustion and, even though their volatility, are still present in CBs. Additives, humectants and flavours are added to the tobacco for its preservation and to make cigarette smoke more palatable and appealing for smokers (Venugopal et al., 2021). Venugopal et al. (2021) measured 7.2, 17, 51, 15000, 430, 420, and 6700 µg/g of CBs of acetaldehyde, formaldehyde, phenol, glycerol, ethylene glycol, menthol and propylene glycol, respectively. Dobaradaran et al (2021) showed a concentration of 0.17, 0.9. 0.25, 0.19 and 0.38 µg/L of benzene, toluene, ethylbenzene, o-xylene, and p-xylene after 1 day of CBs exposed to 10 mL of deionized water. The biological effects of these compounds are different: from cytotoxicity and mutagenicity of aldehydes and alcohols (O' Brien et al., 2008) to protein denaturation of phenol

(Meena et al., 2015), while not particular toxic effects are observed for additives, humectants and flavours (Baker et al., 2004), although their possible contribution in increasing the release of nicotine by CBs (Parker & Rayburn, 2017). Moreover, cancer induction, neurological effects and organs damages are observed for BTEX compounds (WHO, 2010; Akinsanya et al., 2020).

1.3. Effects of cigarette butts and associated contaminants on marine organisms

Cigarette butts are one of the most abundant marine litter observed during beach cleaning activities around the world (Ocean Conservancy, 2020). CBs on beaches depend on many factors, such as incorrect behaviour and littering, lack of environmental awareness, frequency of beachgoers, low efficiency of cleaning services, influence of winds, currents and rivers transportation (Araújo & Costa, 2019). The presence of this waste was monitored and documented by several studies on different beaches around the world: an average of 0.2 CBs/m^2 was found in nine beaches of Cyprus in 2 years of sampling (Loizidou et al., 2018), 0.14 CBs/m^2 was the mean concentration detected in 5 beaches along the north-western coast of Adriatic Sea (Munari et al., 2016), while 2 CBs/m^2 was the largest number of cigarette filters collected in one of six beaches along the Slovenian coast (Laglbauer et al., 2014). From 0 to 0.54 butts/m^2 was found in 11 beaches in Lithuania and from 0 to 29 CBs/m^2 in 18 German beaches, in 7 years of the study (Kataržytė et al., 2020), from 2 to 38 CBs/m^2 in nine stations along the northern part of the Persian Gulf (Dobaradarn et al., 2018) and an average of 2.26 CBs/m^2 in two of Thailand's most popular public beaches (Kungskulniti et al., 2018). Considering the total amount of CBs thrown into the environment annually, CBs and associated contaminants that are present in the marine environment, directly left in sand or derived from urban places due to weather conditions or through wastewater, represent a hazard for marine organisms from coastal areas to the bottom of the sea (Asensio-Montesinos et al., 2021). Some studies evaluated the effects of CBs and related contaminants on marine wildlife such as bioluminescent reduction in *Aliivibrio fischeri* (Micevska et al., 2006; Bonanomi et al., 2020; Oliva et al., 2021; Piccardo et al., 2021), changes in microbial community of sediments (Quéméneur et al., 2020), growth inhibition of microalgae (*Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, Oliva et al., 2021; *Phaeodactylum tricornutum* Piccardo et al., 2021; microphytobenthos, Senga Green et al., 2021). Physiology alteration and cellular death was observed in benthic foraminifers *Rosalina globularis*, *Quinqueloculina spp* and *Textularia agglutinans* (Caridi et al., 2020), growth rate of the macroalga *Ulva lactuca* was reduced (Senga Green et al., 2021), behavioural modifications and

mortality were documented in tidepool snails *Austrocochlea porcata*, *Nerita atramentosa* and *Bembicium nanum* (Booth et al., 2015). Senga Green et al. (2021) showed a reduction of clearance rate of mussels *Mytilus edulis*; Santos-Echeandía et al. (2021) reported metal bioaccumulation in gills and digestive gland of oysters *Crassostrea gigas*. Toxicity in crustaceans with different endpoint such as malformations in nauplii of *Artemia sp* (de Souza Abessa et al., 2021), immobilization of *Acartia tonsa* (Oliva et al., 2021) and fecundity in *Nitokra sp* (Lima et al., 2021) were analysed. Different biological effects were also observed in polychaetes: *Hediste diversicolor* showed inhibition of burrowing behaviour, reduction of growth rates and increase of DNA damage (Wright et al., 2015); *Ficopomatus enigmaticus* presented effects on larval development (Oliva et al., 2021). Sea urchins presented polyspermy (Limatola et al., 2020) and reduction of adult fertilization success and larval development (Piccardo et al., 2021). Finally, mortality in fishes *Atherinops affinis* (Slaughter et al., 2011) and ingestion and contamination in sea birds, turtles and marine mammals (Wilcox et al., 2016) were documented. Most of these studies evaluated the toxicity of CB leachates (i.e. solutions containing substances eluted from CBs maintained in seawater for a certain time) while only Senga Green et al. (2021) exposed organisms to entire CBs. All the biological effects were evaluated in laboratory conditions except for Wilcox et al. (2016) who show CBs ingestion by wild sea birds, turtles and marine mammals. Considering the works, presented in literature, the concentrations of CBs in water were heterogeneous and this discrepancy is mainly due to the lack of information about the real abundance of CBs in the marine environment. Depending on the principal aim of the research, CBs concentrations are ranging between 0.25 to 400 CBs/L for studies on biological effects and/or chemical release of CBs (Micevska et al., 2006; Slaughter et al., 2011; Wright et al., 2015; Parker & Rayburn, 2017; Chevalier et al., 2018; Xu et al., 2019; Santos-Echeandía et al., 2021). The toxicity was evaluated in different CBs typologies: entire CBs (Slaughter et al., 2011) or only the filters (Wright et al., 2015; Quéméneur et al., 2020); collected from public ashtrays (Caridi et al., 2020; de Souza Abessa et al., 2021) or artificially smoked (Lima et al.,2021; Piccardo et al., 2021; Senga Green et al., 2021). A further problem was related to the absence of a standard protocol for CBs leachate preparation: different concentrations of CBs per litre of solvent, different soaking times and filtration with different mesh sizes are reported. So, a huge diversity of types and concentrations of eluted chemicals and the consequent exerted biological effects were described, which in most cases were difficult to compare. Finally, few studies evaluated the ecological impacts of CBs on a realistic scale and in species naturally living in marine environments (i.e. surface, water column and bottom, Quéméneur et al., 2020; Senga Green et al., 2021). The permanence of CBs in

different marine environments is fundamental for understanding the elution and kinetics of their pollutants and toxicity in marine organisms. CBs float on the surface and water column for a variable period (3-20 days) before becoming saturated with liquid and sinking into the sediment (Lima et al., 2021). This variability depends on the porosity of the wrapping paper and of the cellulose acetate filter (Hoffmann & Hoffmann, 1997). So, the consideration of all these aspects is fundamental to determine and assess the CBs toxicity in marine biota through an ecotoxicological approach.

2. Aims of the PhD study

The overall aim of this thesis was to increase knowledge about the impact of cigarette butts (CBs) in marine organisms through an ecotoxicological approach based on the integration of several Line of Evidence (LOE). Research activities were performed for detecting chemicals eluted from cigarette butts in seawater, their bioavailability and the molecular, cellular, and biochemical effects caused by these substances in some marine sentinel species. In addition, an investigation of natural photodegradation process was done to simulate one year of solar exposure of cellulose acetate microfibres in fresh and marine water.

This PhD project was realized by developing specific laboratory experiments, which are presented in the below three chapters.

The 1st chapter presents a multidisciplinary characterization of the toxicological risks of CBs on marine organisms integrating chemical analyses of released compounds with a wide panel of biological responses, such as ecotoxicological bioassays on species of different trophic positions, molecular responses in an *ex vivo* model (Precision-Cut Tissue Slices, PCTS of mussel's digestive glands), bioavailability and cellular biomarkers in mussels exposed to CBs. The results were then integrated into a quantitative Weight of Evidence model for estimating the environmental hazard level of CBs.

The 2nd chapter describes a study where realistic environmental CBs impact was simulated using three species of high ecological-commercial interest, two invertebrates, the mussel *Mytilus galloprovincialis* and the polychaete *Perinereis aibuhitensis* and a vertebrate the juvenile of seabass *Dicentrarchus labrax*. This experiment was performed for evaluating multispecies cellular responses to CBs associated contaminants, either in exposures with singular species or in a mesocosm approach, for investigating if interactions between species could modify biological responses to CBs.

The 3rd chapter is a study performed for investigating the photodegradation effects of virgin cellulose acetate microfibres of CBs filter in fresh and seawater. The novelty of this experiment was the use of the entire solar spectrum, generated by a solar simulator, in realistic aquatic conditions and performing analyses of samples through a Small-Angle X-ray Scattering technique, usually employed to study the structure of materials dispersed in solution with nanometric size, and finally, the evaluation of degradation process creating a physics model of microfibres.

3. Integrated approach for evaluating biological effects of cigarette butts in marine sentinel species: chemicals analyses, ecotoxicological bioassays, *ex vivo* and *in vivo* experiments

This chapter is based on the following paper:

Lucia G., Giuliani M. E., d'Errico G., Booms E., Benedetti M., Di Carlo M., Fattorini D., Gorbi S., Regoli F. (2023). Toxicological effects of cigarette butts for marine organisms, *Environment International*, 171, 2023, 107733. https://doi.org/10.1016/j.envint.2023.107733

3.1. Introduction

Studies on the biological responses and behavioural changes in marine organisms due to cigarette butts (CBs) are still present but few of them have tried to associate the typology and concentration of chemicals eluted from CBs with these effects (Araújo & Costa, 2019; Torkashvand et al 2020; Moroz et al., 2021). In this chapter multidisciplinary studies integrating chemical analyses and the onset of adverse biological effects are present to determine a more realistic hazard and risk assessment of this environmental contaminant. In this respect analyses of chemical substances released from CBs, their bioavailability and toxicological effects considering different species and levels of biological organization, from molecular to cellular and organismic response, were evaluated.

Standardized ecotoxicological bioassays were chosen as useful tools to assess the acute and chronic toxicity of pollutants and chemical mixtures: their practical importance is internationally recognized by several monitoring agencies and expert working groups (US-EPA, ICES and OSPAR), which recommend the use of a battery of at least three selected living species, belonging to different trophic levels. Bioassays are commonly applied to characterize environmental matrices and was recently included in Italian normative on dredged sediments (Morroni et al., 2020), due to multiple advantages such as short-time tests, cost-effectiveness and ease of operation. The importance of these bioassays was confirmed by several studies evaluating the toxicity of marine sediment and water (Prato et al., 2012; Manzo et al., 2014; Costa et al., 2016; Regoli et al., 2019; Maradonna et al., 2020; Morroni et al., 2020). In this study, the toxicity of CB leachates was assessed using a battery of assays composed of five species (*Aliivibrio fischeri*, *Phaeodactylum tricornutum*, *Skeletonema costatum*, *Dunaliella tertiolecta* and *Crassostrea gigas* embryos) from three different trophic levels (decomposer bacterium, primary producers and primary consumers). Bioluminescence inhibition bioassay performed with the Gram-negative bacterium *A. fischeri* was utilized largely in ecotoxicological investigations (Abbas et al., 2018). Marine algal growth inhibition tests are often

carried out for the same objective. The standardized method requires the usage of two recommended species of microalgae, the diatoms *P. tricornutum* and *S. costatum*, but the green algae *D. tertiolecta* is frequently used (ISO, 2016; Gallo et al., 2020). In this study, these three species were used because the sensitivity to single substances (Levy et al., 2007) and environmental samples such as water or sediment elutriates can considerably vary between microalgal species (Mucha et al., 2003; Gallo et al., 2020; Pelusi et al., 2020). Finally, *C. gigas* embryotoxicity bioassay was chosen because different studies have shown that some compounds create malformations during embryonal development. For example, this assay was utilized to assess the quality status of marine sediment (Libralato et al., 2008; Mamindy-Pajany et al., 2020; Maradonna et al., 2020; Morroni et al., 2020), emerging pollutants toxicity (Moreira et al., 2020; Bringer et al., 2021 a, b) and multiple stressors impacts (Gamain et al., 2016; Moreira et al., 2018).

Sublethal effects and cytotoxicity of chemicals released by CBs were also evaluated on an *ex vivo* model using Precision-Cut Tissue Slices (PCTS) obtained from the digestive glands of the Mediterranean mussel *Mytilus galloprovincialis* (Giuliani et al., 2019): molecular responses of key genes allowed to investigate the early activation of biotransformation pathways, metal detoxification response, peroxisomal proliferation and oxidative stress. PCTS are viable sections of tissue that can be maintained in a culture medium and used for acute laboratory exposures, allowing investigations of toxicological effects and mechanisms of action of pure chemical substances or environmental matrices (Giuliani et al., 2019). Cell viability and specific molecular responses of mussel PCTS, under chemical exposures, were previously assessed, suggesting their suitability for ecotoxicological applications (Giuliani et al., 2019). Moreover, from an ecotoxicological point of view, the use of PCTS may be considered a good compromise between the *in vivo* studies and the *in vitro* experiments with cultured cells, because PCTS could reduce the number of organisms used for ecotoxicological analyses and the quantity of exposure compounds for experiments. This determined advantages on ethical issues and laboratory costs like *in vitro* experiments but maintaining physiology and natural structure of organs preserving tissue heterogeneity, cell-cell and cell-matrix interactions, reflecting more closely the *in vivo* organization of the organ than *in vitro* studies (de Kanter et al., 2002; Cho & Yoon, 2017).

To further enrich our knowledge on the bioavailability and cellular toxicity of CB chemicals in aquatic organisms, an *in vivo* exposure was anyway carried out with *M. galloprovincialis*, a widely accepted bioindicator species to assess the presence and impact of environmental pollution. Bioaccumulation in tissues of exposed mussels was integrated with the assessment of a wide panel of biological

effects reflecting immunological responses, lipid metabolism, antioxidant defences, neurotoxic and genotoxic effects. The correlation between chemicals absorption from the environment and consequent biological responses in organisms is fundamental to investigate the toxicity of environmental contaminants on marine biota. *M. galloprovincialis* is a species that is largely used for evaluating this aspect because of its ecological and physiological characteristics (Rey-Salgueiro et al., 2017). Mussels are filter feeders at the base of the food chain so they could accumulate a wide range of contaminants reflecting the environmental status (Galloway et al., 2002). These organisms are also species of commercial interest, especially for Mediterranean Sea countries, so is important to know their health status to reduce potential risks, derived from contaminants accumulation, for humans' health. Moreover, biological effects and their correlated biomarkers analysed in mussels' organs and tissues are widely used to investigate environmental pollution (Curpan et al., 2022).

The results on ecotoxicological bioassays, bioavailability and biomarker responses were integrated through a Weight Of Evidence (WOE) approach, using the Sediqualsoft model, which applies weighted criteria to elaborate specific hazard indices for individual typologies of data (Lines Of Evidence, LOEs), before their overall integration into the final WOE risk index (Piva et al., 2011; Regoli et al., 2019; Morroni et al., 2020). In the Sediqualsoft model, different typologies of data are independently elaborated with specific criteria, which weight typology of chemical pollutants and toxicological relevance of measured endpoints, as well as the number and magnitude of observed variations normalized toward specific thresholds. Synthetic and quantitative hazard indices are calculated, before their overall integration in the WOE assessment, assigning the risk to 1 of 5 classes, from absent to severe (Piva et al., 2011; Regoli et al., 2019). In recent years this approach was validated in several case studies for environmental risk assessment associated with polluted sediments, harbour areas, off-shore platforms, marine incidents, management of complex industrial areas or marine installations, as well toward a better assessment of the impacts of specific classes of pollutants and their interactions with multiple stressors on the marine environment (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al. 2014; Bebianno et al., 2015; Mestre et al., 2017; Pittura et al., 2018; Lehtonen et al., 2019; Regoli et al., 2019; Morroni et al., 2020, Cacciatore et al., 2022; Nardi et al., 2022; Pittura et al., 2022). Sediqualsoft criteria for the weighted elaboration of chemical data and ecotoxicological bioassays have been incorporated in the last Italian law dredged marine sediments (DM 173/2016), providing the rationale for different management options associated with the class of environmental risk.

The integration of chemical analyses of compounds released from CBs, their bioavailability and their effects in different models and levels of biological organization was expected as a valuable approach for a more reliable assessment of the impact of these hazardous materials on marine species.

3.2. Materials & Methods

3.2.1. Preparation of cigarette butt leachates

Naturally smoked cigarette butts (CBs: intact filters, covering paper, unsmoked tobacco and ash) of different brands were collected from covered cigarette receptacles in the campus of the Polytechnic University of Marche (Ancona, Italy) within one week after they were smoked and stored at -20°C in plastic bags until they were used for CB leachates preparation and *in vivo* exposure with *M. galloprovincialis*. Despite the choice of collecting CBs from cigarette receptacles was taken to simulate the heterogeneity of real samples, the different compositions might cause a certain variability in exposure conditions, to be considered when interpreting results. Leachates were prepared in triplicate at two different concentrations, 100 CBs/L = LEAC_A (PERC_1, 2 and 3) and 25 CBs/L = LEAC_B (PERC_A, B and C). These concentrations were chosen following the suggestions from the limited available literature, which recommends a concentration ranging between 4 to 400 CBs/L for studies on chemical release and biological effects of CB leachates (Slaughter et al., 2011; Wright et al., 2015). CBs were soaked in artificial sea water (ASW, Instant Ocean® Sea Salt at 35 psu) at room temperature and maintained in agitation on a magnetic stirrer for 24 hours: the room temperature (18-20°C) is in the range of environmental seawater values measured in the Mediterranean from spring to autumn seasons (Figure 3.1). Obtained leachates were pre-filtered using a battery of sieves from 500 µm to 50 µm to remove remaining fractions of CBs, before a vacuum filtration through a 0.45 µm nylon filter to eliminate particulate matter, as it was done in most works presented in the literature. Cigarette-free seawater (blank) was prepared using the same protocol, including agitation for 24 hours at room temperature. Leachates and blank samples were stored at 4°C before the chemical analyses, ecotoxicological bioassays and *ex vivo* exposure.

Figure 3.1. *Cigarette butt leachates preparation: CBs soaked in ASW for 24 hours in agitation on a magnetic stirrer. These leachates are referred to the triplicates of LEAC_A (100 CBs/L) = PERC_1, 2 and 3.*

3.2.2. Chemical analyses in CB leachates

Concentrations of trace metals (Al, As, Cd, Cr, Cu, Hg, Fe, Mn, Ni, Pb, V and Zn), polycyclic aromatic hydrocarbons (PAHs), aliphatic hydrocarbons (AHs) C10-C40, polychlorinated biphenyls (PCBs), organo-halogenated pesticides (OCPs and OPPs), brominated flame retardants (BFRs), nicotine (NIC), cotinine (COT) and trans-3'-hydroxycotinine (3HC) were determined in the obtained leachates. Measurements were carried out through validated methods by gas-chromatography with flame ionization detector (FID) and mass detector (MS), high-performance liquid chromatography (HPLC) with diode array (DAD), fluorimetric detection and atomic absorption spectrophotometry (AAS), UHPLC-MS Triple quadrupole technique and HPLC-ORBITRAP. For all chemical analyses, quality assurance and quality control were monitored by processing blank and reference standard materials (National Institute of Standards and Technology, USA; EC–DG JRC Institute for Reference Materials and Measurements). The concentrations obtained from these reference standard materials were always within the 95% confidence intervals of the certified values.

Details on analytical methods and procedures are given in supplementary materials (SM1).

3.2.3. Ecotoxicological bioassays

Ecotoxicological effects of CB leachates (LEAC_A and LEAC_B) were tested through batteries of bioassays following standardized procedures. The toxicity of the CB leachates to *Aliivibrio fischeri* was determined using the bioluminescence test with *Aliivibrio fischeri* (UNI EN ISO 11348-3), following the basic test 81.9%, a liquid phase protocol. Each Microtox test consisted of five geometrically increasing toxicant concentrations (i.e. 5.12, 10.24, 20.43, 40.90 and 81.90% of CB leachates in triplicate). Luminescence readings were recorded after 5, 15, and 30 minutes of exposure using Microtox® luminometer. The results were elaborated by dedicated software Microtox OmniTM v. 1.16, to determine the dose-response relationship (relation between leachate concentration and inhibition of bioluminescence).

The toxicity of CB leachates on *Phaodactylum tricornutum*, *Dunaliella tertiolecta* and *Skeletonema costatum* was evaluated following the algal growth inhibition test (UNI EN ISO 10253). Each leachate was tested in 6 different concentrations: 100, 50, 25, 10, 5 and 1% of pure CB leachates, diluted in artificial seawater. Three replicates of leachates were tested, using sterile 12-wells culture plates, which were put in a thermostatic chamber at 20 ± 2°C for 72 hours of incubation (continuous light). After this time a solution of Lugol and ethanol was added to each well to fix and preserve microalgae. The algal growth of every replicate was determined under the optic microscope using a Bürker counting chamber. For each sample and blank, cellular growth was registered to determine cell concentration (cells/mL) and the percentage of growth inhibition was calculated by comparing microalgae concentrations of leachate samples to those present in the blank.

The sea oyster embryotoxicity assay with *Crassostrea gigas* was done following Leverett & Thain (2013). Eggs and sperm of female and male specimens were collected and then put together for a fertilization period of 2 hours in the dark. The suspension should be stirred at least every 10-15 minutes to prevent excessive settling of embryos and potential oxygen starvation. Then an aliquot of this suspension was collected to control, microscopically, if there was fertilization and embryo development (i.e. zygotes have reached the 16-32 cell stage). Every leachate was analysed in 8 concentrations: 100, 50, 25, 10, 5, 1, 0.5 and 0.1% of pure CBs solution, diluted in natural seawater (NSW) filtered at 0.45 µm. Three replicates of leachates were tested, using sterile 6-well culture plates which were put in a thermostatic chamber at 24 ± 2°C, in the dark for 24 hours to guarantee that zygotes could reach the larval D-shaped stage. After this time a solution of Lugol and ethanol was added to each well to fix and preserve larvae. Estimation of the percentage of normal and abnormal larvae was done by counting 100 larvae for every sample using an inverted microscope.

Larvae were considered malformed when presented extruded and granulated tissues, looking like "exploded" larvae as illustrated by Beiras & His (1995) and pre-D larvae stage, protruded mantle, indented shell as described by His et al. (1997). The toxic effect of samples was determined by calculating the percentage of malformed larvae.

For each bioassay, EC_{50} values (CBs/L) were calculated using Probit analysis with statistical R-software. Percentage values of bioluminescence reduction, growth inhibition, larvae malformation and EC_{50} values were expressed as mean ± standard error or standard deviation.

3.2.4. *Ex vivo* study

3.2.4.1. Exposure of PCTS from *M. galloprovincialis* digestive gland

Mussels (*M. galloprovincialis*) were obtained from a local farm (Ancona, Adriatic Sea) and acclimatized with ASW (2 individuals/L), at 18°C and 35 psu for at least 2 weeks, fed with a commercial mixture of zooplankton for filter-feeding organisms (Brightwell Zooplanktos-S, size range 50-300 µm). Precision-Cut Tissue Slices (PCTS) were obtained from the digestive gland of 6 mussels following the protocol described in Giuliani et al. (2019). Briefly, digestive glands were excised, cleaned and immediately placed in cold (4°C), sterilized ASW (supplemented with 1% penicillin/streptomycin antibiotic mix). After inclusion of the tissue in 2.5% agarose, slices of 400 µm thickness were cut using a motorized vibrating blade vibratome (VT1200S, Leica, Wetzlar, Germany). 27 PCTS were produced from each of 6 digestive glands and pools of 3 PCTS were homogeneously distributed in 12-well plates (3 PCTS/well). PCTS were pre-incubated for 1 h in 1 mL Leibovitz's L-15 medium (Lonza; adjusted to NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, supplemented with L-glutamine 2 mM and 1% penicillin/streptomycin mix).

After the pre-incubation phase, PCTS pools were exposed to the three replicates of LEAC_A (100 CBs/L), diluted to 50% in L-15 medium. PCTS incubated in L-15 medium only or in 50% ASW with L-15 were used as control (C) and blank control (CTRL), respectively. Incubations were carried out in 1 mL medium, at 18°C, under normal atmosphere and static conditions to avoid mechanical stress. The exposure was performed at different times (24, 48 and 72 h) (Figure 3.2), after which the PCTS were rinsed with sterile physiological solution (NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, pH 7.3, supplemented with D-glucose 10 mM and 1% penicillin/streptomycin mix), and either used for viability tests (PCTS pools from 3 individuals) or snap-frozen in liquid nitrogen and stored at −80°C until molecular analyses (PCTS pools from 3 individuals).

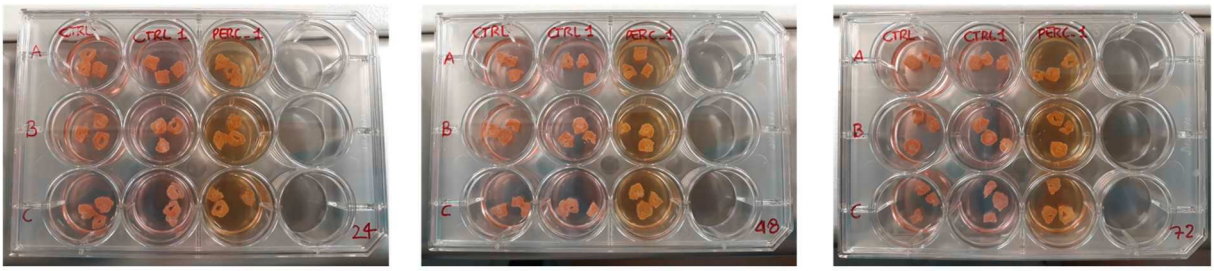

Figure 3.2. *Precision-Cut Tissue Slices of M. galloprovincialis digestive gland exposed to LEAC_A (100 CBs/L) for 24, 48 and 72 hours. Images referred to PERC_1 which is one replicate of LEAC_A.*

3.2.4.2. Viability tests

The viability of PCTS was estimated through the Alamar Blue assay, resazurin-based (TOX-8, Sigma-Aldrich). PCTS pools were incubated with resazurin solution (10% in physiological solution), in shaking conditions, for 2 hours at 18°C. The amount of resazurin reduced by oxidoreductases of viable cells was measured as a decrease in absorbance at 600 nm, with background subtraction at 690 nm, according to the manufacturer's instructions. Absorbance values for each PCTS pool were subtracted from the blank reading (i.e. the initial resazurin content) and expressed as net absorbance per g of wet weight (Giuliani et al., 2019). The data were expressed as viability percentage compared to C (mean ± standard deviation).

3.2.4.3. Molecular analyses: RNA extraction and mRNA levels

Total RNA was purified from PCTS pools using the Hybrid-R™ purification kit (GeneAll®), according to the manufacturer's instructions. Total RNA concentrations were measured using Nano-Drop ND-1000 Spectrophotometer. cDNA was synthesized from 1 µg of each RNA sample, using iScript cDNA Synthesis Kit (Bio-Rad). Absolute quantitative real-time PCR (qPCR) was performed for quantification of mRNA levels of the following target genes: catalase (*cat*), glutathione S-transferase pi-isoform (*gst-pi*), selenium-dependent glutathione peroxidase (*Se-gpx*), 70 kDa heat shock proteins (*hsp70*), Cu, Zn superoxide dismutase (*Cu/Zn-sod*), Acyl-CoA oxidase 1 (*acox1*), isoform 10 and 20 of metallothionein (*mt10* and *mt20*) and cytochrome P450 3A1 (*cyp3A1*). qPCRs were conducted through the SYBR green method in StepOnePlus® Real-Time PCR System (Applied Biosystems). Every qPCR reaction contained 7.5 µL of SYBR Select Master Mix (Life Technologies), 5 µL of total cDNA (diluted 1:5) and 200 nM of forward and reverse primers (Table SM2), in a final volume of 15 µL. The annealing temperatures for all genes are given in Table SM2. The absence of DNA contamination was checked by including negative controls lacking the cDNA template. For each

target gene, the standard curve was obtained by serial dilutions of known amounts of plasmid containing the amplicon of interest. Samples and standards were run in duplicate in the same run. A calibration curve was built by plotting cycle threshold (Ct) values of standards versus $\log_{10}$ copy numbers. Ct values of cDNA samples were converted into copy numbers by interpolating the calibration curve. Data were expressed as fold-change related to C and averaged (n=3).

3.2.5. *In vivo* exposure

3.2.5.1. Exposure of mussels, *M. galloprovincialis*, to cigarette butts

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length) were acclimatized as previously described and exposed to 3 different levels of CBs directly added into exposure tanks. A total of 240 mussels were divided into 4 tanks (each filled with 20 L of ASW) containing 0, 0.5, 1 or 5 CBs/L, respectively (Fig. 3.3). This number of mussels (60 organisms/tank) was chosen to have enough tissue for all the analyses. The exposure time was 14 days and water and CBs were changed every 72 hours: despite a certain variability of exposure conditions might be expected using different CBs, we consider this choice as a more realistic exposure scenario with greater environmental relevance. After changing water, mussels were fed with a commercial mixture of zooplankton for filter-feeding organisms (Brightwell Zooplanktos-S, size range 50-300 µm) and let 2 hours without CBs before their new addiction. At the end of the experiment, organisms were sampled: 3 pools constituted by whole tissues of 5-10 organisms (approximately 25 g) were obtained from each tank and stored at -20°C for bioaccumulation analyses. Gills and digestive glands were dissected and pooled in 5 replicates, each with tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80°C for biomarkers analyses. At the same time, aliquots of haemolymph, taken from adductor muscle, were used for *in vivo* analyses of haemocytes lysosomal membrane stability, granulocytes-hyalinocytes ratio and DNA damage; aliquots of haemolymph were also fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency, or rapidly frozen in liquid nitrogen and maintained at -80°C for acetylcholinesterase activity.

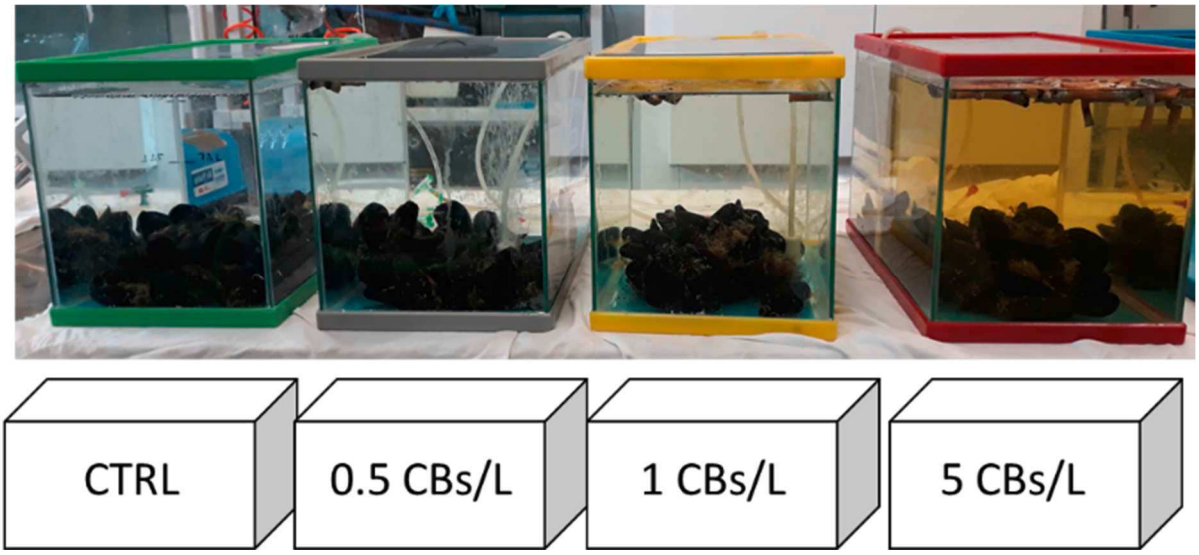

Figure 3.3. *Mytilus galloprovincialis exposed to 0.5, 1 and 5 CBs/L.*

3.2.5.2. Bioaccumulation

Trace metals, PAHs, NIC and COT were measured in the whole tissues of *M. galloprovincialis* following the same analytical methods used for CB leachates and detailed in the supplementary material (SM3).

3.2.5.3. Biomarkers analyses

Validated protocols were followed for the measurement of biomarkers in tissues of *M. galloprovincialis*. Lysosomal membrane stability (Neutral Red Retention Time, NRRT) and granulocytes/hyalinocytes ratio (G/H ratio) were evaluated in haemocytes for immunological responses, while loss of DNA integrity (COMET assay) and micronuclei frequency (MN) for genotoxic damage; acetylcholinesterase activity (AChE) was analysed as a marker for neurotoxic effects in haemolymph and gills. Biomarkers analysed in the digestive gland were metallothioneins (MT) as an inducible metal detoxification system, acyl-CoA oxidase (ACOX) for peroxisomal proliferation and malondialdehyde (MDA) as a typical lipid peroxidation product; oxidative stress biomarkers included catalase (CAT), glutathione S-transferases (GST), Se-dependent glutathione peroxidases (Se-dep. GPx), total GPx, glutathione reductase (GR), total glutathione (TGSH) as single antioxidant defences, integrated with the analysis of total oxyradical scavenging capacity (TOSC) toward peroxyl radical ROO• and hydroxyl radical HO•. The results obtained from replicates were expressed as mean and

standard deviations or errors. Details of analytical methods and procedures are given in supplementary materials (SM4).

3.2.6. Statistical analyses and Weight Of Evidence (WOE) integration

Analysis of variance (one-way ANOVA; levels of significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$) and post-hoc Student Newman Keuls Test were performed on chemical concentrations between blank, LEAC_A and LEAC_B. The same analysis was done for viability and gene expression analyses in exposed PCTS after evaluating normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. A non-metric multidimensional scaling (nMDS) was applied to bioassay results in relation to the chemical parameters revealed in leachates (LEAC_A and LEAC_B).

Analysis of variance (one-way ANOVA) was applied on *in vivo* exposure to determine the significant differences obtained for bioaccumulation and biomarker analyses between control and exposed mussels (levels of significance at $p < 0.001$, $p< 0.01$ and $p< 0.05$); homogeneity of variance was tested by Cochran C, and post-hoc Student-Newman-Keuls test was used for comparisons among these groups. Correlation analyses were performed to examine the relationships between CBs exposure levels, bioaccumulation and biomarkers responses in mussels, accepting correlation coefficients with P values of <0.05 as significant. All statistical analyses were performed using R-software.

To summarize in specific hazard levels the effects of CBs on ecotoxicological bioassays, bioaccumulation and cellular responses, the overall results were elaborated within a quantitative Weight Of Evidence (WOE) model, using the largely validated Sediqualsoft approach (Piva et al., 2011; Regoli et al., 2019; Morroni et al., 2020). This approach can integrate the results obtained from different types of investigations (or lines of evidence, LOEs), in the ecological risk assessment. This model contains specific algorithms and flowcharts that, based on precise objectives and expert judgement assumptions, allow to apply weighted integration criteria to large datasets of data obtained up to different LOEs. Specifically for this experiment, the LOEs integrated in this model were bioavailability of contaminants in bioindicator organisms (LOE-2), sublethal effects measured by biomarker batteries (LOE-3) and toxic effects measured by application of ecotoxicological assays (LOE-4). Each LOE has been developed within a single module able to provide for each type of data, both a quantitative hazard index (HQ, Hazard Quotient), and a synthetic judgment of the level of danger (divided into 5 classes from absent to severe). These calculation procedures have been further developed within a dedicated software that, despite the processing of complex data in

synthetic indices, nevertheless maintains information of significant importance and scientific value useful for any further study.

The elaboration of HQ for bioaccumulation (LOE-2) allows to establish the danger associated with the possible transfer of environmental contaminants to the biotic compartment. The bioavailability hazard (HQBA) for each parameter is calculated considering the variation in concentration with respect to the controls (RTR), adjusted once again according to the type of contaminant and the statistical significance of the differences (RTRw). The statistical significance of the change with respect to the controls is weighted by applying the coefficient Z, calculated as a function of the value obtained from the T test for data with inhomogeneous variance. The coefficient Z has a value equal to 1 (no reduction of the effect) when the sample is significantly different from the control ($p< 0.05$), while it decreases with decreasing significance, passing linearly from 1 to 0.5 when p grows from 0.05 to 0.06; for values of p greater than 0.06, the coefficient Z decreases rapidly in a nonlinear manner to 0.2 when p tends to 1. This correction progressively reduces the overall weight of a parameter whose average variation is not statistically significant but does not completely eliminate its contribution to the calculation of the HQBA. Depending on the magnitude of the variation, which also considers the natural variability of tissue concentrations of contaminants, the individual parameters analysed are attributed to one of 5 classes of effect (from absent to severe): from absent to slight if RTRw is < 2.6, moderate if 2.6 ≤RTRw< 6.5, major if 6.5 ≤RTRw< 13, and severe if RTR w≥13. The cumulative value of HQBA is then calculated without considering the parameters with RTRw< 1.3 (absent effect), averaging for those with RTRw between 1.3 and 2.6 (slight effect), and adding (Σ) all the RTRw> 2.6 (moderate, major and severe effects): depending on the % distribution of the parameters in the various effect classes, the model indicates the overall hazard class for bioavailability.

For the elaboration of HQ for biomarkers (LOE-3), each response has a weight (between 1 and 3) based on its toxicological relevance of the response and the level of knowledge on the mechanisms involved, and a specific threshold for changes of biological relevance that depend on biphasic responses and tissue responsiveness. To ensure a multi-biomarker approach, the model requires some minimum requirements in terms of number and importance of the biomarkers analysed. To calculate the cumulative hazard index for HQBM biomarkers, the variation measured for each biomarker is compared with the specific threshold and corrected both for the biological importance of the response, and for the statistical significance of the difference with respect to the control by applying the coefficient Z already described for the processing of bioavailability data. Each

biomarker is then assigned to an effect class (from absent to severe) depending on the extent of the variation with respect to the threshold: absent or slight if E< 1, moderate with E between 1 and 2, major if E is between 2 and 3, severe for biomarkers with E> 3. The results of all biomarkers are differently weighted in the calculation of the cumulative hazard HQBM, which does not consider the contribution of biomarkers with no or slight effect, calculates the average of the Ew for responses with moderate effect and adds (Σ) the Ew of responses with major or severe effect. The assignment of one of 5 severity classes derives from the percentage distribution of biomarker responses in the 5 effect classes. The model indicates the number of biomarkers within each of the 5 classes, the cumulative hazard value HQBM and the overall hazard class for biomarkers.

The elaboration of HQ for ecotoxicological bioassay results (LOE-4) considers important aspects and specific characteristics of the biological assays used in the battery, including the toxicological relevance of the measured biological response, the magnitude and statistical significance of the difference in effect between sample and control, the sensitivity of the species tested, the type of exposure (acute or chronic) and the matrix tested. In this sense, for each of the tests provided in the different types of batteries there is an effect "threshold" that represents the minimum variation considered biologically significant for each experimental condition, and of the "weights" that are attributed to each test according to the biological relevance of the measured end-point, the duration of exposure and the tested matrix (Morroni et al., 2020). After verification of the data, for each biological assay the effect (Ei) is calculated, as the percentage change of the endpoint measured and compensated by the Abbott correction with respect to the changes observed in the control. The effect Ei is corrected according to the statistical significance of the variation with respect to controls, applying the coefficient Z already described for the processing of bioavailability data. This correction progressively reduces the overall weight of a non-statistically significant test but does not completely eliminate its contribution to the battery. Each effect (Ei) multiplied by its coefficient Z, is compared with the specific "threshold" for that rate; the corrected effect (Eiw) thus obtained indicates how many times the change measured in a test exceeds that considered biologically relevant. Only for certain assays, when it is possible to obtain a possible hormetic effect, an Eiw value equal to 0 is assigned if the hormetic effect is < 40%, 1.25 if the hormetic effect is > 40% but < 100%, equal to 1.5 if the hormetic effect is > 100%. The overall hazard index of the battery of ecotoxicological tests (Hazard Quotient, HQBactery) is calculated as the sum of the weighted effects (Eiw) of the individual tests, further corrected according to the factor W2 which corresponds to the product of the weights assigned according to the biological relevance of the endpoint

considered, the ecological relevance of the tested matrix, the acute or chronic exposure of the organisms. For the assignment of the battery hazard level of ecotoxicological tests, the value obtained for the HQBactery index is normalized to a scale between 0 and 10, where 1 corresponds to the battery threshold value (i.e. the HQ value that would be obtained if all battery tests showed an effect equal to the respective threshold) and 10 corresponds to the maximum battery value (when all tests show 100% effect). Depending on the value of the standardized HQBactery, the level of ecotoxicological danger is attributed to a severity class (from absent to severe).

The HQs elaborated from individual typologies of data (LOEs) are normalized to a common scale and integrated within a WOE approach which assigns a different weight to each LOE. After normalization of hazard indices to a common scale the overall WOE elaboration leads to a risk characterization that provides both a quantitative value and a qualitative assignment at a level ranging from Absent to Severe (Piva et al., 2011). Further elaboration procedures are fully detailed in Regoli et al. (2019).

3.3. Results

3.3.1. Chemical analyses in CB leachates

Chemical analyses highlighted that CBs release chemical compounds after 24 h in ASW, with generally higher concentrations of trace metals, aliphatic hydrocarbons (AHs), polycyclic aromatic hydrocarbons (PAHs), nicotine and cotinine in LEAC_A compared to LEAC_B (Tables 3.1 and SM5). Considering metals, LEAC_A exhibited the highest concentrations for Zn and Mn (mean values of 720 and 711 µg/L, respectively) and the lowest for Cd (mean value of 0.718 µg/L). Similarly, in LEAC_B, the highest concentrations were measured for Fe and Mn (mean value of 259 and 210 µg/L, respectively) and the lowest for Cd (mean value of 0.244 µg/L). Hg and V were always below the detection limit (bdl) (Table 3.1).

Levels of AHs and PAHs were 848 µg/L and 0.515 µg/L in LEAC_A, while 3400 µg/L of AHs and 0.216 µg/L of PAHs were detected in LEAC_B (Table 3.1). Mean values of individual AHs and PAHs congeners are reported in supplementary materials (Table SM5).

Nicotine and cotinine revealed elevated concentrations, with mean values of 180000 and 43200 µg/L of nicotine, and 1500 and 402 µg/L of cotinine in LEAC_A and LEAC_B, respectively (Table 3.1). Trans-3'-hydroxycotinine was below the detection limit (bdl) (Table 3.1), as other organic compounds, like PCBs, OCPs, OPPs, and BFR (data not reported).

3.3.2. Ecotoxicological bioassays

The bioassay with *A. fischeri* revealed a mean EC_{50} value for bioluminescence reduction corresponding to 4.47 CBs/L with both LEAC_A and LEAC_B (Table 3.2). Similar EC_{50} when testing both LEAC_A and LEAC_B were obtained also for algal growth inhibition, resulting 12.4 and 8.05 CBs/L for *P. tricornutum*, 4.89 and 5.55 CBs/L for *S. costatum*, and 3.38 and 3.84 CBs/L for *D. tertiolecta*, respectively (Table 3.2). Conversely, embryotoxicity bioassay with *C. gigas* showed that leachate preparation differently affected larval development and survival with mean EC_{50} values of 0.28 CBs/L for LEAC_A and 2.54 CBs/L for LEAC_B (Table 3.2). Malformed larvae often presented extruded and granulated tissues or other types of malformations such as pre-D larvae stage, protruded mantle and indented shell.

Table 3.1. *Concentrations (µg/L) of metals, aliphatic hydrocarbons (AHs), polycyclic aromatic hydrocarbons (PAHs), nicotine (NIC), cotinine (COT) and trans-3'-hydroxycotinine (3HC) in blank (artificial seawater-ASW) and CB leachates (LEAC_A and LEAC_B). Values are expressed as means ± standard deviations (n=3). Asterisks (*) indicate statistically significant variations while letters indicate differences between groups (post-hoc Student Newman Keuls Test).*

Chemical compound	**BLANK_ASW**	**LEAC_A (100 CBs/L)**	**LEAC_B (25 CBs/L)**
Al *	14.3 ± 4.02 [a]	325 ± 10.4 [b]	155 ± 3.40 [c]
As *	2.98 ± 4.14 [a]	8.53 ± 2.61 [b]	2.24 ± 0.489 [a]
Cd *	< 0.1 [a]	0.718 ± 0.098 [b]	0.244 ± 0.020 [c]
Cr	3.11 ± 0.450	6.70 ± 1.20	52.7 ± 66.7
Cu *	<0.1 [a]	146 ± 17.8 [b]	67.2 ± 24.7 [c]
Hg	< 0.5	< 0.5	< 0.5
Fe *	29.8 ± 9.10 [a]	290 ± 79.5 [b]	259 ± 174 [b]
Mn *	4.31 ± 0.795 [a]	711 ± 62.1 [b]	210 ± 17.6 [c]
Ni	1.51 ± 0.541	22.4 ± 6.19	35.6 ± 36.8
Pb	1.74 ± 1.33	3.23 ± 0.452	9.66 ± 10.9
V	< 20	< 20	< 20
Zn *	117 ± 92.0 [a]	720 ± 183 [b]	87.6 ± 25.5 [a]
Total AHs *	523 ± 109 [a]	848 ± 243 [a]	3400 ± 1390 [b]
Total PAHs *	< 0.001 [a]	0.515 ± 0.355 [b]	0.216 ± 0.062 [b]
NIC *	< 0.05 [a]	180000 ± 13600 [b]	43200 ± 3600 [c]
COT *	< 0.005 [a]	1500 ± 173 [b]	402 ± 36.6 [c]
3HC	< 0.05	< 0.05	< 0.05

Table 3.2. *EC_{50} values for CB leachates expressed in CBs/L for bioluminescence reduction bioassay, algal growth inhibition test and embryotoxicity bioassay. Values are expressed as means ± standard deviations (n=3).*

Species	LEAC_A EC_{50}	LEAC_B EC_{50}
Aliivibrio fischeri	4.47 ± 0.44	4.47 ± 4.08
Phaeodactylum tricornutum	12.4 ± 3.98	8.05 ± 3.06
Skeletonema costatum	4.89 ± 1.47	5.55 ± 2.59
Dunaliella tertiolecta	3.38 ± 0.66	3.84 ± 1.12
Crassostrea gigas	0.28 ± 0.02	2.54 ± 0.96

Non-metric multidimensional scaling (nMDS), based on results of bioassays and chemical analyses of LEAC_A and B, showed a clear separation between the five species, particularly evident for *C. gigas*; this separation was mostly related to Al, As, Cd, Cu, Mn, Zn, PAHs, nicotine and cotinine levels in LEAC-A and LEAC-B (Fig. 3.4). *S. costatum* showed further segregation from the other microalgae, mainly due to Cr, Fe, Ni and Pb concentrations in tested leachates (Fig. 3.4).

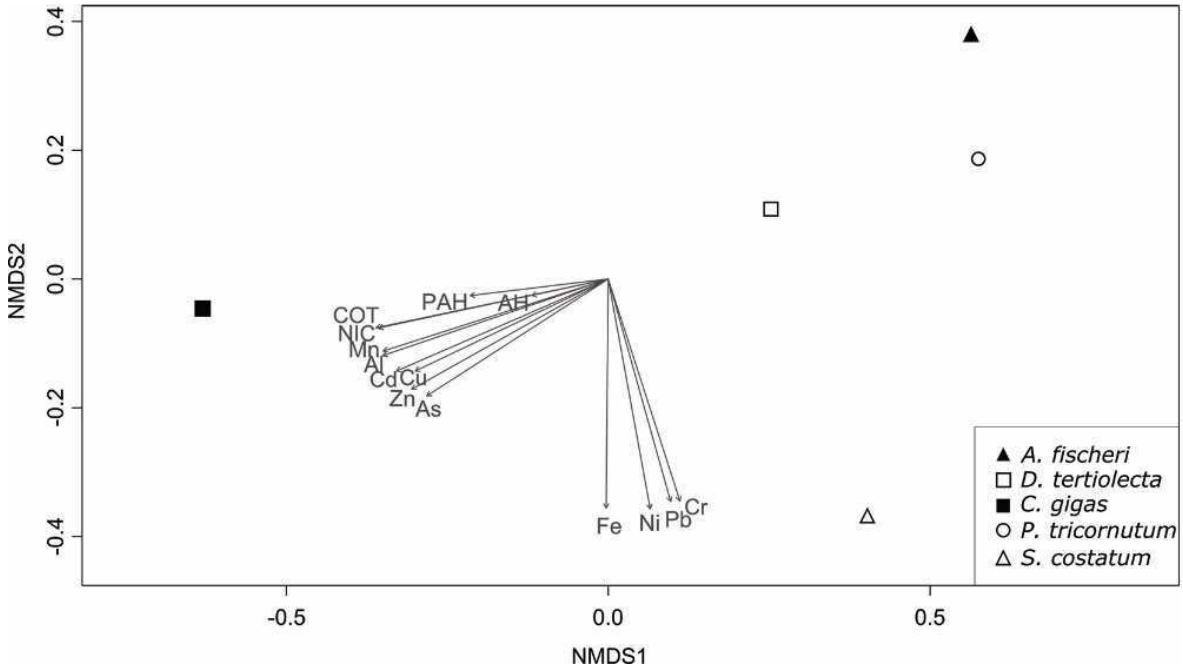

Figure 3.4. *Non-metric multidimensional scaling plot of bioassays results obtained in A. fischeri, S. costatum, P. tricornutum, D. tertiolecta and C. gigas exposed to LEAC_A and LEAC_B. Vectors represent chemicals (concentrations in leachates) correlated to observed endpoints (EC_{50} values).*

3.3.3. *Ex vivo* study: viability and mRNA analyses

The viability test carried out on PCTS of *M. galloprovincialis* digestive glands exposed to LEAC_A (diluted to 50%), did not show any significant difference neither between control and exposed

groups nor among different exposure times (Table SM6). Transcript levels of selected genes showed variable responses. *Acox1* transcription decreased significantly in exposed PCTS, with a progressive reduction over time (Fig. 3.5 A). Among antioxidant genes, mRNA levels of *gst-pi*, *cat* and *Cu/Zn-sod* decreased with a significant time-dependent trend (Fig 3.5 B, C and E). No significant differences were detected for *Se-gpx*, *cyp3A1*, *mt10* and *hsp70*, although a general reduction of transcription levels was often observed after 72 h of exposure (Fig. 3.5 D, F, G, H). The mRNA levels of *mt20* were below the detection limit (data not reported).

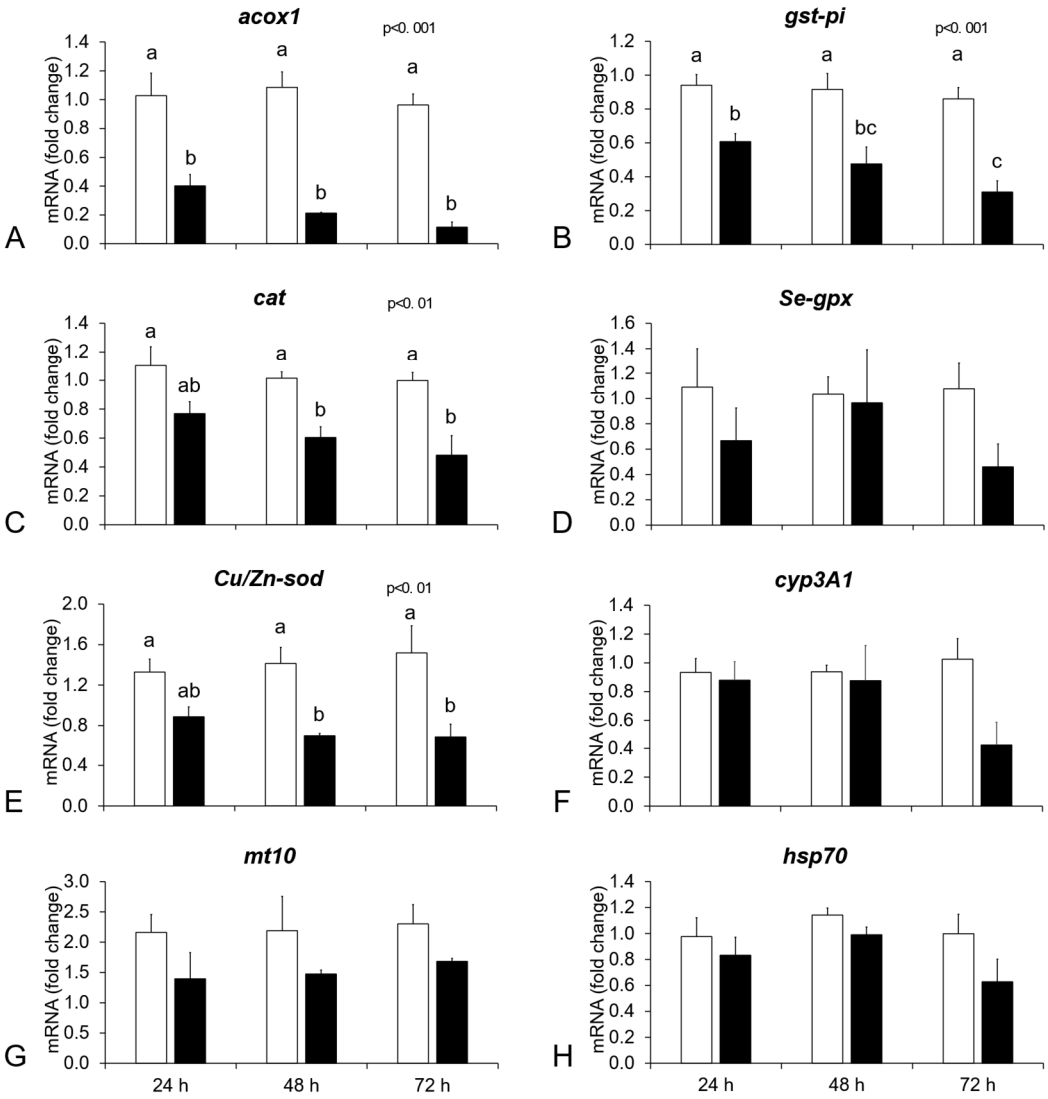

Figure 3.5. *mRNA levels of acox1 (A), gst-pi (B), cat (C), Se-gpx (D), Cu/Zn-sod (E), cyp3A1 (F), mt10 (G) and hsp70 (H) in PCTS exposed, for 24, 48 and 72 h, to CTRL (white) and LEAC_A diluted 50% (black). Data are expressed as mean values ± standard errors (n=3).*

3.3.4. *In vivo* study: chemicals bioaccumulation and biological responses

The *in vivo* exposure of mussels to CBs revealed a limited accumulation of some metals (Cr, Cu, Fe, Hg, Ni, V, Zn) at higher CB concentrations (Table 3.3 and SM8). Concentrations of PAHs significantly

increased with a dose-dependent trend mostly determined by low molecular weight congeners (LMW PAHs) (Table 3.3 and SM7). Bioavailability of NIC was evident for mussels with tissue levels increasing from b.q.l. in controls to 0.124, 0.266 and 3.44 μg/g in organisms exposed to 0.5, 1 and 5 CBs/L, respectively. COT was always below quantification limit (Table 3.3).

Table 3.3. *Concentrations of trace metals, low molecular weight polycyclic aromatic hydrocarbons (LMW PAHs), high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs), total PAHs, nicotine (NIC) and cotinine (COT) in tissues of M. galloprovincialis exposed to various experimental conditions. Values are given in µg/g dry weight (mean values ± standard deviations, n=3). Asterisks (*) indicate statistically significant variations while letters indicate differences between groups (post-hoc Student Newman Keuls Test).*

Chemical compound	CTRL	0.5 CBs/L	1 CBs/L	5 CBs/L
Al	54.5 ± 28.9	69.2 ± 16.8	71.1 ± 47.5	44.8 ± 8.75
As	15.3 ± 4.13	15.0 ± 1.96	15.5 ± 4.28	14.85 ± 1.50
Cd	0.343 ± 0.068	0.364 ± 0.157	0.477 ± 0.107	0.439 ± 0.146
Cr	0.985 ± 0.734	1.08 ± 0.212	1.53 ± 0.734	1.22 ± 0.626
Cu	2.59 ± 0.362	2.61 ± 0.231	2.60 ± 0.730	3.95 ± 0.900
Fe	196 ± 29.5	196 ± 23.0	216 ± 65.3	290 ± 80.6
Hg	0.061 ± 0.012	0.059 ± 0.018	0.070 ± 0.017	0.084 ± 0.025
Mn	4.88 ± 0.431	2.50 ± 0.163	3.16 ± 0.568	3.25 ± 0.795
Ni	0.941 ± 0.619	0.816 ± 0.061	1.33 ± 0.489	1.60 ± 0.453
Pb	0.762 ± 0.039	0.567 ± 0.128	0.763 ± 0.168	0.875 ± 0.361
V	0.715 ± 0.154	0.757 ± 0.012	0.859 ± 0.228	0.963 ± 0.424
Zn	111 ± 8.32	119 ± 68.4	150 ± 17.5	130 ± 41.4
LMW PAHs *	0.201 ± 0.073 ^{a}	0.294 ± 0.094 ^{ab}	0.450 ± 0.032 ^{bc}	0.602 ± 0.162 ^{c}
HMW PAHs *	0.020 ± 0.003 ^{a}	0.018 ± 0.002 ^{a}	0.030 ± 0.005 ^{b}	0.023 ± 0.001 ^{a}
Total PAHs *	0.220 ± 0.067 ^{a}	0.312 ± 0.095 ^{a}	0.480± 0.029 ^{c}	0.625± 0.161 ^{c}
NIC *	< 0.01 a	0.124 ± 0.014 ^{a}	0.266 ± 0.068 ^{a}	3.44 ± 0.560 ^{b}
COT	< 0.01	< 0.01	< 0.01	< 0.01

Data on biomarker responses in mussels exposed to CBs are summarized in Figures 3.6-3.7. Results of immunological biomarkers indicated a significant decrease of lysosomal membrane stability and reduced average values of granulocytes/hyalinocytes ratio in 5 CBs/L exposed group (Fig. 3.6 A and B). Considering biomarkers of genotoxic damage, the frequency of micronuclei increased in mussels exposed to 1 and 5 CBs/L, while a significant effect on DNA fragmentation was observed only at 5 CBs/L exposure (Fig. 3.6 C and D). Neurotoxic effects revealed a marked decrease of acetylcholinesterase activity in haemocytes of mussels exposed to 5 CBs/L and in gills of those exposed to 1 and 5 CBs/L, (Fig. 3.6 E and F). The activity of ACOX increased at 5 CBs/L while no significant variations were visible for metallothioneins (Fig. 3.6 G and H).

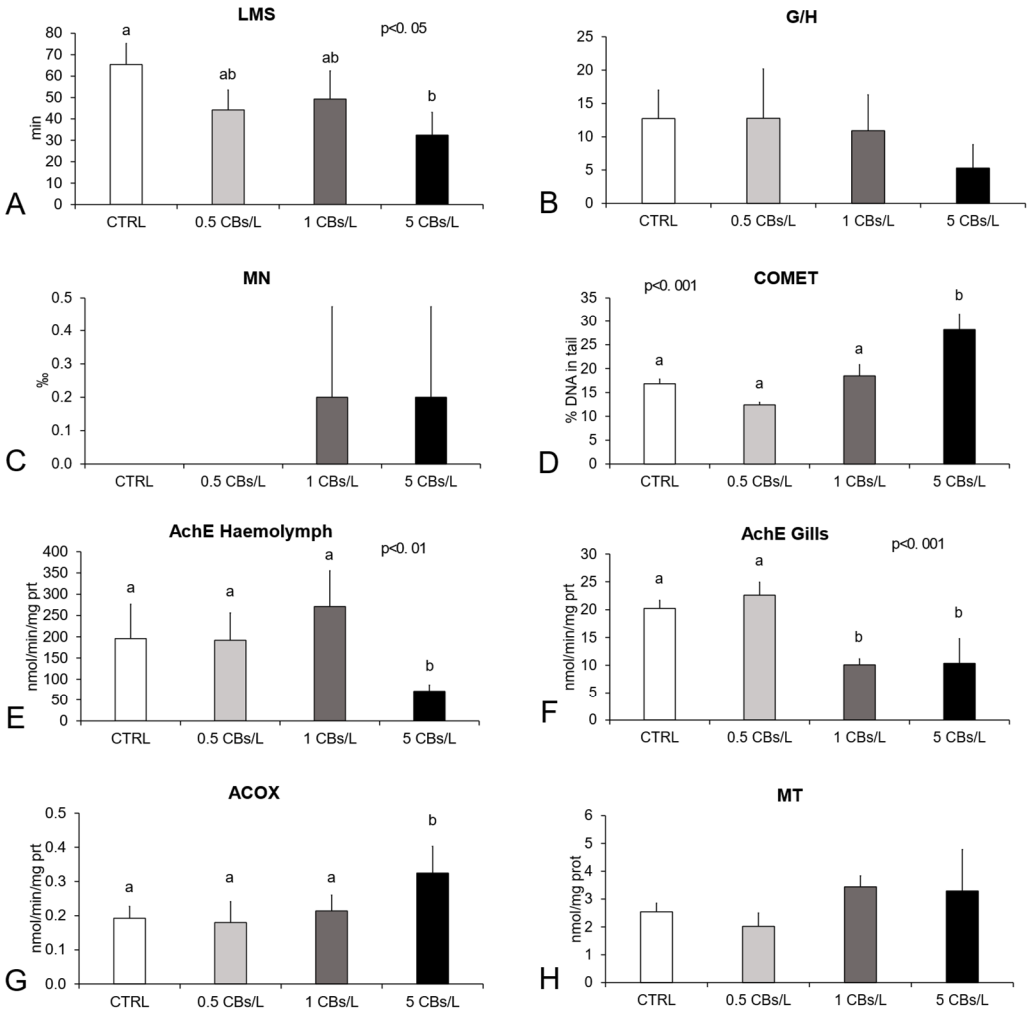

Figure 3.6. *Lysosomal membrane stability (A), granulocytes/hyalinocytes ratio (B), frequency of micronuclei (C), DNA damage (D) in haemocytes; acetylcholinesterase activity in haemolymph (E) and in gills (F), acyl-CoA oxidase activity (G) and metallothioneins (H) in digestive gland of mussels exposed to CBs in various experimental conditions. Data are given as mean values ± standard deviations or standard error (D), n=3; p-values are given for statistically significant effects, while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).*

Among antioxidant defences, significant variations were the increase of glutathione reductase and total glutathione peroxidases in mussels exposed to 5 CBs/L, and the decrease of glutathione in all the groups (Fig. 3.7 B, D and F). A generally limited oxidative pressure was reflected by the lack of effects on total oxyradical scavenging capacity and a decreasing, not significant, trend for malondialdehyde levels (Fig. 3.7 G, H and I).

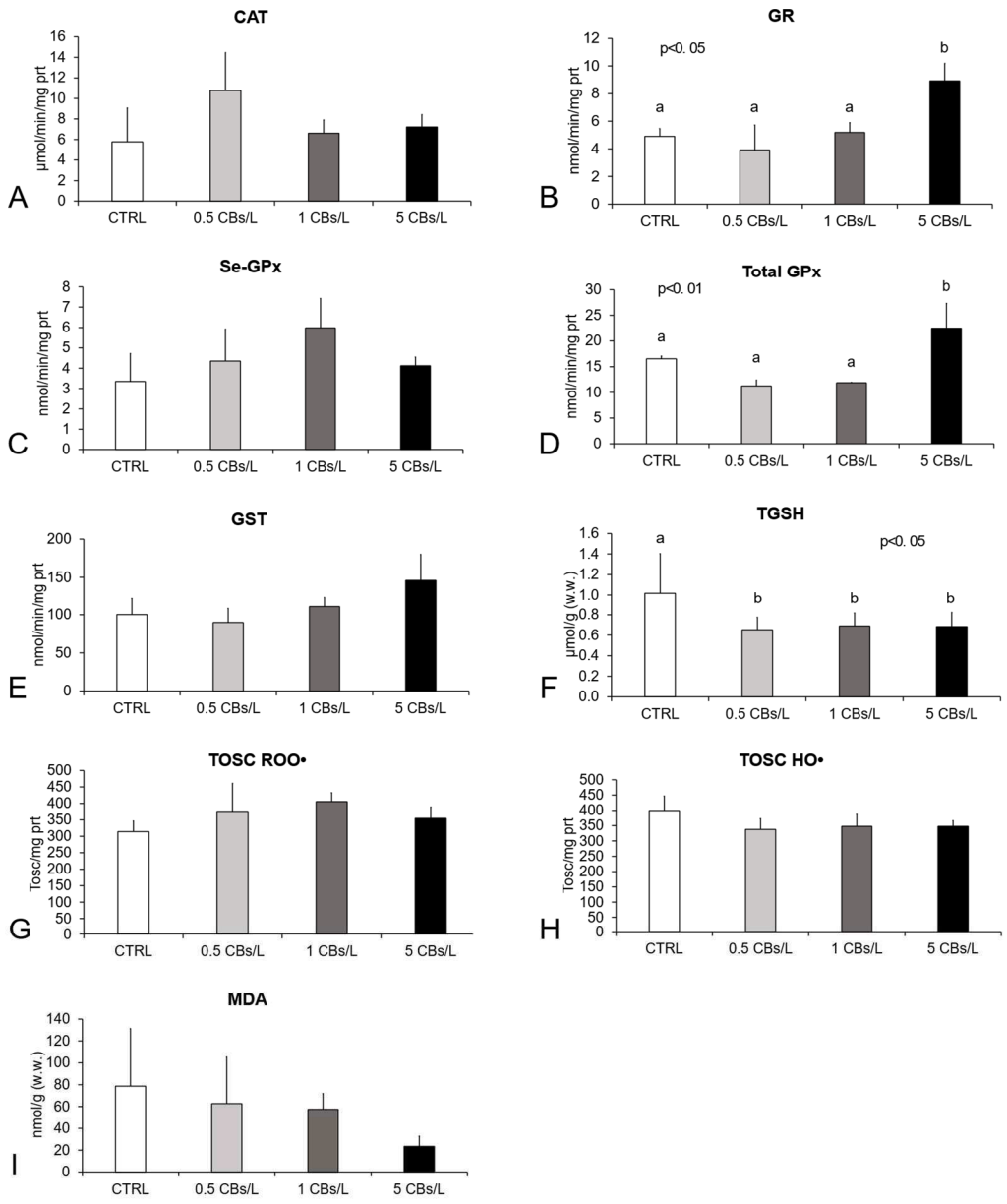

Figure 3.7. *Catalase (A), glutathione reductase (B), Se-dependent and total glutathione peroxidases (C and D), glutathione S-transferase (E), glutathione (F), total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (G and H), malondialdehyde (I) in digestive gland of mussels exposed to CBs in various experimental conditions. Data are given as mean values ± standard deviations (n=3). p-values are given for statistically significant effects, while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).*

3.3.5. Weight of Evidence integration

To summarize the overall biological relevance of obtained results and provide a quali-quantitative assessment of hazard from CBs to marine organisms, the results on ecotoxicological bioassays, bioavailability of chemicals and biomarker responses were integrated and elaborated using the weighted criteria of the Sediqualsoft model. The bioavailability hazard was classified as Slight for

mussels exposed to 0.5 CBs/L and Moderate for 1 and 5 CBs/L, with PAHs and especially nicotine mostly contributing to the calculated HQ in tissues of exposed mussels. The biomarker results, elaborated in terms of magnitude of variations and toxicological relevance of analysed endpoints, provided a level of hazard classified as Slight for mussels exposed to 0.5 and 1 CBs/L and Moderate for 5 CBs/L. Finally, ecotoxicological hazard, evaluated with the battery of *A. fischeri*, *P. tricornutum* and *C. gigas*, was Absent at 0.5 CBs/L, Moderate at 1 CBs/L and Major at 5 CBs/L, with *C. gigas* development effects mostly contributing to the HQ. The integration of single HQs elaborated for the LOEs of bioavailability, biomarkers and bioassays was synthesized in a WOE risk SLIGHT for exposures to 0.5 CBs/L, MODERATE for 1 CBs/L and MAJOR for 5 CBs/L (Fig 3.8).

Sample	LOE Bioavailability	LOE Biomarkers	LOE Bioassays	WOE
CTRL	Absent	Absent	Absent	ABSENT
0.5 CBs/L	Slight	Slight	Absent	SLIGHT
1 CBs/L	Moderate	Slight	Moderate	MODERATE
5 CBs/L	Moderate	Moderate	Major	MAJOR

Figure 3.8. *Elaboration of specific hazards for LOEs on bioavailability, biomarkers, bioassays data and integrated Weight of Evidence (WOE) classification of risk for different concentrations of CBs.*

3.4. Discussion

CBs are one of the most abundant litter items found in the environment, representing a potential risk for organisms due to chemicals contained in filter, ash and tobacco which are released in the aquatic compartment (WHO 2017; Araújo & Costa, 2019; Torkashvand et al., 2020). Previous investigations demonstrated that CBs could elute a complex panel of organic and inorganic chemicals (Hernandez, 2018; Dobaradaran et al., 2020, 2021), but only a few studies presented an integrated approach combining chemical characteristics of CB leachates and their biological effects and cellular responses on aquatic organisms (Micevska et al., 2006; Wright et al., 2015; Montalvão et al., 2019; Xu et al., 2019; Quéméneur et al., 2020). Using a battery of cell-based assays, CB leachates were shown to modulate different biological pathways, such as Ah receptor, estrogen receptor and p53, while effect-directed analysis coupled with nontargeted chemical analysis

allowed to identify compounds potentially responsible for the Ah receptor response (Xu et al., 2019).

Our results revealed that CBs release metals and organic compounds (AHs, PAHs, nicotine and cotinine) in seawater, and higher levels were detected in more concentrated leachate except for Cr, Ni, Pb and AHs. Compared to blank samples, the amounts of chemicals released by CBs were particularly elevated for Al, As, Cr, Cu, Fe, Mn, Ni, Zn, AHs, nicotine and cotinine. However, it has been reported that the typology and quantity of chemicals released from CBs can be influenced both by procedures for leachate production (e.g. concentrations of CBs per litre of solvent, soaking times and filtration mesh size) and the intrinsic variability of CBs (different brands and length, smoker puffs and the possible effect of smokers lip, hands or mouth (Poppendieck et al., 2016). In our study, smoked CBs were from cigarette receptacles, simulating the heterogeneity of real samples but causing a certain variability in experimental conditions. In this respect, caution should be used when comparing different results. Using CB normalization, levels of metals detected in this work were up to 3 orders of magnitude lower than those presented in Lawal & Ologundudu (2013), but up to 30 times higher than those obtained by Moerman & Potts (2011). The study by Xu et al. (2019) was the first to document the release of AHs from CBs in seawater. Concentrations of C12–C20 and C22 alkanes were previously reported for 4 CBs/L eluted in freshwater after 24 hours (Micevska et al.,2006). The release of 15 PAHs, mainly of low molecular weight (such as naphthalene, acenaphthylene, acenaphthene, fluorene and fluoranthene) was observed by Dobaradaran et al. (2020), while those congeners were below the detection limit in our study.

Nicotine, the principal alkaloid naturally present in tobacco leaves (Benowitz et al., 2009) was the most abundant chemical measured in leachates. Similar concentrations were reported by Caridi et al., (2020) in leachates obtained with the same number of CBs, while Wright et al. (2015) revealed a concentration 20 times lower. Our study provided also the first evidence of the release of cotinine, the main nicotine metabolite, already suggested as a marker of tobacco consumption in wastewater (Gracia-Lor et al., 2020). Other organic chemicals in CBs leachate were determined by nontargeted analysis demonstrating, however, the complexity to assess the toxicological contribution of individual tobacco constituents (Xu et al., 2019).

Toxic effects of chemicals eluted from CBs were evaluated through batteries of ecotoxicological bioassays. CB leachates determined the reduction of *A. fischeri* bioluminescence with mean EC_{50} values of 4.47 CBs/L, slightly higher than those reported in other studies and ranging between 0.3

and 2.7 CBs/L for leachates of different CBs brands (Micevska et al., 2006; Oliva et al. (2021); on the other hand, Piccardo et al. (2021) revealed a 35% inhibition of bioluminescence for 10 CBs/L leachate, lower than that found in the present work with about 55 and 70% inhibition for 6.25 and 12.5 CBs/L, respectively.

The three microalgae showed different sensitivities to CB leachates, with *P. tricornutum* being less sensitive than *S. costatum* and *D. tertiolecta*. These results were similar to those of Oliva et al. (2021) with an EC_{50} of 11.8 CBs/L for *P. tricornutum* and 6.2 CBs/L for *D. tertiolecta*. A species-specific sensitivity of these microalgae was also observed by Gallo et al. (2020), but with a different rank. *D. tertiolecta* was the most sensitive and *S. costatum* was the most resistant to the contaminant mixture. The study by Piccardo et al. (2021) reported a lower effect of CB leachates also for algal growth with an inhibition of 32% in *P. tricornutum* exposed to 10 CBs/L leachate compared to 72, 92 and 96% observed in this study for *P. tricornutum*, *S. costatum* and *D. tertiolecta.*

Crassostrea gigas was the only species to show a marked difference in ecotoxicological response according to leachate preparation, with LEAC_A causing an EC_{50} of 0.28 CBs/L, approximately 10 times lower than that obtained with LEAC_B which had an EC_{50} of 2.54 CBs/L, and thus suggesting some synergistic effects or additional chemicals released in the more concentrated leachate. This is the first study documenting the toxic effects of CB leachates on larvae of *C. gigas*, but abnormal embryo development was previously described in other aquatic species: malformation in *plutei* of sea urchin *Paracentrotus lividus* (Piccardo et al., 2021); an LC_{50} of 4.5 CBs/L on *nauplii* of crustacean *Artemia sp* (de Souza Abessa et al., 2020); an EC_{50} of 7 CBs/L on larvae of polychaete *Ficopomatus enigmaticus* (Oliva et al., 2021), malformed eyes and reduced sizes of embryos of medaka fish *Oryzias latipes* at 5 and 10 CBs/L (Lee & Lee., 2015). Comparing these data with our results, *C. gigas* seems very sensitive to substances released in CB leachates, possibly for the fast embryo development. Despite the nMDS seems to indicate the main contribution of some chemicals, mixture effects are more likely to explain the observed toxicity exerted by CB leachates since the reported EC_{50} values of individual compounds (Table SM8) did not match those analysed in our samples.

To evaluate the toxicity of CBs at a lower level of biological organization, sublethal effects and molecular responses were analysed on Precision-Cut Tissue Slices (PCTS) exposed at the final concentration of 50 CBs/L. The major advantages of mussel PCTS include the possibility to set up controlled and reproducible conditions for up to 72 h, reducing the inter-individual variability

similarly to *in vitro* models, but also maintaining the physiological architecture of the tissue, thus ensuring cellular outcomes more similar to those of *in vivo* models (Giuliani et al., 2019). The use of human PCTS has been a valuable tool in the study of the acute response to cigarette smoke in lungs, demonstrating dose-dependent cytotoxicity for concentrations higher than 10 cigarettes/L after 12 h exposure (Mondoñedo et al., 2020).

In our study, 50 CBs/L leachate had no effect on the viability of mussel PCTS but several gene transcripts were reduced, especially after 72 h of exposure to CBs mixture: this result is consistent with transcriptomic studies on *in vivo* and *in vitro* mammalian models, where a massive presence of inhibited genes allowed to conclude that transcriptional down-regulation is a major effect of cigarette toxicity (Maunders et al., 2007). Despite this general evidence, the down-regulation of *Cu/Zn-sod*, *cat* and *gst-pi* mRNA in mussel PCTS was partly unexpected, since the induction of antioxidant genes by cigarette smoke has also been often reported (Spira et al., 2004), confirming complex mechanisms of transcriptional and post-transcriptional regulation of antioxidants (Regoli & Giuliani, 2014). Concerning metallothioneins, the lack of *mt20*, the isoform specifically induced by Cd (Dondero et al., 2005), can be attributed to the low levels of this element measured in the CBs leachate. On the other hand, the constitutive *mt10* isoform is known to be induced in mussel digestive cells by Cu (45 µg/L) and Zn (300 µg/L) (Dondero et al., 2005), while a rather decreasing trend was observed in our study despite the elevated concentration of both Cu and Zn measured in CBs leachate, which supports a possible interference or antagonistic effect of other chemicals or mixture on metallothionein pathway. CBs leachate appeared to modulate also lipid metabolism in mussel PCTS by the downregulation of *acox1*. The impairment of fatty acid metabolism has already been observed in different mammalian organs and cell types, with reduced activity of β-oxidation enzymes and increased lipid accumulation after *in vivo* and *in vitro* exposure to cigarette smoke or extracts (Gong et al., 2019; Gupta et al., 2021); conversely, other studies reported an increase of the same enzymes, reflecting the high complexity of cigarette butts toxicity on fatty acid metabolism (Li et al., 2021). The low levels of PAHs, measured in CB leachates in this study may account for the lack of transcriptional regulation of *cyp3a1,* which is not strongly regulated by Ah receptor, particularly in invertebrates, but known to be responsive to such chemicals in mussels' digestive gland (Cubero-Leon et al., 2012; Giuliani et al., 2013). In mammalian models, *hsps* members were induced by cigarette smoke in *in vitro* but not in *in vivo* exposure (Gebel et al., 2004), and the absence of effects observed in this study on *hsp70* could confirm that the PCTS *ex vivo* model better reflects the physiological response of the entire organism, rather than of individual cells. Our biological model

is not particularly suitable for investigating receptor-based pathways like Ah and estrogenic ones which were shown to respond in specific cell-lines assays (Xu et al., 2019). Future studies integrating additional vertebrate species, approaches, experimental designs and methodologies will certainly contribute to better understand molecular mechanisms and mode of action of CB leachates on aquatic organisms.

In accordance with the chemical characterization of the CB leachate, mussels exposed to CBs showed a limited accumulation of some metals (Cr, Cu, Fe, Hg, Ni, V, Zn), this low accumulation of metals was already observed by Santos-Echeandía et al. (2021) evaluating the bioavailability of metals eluted from 1 CBs/L concentration derived from a previous leachate stock of 46 CBs/L (maintaining CBs 24 h in movement and then filtered at 0.45 µm mesh size) in *C. gigas* tissues. This could depend on the fact that some of these elements (i.e. Co, Cu, Fe, Zn, Mo, V) are used by bivalves for their metabolism as micronutrients. Moreover, mussels could absorb some metals from the environment but after a brief period excrete them into the water, showing a low bioaccumulation rate, independently from CBs concentration. On the contrary, a significant increase of PAHs mostly as low molecular weight congeners, which are typically characterized by higher water-solubility and bioavailability, was observed in mussels exposed to CBs with an upward trend positively correlated with CBs concentrations. Finally, nicotine, largely detected in CB leachates, was markedly accumulated in mussel tissues, confirming its absorption through biological membranes at a relatively high pH of the aqueous solution as that of the present study, 8.2 (Yildiz, 2004). Cotinine was always below quantitation limit (0.01 µg/g dry weight) in all the exposure groups, indicating that mussels do not uptake this chemical released from CBs and/or do not metabolize nicotine which in humans is converted to cotinine at a rate of 70-80%; this transformation is reported to be catalysed by CYP2A6 and aldehyde oxidase (Benowitz et al., 2009), none of which have ever been detected in mussel tissues.

Exposure to CBs provoked measurable effects on haemocytes physiology and immune defences, such as a significant decrease of lysosomal membrane stability, a decrease of granulocytes, the cells involved in cellular immunity and phagocytosis, and an increase of hyalinocytes, less active cells in immune defences (Nardi et al., 2021). Considering the genotoxic effects of CBs, our results demonstrate the onset of DNA fragmentation and an increase of micronuclei frequency in mussels exposed to 1 and 5 CBs/L. No immune responses but nuclear abnormalities and micronuclei were observed in haemocytes of *Anodontites trapesialis* and related to metals (Cr, Ni, Pb, Zn, Mn and Na) eluted from CBs (Montalvão et al., 2019), while nicotine was proposed as a potential cause of

genotoxicity in coelomocytes of *H. diversicolor* exposed to 8 CBs filters/L (Wright et al., 2015): in our experiment, Cr, Cu, Fe, Hg, Ni, V, Zn were accumulated to a limited extent, while a marked increase of nicotine was measured in tissues of exposed mussels.

Neurotoxic effects were reflected by the significant decrease of acetylcholinesterase activity in haemolymph at 5 CBs/L and in gills at 1 and 5 CBs/L. This effect could be explained by the competitive binding of nicotine to nicotinic acetylcholine receptors, thus modifying their conformation and physiology of the cholinergic system (Xiao et al., 2020). Despite mechanisms of action would remain to be clarified especially in invertebrate species, the influence of nicotine on functions of AChE has been investigated in several mammalian models, showing that the effects of nicotine exhibit many similarities to those of AChE inhibitors (Slotkin, 1999). As an additional hypothesis, the presence of some pesticides specific to tobacco crops might also contribute to AChE inhibition observed in mussels exposed to CBs (Soleimani et al., 2022). The increase of ACOX activity to 5 CBs/L could be associated with the accumulation of PAHs in mussel tissues: this enzyme, involved in peroxisomal proliferation and β-oxidation of fatty acids, has been largely used as a biomarker of exposure to organic pollutants in aquatic organisms such hydrocarbons, phthalates, plasticizers (Cajaraville & Ortiz-Zarragoitia, 2006). At the same time, the lack of metallothioneins variations is also consistent with bioaccumulation results which did not highlight marked variations in tissue levels of trace elements, particularly for Cd.

Among antioxidant defences, glutathione showed a significant decrease in all exposure groups, partly compensated in mussels exposed to 5 CBs/L by the increase of GR, responsible for regenerating the functional form of reduced GSH. Glutathione has a great importance against prooxidant chemicals and reactive oxygen species (ROS), acting as a scavenger of ROS and as a cofactor of antioxidant enzymes (Regoli & Giuliani, 2014). Oxidative pressure in mussels exposed to 5 CBs/L was further confirmed by the significant increase of total GPx, which protects cells from oxidative damage caused by organic and inorganic hydroperoxides (H_2O_2): other important antioxidants like GST and catalase did not exhibit significant variations in all exposure groups. The rather constant TOSC values and the decreasing levels of malondialdehyde, a typical marker of oxidative damage, highlighted a limited prooxidant impact of the mix of substances released from the CBs on mussels' capability to counteract oxidative pressure.

The overall results obtained in this study highlight a complex network of effects that can influence the impact of CBs on marine species, confirming the challenge of assessing the risks from emerging

pollutants. The importance of integrating chemical analyses with the measurement of biological effects has gradually risen in monitoring strategies. Multidisciplinary approaches for the characterization of aquatic environment quality are now recommended by European Directives such as the Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MFSD, Directive 2008/56/EC). However, the lack of standardized procedures for the integration of complex datasets of heterogeneous results often prevents the adoption of such multidisciplinary approaches in decision-supporting procedures (Dagnino et al., 2008; Linkov et al., 2009; Semenzin et al., 2008; Piva et al., 2011; Benedetti et al., 2012). In this study, the Sediqualsoft model allowed synthesizing the biological significance of the results observed in ecotoxicological bioassays and in mussels exposed to CBs, for an easier qualitative and quantitative comparison of different conditions. With such approach, the weighted elaboration of ecotoxicological bioassay, bioaccumulation and biomarker results provided specific hazard indices increasing with exposure dose from “Slight” to “Moderate” for both bioavailability and cellular effects while from “Absent” to “Major” for bioassay (Fig. 5): accordingly, the integrated WOE risk index was Slight for 0.5 CBs/L, Moderate for 1 CBs/L and Major for 5 CBs/L. The evident increase of the overall biological impact at higher CBs exposure levels reinforces the ecological risk caused by these items which should be considered as true emerging pollutants.

The presented WOE approach and elaboration procedure, besides the integration of different LOEs for a more complex level of risk assessment, has also a great importance in terms of communication and risk management, still maintaining scientifically robust info derived from the weighted elaboration of various results.

3.5. Conclusions

This study presented a multidisciplinary assessment of the impact of CBs on marine organisms, demonstrating the capability of the hazardous items to release chemical compounds in seawater, to induce effects on a battery of ecotoxicological bioassays, to modulate gene expression in *ex vivo* models (PCTS) of *M. galloprovincialis*, but also a significant accumulation of PAHs and nicotine, paralleled by several alterations of the immune system, antioxidant responses, lipid metabolism, neurotoxic and genotoxic responses in *in vivo* exposed mussels. Considering the complexity of results obtained by each typology of data, the results of this study corroborate the importance of an integrative approach based on multiple LOEs and their weighted elaboration to better address

and communicate the impact and risks of cigarette butts on the marine environment, raising smokers' environmental awareness and more efficient public management of this typology of waste. The clear evidence of an increasing dose-dependent risk from CBs on marine organisms, corroborates their role as emerging pollutants, highlighting the need to prioritize their removal from the beach not only as an aesthetic problem but to rather limit a pollution source for the marine environment.

4. Multispecies responses to cigarette butts associated contaminants: evidence from singular exposures to mesocosm approach

4.1. Introduction

Toxic effects of CBs associated chemicals were demonstrated by multiple studies, which analysed biological responses (e.g. molecular, cellular, physiological and/or behavioural alterations) in freshwater and seawater organisms, from bacteria to vertebrates, but often evaluating concentrations of CBs that were non-environmentally realistic. Considering the results of exposure with *M. galloprovincialis*, described in the previous *in vivo* experiment, the study presented in this chapter is focused on assessing the bioaccumulation of chemicals released by CBs and resulting biological effects on three marine species (*M. galloprovincialis*, *Perinereis aibuhitensis* and *Dicentrarchus labrax*) exposed alone and in a mesocosm approach. Mesocosm was defined in 1984 by Odum as ‘‘a bounded and partially enclosed outdoor experimental unit, which simulates the natural environment, particularly the aquatic environment”. So, it is an experimental system that examines the natural environment under laboratory-controlled conditions which can guarantee reliable references and replications (Watts & Bigg, 2001). Moreover, it could be a good compromise between the natural ecosystem and the artificial laboratory experiments, permitting the evaluation of how organisms or communities could react to environmental-realistic stressors (e.g. pollutants, pathogens, climate change simulations; Sharma et al., 2021). Some studies and reviews, in the scientific literature, used this approach for assessing the effects of contaminants in marine organisms (heavy metals, Foekema et al., 2021; climate change, Hall & Lewandowska, 2022, Moreno et al., 2022, Zhou et al., 2022; microplastics, Lott et al. 2020, Quade et al., 2022, Foekema et al. 2022, Galgani et al. 2022; marine sediment toxicity, Albarano et al. 2019). Focalizing on the evaluation of CBs toxicity, only two works performed this approach: Senga Green et al. (2021) compared the impacts of two filter typologies of CBs (cellulose acetate VS biodegradable) using a benthic community of filter feeder *Mytilus edulis*, primary producer *Ulva lactuca* and sediment microphytobenthos, while Quéméneur et al. (2020) evaluated the effects of cigarette filters (smoked and unsmoked) on the microbial diversity of coastal sediments performing a microcosm experiment. So, the study presented in this chapter can increase the knowledge of the biological and behavioural responses of *M. galloprovincialis, P. aibuhitensis* and *D. labrax* exposed to CBs and in natural-like conditions. These three species were chosen because colonized different ecological niches: the bivalve *M. galloprovincialis* is a hard-bottom filter feeder, the polychaete *P. aibuhitensis*,

is a soft-bottom deposit feeder, and the fish *D. labrax* is a predator which could live in shallow water. Furthermore, they are widely used as environmental pollution bioindicators and for their importance as commercial species. In particular, *P. aibuhitensis* is a deposit-feeder which is abundant in coastal ecosystems of Asian countries like China, South Korea, Philippines and India (Jørgensen et al., 2008; Koo & Seo, 2017) but is largely imported in the European countries as marine fishing bait. This species was chosen, also because is more tolerant to sandy sediment (like the substrate used in the work presented in this chapter) and easier to maintain in laboratory conditions than *Hediste diversicolor*, the polychaete normally used as a bioindicator in several biomonitoring programmes and presented in some protocol of sediment quality assessment (Perez et al., 2004; ASTM, 2007; Durou et al., 2007; Dean, 2008; Bouraoui et al., 2009, 2010). Furthermore, *P. aibuhitensis* was largely used as a model for ecotoxicological and environmental monitoring studies, evaluating the biological effects of contaminants, such as crude oil and PAHs (Zhao et al.,2017, Yuan et al., 2021), heavy metals (Tian et al., 2014, Gong & Zang, 2022), nano and microplastics (Jiang et al., 2019, Leung & Chan, 2018), antifouling biocide (Eom et al., 2019). In these works, enzymes activity related to the antioxidant system (Tian et al., 2014; Zhao et al., 2017, Eom et al., 2019; Yuan et al., 2021), neurotoxic responses (Eom et al., 2019), organic compounds biotransformation (Yuan et al., 2021), bioaccumulation (Jiang et al., 2019) and toxic effects (Tian et al., 2014; Leung & Chan, 2018; Eom et al., 2019; Gong & Zang, 2022) were analysed.

D. labrax is chosen because is a marine species (non-salmonid) largely commercialized and raised in Europe, in particular in Mediterranean areas (FAO, 2013). It is also a predatory fish species, that inhabits coastal areas (FAO, 2018), easy to breed and maintain in laboratory conditions. It is a well-known species and is frequently used as a bioindicator, in ecotoxicological studies, to evaluate the biological effects of various classes of environmental contaminants and stressors (Conti et al., 2015). For example, organochlorine pesticides and polychlorinated biphenyls (Greco et al., 2010); marine polluted sediments (De Domenico et al., 2011; Kerambrun et al., 2011); diclofenac and climate change (Maulvault et al., 2018); microplastics (Antão Barboza et al., 2018; Herrera et al., 2022; Montero et al., 2022; Pedà et al., 2022;) and PAHs (Martins et al., 2016). Some of these works (De Domenico et al., 2011; Kerambrun et al., 2011; Martins et al., 2016; Antão Barboza et al., 2018; Maulvault et al., 2018) present experiments performed with juveniles of *D. labrax*, because is the life stage more susceptible to environmental stressors that could affect species recruitment, ecological success and impact negatively to fisheries and aquaculture (Maulvault et al., 2018).

Finally, *M. galloprovincialis* was chosen for the reasons explained deeply in the previous chapter of this thesis.

The effects of CBs on mussels, polychaetes and juveniles of seabass were assessed by evaluating the survival of specimens, the bioavailability of CBs contaminants and several biomarkers such as lipid metabolism, neurotoxic and genotoxic effects and antioxidant defences. The integration of these responses in environmental-realistic CBs pollution might be the starting point for assessing the real impact of CBs on the marine ecosystem.

4.2. Materials & methods

4.2.1. Cigarette butts and animals' collection

Cigarette butts (CBs) of both roll-your-own cigarettes and industrial cigarettes of different commercial brands were collected from smokers of the Department of Life and Environmental Sciences of Polytechnic University of Marche (Ancona, Italy) and stored, at -20°C, in plastic bags until they were used. Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length) obtained from a local farm (Ancona, Adriatic Sea), were acclimatized with artificial seawater (ASW) at room temperature (T_R) and 35 psu for three weeks. Polychaetes, *Perinereis aibuhitensis* (10 cm length) were taken from a commercial bait supplier (4nodi, Nautica Retail Srls), gently washed and acclimatized in ASW at 35 psu and T_R for 48 hours in natural marine sediment (NMS), formed by 98.5% of sand, collected from a location (43°36'50.59"N- 13°26'29.20"E) at 1 m depth. Seabass, *Dicentrarchus labrax* (5-9 cm length) was taken from a commercial aquaculture and maintained in aquaria in constant conditions of temperature (T_R), salinity (35 psu) and fed one time a day until they were used for the experiments.

4.2.2. Experimental design and exposures preparation

The experimental plan consisted of four consecutive experiments: three different experiments in which each species was exposed alone, while a further experiment included all the three species, exposed together in a mesocosm scenario. The experimental plans were carried out in the Aquaria structure of the Department of Life and Environmental Sciences of Polytechnic University of Marche (Ancona, Italy) (Fig. 4.1). Two tanks of 200 L capacity (61x98x33.5 cm^3) were filled up to 5 cm depth

of NMS and ASW for a final volume of 100 L for mussels and polychaetes exposed alone, 120 L for seabass exposed alone and 150 L for all the three species exposed together. Each tank was constantly aerated; in each tank, five PVC grids (30x30x1 cm^3) were inserted for creating a hard substrate for mussels, and they were left in tanks for all four experiments, also without mussels, to maintain the same environmental conditions in every singular experiment. The exposure time of each experiment was 10 days. At the end of every experiment, sediment of both tanks was washed three times using ASW, resuspending sand in water, letting its deposit and removing the water every time.

Figure 4.1. *Aquaria structure of the Department of Life and Environmental Sciences of the Polytechnic University of Marche.*

4.2.2.1. Mussels exposed to cigarette butts

100 mussels were introduced in each tank and CBs were directly put in the water at the concentration of 0.5 CBs/L (Fig. 4.2 A). The number of mussels for this experiment was chosen to have enough tissue for all the analyses. Moreover, in respect of the *in vivo* experiment presented in the previous chapter, the number of individuals was higher and the concentration of CBs lower. This choice was done to simulate environmental realistic conditions. Water and CBs were changed every 72 hours. After changing water, mussels were fed, with a commercial mixture of marine plankton (Easy Reefs EasySPS EVO, size range 0.2-400 µm) and left for 30 minutes without CBs, then new CBs were put in the exposure tank. At the end of the experiment, organisms' tissues were collected as

follows: 3 pools of 25 g of whole soft tissues were gathered from each experimental tank and stored at -20°C until bioaccumulation analyses. For biomarkers analyses, gills and digestive glands were excised from mussels, pooled in 5 groups each constituted by the tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80°C until analyses. At the same time, aliquots of haemolymph withdrawn from the adductor muscle were used for *in vivo* analyses of DNA damages (COMET assay), fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency or rapidly frozen in liquid nitrogen and maintained at -80°C for acetylcholinesterase activity.

4.2.2.2. Polychaetes exposed to cigarette butts

100 polychaetes were introduced in each tank and CBs were directly put in the water at the concentration of 0.5 CBs/L (Fig. 4.2 B). The number of polychaetes for this experiment was chosen to have enough tissue for all the analyses and to simulate environmental realistic conditions either considering number of individuals or the concentration of CBs. Water and CBs were changed every 72 hours. After changing water, polychaetes were fed, with a commercial mixture of marine plankton (Easy Reefs EasySPS EVO, size range 0.2-400 µm) and left for 30 minutes without CBs, then new CBs were put in the exposure tank. At the end of the experiment, organisms were taken, rinsed and put in clean tanks with ASW at T_R for 24 hours adding glass tubes for simulating sediment galleries and preventing cannibalism events. This procedure allowed the elimination of the sediment inside the gastrointestinal tube that could compromise the results of biochemical and chemical analyses. After this, polychaetes were collected as follows: 3 pools of 25 g of whole soft tissues were gathered and stored at -20°C until bioaccumulation analyses. For biological analyses, 15 organisms were collected from each tank, pooled in 5 groups of 3 individuals each, and rapidly frozen in liquid nitrogen and maintained at -80°C until analyses. Other 15 specimens, pooled in 5 groups of 3 individuals, were used for coelomic fluid extraction: organisms were left alive in glass Petri dish with 5-10 mL (depending on the size of the polychaete) of physiologic solution (NaCl 137mM, KCl 2,7mM, Na_2HPO_4 10mM, KH_2PO_4 1,8mM + EDTA 500 mM pH 8), and subjected to electric stimulation (4.5 V for 1 minute) to facilitate the expulsion of coelomic fluid. Then the medium in the Petri dish (physiologic solution containing coelomic fluid) was collected and centrifuged for 5 minutes at 300 x g at 4°C. The supernatant was rejected and the pellet containing coelomocytes resuspended in 200 µL of physiologic solution. The samples prepared using this approach were used for *in vivo*

analyses of DNA damages (COMET assay), fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency or rapidly frozen in liquid nitrogen and maintained at -80°C for acetylcholinesterase activity.

4.2.2.3. Seabass exposed to cigarette butts

20 juveniles of seabass were introduced in each tank and CBs were directly put in the water at the concentration of 0.05 CBs/L (Fig. 4.2 C), chosen according to results of preliminary investigations that determined 50% of individuals mortality with 0.5 CBs/L. The number of seabass for this experiment was chosen to have sufficient quantity of tissue for all the analyses and to simulate environmental realistic conditions either considering number of individuals or the concentration of CBs. Every 24 hours, water was completely renewed, and CBs removed and re-dosed: after the water change, organisms were fed with a commercial feed specific for warm saltwater fishes (Aller Aqua), left for 30 minutes without CBs and then new CBs dosed at the nominal concentration. At the end of the experiment organisms were collected as follows. For bioaccumulation analyses, 5 individuals were gathered from each tank, anesthetized in ice, lyophilized and maintained at T_R until analyses; for biological responses, 15 organisms were collected, anesthetized in ice and rapidly dissected: blood was extracted from gills using heparin solution, partially used for *in vivo* analyses of DNA damages (COMET assay) and partially fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency; gills, muscle, head and liver were collected, rapidly frozen in liquid nitrogen and maintained at -80°C for different biomarkers analyses. Each sample prepared as previously described was made up pooling the tissues of 3 organisms.

4.2.2.4. Mesocosm exposed to cigarette butts

20 juveniles of seabass, 80 mussels and 80 polychaetes were introduced in each tank and CBs were directly put in the water at the concentration of 0.1 CBs/L (Fig. 4.2 D), chosen because was in the range of tolerance for seabass. The number of organisms co-exposed for this experiment was chosen to have enough tissue for all the analyses and to simulate environmental realistic conditions either considering number of individuals or the concentration of CBs. Water and CBs were changed every 24 hours. The experimental procedure was the same as previously described for species exposed alone.

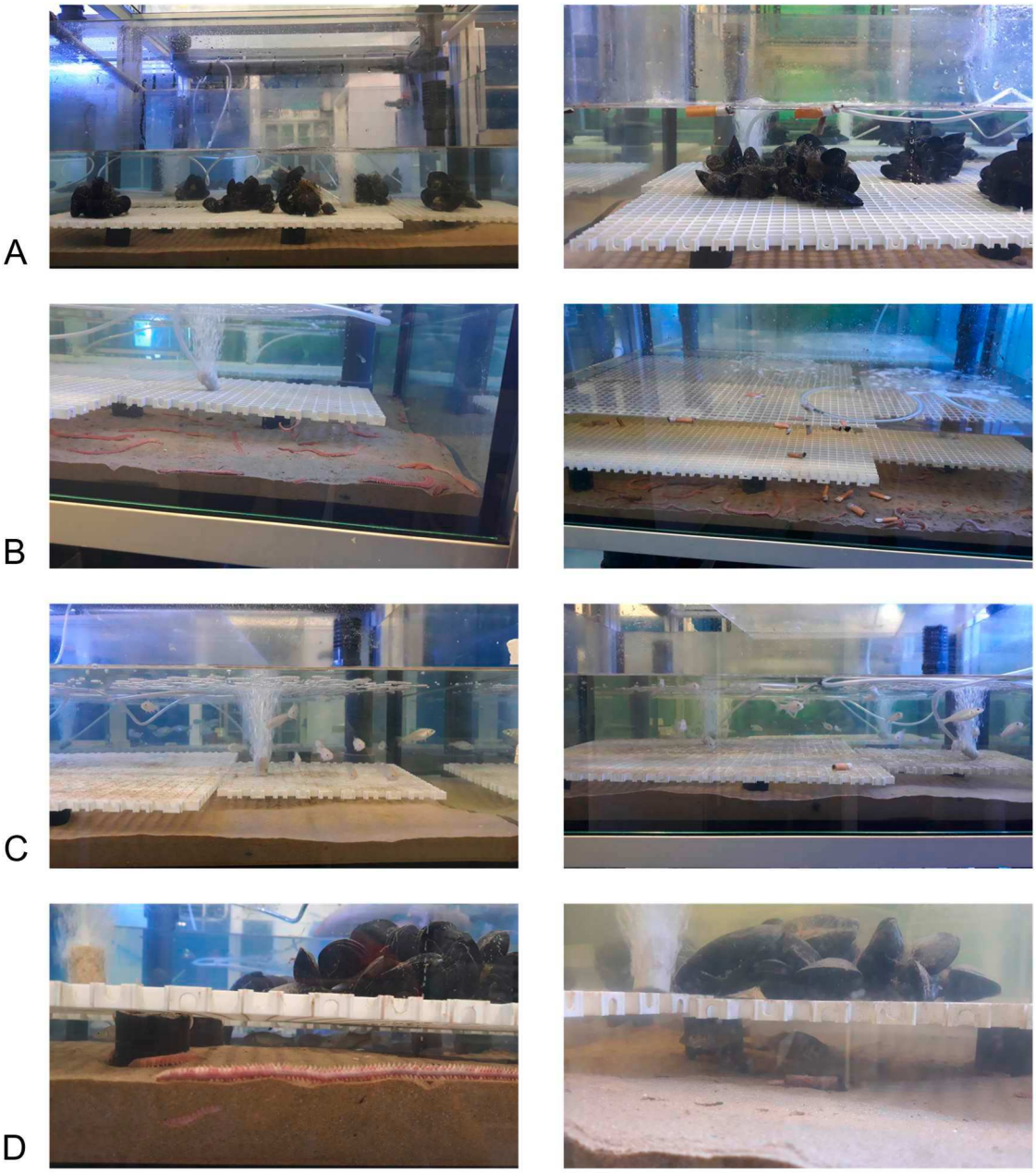

Figure 4.2. *Four exposure plans: M. galloprovincialis alone (A), P. aibuhitensis alone (B), juveniles of D. labrax alone (C) and mesocosm (D). The control group is on the left and the exposure group is on the right.*

4.2.3. Chemical analyses

Concentrations of trace metals (Al, As, Cd, Cr, Cu, Hg, Fe, Mn, Ni, Pb, V and Zn), polycyclic aromatic hydrocarbons (PAHs), were determined in all three species, while nicotine (NIC) and cotinine (COT) were detected only in mussels and polychaetes due to the limited amount of fish tissues. Measurements were carried out through validated methods by gas-chromatography with flame ionization detector (FID) and mass detector (MS), high-performance liquid chromatography (HPLC) with diode array (DAD), fluorimetric detection and atomic absorption spectrophotometry (AAS), and

UHPLC-MS Triple quadrupole technique. For all chemical analyses, quality assurance and quality control were monitored by processing blank and reference standard materials (National Institute of Standards and Technology, USA; EC–DG JRC Institute for Reference Materials and Measurements). The concentrations obtained from these reference standard materials were always within the 95% confidence intervals of the certified values.

Details on analytical methods and procedures are given in supplementary materials (SM1).

4.2.4. Biomarkers analyses

Validated protocols were followed for the measurement of biomarkers in tissues of *M. galloprovincialis*, *P. aibuhitensis* and *D. labrax*. Loss of DNA integrity (COMET assay) and micronuclei frequency (MN) for genotoxic damage were evaluated in haemocytes, coelomocytes and blood cells; acetylcholinesterase activity (AChE) was analysed as a marker for neurotoxic effects in haemocytes and gills of mussels, in whole tissue and coelomocytes of polychaetes, in head, muscle and gills of seabass. Acyl-CoA oxidase (ACOX) for peroxisomal proliferation was analysed in digestive gland of mussels and whole tissue of polychaetes; oxidative stress biomarkers included catalase (CAT), glutathione S-transferases (GST), Se-dependent glutathione peroxidases (Se-dep. GPx), total GPx, glutathione reductase (GR), total glutathione (TGSH) as single antioxidant defences, integrated with the analysis of total oxyradical scavenging capacity (TOSC) toward peroxyl radical ROO• and hydroxyl radical HO• were investigated in digestive gland of mussels, whole tissue of polychaetes and liver of seabass. Finally, ethoxyresorufin O-deethylase (EROD) was analysed in seabass' liver as a biomarker of xenobiotic metabolization (CYP1A) and exposure to substances that bind the aryl hydrocarbon (Ah) receptor. The results obtained from replicates were expressed as mean and standard deviations or errors. Details of analytical methods and procedures are given in supplementary materials (SM4).

4.2.5. Statistical analyses

Analysis of variance (one-way ANOVA) was applied to determine the significant differences obtained for biomarker analyses between control and exposed organisms in both typologies of exposure (levels of significance at $p < 0.001$, $p< 0.01$ and $p< 0.05$); homogeneity of variance was tested by

Levene test for all the parameters except MN where Mann Whitney test was used. All statistical analyses were performed using R software.

4.3. Results

At the end of mesocosm exposure, was observed a mortality of 85 and 98.75% of polychaetes in control and CBs exposure, respectively because, unexpectedly juvenile seabass ate some polychaetes or killed them ripping off their tail and they died after a few days. So, it was not possible to analyse the bioavailability and biological effects of polychaetes exposed in the mesocosm approach.

The exposure of organisms to CBs revealed a limited accumulation of chemicals: Cd, Fe, Mn and PAHs for mussels exposed alone (0.5 CBs/L) while Cr, Cu, Fe and PAHs exposed in mesocosm approach (0.1 CBs/L); Al, Cr, Fe, PAHs and NIC for polychaetes exposed alone (0.5 CBs/L); Mn for seabass exposed alone (0.05 CBs/L) while Mn and Fe expose in mesocosm approach (0.1 CBs/L) (Table 4.1, 4.2, SM9 and SM10). For mussels and seabass NIC concentrations are below quantitation limit and data not available, respectively (Table 4.1 and 4.2).

Table 4.1. *Concentrations of trace metals, low molecular weight polycyclic aromatic hydrocarbons (LMW PAHs), high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs), total PAHs, nicotine (NIC) and cotinine (COT) in tissues of M. galloprovincialis, P. aibuhitensis and juveniles of D. labrax exposed alone to CBs. Values are given in µg/g dry weight (mean values ± standard deviations, n=3 for CBs exposure; n=1, for control).*

Chemical compound	***Mytilus galloprovincialis***		***Perinereis aibuhitensis***		***Dicentrarchus labrax***	
	CTRL	**0.5 CBs/L**	**CTRL**	**0.5 CBs/L**	**CTRL**	**0.05 CBs/L**
Al	24.3	20.7 ± 6.97	14.5	37.4 ± 6.38	73.3	70.7
As	8.74	9.97 ± 0.295	3.15	3.51 ± 0.391	2.13	0.673
Cd	0.889	1.12 ± 0.039	0.209	0.233 ± 0.046	0.069	0.057
Cr	0.797	0.698 ± 0.046	0.175	0.764 ± 0.236	1.83	0.606
Cu	3.96	3.89 ± 0.784	4.87	4.90 ± 1.42	3.52	4.11
Fe	135	178 ± 12.5	304	347 ± 21.5	104	109
Hg	0.061	0.057 ± 0.017	0.012	0.005 ± 0.001	0.013	0.029
Mn	6.33	8.83 ± 0.707	14.3	13.1 ± 3.91	3.87	8.35
Ni	2.19	2.39 ± 0.075	1.63	2.06 ± 0.526	1.26	1.06
Pb	0.786	0.678 ± 0.299	0.259	0.307 ± 0.028	0.180	0.152
V	4.51	4.73 ± 0.347	1.10	1.02 ± 0.089	0.852	0.853
Zn	65.9	108 ± 65.4	37.2	73.3 ± 74.8	85.1	88.1
LMW PAHs	0.100	0.117 ± 0.020	0.095	0.128 ± 0.037	0.066	0.067
HMW PAHs	0.001	0.003 ± 0.002	0.004	0.003 ± 0.002	0.004	0.004
Total PAHs	0.101	0.120 ± 0.021	0.099	0.132 ± 0.039	0.070	0.072
NIC	< 0.01	< 0.01	< 0.01	0.250± 0.014	NA	NA
COT	< 0.01	0.043 ± 0.006	< 0.01	< 0.01	NA	NA

Table 4.2. *Concentrations of trace metals, low molecular weight polycyclic aromatic hydrocarbons (LMW PAHs), high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs), total PAHs, nicotine (NIC) and cotinine (COT) in tissues of M. galloprovincialis and juveniles of D. labrax exposed to CBs in mesocosm approach. Values are given in µg/g dry weight (mean values ± standard deviations, n=3 for CBs exposure; n=1, for control).*

Chemical compound	***Mytilus galloprovincialis***		***Dicentrarchus labrax***	
	CTRL	**0.1 CBs/L**	**CTRL**	**0.1 CBs/L**
Al	25.5	29.4 ± 7.91	59.6	43.5
As	8.54	7.97 ± 0.374	1.83	2.36
Cd	0.418	0.454 ± 0.016	0.122	0.095
Cr	0.664	0.932 ± 0.165	1.95	1.98
Cu	0.706	2.38 ± 0.318	2.21	2.86
Fe	100	108 ± 4.67	95.1	119
Hg	0.031	0.029 ± 0.002	0.023	0.020
Mn	5.66	5.93 ± 0.667	2.64	14.9
Ni	1.42	1.24 ± 0.230	1.78	1.99
Pb	1.20	1.32 ± 0.161	0.244	0.186
V	1.36	1.37 ± 0.117	1.29	0.248
Zn	30.5	42.1 ± 11.3	75.0	73.6
LMW PAHs	0.087	0.124 ± 0.024	0.094	0.100
HMW PAHs	0.002	0.002 ± 0.001	0.003	0.004
Total PAHs	0.089	0.127 ± 0.023	0.097	0.104
NIC	< 0.01	< 0.01	NA	NA
COT	< 0.01	0.043 ± 0.006	NA	NA

Data on biomarker responses in mussels exposed to CBs are summarized in Figure 4.3. Results of genotoxic damage indicated an increase in micronuclei frequency in mussels exposed to CBs alone, while a significant effect on DNA fragmentation was observed only in those exposed to CBs in mesocosm (Fig. 4.3 A and B). Neurotoxic effects revealed a significant increase of acetylcholinesterase activity in haemolymph of mussels exposed to CBs alone while in gills of both conditions the AChE activity is similar between control and CBs exposure (Fig. 4.3 C and D). The activity of ACOX is lower in mussels with CBs in mesocosm (Fig. 4.3 E). Among antioxidant defences, glutathione reductase and glutathione S-transferase activities increased in mussels exposed to CBs alone, while higher levels of total glutathione were observed in mussels exposed to CBs in mesocosm condition respect to the control (Fig. 4.3 G, H and K). Catalase showed similar activity in both conditions and between control and exposure groups; Se-dependent and total glutathione peroxidases revealed a low decrease of their activity in the exposure group related to control either in mussels exposed alone or in mesocosm scenario (Fig. 4.3 F, I and J). A generally limited oxidative

pressure was reflected by the lack of significant variation on total oxyradical scavenging capacity, with a limited increase of responses toward peroxyl radical in mussels exposed to CBs alone and in mesocosm (Fig. 4.6 L and M).

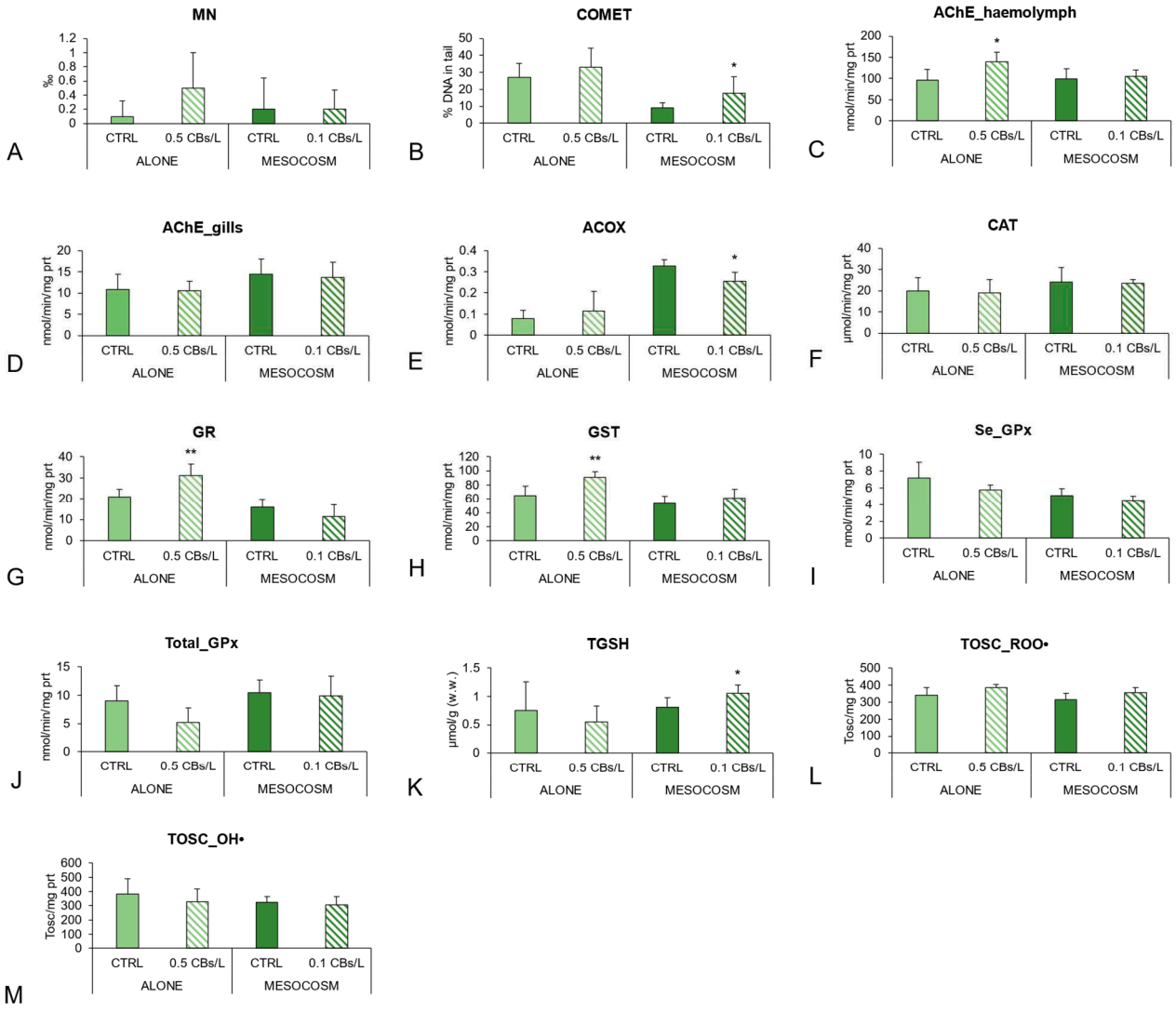

Figure 4.3. *Frequency of micronuclei (A), DNA damage (B) in haemocytes; acetylcholinesterase activity in haemolymph and gills (C and D); acyl-CoA oxidase activity (E), catalase (F), glutathione reductase (G), glutathione S-transferase (H), Se-dependent and total glutathione peroxidases (I and J), total glutathione concentration (K) and total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (L and M) in digestive gland of M. galloprovincialis exposed to CBs alone and in mesocosm approach. Data are given as mean values ± standard deviations (n=5). *= p-value< 0.05 and **= p-value< 0.01.*

Data on biomarker responses in polychaetes exposed to CBs are summarized in Figure 4.4. In general, no significant differences were present for all the analyses, but some effects related to CBs are present with an increase or decrease of some biomarkers activity. Results of genotoxic damage indicated the presence of micronuclei in exposed group (Fig 4.4 A). Neurotoxic effects revealed an increase of acetylcholinesterase activity measured in whole tissues of worms exposed to CBs (Fig.

4.4 D). The activity of ACOX is greater in the exposure group respect to control (Fig. 4.4 E). Among antioxidant defences, there was an increase of glutathione reductase, glutathione S-transferase and total glutathione peroxidases activities (Fig. 4.4 G, H and J). Catalase and Se-dependent glutathione peroxidase revealed a decrease in their activity in the exposure group (Fig. 4.4 F, and I). A generally limited oxidative pressure was reflected by the lack of significant variations on total oxyradical scavenging capacity with a limited no significative increase of responses toward hydroxyl radical in polychaetes exposed to CBs (Fig. 4.4 M).

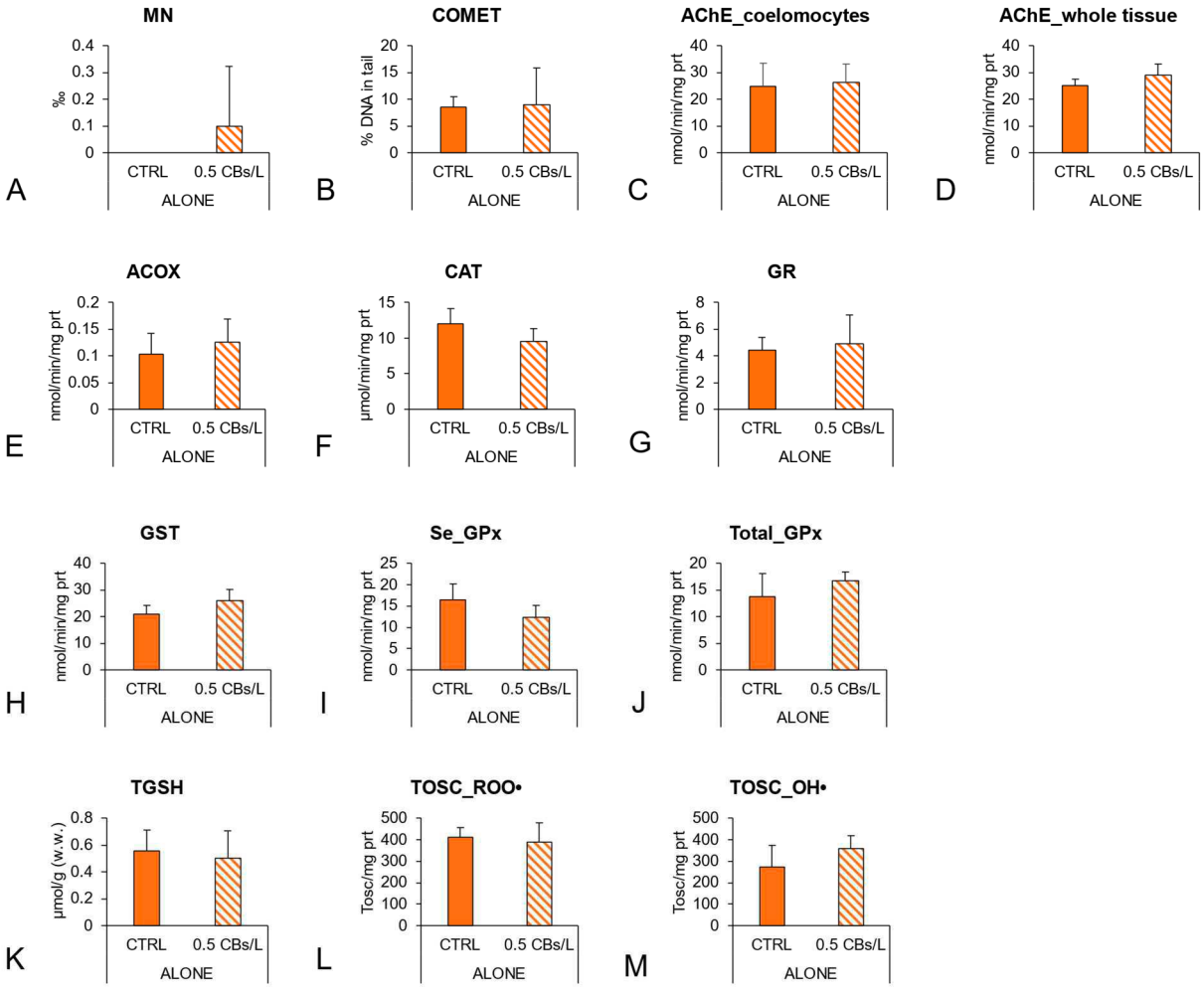

Figure 4.4. *Frequency of micronuclei (A), DNA damage (B) in coelomocytes; acetylcholinesterase activity in coelomocytes and whole tissue (C and D); acyl-CoA oxidase activity (E), catalase (F), glutathione reductase (G), glutathione S-transferase (H), Se-dependent and total glutathione peroxidases (I and J), total glutathione concentration (K) and total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (L and M) in whole tissue of P. aibuhitensis exposed to CBs alone. Data are given as mean values ± standard deviations (n=5).*

Data on biomarker responses in seabass exposed to CBs are summarized in Figure 4.5. Results of genotoxic damage indicated an increase in micronuclei frequency in seabass exposed to CBs in mesocosm, while a significant effect on DNA fragmentation was observed only in those exposed to

CBs alone (Fig. 4.5 A and B). Ethoxyresorufin O-deethylase (EROD) showed an increase in its activity in fishes exposed to CBs in mesocosm while no differences were present in those exposed alone (Fig. 4.5 C). Neurotoxic effects did not show significant differences between the control and exposure groups in both scenarios (Fig. 4.5 D, E and F). Among antioxidant defences, significant variations included the decrease of glutathione reductase and the increase of total glutathione in fishes exposed alone (Fig. 4.5 H and L). Catalase showed an increase of activity while glutathione S-transferase a decrease in the exposure group in mesocosm (Fig 4.5 G and I); Se-dependent and total glutathione peroxidases revealed a low decrease of their activity in the exposure group in seabass exposed alone (Fig. 4.5 J and K). A generally limited oxidative pressure was reflected by the lack of significant variations on total oxyradical scavenging capacity in both exposures (Fig. 4.5 M and N).

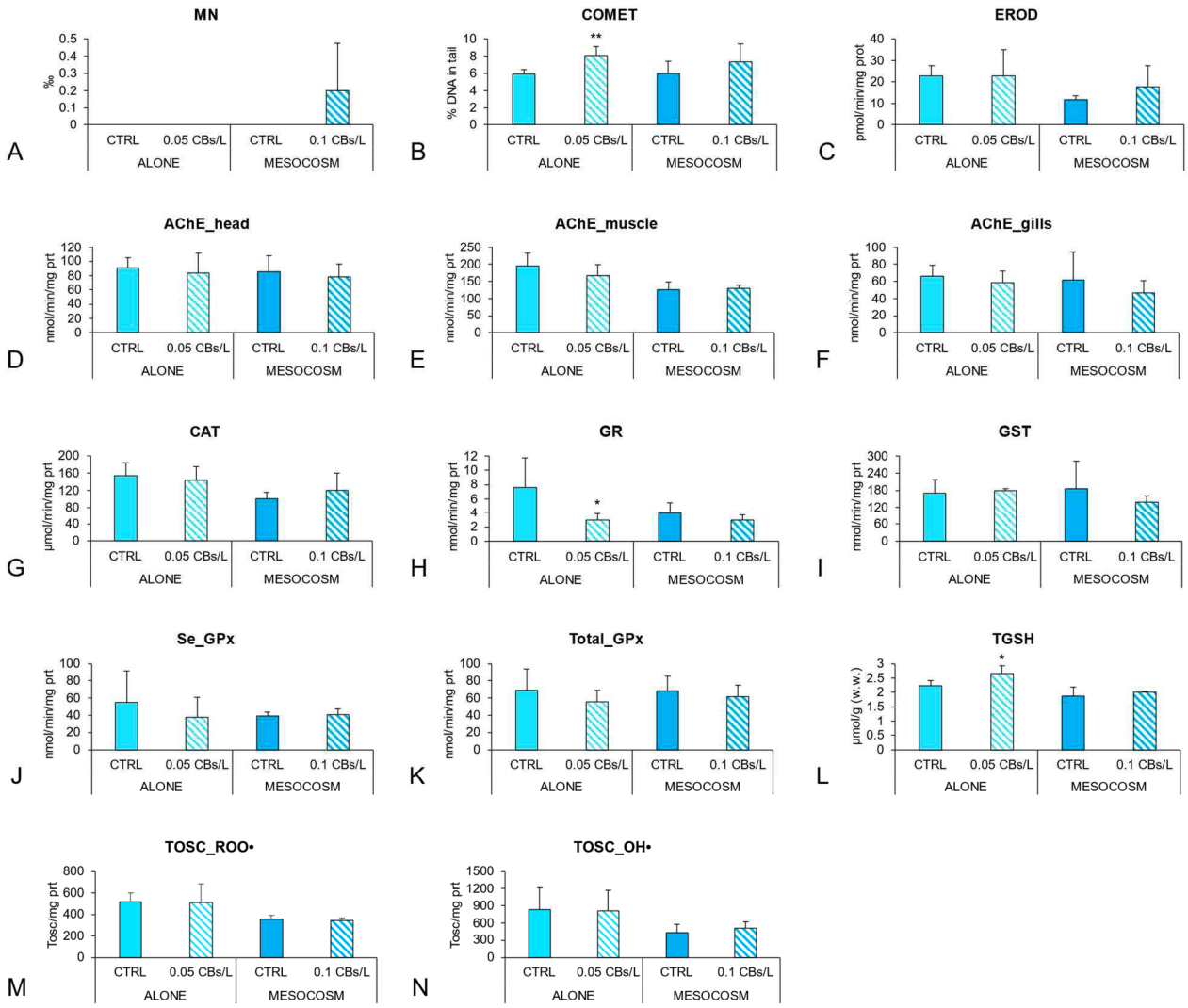

Figure 4.5. *Frequency of micronuclei (A), DNA damage (B) in blood cells; ethoxyresorufin O-deethylase in liver (C), acetylcholinesterase activity in head, muscle and gills (D, E and F); catalase (G), glutathione reductase (H), glutathione S-transferase (I), Se-dependent and total glutathione peroxidases (J and K), total glutathione concentration (L) and total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (M and N) in liver of D. labrax exposed to CBs alone and in mesocosm approach. Data are given as mean values ± standard deviations (n=5). *= p-value< 0.05 and **= p-value< 0.01.*

4.4. Discussion and conclusions

The huge number of CBs thrown into the environment annually and the abundance of this litter detected during sampling and beach cleaning activities all over the world have been growing the research interest to investigate the possible toxic effects of CBs in marine organisms (Green et al., 2022). Most studies detected and evaluated the typology of chemicals eluted from CBs in water, revealing the biological risk for the aquatic exposed to these substances (Venugopal et al., 2021; Dobaradaran et al., 2018, 2019, 2020, 2021, 2023). Some studies presented the biological effects in

marine organisms from different trophic levels (e.g., Booth et al., 2015; Quéméneur et al., 2020; de Souza Abessa et al., 2021; Oliva et al., 2021) but in most works, the concentrations of CBs used for the analyses are not environmentally realistic. The study presented in this chapter simulate, the CBs contamination using three species of different ecological levels (*M. galloprovincialis*, *P. aibuhitensis* and *D. labrax*), exposing them to low concentrations of CBs, both alone (0.5 CBs/L for mussels and polychaetes and 0.05 CBs/L for seabass) and in mesocosm conditions (0.1 CBs/L). Our results revealed that mussels, polychaetes and seabass exposed to CBs showed a limited accumulation of chemicals (Al, Cr, Cu, Fe, Mn, PAHs and nicotine). Low accumulation of metals in mussels was already observed in the *in vivo* experiment, also at higher CBs concentrations. Also, in this case, CBs did not release metals in concentrations such that can be bioavailable and bioaccumulated in elevated concentrations. In the previous experiment mussels accumulated a significantly higher quantity of PAHs and nicotine at the same CBs concentration (0.5 CBs/L), while in the present work, PAHs have similar concentrations between the control and exposure group and nicotine is below quantification limit in both the exposure conditions (alone and mesocosm approach). This revealed the low bioavailability and accumulation capacity of these organic compounds in mussel tissues. Polychaetes revealed low accumulation of metals, with a little increase of Al, Cr and Fe in the whole tissue of those exposed to CBs, but polychaetes can detoxify metals accumulated with the food or absorbed through the body wall and transform into less toxic forms (Fan et al., 2014). Moreover, polychaetes had an average concentration of nicotine of 0.25 µg/g in their whole body. Wright et al. (2015) have already investigated the CBs toxicity with the ragworm *Hediste diversicolor* exposed for 96 h to serial dilutions of CBs leachate prepared from 8 CBs/L stock (putting CBs in ASW for 24 hours on an orbital shaker and then filtered at 11 µm of mesh size) and the leachate was renewed after 48 h. Comparing the results of the same CBs concentration (0.5 CBs/L), *H. diversicolor* accumulated 1.901 µg/g of nicotine in their tissues, more than 7 times higher than the quantity detected in *P. aibuhitensis*. At the same time, the ragworms were also exposed to CB microfibers directly putting the fibres into the sediment and nicotine content was measured after 96 h and 28 days with a concentration of 0.186 and 0.041 µg/g, respectively, which are lower than those obtained in our study and worms exposed to leachate. The different concentrations of nicotine could be explained by the utilization of CB leachates and their preparation which advantaged the releasing of substances from CBs into the water and the immediate availability of nicotine via epidermis. Nicotine is more absorbable by biological membranes in basic conditions like a pH of 8 of ASW (Yildiz, 2004), but a moderate acidity in polychaetes gut could limit its bioavailability.

Moreover, worms have a mucus burrow that could act as a physical barrier, limiting the accumulation of nicotine. Finally, these organisms did not encounter the entire volume of the sediment and so only a fraction of contaminants, such as microfibers for Wright et al. (2015) or particles of ash, tobacco and microfibers in our study, could be ingested, determining a low accumulation of nicotine. Considering seabass, only Mn shows an increase in the whole body of them either alone or in the mesocosm approach and in the second scenario also Fe was more present in CBs exposure. The low levels of compounds found in fishes might be due to several reasons: the first could be related to the obligated choice of analysing the entire organisms because it was not possible to select specific target organs for bioaccumulation analyses, such as liver, gills or muscles; the second could depend on the low concentrations of substances eluted from CBs and the consequent low availability of them and the third could be the short time of exposure (10 days), which in the case of fishes could not permit to accumulate substances in their muscles.

Although the low concentrations of CBs and bioaccumulation rate, exposure to CBs provoked some measurable biological effects in these three species. Considering genotoxic effects, CBs increase micronuclei frequency in mussels and polychaetes exposed alone and in mussels and seabass in mesocosm; DNA fragmentation was present in mussels and seabass exposed in both conditions, while coelomocytes of polychaetes did not show this effect related to CBs presence as Wright et al. (2015) demonstrated at the same concentration in *H. diversicolor*. Few effects on neural signalling were reflected by the significant increase of acetylcholinesterase activity in haemolymph of mussels exposed alone while mesocosm mussels were not affected; also AChE measured in gills did not show significant changes in its activity in both conditions. These results are in line with those found in mussels exposed to 0.5 CBs/L in the experiment presented in the previous chapter. Polychaetes showed an increase of AChE activity in the whole tissue while coelomocytes did not present alteration of this enzyme due to CBs presence. To our knowledge, this is the first time that the AChE activity was measured in these cells, and it was interesting to discover a similar value of its activity between whole tissue and coelomocytes. Finally, seabass showed a no significant decrease of activity in all the tissue when exposed to CBs. ACOX activity presented a significant decrease in mussels exposed in mesocosm while those exposed alone did not show differences between control and CBs exposure, as demonstrated in the previous chapter. This unchanged activity could be explained by the level of PAHs measured in mussels' tissues with similar concentrations between control and exposure groups in both conditions. On the contrary, polychaetes exhibited an increase in ACOX activity related to CBs probably due to an accumulation of PAHs in their tissues which is

higher than control. The EROD activity, measured in seabass, did not show differences between control and CBs exposed group in both conditions due to low concentrations of PAHs eluted from CBs. Considering antioxidant defences, mussels were able to respond to prooxidant substances eluted from CBs in both exposure conditions. TOSC values remain constant and mussels exposed alone, largely increase GR activity for transforming the oxidative form of glutathione in the reductive form utilized in detoxification and metabolization of organic xenobiotics (Regoli & Giuliani, 2014) by GST. On the contrary, mussels exposed to CBs in mesocosm showed a high TGSH level and a reduction of GR activity. All the other important antioxidants like CAT and GPx did not exhibit significant alterations due to CBs presence. Also, polychaetes were not affected by oxidative stress with responses that are very similar to mussels exposed alone. Seabass present high responsiveness against oxidative pressure in both scenarios with a trend of induction, inhibition and concentration that are similar to mussels exposed in mesocosm. Fishes manifested a significant increase of TGSH and a decrease of GR, especially when exposed alone. All other tested antioxidant biomarkers presented similar results between control and exposure groups.

This study was one of the first that proposed a laboratory exposure with low concentrations of CBs directly put into water, using three species of different trophic levels and comparing the responses of these species exposed alone or in a mesocosm scenario; moreover for the first time, the impact of CBs was evaluated in *Perinereis aibuhitensis* and *Dicentrarchus labrax*. A deep investigation was done detecting chemicals eluted from CBs which were available for these organisms and caused some biological effects, in terms of animals' survival, cellular and biochemical responses. Although the different concentrations of CBs tested in species exposed alone or in mesocosm condition, little changes in bioaccumulation and biological effects were observed. The mesocosm approach was a useful tool for assessing the impact of this contaminant in environmental-like conditions showing some possible interaction between species belonging to different trophic levels and different responses due to the contaminants. Considering the results of this study, it might be interesting to expose organisms for at least one month and see if a long period of low CBs concentrations could cause chronic toxicity in the same species. Moreover, an experiment of trophic transfer may be performed to assess the biomagnification of substances eluted from CBs in sediment in a mesocosm with polychaetes and either juveniles or adults of seabass.

5. Photodegradation of cellulose acetate microfibres in fresh and seawater: an investigation of degradation process under one year of simulated solar irradiation

5.1. Introduction

CBs filter can contain about 15000 microfibers (MFs) of cellulose acetate (CA), an artificial plastic polymer. Considering the number of CBs thrown into the environment every year, thousands of trillion of these MFs are potentially released in the aquatic ecosystem annually. Due to its chemical characteristics, CA is a resistant material that in certain conditions, such as marine environment, could be completely degraded in 30 years (Niaounakis, 2017; Joly & Coulis, 2018). Woodall et al. (2014), analysing samples of plastic MFs collected in deep-sea sediment, found that 56.9% of MFs (called rayon) were made by cellulose acetate, which can be attributed partially to CB MFs. So, there is a potential risk for marine organisms to enter in contact with or ingest these MFs. The environmental degradation process of CA is a synergism of biological degradation exerted by microorganisms, the mechanical and physical weathering developed by waves motion and abrasion with the bottom of the sea, and photodegradation performed by the UV component of solar light (Puls et al., 2011). Photodegradation is fundamental for decomposing CA materials in the environment because UV irradiation reacts with carbon bonds of cellulose acetate structure, causing the formation of several free radicals' species which contribute to breaking polymer chain and facilitate the action of degradation enzymes of microorganisms. Pure CA alone has limited photodegradation in sunlight because absorbs UV light region with wavelengths of approximately 260 nm, which can't arrive on the hearth surface due to the atmosphere. The presence of titanium dioxide, commonly added to bleach CA materials, contributes to the degradation of CA because is a photo catalyst that absorbed UV light at about 390 nm, which is the UV component that arrives on the hearth surface (Puls et al., 2011). During this degradation process, MFs and nanofibers (NFs) of CA could be released by CBs filter into the aquatic environment continuously with consequences for the biota. Few studies have already evaluated the degradation rate of CBs and their MFs in aquatic environments, simulating real natural degradation processes (Gerritse et al., 2020; Belzagui et al., 2021).

In this chapter, an experiment of degradation of CA MFs in fresh and seawater was done by simulating one year of solar irradiation and natural hydrodynamic conditions. A cutting-edge technique was used to investigate the weathering processes on CA MFs: the Small-Angle X-ray

Scattering (SAXS) is a technique, based on X-ray, used to study the nanometric structure of materials in solution (Glatter & Kratky, 1982; Feigin & Svergun, 1987). SAXS is largely employed for studying biological macromolecules dispersed in their physiological environment and allows to get information about the folding and unfolding state of molecules, particle size and shape, particle-particle interaction, their hydration properties, and conformational changes that take place in solution (Putnam et al., 2007). In addition to biological samples, SAXS can be applied to other materials, such as polymers and could reveal the presence of particles of nanometric dimensions (1-100 nm) spread in a natural environment like water (Schnablegger & Singh, 2017). SAXS technique is available in large-scale facilities such as synchrotrons, where a powerful and continuous source of electromagnetic waves of a wide range of wavelengths, including X-rays, is available. The synchrotron light is channelled into specific lines called beamlines, where the exact wavelength will be selected and used to irradiate a sample. The advantages of synchrotrons are multiples such as the extremely powerful light which allows extracting information much more detailed the one derived by common laboratory X-ray production. To note samples don't need to be highly concentrated, and the analysis time required is short. On the contrary, one disadvantage is that, due to intense light irradiation, samples could be damaged and can’t be used again for other analyses.

5.2. Materials & methods

5.2.1. Microfibres production and experimental plan

Ten unsmoked cigarette filters (USFs), made of cellulose acetate, were taken to obtain a heterogeneous sample of USFs: 5 typically used for produced roll-your-own cigarettes and 5 separated from unsmoked cigarettes of five different commercial brands. Then these USFs were soaked in deionized water to accumulate water and after that frozen at -80°C. USFs were cut by cryostat for producing cellulose acetate microfibres (CA) of 10 µm length, which were collected in glass Petri dishes and stored at room temperature until the photodegradation experiment. To compare the degradation rate of CA, four textile microfibres, two natural (cotton, C and linen, L) and two synthetic (polyester, PE and polyamide, PA), were prepared from a standard fabric Retsch mill with a 500 µm grid by IPCB-CNR Pozzuoli (for much information and specific characteristics of MFs referring to Pittura et al., 2022).

The photodegradation experiment was performed using a solar simulator kindly offered by the Department of Industrial Engineering and Mathematical Sciences at the Polytechnic University of

Marche (Ancona, Italy). To know how many days of photo exposition we need to simulate one year of solar irradiation, different parameters were taken into account: the annual mean solar irradiation sea surface of 1200 kWh/m^2 (Joint Research Centre); 5% of solar irradiation is UV radiation (Joint Research Centre), so its amount is 60 kWh/m^2; the UV component of solar simulator irradiance used in this experiment is 0.06 kW/m^2; the exposure time of the solar simulator is 24 hours/day. Considering these parameters, the exposure time of the solar simulator corresponding to the annual mean solar irradiation on the surface is found to be 42 days (Table 5.1).

Table 5.1. *Parameters considered to evaluate the time exposure of microfibres under the solar simulator.*

Annual mean solar irradiation on sea surface (kWh/m^2)	1200
5% of UV component of annual mean solar irradiation on sea surface (kWh/m^2)	60
UV irradiance of solar simulator in this experiment (kW/m^2)	0.06
Exposure time per day in this experiment (h)	24
Total UV irradiation per day in this experiment (kWh/m^2)	1.44
Total exposure time for simulating one year of mean solar irradiation on sea surface (days)	41.67

For the solar exposure, twelve pyrex bottles of 250 mL, cleaned with compressed air to eliminate any previous MFs and microplastics contamination, were used. All bottle cups were cut to form a circular hole for inserting a circular quartz glass which didn't absorb the UV component of the solar radiation (280-400 nm), allowing UV rays to pass through the bottle cup (Fig 5.1 A). Moreover, each remaining plastic part of the cup bottles was accurately covered by aluminium to reflect the solar radiation and avoid the risk of plastic degradation inside the samples and consequent contamination (Fig 5.1 B). Two samples per type of MFs were weighted: one for seawater and one for freshwater. 100 mg of samples were put into the bottles under a laminar air hood for preventing environmental contamination of MFs and after that 100 mL of artificial seawater (ASW, Instant Ocean® Sea Salt at 35 psu) and freshwater (FW, tap water) (Fig 5.1 C), both filtered using a vacuum filtration apparatus with a polyether sulfone membrane of 0.22 µm mesh size, were added. The concentration of MFs for all 10 samples was 1 g/L. Two controls (CTRL), one in seawater and one in fresh water, were prepared in the same conditions as MF samples. To simulate the hydrodinamism of the aquatic environment, the 12 bottles were put over a flat orbital shaker, set under the solar simulator (Fig 5.1 D). Two air fans were used to maintain the temperature constant at 22°C preventing the increase of T due to heat flow generated by the lamps of the solar simulator.

Samplings were carried out before the starting of exposure (t=0) and 4 times a week during the 42 days of photodegradation experiment, for a total of 300 collected samples. 1000 µL micropipette with the tip half cut, to have a bigger diameter hole and suck a more homogeneous quantity of sample, was used to take samples, which were inserted in glass vials of 2 mL, previously cleaned with compressed air. Then samples were stored in covered boxes at T_R until their analysis.

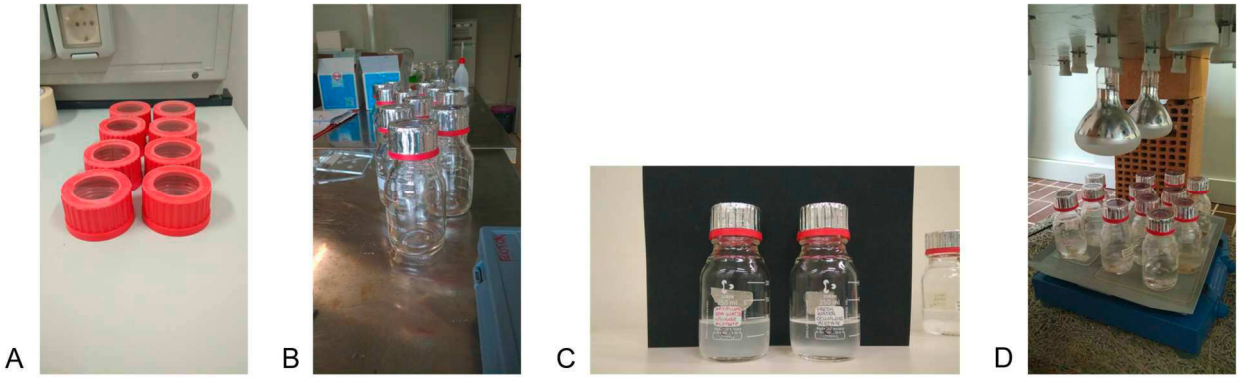

Figure 2.1. *Modified caps of the bottles with the top surface with quartz glass (A) and plastic parts covered with aluminium for preventing external plastic contamination (B). Bottle with CA MFs in fresh and seawater at a concentration of 1 g/L (C) and solar simulator used during the experiment with samples on a flat orbital shaker (D).*

5.2.2. Microfibres analyses and microfibres model

Samples were analysed at the Austrian SAXS beamline of the Elettra synchrotron in Trieste in December 2021. MFs dispersed in water were put in SAXS cell (Fig 5.2 A), and immediately inserted in the sample holder of SAXS beamline for starting the measurement that were taking place at room temperature (Fig 5.2 B and C). For each sample, eighteen frames of 10 s duration were collected and subsequently treated with both FIT2D, a data analysis program used in European Synchrotron Research Facility beamlines (Hammersley, 2016), and with a IGOR Pro macro (WaveMetrics, Portland, OR 97223, USA) in order to obtain the final scattering curves. These experimental curves were fitted with a novel model specifically developed for describing the structure of microfibers. According to Oehme et al. (2015), the internal structure of a microfiber is considered to be composed of several fibres of decreasing dimensions twisted from one to another. Each microfibre was composed of *macrofibrils* (MAFs) and each of them contained *microfibrils* (MIFs) surrounded by water (Fig 5.3 A and B). Both fibrils have dimensions that are in the nanometre range, which is measurable by SAXS (1-100 nm). Different parameters of MAFs were considered for evaluating the effect of one year of photodegradation, either on the longitudinal axis or on the cross-section. In particular, for the longitudinal axis, L is the total length of MIF and L is made by a number (n_b) of rigid segments, whose length is indicated with b, so L is simply given by $L=n_b b$ (Fig. 5.3 C). Considering

the transversal section of MAF, the MIFs are organized in a two-dimensional hexagonal lattice where a is the distance between MIFs (Å = 0.1 nm), R is the radius of MIFs (Å), N_a is the number of MIFs present along each radius (defined as $D/2$) of microfibril and g_a is the order degree of MIFs in the hexagonal lattice (Fig 5. 3 B and D), finally, D is the diameter of MAF (nm). This model, inspired by a previous model proposed by Penttilä et al. (2019), has been implemented in the software GENFIT, developed in the Molecular Biophysics Laboratory of the Department of Life and Environmental Sciences (DISVA) of the Polytechnic University of Marche (Ancona, Italy), which can fit small-angle scattering data of randomly oriented macromolecular or nanosized systems according to a wide list of models (Spinozzi et al., 2014).

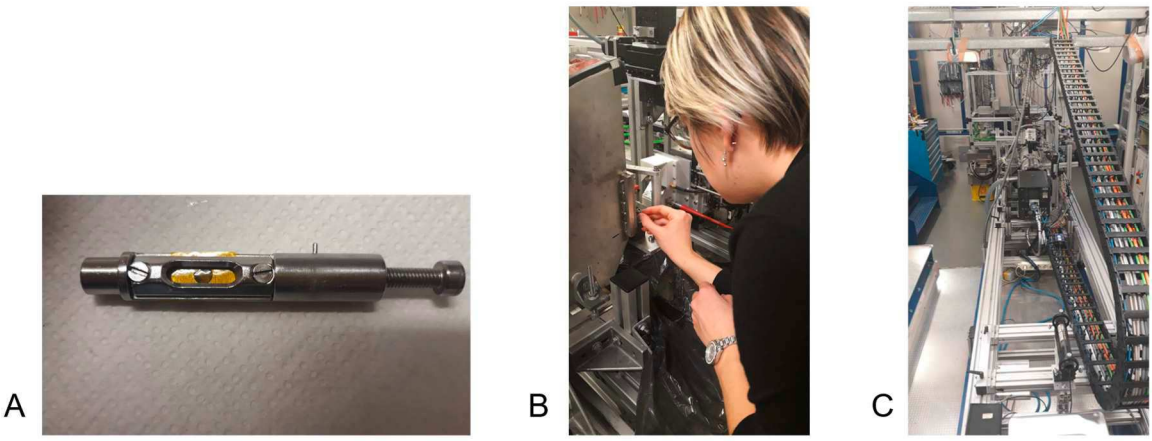

Figure 5.2. *SAXS cell used during the SAXS experiment of MFs analyses at Elettra synchrotron in Trieste (A), insertion of cell into the SAXS beamline (B) and overview of the beamline (C).*

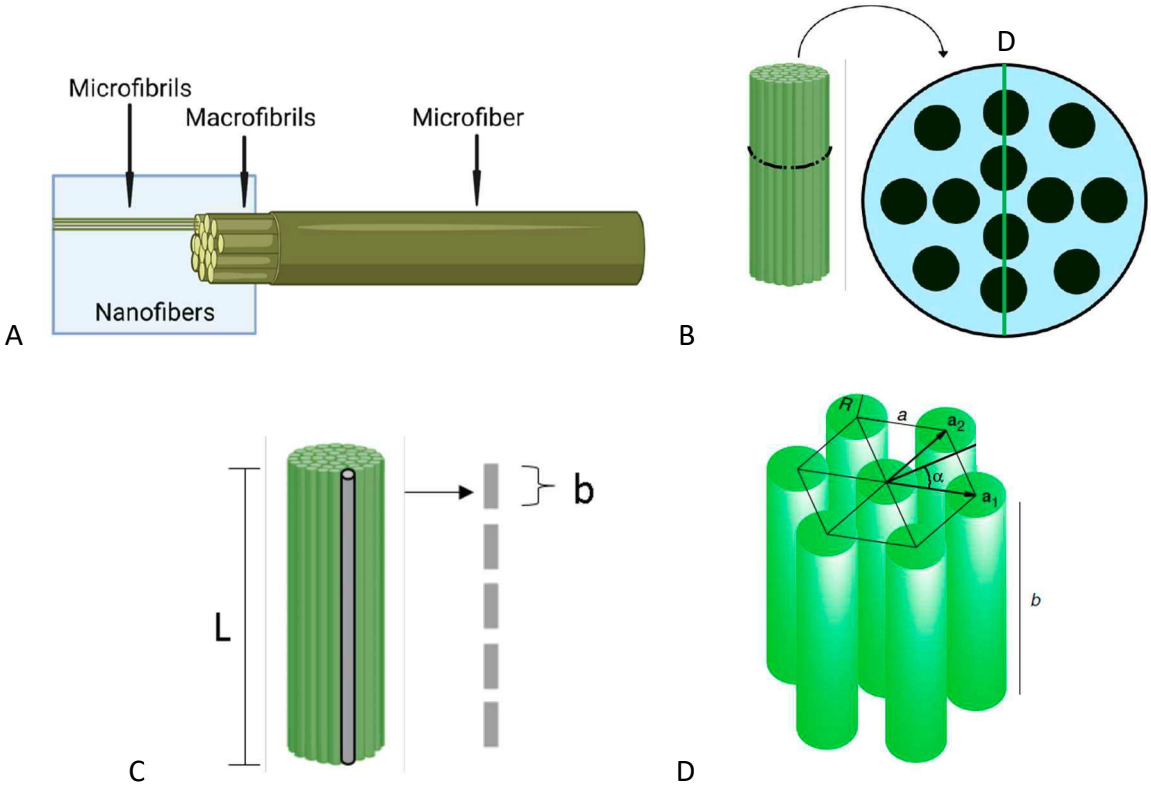

Figure 5.3. *Microfibres model created for this experiment: representation of the internal and external structure of one microfibre (A); longitudinal macrofibril structure with considered parameters (B); transversal section of macrofibril with the two-dimensional hexagonal lattice of several microfibrils (black circle) surrounding by water (C) and transversal section of microfibrils with considered parameters (D).*

5.3. Results

All data measured at the SAXS beamline of the Elettra synchrotron for the MFs and CTRL samples were systematically and successfully analysed with the GENFIT software by using the microfibers model previously described (Fig 5.4 and Fig 5.6). This thesis described in detail only the results for cellulose acetate MFs (CA) in ASW and FW.

Macrofibrils (MAFs) of CA in ASW have a diameter *D* of about 271 nm (Fig 5.5 J-P), which during the irradiation time didn't change (Fig 5.5 J-P). Considering microfibrils' total length *L*, the length of rigid segments *b* and the number of segments n_b remain the same: L ≈ 117 µm, b ≈ 2000 Å =200 nm and n_b=585. They didn't show also modification along the irradiation time: the average radius *R* is from 40 to 41 Å (4-4.1 nm) from t_0 to t_{42}, the number of MIFs N_a, their distance *a* and distribution in lattice g_a are the same (Fig 5.5 A-I).

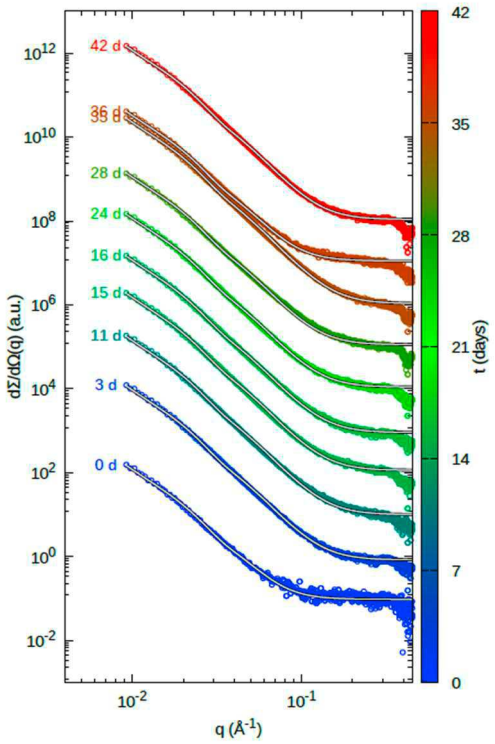

Figure 5.4. *SAXS curves of the microfibers of cellulose acetate in artificial seawater were recorded as a function of the irradiation time (from 0 to 42 d). The colour of the points is selected according to the vertical colour box representing the irradiation time (days). Solid grey lines over dots are the best fits obtained with the software GENFIT.*

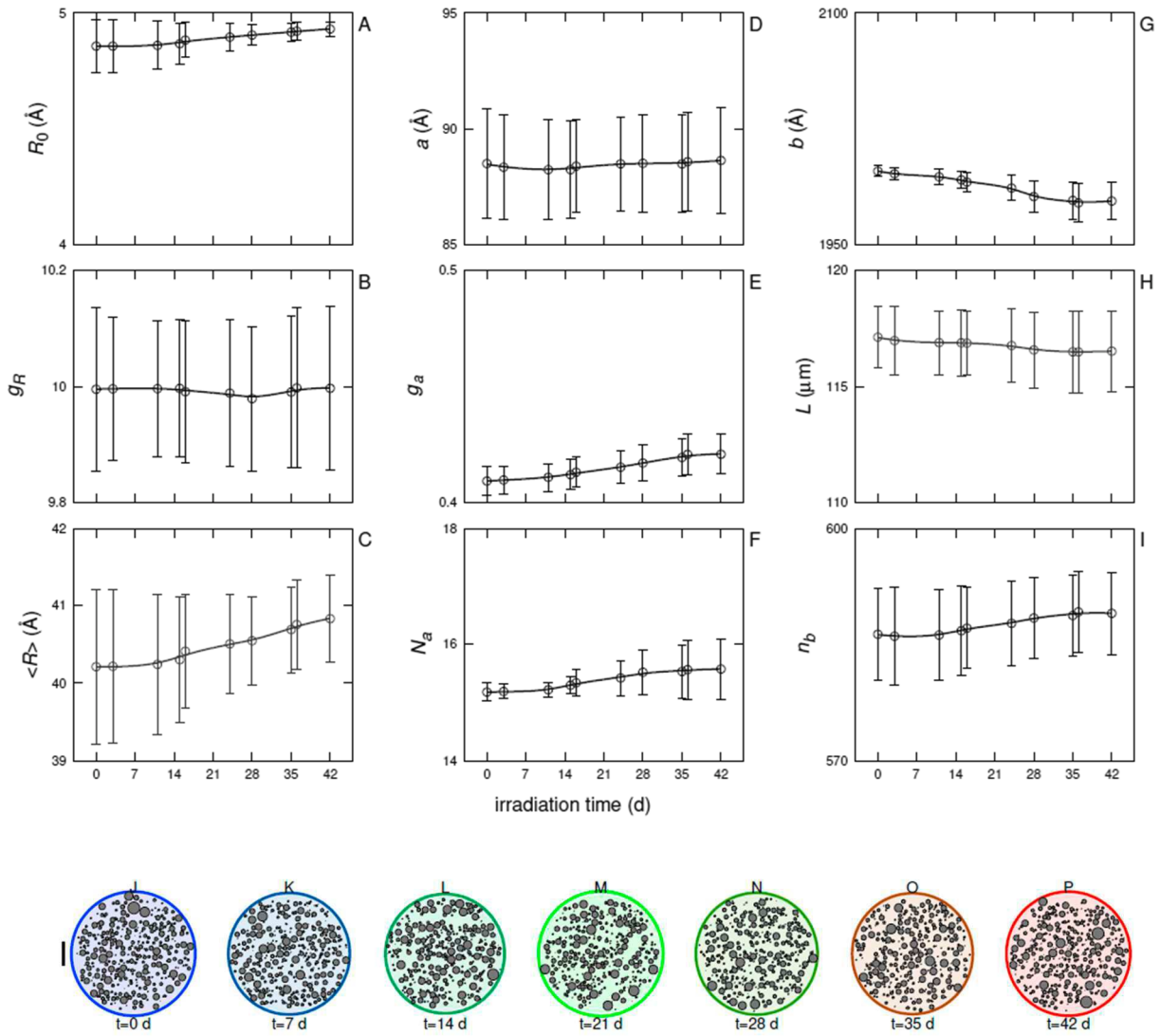

Figure 5.5. *Parameters of the microfibers model used for cellulose acetate MFs in artificial seawater reported as a function of the irradiation time. Curves among the points have been obtained with cubic splines weighted with uncertainties of the parameters (Panels A-I). Representation of the average microfibril transversal section according to the parameters. The small grey circles, representing the microfibrils, which have been drawn with a radius sampled by the fitted Gaussian poly-dispersed distribution and in a position sampled according to the bi-dimensional para-crystal fitting parameters a (D), g_a (E) and N_a (F). The vertical black line on the left of panel J represents the length of 500 Å= 50 nm (J-P).*

A completely different situation is present in CA in FW. The macrofibril diameter is 50 nm at t_0 (5 times smaller than D_{t0} of CA in ASW) and increases linearly along exposure time until 360 nm at t_{42} (1.3 times bigger than D_{t42} of CA in ASW) (Fig. 5.7 J-P). Considering microfibrils *L*, *b* and n_b remain constant along time (*L* = 90-91 µm, *b* = 1600 Å =160 nm and n_b=570). Their average radius *R* increase from 18 Å (1.8 nm) to 50 Å (5 nm), the number of MIFs N_a starts with 12, at t_{14} there is a weak reduction and then an increase until t_{14}, the distance between MIFs (*a*) increases from 20 Å (2 nm) to 120 Å (12 nm), and the g_a decreases slowly when the irradiation time passes, showing a more ordered distribution of microfibrils (Fig. 5.7 A-I).

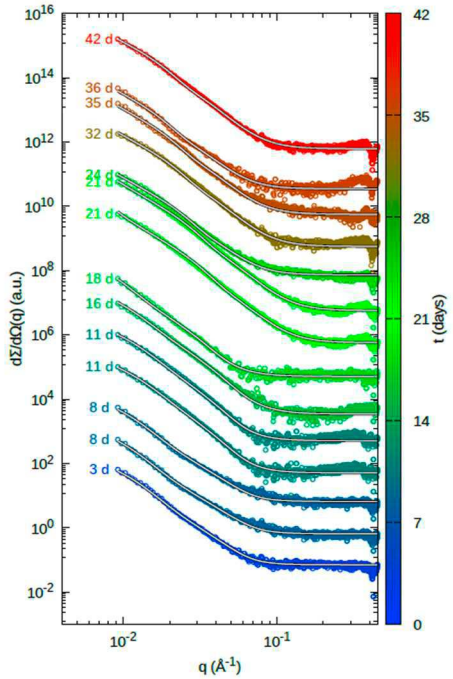

Figure 5.6 *SAXS curves of the microfibers of cellulose acetate in artificial freshwater recorded as a function of the irradiation time (from 0 to 42 d). The colour of the points is selected according to the vertical colour box representing the irradiation time (days). Solid grey lines over dots are the best fits obtained with the software GENFIT.*

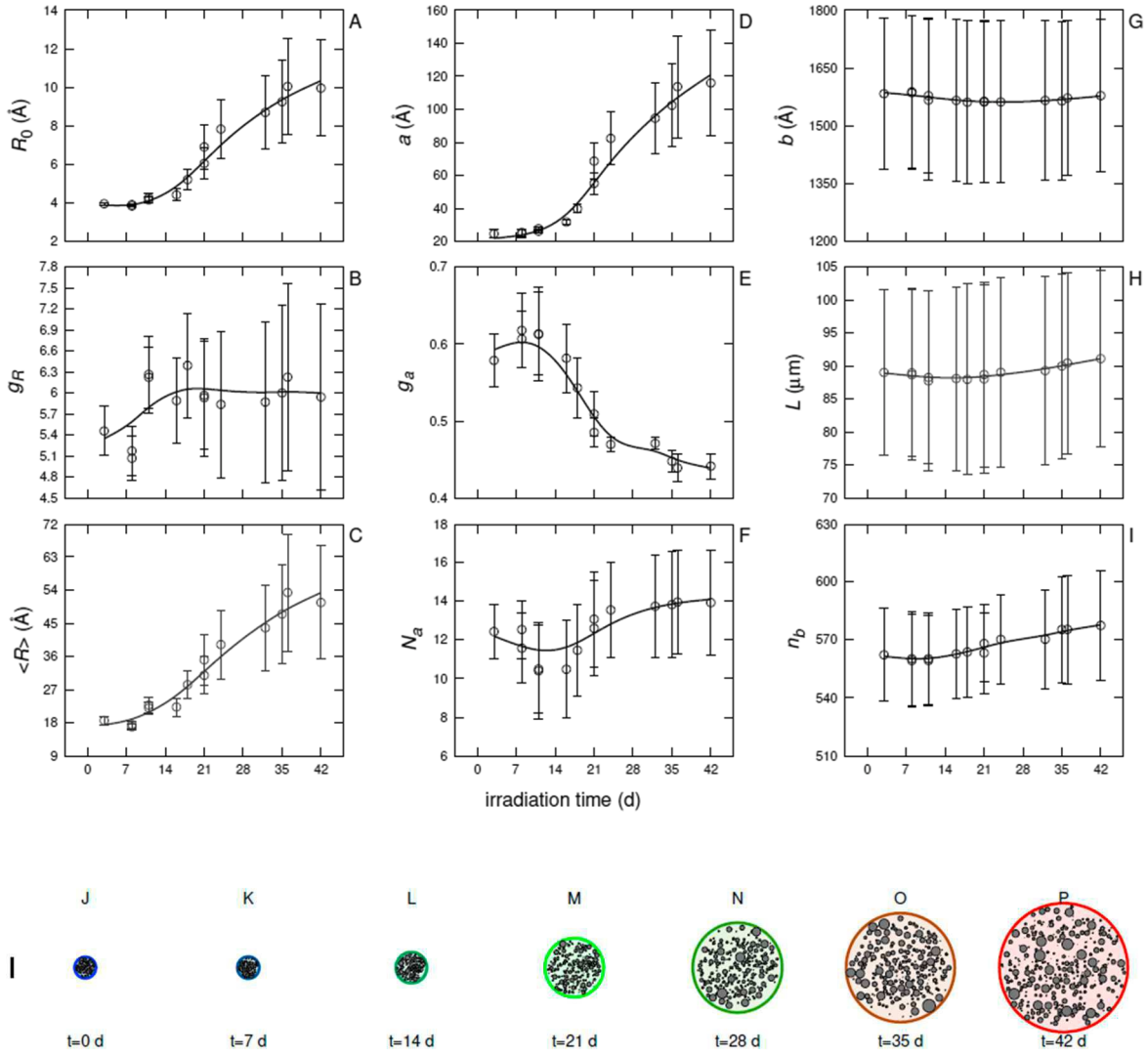

Figure 5.7. *Parameters of the microfibers model used for cellulose acetate MFs in freshwater reported as a function of the irradiation time. Curves among the points have been obtained with cubic splines weighted with uncertainties of the parameters (A-I). Representation of the average microfibril transversal section according to the parameters. The small grey circles, representing the microfibrils, which have been drawn with a radius sampled by the fitted Gaussian poly-dispersed distribution and in a position sampled according to the bi-dimensional para-crystal fitting parameters a (D), g_a (E) and N_a (F). The vertical black line on the left of panel J represents the length of 500 Å= 50 nm (J-P).*

5.4. Discussion and conclusions

The simulation of one year of natural solar irradiation, normally present on European region, revealed different effects on MFs of CB filters which depend on the typology of water in which they are spiked. In seawater, CA didn't show morphological/structural modification related to photodegradation, while in freshwater morphological changes were presented during the exposure time. SAXS results demonstrated that macrofibrils of CA, in seawater, immediately increased their diameter due to the permeation of water inside which cause the distancing between microfibrils. The entrance of water might deposit some particles of salt forming crystals which didn't allow UV rays to arrive on the surface of MFs and react with double bonds of CA structure with consequent free radicals' generation. Moreover, less concentration of oxygen dissolved in seawater could contribute to a less photooxidation process, which in other environments, contributes largely during the degradation of polymer (Cooper & Corcoran, 2010). On the contrary, MFs in freshwater showed changes in the internal structure with an increase of macrofibrils diameter along solar exposure and some modification of microfibrils conformation. The combined action between water absorption and photooxidation, which is favourite by a higher concentration of oxygen in FW, caused the increase of microfibrils distance and number. Another hypothesis for justifying this different behaviour of CA in FW and ASW is the contribution of chemical hydrolysis which is usually not a significant mechanism for the degradation of most commercial plastics in seawater (Andrady, 2011). The hydrolysis of acetate groups of CA and photooxidation are essential for enhancing the biodegradation process made by microorganisms, permitting cellulases and deacetylases of some microbes to modify CA structure. The microbial species (such as bacteria, fungi and yeast) that can metabolize this polymer are present in nature, also in the marine environment (Yadav & Hakkarainen, 2021), but their activities depend on many factors such as the degree of acetylation of cellulose, external temperature and organic content in the environment (Bonanomi et al., 2015; Yadav & Hakkarainen, 2021). In our study, we have not analysed the proliferation of microorganisms in samples and their effects on MFs but, due to the absence of aseptic conditions, during the photodegradation experiment, their contribution to different degradation of CA in FW and ASW could be possible. All of these considerations suggest a longer degradation time of CA in seawater than in freshwater which was already supposed by Kadir & Sarani (2015) and Niaounakis (2017). Considering all these aspects CB filters can be consider as a source of CA MFs in the aquatic environment and their complete degradation could be influenced by many factors (Puls et al., 2011; Joly & Coulis, 2018; Gerritze et al., 2020; Belzagui et al., 2021).

Woodall et al. (2014) reported CA MFs, attributable much more to CB filters than other sources of contamination, as the most abundant synthetic polymers analysed in several sediments around the world. Despite this evidence, most publications considered only textile MFs as contaminants of emerging concern (Kwak et al., 2022), mentioning CBs as only an example of bad littering behaviour (SAPEA, 2019). Therefore, given the slow degradation rate of cigarette filters, especially in marine water, and the constant and incorrect attitude of smokers to throw CBs into the environment, it is fundamental to give adequate importance to CBs as a potential enormous source of MFs. In this regard, this study offered another point of view about their permanence for a long time in the aquatic environment, posing a possible threat to organisms because entire CBs or their MFs could be ingested. Moreover, the slow degradability of CBs permits them to continue releasing chemical substances trapped in their filter and spread CA MFs and possibly nanoplastics, which are considered highly dangerous for aquatic organisms (Gong et al., 2023).

In conclusion, this work was an overview to understand the mechanisms of degradation of CA MFs in marine and freshwater, simulating a realistic solar irradiation and analysing samples with the advanced technique SAXS, never used before for this application. Additional studies will be needed to investigate how microorganisms can contribute to the degradation process in a more environmentally realistic scenario, simulating either more than one year of natural solar irradiation or the photodegradation at different death of the water column. In fact, this degradation process is the first and one of the most important for degrading polymers but can act when plastics are in surface or at first metres of water column where light can arrive. It could be, also, interesting to know the chemical kinetics of substances eluted from MFs of smoked and unsmoked CBs during photodegradation and evaluate if these MFs can cause ecotoxicological effects on marine organisms.

6. General conclusions and future perspectives

The general objective of this PhD thesis was the assessment of CBs impact in marine ecosystems, following an ecotoxicological approach based on the integration of several parameters: chemical, biological and physical analyses.

Laboratory experiments showed that cigarette butts can release metals and organic compounds, such as nicotine, cotinine, PAHs and AHs in seawater after 24 hours, with concentrations that are not always related to the number of CBs used for prepared CB leachates (25 and 100 CBs/L) but influenced by different factors, such as CBs brands, smokers' behaviour and possible environmental contamination. The released chemicals can induce significant toxic effects on different species causing a reduction of bioluminescence in *Aliivibrio fischeri*, alteration of larvae development of *Crassostrea gigas* and growth inhibition of *Phaeodactylum tricornutum*, *Skeletonema costatum* and *Dunaliella tertiolecta*. CBs leachate can determine significant modulation of expression of genes involved in lipid peroxidation and oxidative stress responses in PCTS of *Mytilus galloprovincialis* digestive gland. Moreover, mussels exposed for 14 days to different concentrations of CBs accumulate significant levels of PAHs and nicotine, positively correlated with CBs concentrations and the substances and show several alterations of immunological efficiency, antioxidant defences, lipid metabolism, genotoxicity and neurotoxic system. The use of Weight of Evidence elaboration was helpful to clarify the CBs risk for marine organisms considering the bioassays results, the bioavailability of CBs substances and biomarkers in mussels.

The exposure of the three species *M. galloprovincialis*, *Perinereis aibuhitensis* and juveniles of *Dicentrarchus labrax* to low concentrations of CBs and in environmental-like conditions was a further step towards the comprehension of CBs environmental impact and to observe if the interaction of species could modulate the effects of this waste. Even at low CBs concentrations, species present some responses due to CBs exposure, with bioaccumulation of chemicals and biological effects related to CB eluted substances.

The experiment of one year of solar irradiation simulation was carried out for evaluating how cellulose acetate MFs could be degraded in sea and freshwater. No visible effects on one year of photodegradation are presented in MFs in seawater, confirming the estimations of their slow degradability in marine environment compared to other conditions like freshwater or soil, with a possibly increase of threat for marine organisms related to their ingestions.

Observing the research results, the ecotoxicological approach based on integration of different Line of evidence analyses is useful to investigate and clarify the impact of cigarette butts on marine organisms. Further analyses are needed to grow the knowledge about this contaminant of emerging concern including the assess of CBs concentrations, levels of released chemicals and MFs in marine environmental compartments (surface, water column and sediment), investigating possible chronic effects of CBs exposure on different marine species belonging to several trophic levels and studying the interactions of this litter with other marine contaminants or multiple stressors.

All these considerations about CBs impacts are important for raising smokers' environmental awareness, informing society about the risk to human and environmental health and suggesting more efficient public management of this waste for preventing and reducing their abandonment and accumulation in the environment, especially in the sea.

7. References

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8. Supplementary materials

SM1 Analytical procedures of chemical analyses in CB leachates

For trace metals detection, CB leachates were acidified with 20% of nitric acid (to 20 mL of sample were added 5 mL of nitric acid). Aluminium, arsenic, cadmium, chromium, nickel, lead and vanadium were determined by atomic absorption spectrophotometry with graphite furnace atomization and Zeeman effect (Varian SpectrAA 240Z). copper, iron, manganese and zinc are measured by flame atomization (Varian SpectrAA 2420FS), while mercury (Hg) by specific analyzer based on the formation of mercury cold vapors (Cetac Quick Trace Mercury Analyzer M6100).

For polycyclic aromatic hydrocarbons (PAHs) determination, aliquots of 50 mL of leachates were concentrated and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric and diode array detection. The PAHs were identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, acenaphthylene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight (HMW: fluoranthene, pyrene, benzo(a)anthracene, chrysene, 7,12-dimethyl benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3,c,d)pyrene).

For aliphatic hydrocarbons (AHs) detection, 50 mL of CBs leachates were purified with solid-phase extraction (Phenomenex Strata-X, 500 mg × 6 mL plus Phenomenex Strata-FL, 1000 mg × 6 mL) and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dryness. Samples were finally recovered with 1 mL of pure, analytical GC grade *n*-hexane and analysed with a gas chromatograph (Perkin Elmer) equipped with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25 µm-df) and a flame ionization detector. For quantitative determination, the system was calibrated with an unsaturated pair *n*-alkane standard mixture according to EN ISO 9377-3 (Fluka 68281).

Polychlorinated biphenyls, organo-halogenated pesticides and brominated flame retardants were analysed by gas chromatography-mass spectrometry, samples preparation was similar to that described for AHs analysis. For quantitative determination, specific solutions of different concentrations of pure analytical standards and mixtures of pure, known concentration of standards of every type of compounds, were used.

Determination of nicotine (NIC), cotinine (COT) and trans-3'-hydroxycotinine (3HC) in CB leachates was performed using UHPLC-MS Triple quadrupole technique. In brief, volumes of leachates (100 µL) were extracted and concentrated using solid phase extraction procedures (SPE), using Phenoenex Strata C18-E (500mg, 6mL), using standard procedure (water-methanol extraction and recovery in methanol), then samples were analysed using a Perkin Elmer QSight Triple Quadrupole system (220 series, Perkin Elmer). Quantitative determination was carried out by injecting 2 µl of supernatant and calibrating the system through the use of solutions at different concentrations of the pure analytical standards.

Table SM2. Sequences of forward and reverse primers and annealing temperatures used for qPCR analysis, amplicon sizes and accession numbers of genes

Gene	Primer sequences	Annealing T	Amplicon size	Accession n.
cat	F: CGACCAGAGACAACCCACC[a] R: GCAGTAGTATGCCTGTCCATCC[a]	55°C	132 bp	AY743716
gst-pi	F: TCCAGTTAGAGGCCGAGCTGA[b] R: CTGCACCAGTTGGAAACCGTC[b]	55°C	172 bp	AF527010
Se-gpx	F: AGCCTCTCTCTGAGGAACAACTG R: TGGTCGAACATGCTCAAGGGC	55°C	166 bp	HQ891311
hsp70	F: GGTGGTGAAGACTTTGACAACAG[c] R: CTAGTTTGGCATCGCGTAGAGC[c]	62°C	295 bp	AY861684
Cu/Zn-sod	F: AGCCAATGCAGAGGGAAAAGCAGA[d] R: CCACAAGCCAGACGACCCCC[d]	65°C	177 bp	FM177867
acox1	F: ACAGTCGTGCAAAACAGGGAC R: CTGCTGCTTCAACCAACCTGG	62°C	153 bp	EF525542
mt10	F: GGGCGCCGACTGTAAATGTTC[e] R: CACGTTGAAGGCCCTGTACACC[e]	55°C	93 bp	AY566248
mt20	F: TGTGAAAGTGGCTGCGGA[e] R: GTACAGCCACATCCACACGC[e]	55°C	80 bp	AY566247
cyp3A1	F: TGAACTCGCAAAAAGAACCA[f] R: GGAACACTGGAGCCTTGAAC[f]	55°C	80 bp	AY566247

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SM3 Analytical methods for measurements of chemicals in animals' tissues

For trace metals detection, 3 g (wet weight) of tissues were homogenized, dried to constant weight at 60°C and digested under pressure with nitric acid and hydrogen peroxide (7:1) with microwave. Aluminium, arsenic, cadmium, chromium, copper, iron, lead, manganese, nickel, vanadium and zinc were analysed by atomic absorption spectrophotometry, with flame (Varian, SpectrAA 220FS) and flameless atomization (Varian SpectrAA 240Z); the mercury content was quantified by cold vapor atomic absorption spectrometry (Cetac QuickTrace M-6100 Mercury Analyzer).

For analysis of polycyclic aromatic hydrocarbons (PAHs), about 3 g (wet weight) of tissues were homogenized and extracted in 10 mL 0.5 M potassium hydroxide in methanol with microwave at 55°C for 20 min (800 Watt) (CEM, Mars System). After centrifugation at 3000 × g for 10 min, the methanolic solutions were concentrated in a SpeedVac and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric detection and diode array detection. The PAHs were identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight (HMW: fluoranthene, pyrene, benzo[a]anthracene, chrysene, 7,12-dimethyl benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3,c,d]pyrene). For quantitative determination, specific solutions of different concentrations of pure analytical standards and mixtures of pure, known concentration of standards of every type of compounds, were used.

For nicotine (NIC) and cotinine (COT) quantification, 20 g (wet weight) of tissues were homogenized and extracted in water and methanol and then filtered. The samples were injected directly in the

instrument (HPLC-ORBITRAP) where exact masses of NIC and COT were monitored and confirmed with MS/MS spectrum. NIC and COT were identified and quantified according to an appropriate calibration of the instrument with standard solutions.

SM4 Biomarker analyses

Lysosomal membrane stability (NRRT) was evaluated after the haemocytes incubation on a glass slide with a freshly prepared Neutral Red (NR) working solution (2µl/ml filtered sea water from a stock solution of 20 mg NR dye dissolved in 1 ml of dimethyl sulfoxide) and microscopically examined at 20 min intervals to determine the time at which 50% of cells had lost into the cytosol the dye previously taken up by lysosomes.

For the analysis of granulocytes-hyalinocytes ratio, aliquot of haemolymph was dispersed on a glass slide and, after drying, fixed in Beker's fixative. The slides were washed with water and stained with May-Grunwald Giemsa before mounting in glycerol gelatine and observed with a light microscope (1,000 ×).

The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the Comet assay, and at chromosomal level by the micronucleus test. The comet assay was carried out on mussels haemocytes, polychaetes coelomocytes and seabass blood cells included in 1% normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with 1 µg/ml 4',6-diamidino-2-phenylindole; 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 x magnification; Olympus BX-51), and the captured images (Image-Pro-Plus package) were analysed by the Comet Score software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

Micronuclei (MN) frequency was measured in haemocytes, coelomocytes and blood cells fixed in Carnoy's solution (3:1 ethanol, acetic acid), dispersed on glass slides and stained with the fluorescent dye 40,6-diamidino-2-phenylindole (DAPI) at 100 ng ml-1. For each specimen, 2000 cells with preserved cytoplasm were scored for the presence of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter, on the same optical plan and clearly separated from it.

Acetylcholinesterase activity (AChE) was spectrophotometrically assayed in mussels haemolymph and polychaetes coelomocytes (centrifuged at 3,000 x g for 5 min) and mussels gills, polychaetes

whole tissue and seabass head, gills and muscle (homogenized in 0.1 M Tris-HCl buffer pH 7.2, 0.25 M sucrose and centrifuged at 10000 x g for 10 min). Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18±1°C. For all the samples, AChE activity (normalized to content of protein) was calculated by dividing the AChE values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

For the activity of Acyl CoA oxidase (ACOX) samples of mussels digestive gland and whole tissue of polychaetes were homogenized in 1mM sodium bicarbonate buffer (pH 7.6) containing 1mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 x g for 15 min at 4 °C. The H_2O_2 production was measured in a coupled assay by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalysed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 µM sodium azide, 0.01% Triton X-100, 1.2 U/ml HRP in a final volume of 1 ml. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrate Palmitoyl-CoA, at final concentrations of 30 µM; readings were carried out against a blank without the substrate at 502 nm. For all the samples, ACOX activity (normalized to content of protein) was calculated by dividing the ACOX values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

For the analysis of ethoxyresorufin O-deethylase (EROD), seabass livers were homogenized in 100 mM K-phosphate buffer pH 7.5, 0.15M KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)and centrifuged at 12000 x g for 15 min. The resulting supernatants (S9) were immediately incubated at 30°C in a final volume of 1 ml containing 100 mM K-phosphate buffer pH 7.5, 4µM 7-ethoxyresorufin, and 0.25 mM β-nicotinamide adenine dinucleotide (NADPH) for 5 min, before addition of 2 ml acetone to stop the reaction. Incubation mixtures as mentioned previously but stopped at time zero were used as blank value and subtracted from the sample fluorescence. Fluorimetric analyses (535/585 nm) were quantified by reference to resorufin standards (0.02–1µM). For all the samples, EROD activity (normalized to content of protein) was calculated by dividing the EROD values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

Malondialdehyde (MDA) was quantified in mussels digestive glands homogenized in 20 mM Tris–HCl (pH 7.4), centrifuged at 3000 × g for 20 min. A conjugation reaction was performed in 1 ml

reaction mixture (45 °C, 40 min), containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1), 32% HCl, 100 µl water and 100 µl of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl [pH 7.4]). Samples were finally cooled on ice, centrifuged at 15000 × g for 10 min and spectrophotometrically analysed at 586 nm. MDA concentrations were determined as a function of the 1, 1, 3, 3-tetramethoxypropane standard curve and expressed as nmol/g tissue.

For enzymatic antioxidants, samples of mussels digestive gland, polychaetes whole tissue and seabass liver were homogenized in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 mg/ml leupeptin, 0.5 mg/ml pepstatin, NaCl 2.5%, and centrifuged at 110000 x g for 1 h at 4 °C. Measurements were made with spectrophotometer at a constant temperature of 18 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm due to the consumption of 12 mM H2O2 in 100 mM K-phosphate buffer pH 7.0. Glutathione reductase (GR) was determined at 340 nm, from NADPH oxidation during the reduction of 1 mM GSSG in 100 mM K-phosphate buffer pH 7.0 and 60 mM NADPH. Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates respectively for the Se-dependent and for the sum of Se-dependent and Se-independent forms. Glutathione S-transferases (GST) were determined at 340 nm using 1.5 mM 1-chloro-2,4-dinitrobenzene as substrate (CDNB) and 1 mM GSH, in 100 mM K-phosphate buffer pH 6.5. For all the samples, CAT, GR, GST and GPx activity (normalized to content of protein) was calculated by dividing the enzymatic activity values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

Total glutathione was enzymatically assayed in supernatant samples obtained after the homogenization in 5% sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37000 x g for 15 min.

The Total Oxyradical Scavenging Capacity (TOSC) was measured in mussel digestive glands, polychaetes whole tissue and seabass liver homogenized as previously reported for enzymatic

antioxidants, without PMSF in the homogenization buffer. The artificially generated radicals (ROO·and OH) were obtained from the thermal homolysis of 2-2-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) and the iron (plus ascorbate)-driven Fenton reaction, respectively. The absorption of artificially generated oxyradicals by cellular antioxidants was measured by the quantification of inhibited oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Ethylene formation was determined by gas-chromatographic analyses and TOSC values were quantified from the equation: TOSC = 100-(∫SA/ ∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

Metallothioneins (MTs) were analysed in mussels digestive gland samples homogenized (1:5 w/v) in 20 mM Tris–HCl buffer, pH 8.6, 0.5M sucrose, 0.006mM leupeptin, 0.5 mM PMSF (phenylmethylsulphonylfluoride), and 0.01% b-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MTs were quantified by the spectrophotometric assay using GSH as standard. For all the samples, MT activity (normalized to content of protein) was calculated by dividing the MT values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

Table SM6. *Concentrations (µg/L) of individual congeners of aliphatic hydrocarbons (C10-C40) and polycyclic aromatic hydrocarbons (from naphthalene to indeno(1,2,3,c,d)pyrene) in blank and CB leachates. Values are expressed as mean ± standard deviation.*

Chemical compound	**BLANK**	**LEAC_A**	**LEAC_B**
C10-C12	<10	176± 37.0	8.57 ± 2.93
C12-C14	<10	89.3 ± 88.8	2502 ± 1318
C14-C16	17.9 ± 6.51	78.9 ± 27.8	77.4 ± 23.4
C16-C18	172 ± 38.3	189 ± 156	380 ± 40.8
C18-C20	128 ± 7.43	167 ± 79.4	202 ± 8.58
C20-C22	50.3 ± 0.558	81.8 ± 68.7	77.6 ± 13.7
C22-C24	25.0 ± 3.29	36.6 ± 21.7	39.7 ± 2.61
C24-C26	18.2 ± 2.15	23.5 ± 19.8	22.6 ± 6.06
C26-C28	<10	29.0 ± 20.2	23.0 ± 4.72
C28-C30	<10	29.8 ± 23.6	15.6 ± 3.87
C30-C32	76.7 ± 7.75	44.7 ± 32.4	40.7 ± 16.1
C32-C34	<10	<10	<10
C34-C36	<10	<10	<10
C36-C38	<10	31.0	5.76 ± 0.792
C38-C40	<10	<10	<10
naphthalene	<0.002	<0.002	<0.002
acenaphthylene	<0.001	<0.001	<0.001
1-methyl naphthalene	<0.002	<0.002	0.160 ± 0.043
2-methyl naphthalene	<0.002	0.485 ± 0.375	0.062 ± 0.045
acenaphthene	<0.0002	<0.0002	<0.0002
fluorene	<0.0002	<0.0002	<0.0002
phenanthrene	<0.0002	0.019 ± 0.011	0.007 ± 0.001
anthracene	<0.0002	0.002 ± 0.001	0.011
fluoranthene	<0.0002	<0.0002	<0.0002
pyrene	<0.0002	0.013 ± 0.016	0.0007
Benzo[a]anthracene	<0.0002	<0.0002	<0.0002
chrysene	<0.0002	<0.0002	<0.0002
7,12-dimethyl benzo[a]anthracene	<0.001	<0.001	<0.001
Benzo[b]fluoranthene	<0.0002	<0.0002	<0.0002
Benzo[k]fluoranthene	<0.0002	<0.0002	<0.0002
Benzo[a]pyrene	<0.0002	<0.0002	0.001 ± 2.83E-05
Dibenzo[a,h]anthracene	<0.0002	<0.0002	0.007 ± 0.005
Benzo[g,h,i]perylene	<0.0002	<0.0002	<0.0002
Indeno[1,2,3,c,d]pyrene	<0.001	<0.001	<0.001

Table SM7. *Viability of PCTS evaluated after 24 h, 48 h and 72 h incubation in CTRL (Blank Control) and LEAC_A. Mean values ± standard deviation (n=3) are expressed as percentage over C (Control).*

Exposure solution and time	**%**
CTRL 24h	97 ± 16
CTRL 48h	93 ± 11
CTRL 72h	90 ± 15
LEAC_A 24h	102 ± 20
LEAC_A 48h	113 ± 20
LEAC_A 72h	88 ± 25

Table SM8. *Concentrations (ng/g dry weight) of individual congeners of polycyclic aromatic hydrocarbons (from naphthalene to indeno(1,2,3,c,d)pyrene) in the whole tissues of M. galloprovincialis exposed to experimental conditions. Values are given in ng/g dry weight (average ± standard deviation, n=3).*

Chemical compound	**CTRL**	**5 CBs/L**	**1 CBs/L**	**5 CBs/L**
naphthalene	46.6 ± 6.88	96.1 ± 16.1	130 ± 10.8	304 ± 57.8
acenaphthylene	<0.05	<0.05	<0.05	<0.05
1-methyl naphthalene	105 ± 87.0	138 ± 102	259 ± 20.6	220 ± 113
2-methyl naphthalene	25.6 ± 2.80	26.9 ± 2.57	27.4 ± 1.54	27.1 ± 4.05
acenaphthene	<0.01	<0.01	<0.01	<0.01
fluorene	<0.01	6.62 ± 1.36	7.47 ± 1.88	17.1 ± 3.44
phenanthrene	23.0 ± 4.79	24.8 ± 1.14	25.8 ± 2.60	32.3 ± 16.4
anthracene	<0.01	0.518 ± 0.123	0.556 ± 0.260	1.36 ± 0.516
fluoranthene	<0.01	<0.01	8.92 ± 5.71	4.22 ± 1.91
pyrene	2.53 ± 0.178	3.06 ± 0.736	2.43 ± 0.821	2.64 ± 0.347
benzo(a)anthracene	1.66± 0.084	1.72 ± 0.126	1.74 ± 0.112	1.32 ± 0.176
chrysene	1.95 ± 0.100	2.15 ± 0.071	2.17 ± 0.206	1.45 ± 1.22
7,12-dimethyl benzo(a)anthracene	<0.05	<0.05	<0.05	<0.05
Benzo[b]fluoranthene	4.31 ± 0.460	3.88 ± 0.424	4.24 ± 0.402	3.48 ± 0.621
Benzo[k]fluoranthene	3.04 ± 0.376	2.65 ± 0.309	3.13 ± 0.341	2.62 ± 0.400
Benzo[a]pyrene	3.44 ± 0.557	2.91 ± 0.391	3.38 ± 0.602	2.82 ± 0.369
Dibenzo[a,h]anthracene	<0.001	<0.001	<0.001	<0.001
Benzo[g,h,i]perylene	1.93 ± 1.76	1.21 ± 1.34	2.32 ± 1.56	2.46 ± 0.190
Indeno[1,2,3,c,d]pyrene	0.932 ± 0.189	0.911 ± 0.187	1.59 ± 0.983	1.63 ± 1.25

Table SM9. *Review of A. fischeri, P. tricornutum, S. costatum, D. tertiolecta and C. gigas embryos sensitivity to single chemical compound. For microalgae the EC50 values considered were only those calculated after 72 hours of exposure.*

Chemical compound	***A. fischeri***	***P. tricornutum***	***S. costatum***	***D. tertiolecta***	***C. gigas***
As	768 µg/L (Ishaque et al., 2006)				48 h EC_{50} = 326 µg/L (Martin et al., 1981)
Cd	508 µg/L (Ishaque et al., 2006)	5368 µg/L (Horvatić & Peršić, 2007)			24 h EC_{50} = 212 µg/L, Mai et al., 2012
Cu	250 µg/L (Tsiridis et al., 2006)	35 µg/L (Moreno-Garrido et al., 2000); 10 µg/L (Franklin et al., 2001)	27 µg/L (Ward and Boeri, 1990)	530 µg/L (Levy et al., 2007)	24 h EC_{50} = 12.5 µg/L (Mai et al., 2012); 7.35 µg/L (Gamain et al., 2016)
Hg	109 µg/L (Ishaque et al., 2006)				
Ni		7280 µg/L (Horvatić & Peršić, 2007)			48 h EC_{50} = 349 µg/L (Martin et al., 1981)
Pb	480 µg/L (Tsiridis et al., 2006); 455 µg/L (Ishaque et al., 2006)				
Zn	1500 µg/L (Tsiridis et al., 2006)	41850 µg/L (Horvatić & Peršić, 2007)			48 h EC_{50} = 119 µg/L (Martin et al., 1981)

Tsiridis, V., Petala, M., Samaras, P., Hadjispyrou, S., Sakellaropoulos, G., & Kungolos, A. (2006). Interactive toxic effects of heavy metals and humic acids on *Vibrio fischeri*. Ecotoxicology and Environmental Safety, 63 (1), 158-167. https://doi.org/10.1016/j.ecoenv.2005.04.005

Horvatić, J., & Peršić, V. (2007) The effect of Ni2+, Co2+, Zn2+, Cd2+ and Hg2+ on the growth rate of marine diatom *Phaeodactylum tricornutum* Bohlin: microplate growth inhibition test. Bulletin of Environmental Contamination and Toxicology, 79 (5), 494-8. 10.1007/s00128-007-9291-7 Moreno-Garrido, I., Lubián, L.M., & Soares, A. (2000). Influence of cellular density on determination of EC50 in microalgal growth inhibition tests. Ecotoxicology and Environmental Safety, 47 (2), 112–116. https://doi.org/10.1006/eesa.2000.1953

Franklin, N.M., Stauber, J.L., Lim, R.P. (2001). Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. Environmental Toxicology and Chemistry, 20 (1), 160-170.

Ward, T., & Boeri, R. (1990). Acute static toxicity of nonylphenol to the marine alga, *Skeletonema costatum*. Envirosystems Study 8970-CMA. Final Technical Report. Chemical Manufacturers Association, Hampton, NH, USA.

Martin, M., Osborn, K.E., Billig, P., Glickstein, N. (1981). Toxicities of ten metals to Crassostrea gigas and Mytilus edulis embryos and Cancer magister larvae. Marine Pollution Bulletin 12 (9), 305–308. https://doi.org/10.1016/0025-326X(81)90081-3

Mai, H., Cachot, J., Brune, J., Geffard, O., Belles, A., Budzinski, H., & Morin, B. (2012). Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*). Marine Pollution Bulletin, 64(12), 2663–2670. https://doi.org/10.1016/j.marpolbul.2012.10.009

Table SM10. *Concentrations (ng/g dry weight) of individual congeners of polycyclic aromatic hydrocarbons (from naphthalene to indeno[1,2,3,c,d]pyrene) in the M. galloprovincialis, P. aibuhitensis and juveniles of D. labrax exposed to CBs alone. Values are given in ng/g dry weight (average ± standard deviation, n=3).*

Chemical compound	***Mytilus galloprovincialis***		***Perinereis aibuhitensis***		***Dicentrarchus labrax***	
	CTRL	**0.5 CBs/L**	**CTRL**	**0.5 CBs/L**	**CTRL**	**0.05 CBs/L**
naphthalene	62.1	86.8 ± 14.6	54.8	92.4 ± 28.2	43.3	57.0
acenaphthylene	0.564	2.39 ± 0.797	2.09	1.41 ± 0.874	<0.05	<0.05
1-methyl naphthalene	15.0	0.463 ± 0.229	<0.1	10.2 ± 2.38	<0.1	<0.1
2-methyl naphthalene	17.1	17.1 ± 5.81	27.7	16.3 ± 3.70	14.6	6.27
acenaphthene	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
fluorene	1.23	2.37 ± 1.24	2.06	1.66 ± 0.453	1.55	0.701
phenanthrene	4.02	7.50 ± 0.974	7.96	5.80 ± 1.51	6.68	3.46
anthracene	<0.01	0.195 ± 0.069	0.217	0.487 ± 0.404	<0.01	<0.01
fluoranthene	0.172	0.799 ± 0.918	2.63	1.95 ± 1.63	<0.01	3.41
pyrene	0.343	1.04 ± 0.741	0.245	0.472 ± 0.044	2.361	<0.01
benzo(a)anthracene	<0.01	1.08 ± 0.304	<0.01	0.138 ± 0.035	0.560	0.823
chrysene	0.032	<0.01	0.080	0.058 ± 0.009	<0.01	<0.01
7,12-dimethyl benzo[a]anthracene	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Benzo[b]fluoranthene	0.065	<0.001	0.192	0.127 ± 0.047	0.220	0.268
Benzo[k]fluoranthene	0.063	0.129 ± 0.047	0.097	0.063 ± 0.020	<0.001	<0.001
Benzo[a]pyrene	0.056	0.058 ± 0.021	< 0.066	0.071 ± 0.018	<0.001	<0.001
Dibenzo[a,h]anthracene	0.280	< 0.001	0.278	0.0313 ± 0.059	0.412	<0.001
Benzo[g,h,i]perylene	0.163	0.184 ± 0.007	0.183	1.67 ± 0.001	<0.001	<0.001
Indeno[1,2,3,c,d]pyrene	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Table SM11. *Concentrations (ng/g dry weight) of individual congeners of polycyclic aromatic hydrocarbons (from naphthalene to indeno[1,2,3,c,d)pyrene) in tissue of M. galloprovincialis and juveniles of D. labrax exposed to CBs in mesocosm approach Values are given in ng/g dry weight (average ± standard deviation, n=3).*

Chemical compound	***Mytilus galloprovincialis***		***Dicentrarchus labrax***	
	CTRL	**0.1 CBs/L**	**CTRL**	**0.1 CBs/L**
naphthalene	55.6	86.1 ± 18.8	66.3	66.8
acenaphthylene	<0.05	<0.05	<0.05	<0.05
1-methyl naphthalene	0.314	<0.1	<0.1	<0.1
2-methyl naphthalene	21.7	28.0 ± 4.11	19.4	23.8
acenaphthene	<0.01	<0.01	<0.01	<0.01
fluorene	1.59	2.07 ± 0.409	2.05	2.78
phenanthrene	7.25	7.73 ± 1.18	5.57	6.58
anthracene	0.181	0.505 ± 0.308	0.569	0.248
fluoranthene	1.60	0.714 ± 0.640	1.91	3.20
pyrene	0.396	1.22 ± 1.63	0.445	<0.01
benzo(a)anthracene	<0.01	<0.01	<0.01	<0.01
chrysene	0.033	<0.01	<0.01	<0.01
7,12-dimethyl benzo[a]anthracene	<0.05	<0.05	<0.05	<0.05
benzo[b]fluoranthene	<0.001	<0.001	0.114	0.307
enzo[k]fluoranthene	0.112	0.072 ± 0.010	<0.001	<0.001
benzo[a]pyrene	0.056	0.070 ± 0.015	0.059	0.067
dibenzo[a,h]anthracene	<0.001	<0.001	<0.001	0.281
benzo[g,h,i]perylene	0.162	0.190 ± 0.035	<0.001	0.194
Indeno[1,2,3,c,d]pyrene	<0.05	<0.05	<0.05	<0.05

APPENDIX

List of paper

This thesis is partly based on the following publications:

1. Lucia G., Giuliani M. E., d'Errico G., Booms E., Benedetti M., Di Carlo M., Fattorini D., Gorbi S., Regoli F. (2023). Toxicological effects of cigarette butts for marine organisms. Environment International, 171, 107733. https://doi.org/10.1016/j.envint.2023.107733

2. Lucia G., Nardi A., d'Errico G., Quevedo Zabala J., Benedetti M., Di Carlo M., Fattorini D., Regoli F., Gorbi S. (2023). Multispecies cellular responses to cigarette butts associated contaminats. In preparation

3. Piccinini A., Lucia G., Colarossi D., Pittura L., Principi P., Gorbi S., Regoli F., Spinozzi F. (2023). Photodegradation of microfibres in fresh and seawater: an investigation of degradation process under one year of simulated solar irradiation. In preparation

List of outreach activities and dissemination events

List of attended courses/seminars/schools

1. PLS activities: Seminars done for high school students in 2020-2021-2022

2. UNIVPM Orienta: Seminars carried out for high school students in 2020-2021-2022

3. Participation of 7th edition of Contamination LAB 2020-2021, a business school organized by UNOVPM and final victory as "the best business idea" proposing a reverse vending machine for cigarette butts

4. Local selection (1st classified) and national final competition of FameLab 2020, an international scientific communication competition

5. PANDORA Project with realization of a video about Environmental Risk communication "Dove comincia il mare", coordinating a team of 12 PhD students and researchers of DISVA department of UNIVPM

6. Fosforo la Festa della Scienza 2021-2022, a citizen science festival in which I proposed laboratory activities for children called "Raccogli, Setaccia & Ricicla", in which participants assumed the role of plastic researchers', simulating sand sieving in search of meso and microplastics.

7. Sharper 2021-2022-2023 with several activities related to contaminants of emerging concern and sustainable development with Agenda2030 goals

8. SostenibilMENTE, citizen science activities proposed and organized in collaboration with municipality of Ancona, 13-14 October 2021

9. Children laboratory activities organized during the exhibition "La cultura della plastica: arte, design, ambiente" for Museo Omero, Ancona

10. Conference "Sostenibilmente" for UniTRE of Corinaldo, Ancona where I have described my research activities and how much they were useful for contributing to Sostainable Development Goals of Agenda2030

11. Shot of Science (a series of seminars organised by DISVA). I made one called "Toxicological effects of cigarette butts for marine organisms"

12. Research Show, a new event of Sharper 2022, in which a researcher for any university has to prepare and propose a scientific show to students of middle and high school. I was chosen for representing UNVPM and I have prepared a show called “In plastichae menti”

13. Participation to Marevivo campaign «Piccoli gesti, grandi crimini» 2020-2021

14. Representative of DISVA to Sealogy in 2021, a trade fair entirely dedicated to the sea and its resources

15. Organization of laboratory activities for elementary and middle school students during World Water Day 2022

16. Participation to Climbing for Climate 2020-2021-2022, an event organized by the four University of Marche Region, annually, for promoting responsible and sustainable action against climate change and environmental pollution

17. Participation to Moby Litter 2020-2021-2022, an event organized by DISVA department for talking about the activities proposed for reducing plastic dispersion into environment and research results about plastic pollution in the sea

List of conferences/workshops attended and of contributions presented

1. SETAC Europe 31st Annual Meeting. Virtual conference 03 - 06 May 2021. Toxicity Effects of Cigarette Butt Leachates on Marine Sentinel Species. Lucia G., Gorbi S., Giuliani M. E., d’Errico, Di Carlo M., Fattorini D. & Regoli F. Oral presentation

2. 21st International Symposium on Pollutant Responses in Marine Organisms (PRIMO 21). Gothenburg, Sweden, 22 - 25 May 2022. Biological Effects Of Cigarette Butts On Marine Sentinel Species. Lucia G., Gorbi S., Giuliani M. E., d’Errico, Di Carlo M., Fattorini D. & Regoli F. Oral presentation

3. Ocean Hackathon 2020. 09-11 October 2020. Exploring the fate of textile nanofibers: from the washing machine to the sea. Piccinini A., Lucia G., Nardi A., Pittura L., Gorbi S., Spinozzi F. Oral presentation

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Toxicological effects of cigarette butts for marine organisms

Giulia Lucia [a], Maria Elisa Giuliani [a], Giuseppe d'Errico [a], Emily Booms [b], Maura Benedetti [a], Marta Di Carlo [a], Daniele Fattorini [a], Stefania Gorbi [a,c], Francesco Regoli [a,c,*]

[a] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, Ancona, Italy
[b] Ghent University, Krijgslaan, 281/S8 9000 Ghent, Belgium
[c] National Future Biodiversity Center (NFBC), Palermo, Italy

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ABSTRACT

Cigarette butts (CBs), one of the most common litter items found on beaches, represent a still unexplored environmental hazard. This study aimed at a multidisciplinary characterization of their toxicological risks on marine organisms integrating chemical analyses of released compounds with a wide panel of biological responses, such as ecotoxicological bioassays on species of different trophic positions, molecular responses in an *ex vivo* model (Precision-Cut Tissue Slices, PCTS of mussels digestive glands), bioavailability and cellular biomarkers in mussels exposed to CBs in laboratory experiments. Trace metals, aliphatic and polycyclic aromatic hydrocarbons, nicotine and cotinine were released in artificial seawater after 24 h which determined a significant inhibition of bacterial bioluminescence, oyster embryo development and growth in different algal species. Modulation of peroxisomal proliferation and antioxidant gene expression was observed in mussels PCTS, while the *in vivo* exposure determined accumulation of chemicals and significant alterations of immune system, antioxidant and neurotoxic responses, peroxisomal proliferation and genotoxic damage. Using a quantitative Weight of Evidence model, the risks of CBs to the marine environment were summarized, highlighting the importance of integrating chemical analyses, batteries of ecotoxicological bioassays, molecular and cellular biomarkers to assess the impact of these hazardous materials on marine environment.

1. Introduction

Approximately 5.7 trillion cigarettes are consumed worldwide every year and 4.5 trillion smoked cigarette butts (CBs) are thrown into the environment (dos Santos et al., 2017). CBs are the most common form of personal litter found in the streets, urban roads, public places and beaches where, according to the Ocean Conservancy report (2020), more than 4 million CBs were daily collected during cleaning activities in more than 100 countries. The presence of CBs on beaches depends on many factors, such as incorrect behaviour and littering, lack of environmental awareness, frequency of beachgoers, low efficiency of cleaning services, winds, currents and rivers (Araújo & Costa, 2019). Despite their small size, CBs can represent an environmental hazard for aquatic organisms since they contain more than 5000 chemicals such as nicotine, metals, polycyclic aromatic hydrocarbons, benzene, phenols, pesticides, carbon monoxide, nitrogen oxides, ammonia, aldehydes (Torkashvand et al., 2020). Among these, at least 150 compounds (of which 44 are commonly found at elevated levels) are considered highly toxic, mainly because of their carcinogenic and mutagenic potential (Torkashvand et al., 2020). These chemicals are concentrated in the remaining intact filter, covering paper, unsmoked tobacco and ash from which they can be released into the environment (WHO, 2017), possibly becoming bioavailable for aquatic organisms with negative effects on their growth, behaviour and viability (Slaughter et al., 2011). In this respect, smoked CBs leachates were shown to exert molecular effects on *in vitro* exposed cell lines, including responses of Ah receptor, estrogen receptor and p53 (Xu et al., 2019), providing additional evidence of environmental risk posed by littered CBs.

Actual knowledge on hazard and toxicological effects of CBs in marine organisms is still fragmented (Araújo & Costa, 2019). Previously reported responses include decreased bioluminescence of *Aliivibrio fischeri* (Micevska et al., 2006; Bonanomi et al., 2020), microbial community changes in coastal marine sediments (Quéméneur et al., 2020), physiology alteration and cellular death of benthic foraminifers (*Rosalina globularis*, *Quinqueloculina* spp. and *Textularia agglutinans*; Caridi et al., 2020), behavioural modifications in tidepool snails (*Austrocochlea*

* Corresponding author at: Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, Ancona, Italy.
E-mail address: f.regoli@staff.univpm.it (F. Regoli).

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porcata, *Nerita atramentosa* and *Bembicium nanum*; Booth et al., 2015), inhibited burrowing behaviour, reduced growth rates and increased DNA damage in the ragworm (*Hediste diversicolor*; Wright et al., 2015), mortality in the marine topsmelt (*Atherinops affinis*; Slaughter et al., 2011). These investigations can contribute to raise awareness on the impact of CBs on marine organisms, but multidisciplinary studies integrating chemical analyses and onset of adverse biological effects are needed for a more realistic hazard and risk assessment. In this respect, the main objective of the present study was to evaluate the release of chemical substances from CBs, their bioavailability and toxicological effects considering different species and levels of biological organization, from molecular to cellular and organismic response. Standardized ecotoxicological bioassays were chosen as useful tools to assess the acute toxicity of pollutants and chemical mixtures: their practical importance is internationally recognized by several monitoring agencies and expert working groups (US-EPA, ICES and OSPAR). They are commonly applied to quality characterization of environmental matrices, and recently included in Italian normative on dredged sediments (Morroni et al., 2020). In this study, the battery of selected bioassays included five species from three different trophic levels, the bacterium *A. fischeri*, the microalgae *Phaeodactylum tricornutum*, *Skeletonema costatum* and *Dunaliella tertiolecta*, and the bivalve *Crassostrea gigas*.

Sublethal effects and cytotoxicity of chemicals released by CBs were also evaluated on an *ex vivo* model using Precision-Cut Tissue Slices (PCTS) obtained from digestive glands of Mediterranean mussel *Mytilus galloprovincialis* (Giuliani et al., 2019): molecular responses of key genes allowed to investigate the early activation of biotransformation pathways, metal detoxification response, peroxisomal proliferation and oxidative stress.

To further enrich our knowledge on bioavailability and cellular toxicity of CBs chemicals in aquatic organisms, an *in vivo* exposure was carried out with *M. galloprovincialis*, a widely accepted bioindicator species to assess presence and impact of environmental pollution. Accumulation in tissues of exposed mussel was integrated with the assessment of a wide panel of biological effects reflecting immunological responses, lipid metabolism, antioxidant defences, neurotoxic and genotoxic effects. The results on ecotoxicological bioassays, bioavailability and biomarker responses were integrated through a Weight Of Evidence (WOE) approach, using the Sediqualsoft model, which applies weighted criteria to elaborate specific hazard indices for individual typologies of data (Lines Of Evidence, LOEs), before their overall integration into the final WOE risk index (Piva et al., 2011; Regoli et al., 2019; Morroni et al., 2020).

The integration of chemical analyses of compounds released from CBs, their bioavailability and effects in different models and levels of biological organization provided a valuable approach for a more reliable assessment of the impact of these hazardous materials on marine species.

2. Materials & methods

2.1. Preparation of cigarette butt leachates

Naturally smoked cigarette butts (CBs: intact filters, covering paper, unsmoked tobacco and ash) of different brands were collected from covered cigarette receptacles in the campus of Polytechnic University of Marche (Ancona, Italy) within one week after they were smoked and stored at −20 °C in plastic bags until they were used for CB leachates preparation and *in vivo* exposure with *M. galloprovincialis*. Despite the different composition of collected CBs might cause a certain variability in exposure conditions and results, this better simulates natural conditions and the heterogeneity of real samples. Leachates were prepared in triplicate at two different concentrations, 100 CBs/L = LEAC_A and 25 CBs/L = LEAC_B, following the suggestions from the available literature, which recommends a concentration ranging between 4 and 400 CBs/L for studies on chemical release and biological effects of CBs (Slaughter et al., 2011; Wright et al., 2015). CBs were soaked in artificial sea water (ASW, Instant Ocean® Sea Salt at 35 psu) at room temperature and maintained in agitation on a magnetic stirrer for 24 h: the room temperature (18–20 °C) was in the range of Mediterranean seawater values typically occurring from spring to autumn seasons. Obtained leachates were pre-filtered using a battery of sieves from 500 μm to 50 μm to remove remaining fractions of CBs, before a vacuum filtration through a 0.45 μm nylon filter to eliminate particulate matter. Cigarette-free seawater (blank) was prepared using the same protocol, including agitation for 24 h at room temperature. Leachates and blank samples were stored at 4 °C before the chemical analyses, ecotoxicological bioassays and *ex vivo* exposure.

2.2. Chemical analyses in CB leachates

Concentrations of trace metals (Al, As, Cd, Cr, Cu, Hg, Fe, Mn, Ni, Pb, V and Zn), polycyclic aromatic hydrocarbons (PAHs), aliphatic hydrocarbons (AHs) C10-C40, polychlorinated biphenyls (PCBs), organohalogenated pesticides (OCPs and OPPs), brominated flame retardants (BFRs), nicotine (NIC), cotinine (COT) and trans-3′-hydroxycotinine (3HC) were determined in the obtained leachates. Measurements were carried out through validated methods by gas-chromatography with flame ionization detector (FID) and mass detector (MS), high-performance liquid chromatography (HPLC) with diode array (DAD), fluorimetric detection and atomic absorption spectrophotometry (AAS), UHPLC-MS Triple quadrupole technique and HPLC-ORBITRAP. For all chemical analyses, quality assurance and quality control were monitored by processing blank and reference standard materials (National Institute of Standards and Technology, USA; EC–DG JRC Institute for Reference Materials and Measurements). The concentrations obtained from these reference standard materials were always within the 95 % confidence intervals of the certified values.

Details on analytical methods and procedures are given in supplementary materials (SM1).

2.3. Ecotoxicological bioassays

Ecotoxicological effects of CB leachates were tested through a battery of bioassays following standardized procedures on serial dilutions of both LEAC_A and LEAC_B.

The bioluminescence reduction was determined in *A. fischeri* (UNI EN ISO 11348-3), the algal growth inhibition test was evaluated in *P. tricornutum*, *D. tertiolecta* and *S. costatum* (UNI EN ISO 10253), while the embryotoxicity bioassay was performed with *C. gigas* (Leverett & Thain, 2013).

Each bioassay was tested in triplicate and EC_{50} values (CBs/L) were calculated using Probit analysis with statistical R-software. Percentage values of bioluminescence reduction, growth inhibition, larvae malformation and EC_{50} values were expressed as mean ± standard error or standard deviation.

Details on bioassay protocols and procedures are given in supplementary materials (SM3).

2.4. Ex vivo study

2.4.1. Exposure of PCTS from M. galloprovincialis digestive gland

Mussels (*M. galloprovincialis*) were obtained from a local farm (Ancona, Adriatic Sea) and acclimatized with ASW (2 individuals/L), at 18 °C and 35 psu for at least 2 weeks, fed with a commercial mixture of zooplankton for filter-feeding organisms (Brightwell Zooplanktos-S, size range 50–300 μm). Precision-Cut Tissue Slices (PCTS) were obtained from digestive gland of 6 mussels following the protocol described in Giuliani et al. (2019). Briefly, digestive glands were excised, cleaned and immediately placed in cold (4 °C), sterilized ASW (supplemented with 1 % penicillin/streptomycin antibiotic mix). After inclusion of the tissue in 2.5 % agarose, slices of 400 μm thickness were cut using a motorized

vibrating blade vibratome (VT1200S, Leica, Wetzlar, Germany). 27 PCTS were produced from each of 6 digestive glands and pools of 3 PCTS were homogeneously distributed in 12-well plates (3 PCTS/well). PCTS were pre-incubated for 1 h in 1 mL Leibovitz's L-15 medium (Lonza; adjusted to NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, supplemented with L-glutamine 2 mM and 1 % penicillin/streptomycin mix).

After the pre-incubation phase, PCTS pools were exposed to LEAC_A (100 CBs/L), diluted to 50 % in L-15 medium. PCTS incubated in L-15 medium only or in 50 % ASW with L-15 were used as control (C) and blank control (CTRL), respectively. Incubations were carried out in 1 mL medium, at 18 °C, under normal atmosphere and static conditions to avoid mechanical stress. The exposure was performed for different times (24 h, 48 h and 72 h), after which the PCTS were rinsed with sterile physiological solution (NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, pH 7.3, supplemented with D-glucose 10 mM and 1 % penicillin/streptomycin mix), and either used for viability tests (PCTS pools from 3 individuals) or snap-frozen in liquid nitrogen and stored at − 80 °C until molecular analyses (PCTS pools from 3 individuals).

2.4.2. Viability tests

The viability of PCTS was estimated through the Alamar Blue assay, resazurin-based (TOX-8, Sigma-Aldrich). PCTS pools were incubated with resazurin solution (10 % in physiological solution), in shaking conditions, for 2 h at 18 °C. The amount of resazurin reduced by oxidoreductases of viable cells was measured as a decrease in absorbance at 600 nm, with background subtraction at 690 nm, according to manufacturer's instruction. Absorbance values for each PCTS pool were subtracted from the blank reading (i.e. the initial resazurin content) and expressed as net absorbance per g of wet weight (Giuliani et al., 2019). The data were expressed as viability percentage compared to C (mean ± standard deviation).

2.4.3. Molecular analyses: RNA extraction and mRNA levels

Total RNA was purified from PCTS pools using the Hybrid-R™ purification kit (GeneAll®), according to the manufacturer's instructions. Total RNA concentrations were measured using Nano-Drop ND-1000 Spectrophotometer. cDNA was synthesized from 1 μg of each RNA sample, using iScript cDNA Synthesis Kit (Bio-Rad). Absolute quantitative real-time PCR (qPCR) was performed for quantification of mRNA levels of the following target genes: catalase (*cat*), glutathione S-transferase pi-isoform (*gst-pi*), selenium-dependent glutathione peroxidase (*Se-gpx*), 70 kDa heat shock proteins (*hsp70*), Cu, Zn superoxide dismutase (*Cu/Zn-sod*), Acyl-CoA oxidase 1 (*acox1*), isoform 10 and 20 of metallothionein (*mt10* and *mt20*) and cytochrome P450 3A1 (*cyp3A1*). qPCRs were conducted through the SYBR green method in StepOnePlus® Real-Time PCR System (Applied Biosystems). Every qPCR reaction contained 7.5 μL of SYBR Select Master Mix (Life Technologies), 5 μL of total cDNA (diluted 1:5) and 200 nM of forward and reverse primers (Table SM4), in a final volume of 15 μL. The annealing temperatures for all genes are given in Table SM4. The absence of DNA contamination was checked by including negative controls lacking cDNA template. For each target gene, standard curve was obtained by serial dilutions of known amounts of plasmid containing the amplicon of interest. Samples and standards were run in duplicate in the same run. A calibration curve was built by plotting cycle threshold (Ct) values of standards versus $\log_{10}$ copy numbers. Ct values of cDNA samples were converted into copy numbers by interpolating the calibration curve. Data were expressed as fold-change related to C and averaged (n = 3).

2.5. In vivo exposure

2.5.1. Exposure of mussels, M. galloprovincialis, to cigarette butts

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length) were acclimatized as previously described and exposed to 3 different levels of CBs directly added into exposure tanks. A total of 240 mussels were divided in 4 tanks (each filled with 20 L of ASW) containing 0, 0.5, 1 or 5 CBs/L, respectively. The exposure time was 14 days and water and CBs were changed every 72 h: also for these experiments, the variability derived from using different CBs, was considered as a more realistic exposure scenario with greater environmental relevance. After changing water, mussels were fed with a commercial mixture of zooplankton for filter-feeding organisms (Brightwell Zooplanktos-S, size range 50–300 μm) and let 2 h without CBs before their new addiction. At the end of the experiment, organisms were sampled: 3 pools constituted by whole tissues of 5–10 organisms (approximately 25 g) were obtained from each tank and stored at −20 °C for bioaccumulation analyses. Gills and digestive glands were dissected and pooled in 5 replicates, each with tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at −80 °C for biomarkers analyses. At the same time, aliquots of haemolymph, taken from adductor muscle, were used for *in vivo* analyses of haemocytes lysosomal membrane stability, granulocytes-hyalinocytes ratio and DNA damage; aliquots of haemolymph were also fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency, or rapidly frozen in liquid nitrogen and maintained at −80 °C for acetylcholinesterase activity.

2.5.2. Bioaccumulation

Trace metals, PAHs, NIC and COT were measured in the whole tissues of *M. galloprovincialis* following the same analytical methods used for CB leachates and detailed in supplementary material (SM2).

2.5.3. Biomarkers analyses

Validated protocols were followed for measurement of biomarkers in tissues of *M. galloprovincialis*. Lysosomal membrane stability (Neutral Red Retention Time, NRRT) and granulocytes/hyalinocytes ratio (G/H ratio) were evaluated in haemocytes for immunological responses, while loss of DNA integrity (COMET assay) and micronuclei frequency (MN) for genotoxic damage; acetylcholinesterase activity (AChE) was analysed as a marker for neurotoxic effects in haemocytes and gills. Biomarkers analysed in digestive gland were metallothioneins (MT) as inducible metal detoxification system, acyl-CoA oxidase (ACOX) for peroxisomal proliferation and malondialdehyde (MDA) as a typical lipid peroxidation product; oxidative stress biomarkers included catalase (CAT), glutathione S-transferases (GST), Se-dependent glutathione peroxidases (Se-dep. GPx), total GPx, glutathione reductase (GR), total glutathione (TGSH) as single antioxidant defences, integrated with the analysis of total oxyradical scavenging capacity (TOSC) toward peroxyl radical ROO• and hydroxyl radical HO•. The results obtained from replicates were expressed as mean and standard deviations or errors. Details of analytical methods and procedures are given in supplementary materials (SM5).

2.6. Statistical analyses and Weight Of Evidence (WOE) integration

One-way analysis of variance, ANOVA (levels of significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$) and post-hoc Student Newman Keuls Test were performed to compare differences in chemical concentrations between blank, LEAC_A and LEAC_B. A non-metric multidimensional scaling (nMDS) was applied on bioassay results in relation to the chemical parameters revealed in leachates (LEAC_A and LEAC_B).

One-way ANOVA was also applied on PCTS to evaluate variations in viability and gene expression, as well as on *in vivo* exposure to determine the significant differences obtained for bioaccumulation and biomarker analyses between control and exposed mussels (levels of significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$); homogeneity of variance was tested by Cochran C, and post-hoc Student-Newman-Keuls test was used for comparisons among these groups. Correlation analyses were performed to examine the relationships between CBs exposure levels, bioaccumulation and biomarkers responses in mussels, accepting correlation coefficients with P values of < 0.05 as significant. All statistical analyses were performed using R-software.

To summarize in specific hazard levels the effects of CBs on ecotoxicological bioassays, bioaccumulation and cellular responses, the overall results were elaborated within a quantitative Weight Of Evidence (WOE) model, using the largely validated Sediqualsoft approach (Piva et al., 2011; Regoli et al., 2019; Morroni et al., 2020). The elaboration of Hazard Quotient (HQ) for bioaccumulation calculates for each chemicals the statistical significance and magnitude of difference between tissue concentrations of control and exposed mussels, corrected for the typology of each chemical (Regoli et al., 2019). For elaboration of biomarkers, each response has a weight based on its toxicological relevance, and a specific threshold for changes of biological relevance that depend on biphasic responses and tissue responsiveness (Piva et al., 2011): biomarker variations are compared to their specific thresholds, corrected for the weight of the response and the statistical significance of differences compared to controls (Piva et al., 2011). The elaboration of HQ for ecotoxicological bioassay results considers specific thresholds and weights for each bioassay, corrected for the significant differences, depending on biological endpoint, tested matrix and time of exposure (Morroni et al., 2020). The HQs elaborated from individual typologies of data (or Lines Of Evidence, LOEs) are normalized to a common scale and integrated within a WOE approach which assigns a different weight to each LOE before synthesizing one of five classes of risk, from Absent to Severe (Piva et al., 2011). Detailed elaboration procedures, flow-charts of calculation, weight and thresholds are fully detailed elsewhere (Regoli et al., 2019).

3. Results

3.1. Chemical analyses in CB leachates

Chemical analyses highlighted that CBs release chemical compounds after 24 h in ASW, with significantly higher concentrations of several trace metals, polycyclic aromatic hydrocarbons (PAHs), nicotine and cotinine in LEAC_A compared to LEAC_B (Tables 1 and SM6). Considering metals, LEAC_A exhibited the highest concentrations for Zn and Mn (mean values of 720 and 711 μg/L, respectively) and the lowest for Cd (mean value of 0.718 μg/L). Similarly, in LEAC_B, the highest concentrations were measured for Fe and Mn (mean value of 259 and 210 μg/L, respectively) and the lowest for Cd (mean value of 0.244 μg/L). Hg and V were always below the detection limit (bdl) (Table 1).

Levels of aliphatic hydrocarbons (AHs) and PAHs were 848 μg/L and 0.515 μg/L in LEAC_A, while 3400 μg/L of AHs and 0.216 μg/L of PAHs were detected in LEAC_B (Table 1). Mean values of individual AHs and PAHs congeners are reported in supplementary materials (Table SM6).

Nicotine and cotinine revealed elevated concentrations, with mean values of 180000 and 43200 μg/L of nicotine, and 1500 and 402 μg/L of cotinine in LEAC_A and LEAC_B, respectively (Table 1). Trans-3′-hydroxycotinine was below the detection limit (bdl) (Table 1), as other organic compounds, like PCBs, OCPs, OPPs, and BFR (data not reported).

Table 1
Concentrations (μg/L) of metals, aliphatic hydrocarbons (AHs), polycyclic aromatic hydrocarbons (PAHs), nicotine (NIC), cotinine (COT) and trans-3′-hydroxycotinine (3HC) in blank (artificial seawater-ASW) and CB leachates (LEAC_A and LEAC_B). Values are expressed as means ± standard deviations (n = 3). Asterisks (*) indicate statistically significant variations while letters indicate differences between groups (post-hoc Student Newman Keuls Test).

Chemical compound	BLANK_ASW	LEAC_A (100 CBs/L)	LEAC_B (25 CBs/L)
Al *	14.3 ± 4.02 [a]	325 ± 10.4[b]	155 ± 3.40[c]
As *	2.98 ± 4.14 [a]	8.53 ± 2.61[b]	2.24 ± 0.489 [a]
Cd *	< 0.1 [a]	0.718 ± 0.098[b]	0.244 ± 0.020[c]
Cr	3.11 ± 0.450	6.70 ± 1.20	52.7 ± 66.7
Cu *	<0.1 [a]	146 ± 17.8[b]	67.2 ± 24.7[c]
Hg	< 0.5	< 0.5	< 0.5
Fe *	29.8 ± 9.10 [a]	290 ± 79.5[b]	259 ± 174[b]
Mn *	4.31 ± 0.795 [a]	711 ± 62.1[b]	210 ± 17.6[c]
Ni	1.51 ± 0.541	22.4 ± 6.19	35.6 ± 36.8
Pb	1.74 ± 1.33	3.23 ± 0.452	9.66 ± 10.9
V	< 20	< 20	< 20
Zn *	117 ± 92.0 [a]	720 ± 183[b]	87.6 ± 25.5 [a]
Total AHs *	523 ± 109 [a]	848 ± 243 [a]	3400 ± 1390[b]
Total PAHs *	< 0.001 [a]	0.515 ± 0.355[b]	0.216 ± 0.062[b]
NIC *	< 0.05 [a]	180000 ± 13600[b]	43200 ± 3600[c]
COT *	< 0.005 [a]	1500 ± 173[b]	402 ± 36.6[c]
3HC	< 0.05	< 0.05	< 0.05

3.2. Ecotoxicological bioassays

The bioassay with *A. fischeri* revealed a mean EC_{50} value for bioluminescence reduction corresponding to 4.47 CBs/L with both LEAC_A and LEAC_B (Table 2). Similar EC_{50} when testing both LEAC_A and LEAC_B, were obtained also for algal growth inhibition, resulting 12.4 and 8.05 CBs/L for *P. tricornutum*, 4.89 and 5.55 CBs/L for *S. costatum*, and 3.38 and 3.84 CBs/L for *D. tertiolecta*, respectively (Table 2). Conversely, embryotoxicity bioassay with *C. gigas* showed that leachate preparation differently affected larval development and survival with mean EC_{50} values of 0.28 CBs/L for LEAC_A and 2.54 CBs/L for LEAC_B (Table 2). Malformed larvae often presented extruded and granulated tissues or other types of malformations such as pre-D larvae stage, protruded mantle and indented shell.

Non-metric multidimensional scaling (nMDS), based on results of bioassays and chemical analyses of LEAC_A and B, showed a clear separation between the five species, particularly evident for *C. gigas*; this separation was mostly related to Al, As, Cd, Cu, Mn, Zn, PAHs, nicotine and cotinine levels in LEAC_A and LEAC_B (Fig. 1). *S. costatum* showed a further segregation from the other microalgae, mainly due to Cr, Fe, Ni and Pb concentrations in tested leachates (Fig. 1).

3.3. Ex vivo study: viability and mRNA analyses

The viability test carried out on PCTS of *M. galloprovincialis* digestive glands exposed to LEAC_A (diluted to 50 %), did not show any significant difference neither between control and exposed groups, nor among different exposure times (Table SM7). Transcript levels of selected genes showed variable responses. *Acox1* transcription decreased significantly in exposed PCTS, with a progressive reduction over time (Fig. 2A). Among antioxidant genes, mRNA levels of *gst-pi*, *cat* and *Cu/Zn-sod* decreased with a significant time-dependent trend (Fig. 2B, C and E). No significant differences were detected for *Se-gpx*, *cyp3A1*, *mt10* and *hsp70*, although a general reduction of transcription levels was often observed after 72 h of exposure (Fig. 2D, F, G, H). The mRNA levels of *mt20* were below the detection limit (data not reported).

3.4. In vivo study: chemicals bioaccumulation and biological responses

The *in vivo* exposure of mussels to CBs revealed a limited accumulation of some metals (Cr, Cu, Fe, Hg, Ni, V, Zn) at higher CBs concentrations (Table 3 and SM8). Conversely, concentrations of PAHs significantly increased with a dose-dependent trend mostly determined by low molecular weight congeners (LMW PAHs) (Table 3 and SM8).

Table 2
EC_{50} values for CB leachates expressed in CBs/L for bioluminescence reduction bioassay, algal growth inhibition test and embryotoxicity bioassay. Values are expressed as means ± standard deviations (n = 3).

	LEAC_A	LEAC_B
Species	EC_{50}	EC_{50}
Aliivibrio fischeri	4.47 ± 0.44	4.47 ± 4.08
Phaeodactylum tricornutum	12.4 ± 3.98	8.05 ± 3.06
Skeletonema costatum	4.89 ± 1.47	5.55 ± 2.59
Dunaliella tertiolecta	3.38 ± 0.66	3.84 ± 1.12
Crassostrea gigas	0.28 ± 0.02	2.54 ± 0.96

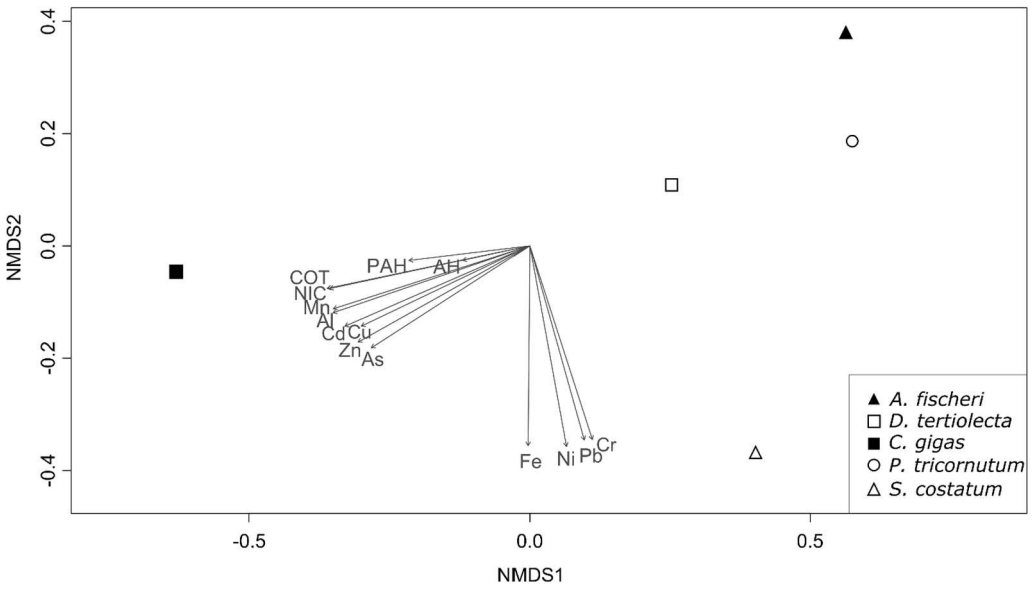

Fig. 1. Non-metric multidimensional scaling plot of bioassays results obtained in *A. fischeri*, *S. costatum*, *P. tricornutum*, *D. tertiolecta* and *Crassostrea gigas* exposed to LEAC_A and LEAC_B. Vectors represent chemicals (concentrations in leachates) correlated to observed endpoints (EC_{50} values).

Bioavailability of NIC was evident for mussels with tissue levels increasing from b.q.l. in controls to 0.124, 0.266 and 3.44 μg/g in organisms exposed to 0.5, 1 and 5 CBs/L, respectively. COT was always below quantification limit (Table 3).

Data on biomarker responses in mussels exposed to CBs are summarized in Figs. 3-4. Results of immunological biomarkers indicated a significant decrease of lysosomal membrane stability and reduced average values of granulocytes/hyalinocytes ratio in 5 CBs/L exposed group (Fig. 3 A and B). Considering biomarkers of genotoxic damage, the frequency of micronuclei increased in mussels exposed to 1 and 5 CBs/L, while a significant effect on DNA fragmentation was observed only at 5 CBs/L exposure (Fig. 3 C and D). Neurotoxic effects revealed a marked decrease of acetylcholinesterase activity in haemolymph of mussels exposed to 5 CBs/L and in gills of those exposed to 1 and 5 CBs/L, (Fig. 3 E and F). The activity of ACOX increased at 5 CBs/L while no significant variations were visible for metallothioneins (Fig. 3 G and H). Among antioxidant defences, significant variations were the increase of glutathione reductase and total glutathione peroxidases in mussels exposed to 5 CBs/L, and the decrease of glutathione in all the groups (Fig. 4 B, D and F). A generally limited oxidative pressure was reflected by the lack of effects on total oxyradical scavenging capacity and a decreasing, not significant, trend for malondialdehyde levels (Fig. 4 G, H and I).

To summarize the overall biological relevance of obtained results and provide a quali-quantitative assessment of hazard from CBs to marine organisms, the results on ecotoxicological bioassays, bioavailability of chemicals and biomarker responses were integrated and elaborated using the weighted criteria of the Sediqualsoft model. The bioavailability hazard was classified as Slight for mussels exposed to 0.5 CBs/L and Moderate for 1 and 5 CBs/L, with PAHs and especially nicotine mostly contributing to the calculated HQ in tissues of exposed mussels. The biomarker results, elaborated in terms of magnitude of variations and toxicological relevance of analysed endpoints, provided a level of hazard classified as Slight for mussels exposed to 0.5 and 1 CBs/L and Moderate for 5 CBs/L. Finally, ecotoxicological hazard, evaluated with the battery of *A. fischeri*, *P. tricornutum* and *C. gigas*, was Absent at 0.5 CBs/L, Moderate at 1 CBs/L and Major at 5 CBs/L, with *C. gigas* development effects mostly contributing to the HQ. The integration of single HQs elaborated for the LOEs of bioavailability, biomarkers and bioassays was synthesized in a WOE risk SLIGHT for exposures to 0.5 CBs/L, MODERATE for 1 CBs/L and MAJOR for 5 CBs/L.

4. Discussion

CBs are one of the most abundant litter items found in the environment, representing a potential risk for organisms due to chemicals contained in filter, ash and tobacco which are released in the aquatic compartment (WHO 2017; Araújo & Costa, 2019; Torkashvand et al., 2020). Previous investigations demonstrated that CBs can elute a complex panel of organic and inorganic chemicals (Hernandez 2018; Dobaradaran et al., 2020, 2021), but only a few studies presented an integrated approach combining chemical characteristics of CB leachates with their biological effects and cellular responses on aquatic organisms (Micevska et al., 2006; Wright et al., 2015; Montalvão et al., 2019; Xu et al., 2019; Quéméneur et al., 2020). Using a battery of cell-based assays, CB leachates were shown to modulate different biological pathways, such as Ah receptor, estrogen receptor and p53, while effect directed analysis coupled with nontargeted chemical analysis allowed to identify compounds potentially responsible for the Ah receptor response (Xu et al., 2019).

Our results revealed that CBs release metals and organic compounds (AHs, PAHs, nicotine and cotinine) in seawater. Compared to blank samples, the amounts of chemicals released by CBs were particularly elevated for Al, As, Cr, Cu, Fe, Mn, Ni, Zn, AHs, nicotine and cotinine. Higher levels of contaminants were generally detected in more concentrated leachate with the exception of Cr, Ni, Pb and AHs. The typology and quantity of chemicals released from CBs can be influenced both by procedures for leachate production (e.g. concentrations of CBs per litre of solvent, soaking times and filtration mesh size) and the intrinsic variability of CBs (different brands and length, smoker puffs and possible effect of smokers lip, hands or mouth (Poppendieck et al., 2016). In our study, smoked CBs were collected from cigarette receptacles to simulate the heterogeneity of real samples, and the resulting variability in experimental conditions might have influenced some of the quantitative differences between leachates. Using CB normalization, levels of metals detected in this work were up to 3 orders of magnitude lower than those presented in Lawal & Ologundudu (2013), but up to 30 times higher than those obtained by Moerman & Potts (2011). The study from Xu et al. (2019) was the first to document the release of AHs from CBs in seawater. Concentrations of C12–C20 and C22 alkanes were previously reported for 4 CBs/L eluted in freshwater after 24 h (Micevska et al.,2006). The release of 15 PAHs, mainly of low molecular weight (such as naphthalene, acenaphthylene, acenaphthene, fluorene

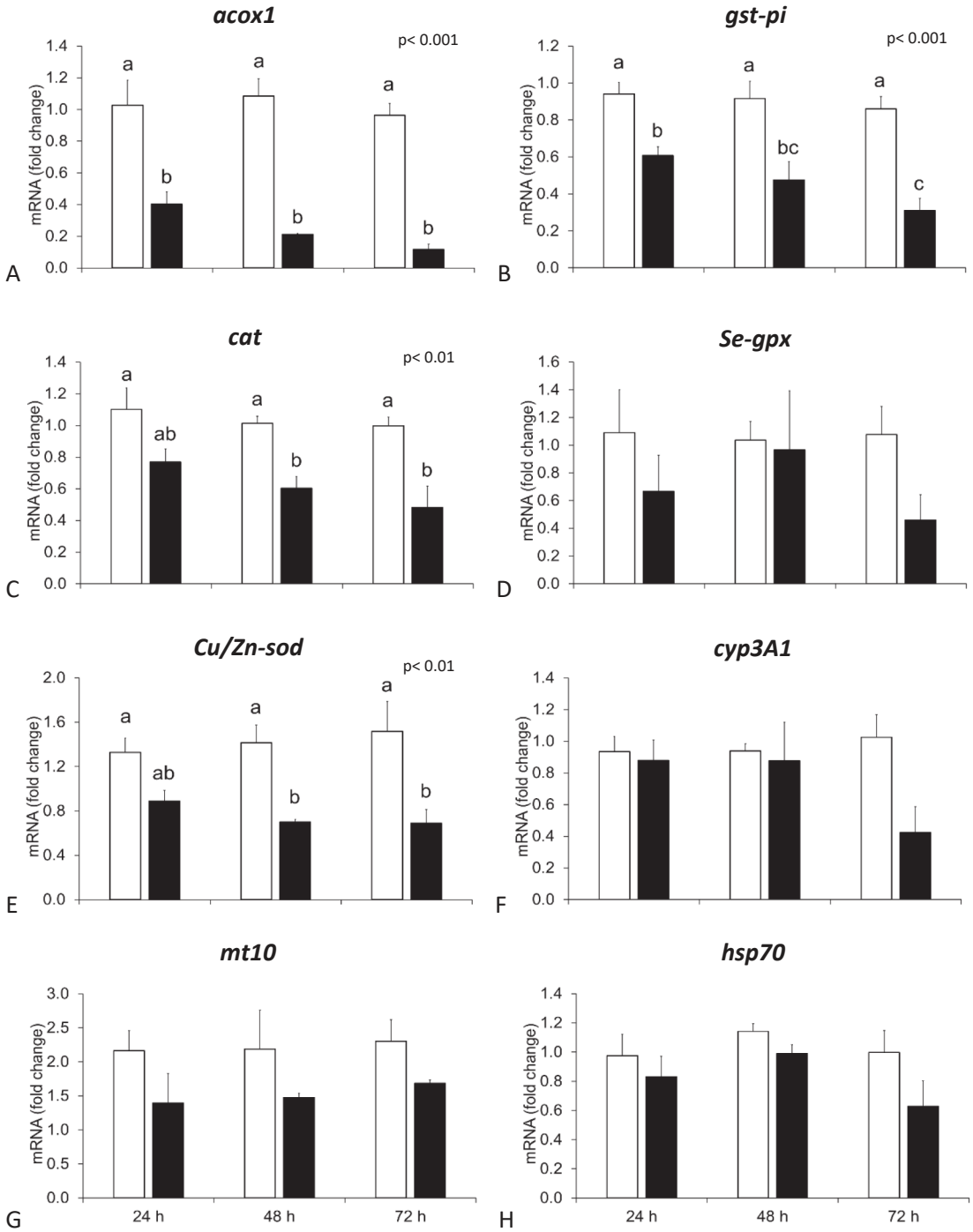

Fig. 2. mRNA levels of *acox1* (A), *gst-pi* (B), *cat* (C), *Se-gpx* (D), *Cu/Zn-sod* (E), *cyp3A1* (F), *mt10* (G) and *hsp70* (H) in PCTS exposed, for 24, 48 and 72 h, to CTRL (white) and LEAC_A diluted 50 % (black). Data are expressed as mean values ± standard errors (n = 3). *p*-values are given for statistically significant effects, while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

and fluoranthene) was observed by Dobaradaran et al. (2020), while those congeners were below the detection limit in our study.

Nicotine, the principal alkaloid naturally present in tobacco leaves (Benowitz et al., 2009) was the most abundant chemical measured in leachates. Similar concentrations were reported by Caridi et al., (2020) in leachates obtained with the same number of CBs, while Wright et al., (2015) revealed a concentration 20 times lower. Our study provided also the first evidence on the release of cotinine, the main nicotine metabolite, already suggested as marker of tobacco consumption in waste waters (Gracia-Lor et al., 2020). Other organic chemical in CB leachate were determined by nontargeted analysis demonstrating, however, the complexity to assess the toxicological contribution of individual tobacco constituents (Xu et al., 2019).

Acutely toxic effects of chemicals eluted from CBs were evaluated through a battery of ecotoxicological bioassays. CB leachates determined the reduction of *A. fischeri* bioluminescence with mean EC_{50} values of 4.47 CBs/L, slightly higher than those reported in other studies and ranging between 0.3 and 2.7 CBs/L for leachates of different CBs brands (Micevska et al., 2006; Oliva et al. (2021); on the other hand, Piccardo et al. (2021) revealed a 35 % inhibition of bioluminescence for

Table 3
Concentrations of trace metals, low molecular weight polycyclic aromatic hydrocarbons (LMW PAHs), high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs), total PAHs, nicotine (NIC) and cotinine (COT) in tissues of *M. galloprovincialis* exposed to various experimental conditions. Values are given in μg/g dry weight (mean values ± standard deviations, n = 3). Asterisks (*) indicate statistically significant variations while letters indicate differences between groups (post-hoc Student Newman Keuls Test).

Chemical compound	CTRL	0.5 CBs/L	1 CBs/L	5 CBs/L
Al	54.5 ± 28.9	69.2 ± 16.8	71.1 ± 47.5	44.8 ± 8.75
As	15.3 ± 4.13	15.0 ± 1.96	15.5 ± 4.28	14.85 ± 1.50
Cd	0.343 ± 0.068	0.364 ± 0.157	0.477 ± 0.107	0.439 ± 0.146
Cr	0.985 ± 0.734	1.08 ± 0.212	1.53 ± 0.734	1.22 ± 0.626
Cu	2.59 ± 0.362	2.61 ± 0.231	2.60 ± 0.730	3.95 ± 0.900
Fe	196 ± 29.5	196 ± 23.0	216 ± 65.3	290 ± 80.6
Hg	0.061 ± 0.012	0.059 ± 0.018	0.070 ± 0.017	0.084 ± 0.025
Mn	4.88 ± 0.431	2.50 ± 0.163	3.16 ± 0.568	3.25 ± 0.795
Ni	0.941 ± 0.619	0.816 ± 0.061	1.33 ± 0.489	1.60 ± 0.453
Pb	0.762 ± 0.039	0.567 ± 0.128	0.763 ± 0.168	0.875 ± 0.361
V	0.715 ± 0.154	0.757 ± 0.012	0.859 ± 0.228	0.963 ± 0.424
Zn	111 ± 8.32	119 ± 68.4	150 ± 17.5	130 ± 41.4
LMW PAHs *	0.201 ± 0.073 ^{a}	0.294 ± 0.094 ^{ab}	0.450 ± 0.032 ^{bc}	0.602 ± 0.162^{c}
HMW PAHs *	0.020 ± 0.003 ^{a}	0.018 ± 0.002 ^{a}	0.030 ± 0.005^{b}	0.023 ± 0.001 ^{a}
Total PAHs *	0.220 ± 0.067 ^{a}	0.312 ± 0.095 ^{a}	0.480 ± 0.029^{c}	0.625 ± 0.161^{c}
NIC *	< 0.01 a	0.124 ± 0.014 ^{a}	0.266 ± 0.068 ^{a}	3.44 ± 0.560^{b}
COT	< 0.01	< 0.01	< 0.01	< 0.01

10 CBs/L leachate, lower than that found in the present work with about 55 and 70 % inhibition for 6.25 and 12.5 CBs/L, respectively.

The three microalgae showed different sensitivities to CB leachates, with *P. tricornutum* being less sensitive than *S. costatum* and *D. tertiolecta*. These results were similar to those of Oliva et al. (2021) with an EC_{50} of 11.8 CBs/L for *P. tricornutum* and 6.2 CBs/L for *D. tertiolecta*. A species-specific sensitivity of these microalgae was also observed by Gallo et al. (2020), but with a different rank. *D. tertiolecta* was the most sensitive and *S. costatum* the most resistant to the contaminant mixture. The study by Piccardo et al. (2021) reported a lower effect of CB leachates also for algal growth with an inhibition of 32 % in *P. tricornutum* exposed to 10 CBs/L leachate compared to 72, 92 and 96 % observed in this study for *P. tricornutum*, *S. costatum* and *D. tertiolecta*.

Crassostrea gigas was the only species to show a marked difference in ecotoxicological response according to leachate preparation, with LEAC_A causing an EC_{50} of 0.28 CBs/L, approximately 10 times lower than that obtained with LEAC_B which had an EC_{50} of 2.54 CBs/L, and thus suggesting some synergistic effects or additional chemicals released in the more concentrated leachate. This is the first study documenting toxic effects of CB leachates on larvae of *C. gigas*, but abnormal embryo development was previously described in other aquatic species: malformation in *plutei* of sea urchin *Paracentrotus lividus* (Piccardo et al., 2021); an LC_{50} of 4.5 CBs/L on *nauplii* of crustacean *Artemia sp* (de Souza Abessa et al., 2020); an EC_{50} of 7 CBs/L on larvae of polychaete *Ficopomatus enigmaticus* (Oliva et al., 2021), malformed eyes and reduced sizes of embryos of medaka fish *Oryzias latipes* at 5 and 10 CBs/L (Lee and Lee, 2015). Comparing these data with our results, *C. gigas* seems very sensitive to substances released in CB leachates, possibly for the fast embryo development. Despite the nMDS seems to indicate the main contribution of some chemicals, mixture effects are more likely to explain the observed acute toxicity exerted by CB leachates, since the reported EC_{50} values of individual compounds (Table SM9) did not match those analysed in our samples.

To evaluate toxicity of CBs at a lower level of biological organization, sublethal effects and molecular responses were analysed on Precision-Cut Tissue Slices (PCTS) exposed at the final concentration of 50 CBs/L. The major advantages of mussels PCTS include the possibility to setup controlled and reproducible conditions for up to 72 h, reducing the inter-individual variability similarly to *in vitro* models, but also maintaining the physiological architecture of the tissue, thus ensuring cellular outcomes more similar to those of *in vivo* models (Giuliani et al., 2019). The use of human PCTS has been a valuable tool in the study of the acute response to cigarette smoke in lungs, demonstrating a dose-dependent cytotoxicity for concentrations higher than 10 cigarette/L after 12 h exposure (Mondoñedo et al., 2020).

In our study, 50 CBs/L leachate had no effect on the viability of mussel PCTS but several gene transcripts were reduced, especially after 72 h of exposure to CBs mixture: this result is consistent with transcriptomic studies on *in vivo* and *in vitro* mammalian models, where a massive presence of inhibited genes allowed to conclude that transcriptional down-regulation is a major effect of cigarette toxicity (Maunders et al., 2007). Despite this general evidence, the down-regulation of *Cu/Zn-sod*, *cat* and *gst-pi* mRNA in mussel PCTS was partly unexpected, since induction of antioxidant genes by cigarette smoke has also been often reported (Spira et al., 2004), confirming complex mechanisms of transcriptional and post-transcriptional regulation of antioxidants (Regoli & Giuliani, 2014). Concerning metallothioneins, the lack of *mt20*, the isoform specifically induced by Cd (Dondero et al., 2005), can be attributed to the low levels of this element measured in the CBs leachate. On the other hand, the constitutive *mt10* isoform is known to be induced in mussel digestive cells by Cu (45 μg/L) and Zn (300 μg/L) (Dondero et al., 2005), while a rather decreasing trend was observed in our study despite the elevated concentration of both Cu and Zn measured in CBs leachate, which support a possible interference or antagonistic effect of other chemicals or mixture on metallothionein pathway. CBs leachate appeared to modulate also lipid metabolism in mussels PCTS by the downregulation of *acox1*. The impairment of fatty acid metabolism has already been observed in different mammalian organs and cell types, with reduced activity of β-oxidation enzymes and increased lipid accumulation after *in vivo* and *in vitro* exposure to cigarette smoke or extracts (Gong et al., 2019; Gupta et al., 2021); conversely, other studies reported an increase of the same enzymes, reflecting the high complexity of cigarette butts toxicity on fatty acid metabolism (Li et al., 2021). The low levels of PAHs, measured in CB leachates in this study may account for the lack of transcriptional regulation of *cyp3a1,* which is not strongly regulated by Ah receptor particularly in invertebrates, but known to be responsive to such chemicals in mussels digestive gland (Cubero-Leon et al., 2012; Giuliani et al., 2013). In mammalian models, *hsps* members were induced by cigarette smoke in *in vitro* but not in *in vivo* exposure (Gebel et al., 2004), and the absence of effects observed in this study on *hsp70* could confirm that the PCTS *ex vivo* model better reflects the physiological response of the entire organism, rather than of individual cells. Our biological model is not particularly suitable for investigating receptor-based pathways like Ah and estrogenic ones which were shown to respond in specific cell-lines assays (Xu et al., 2019). Future studies integrating additional vertebrate species, approaches, experimental designs and methodologies will certainly contribute to better understand molecular mechanisms and mode of action of CB leachates on aquatic organisms.

In accordance with the chemical characterization of the CB leachate, mussels exposed to CBs showed a limited accumulation of some metals (Cr, Cu, Fe, Hg, Ni, V, Zn) and a significant increase of PAHs mostly as low molecular weight congeners, which are typically characterized by higher water-solubility and bioavailability. Nicotine, largely detected in CB leachates, was markedly accumulated in mussel tissues, confirming

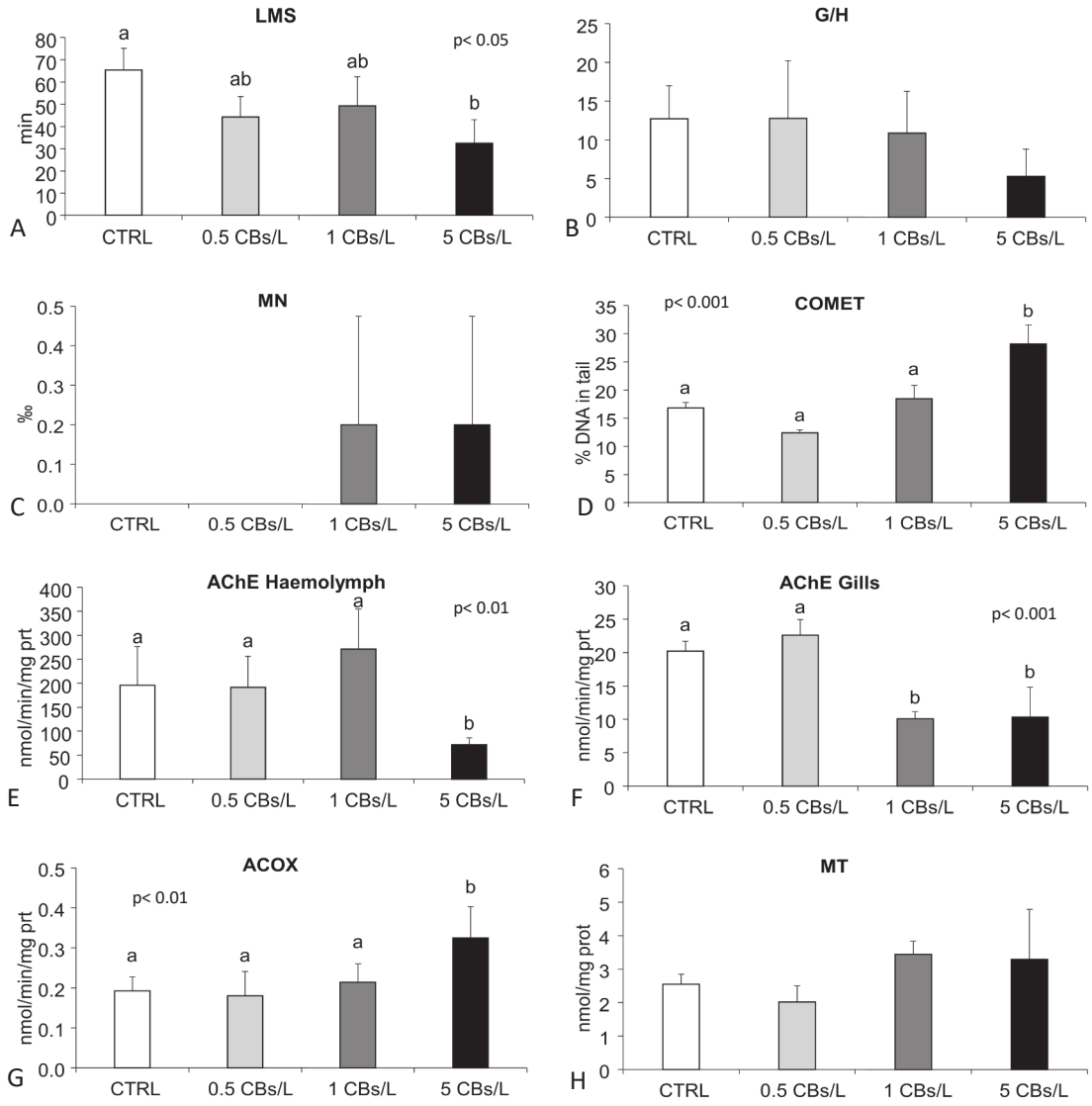

Fig. 3. Lysosomal membrane stability (A), granulocytes/hyalinocytes ratio (B), frequency of micronuclei (C), DNA damage (D) in haemocytes; acetylcholinesterase activity in haemolymph (E) and in gills (F), acyl-CoA oxidase activity (G) and metallothioneins (H) in digestive gland of mussels exposed to CBs in various experimental conditions. Data are given as mean values ± standard deviations or standard error (D), n = 3; letters indicate statistical differences between groups. *p*-values are given for statistically significant effects, while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

its absorption through biological membranes at relatively high pH of the aqueous solution as that of the present study, 8.2 (Yildiz, 2004). Cotinine was always below quantitation limit (0.01 μg/g dry weight) in all the exposure groups, indicating that mussels do not uptake this chemical released from CBs and/or do not metabolize nicotine which in humans is converted to cotinine at a rate of 70–80 %; this transformation is reported to be catalysed by CYP2A6 and aldehyde oxidase (Benowitz et al., 2009), none of which have ever been detected in mussel tissues.

Exposure to CBs provoked measurable effects on haemocytes physiology and immune defences, such as a significant decrease of lysosomal membrane stability, a decrease of granulocytes, the cells involved in cellular immunity and phagocytosis, and an increase of hyalinocytes, less active cells in immune defences (Nardi et al., 2021). Considering genotoxic effects of CBs, our results demonstrate the onset of DNA fragmentation and an increase of micronuclei frequency in mussels exposed to 1 and 5 CBs/L. No immune responses but nuclear abnormalities and micronuclei were observed in haemocytes of *Anodontites trapesialis* and related to metals (Cr, Ni, Pb, Zn, Mn and Na) eluted from CBs (Montalvão et al., 2019), while nicotine was proposed as a potential cause of genotoxicity in coelomocytes of *H. diversicolor* exposed to 8 CBs filters/L (Wright et al., 2015): in our experiment, Cr, Cu, Fe, Hg, Ni, V, Zn were accumulated to a limited extent, while a marked increase of nicotine was measured in tissues of exposed mussels.

Neurotoxic effects were reflected by the significant decrease of acetylcholinesterase activity in haemolymph at 5 CBs/L and in gills at 1 and 5 CBs/L. This effect could be explained by the competitive binding of nicotine to nicotinic acetylcholine receptors, thus modifying their conformation and physiology of the cholinergic system (Xiao et al., 2020). Despite mechanisms of action would remain to be clarified especially in invertebrate species, the influence of nicotine on functions of AChE has been investigated in several mammalian models, showing that effects of nicotine exhibit many similarities to those of AChE inhibitors (Slotkin, 1999). As additional hypothesis, the AChE inhibition observed in mussels exposed to CBs might also be related to the presence of some pesticides specific for tobacco crops (Soleimani et al., 2022).

The increase of ACOX activity to 5 CBs/L could be associated to the accumulation of PAHs in mussel tissues: this enzyme, involved in peroxisomal proliferation and β-oxidation of fatty acids, has been largely

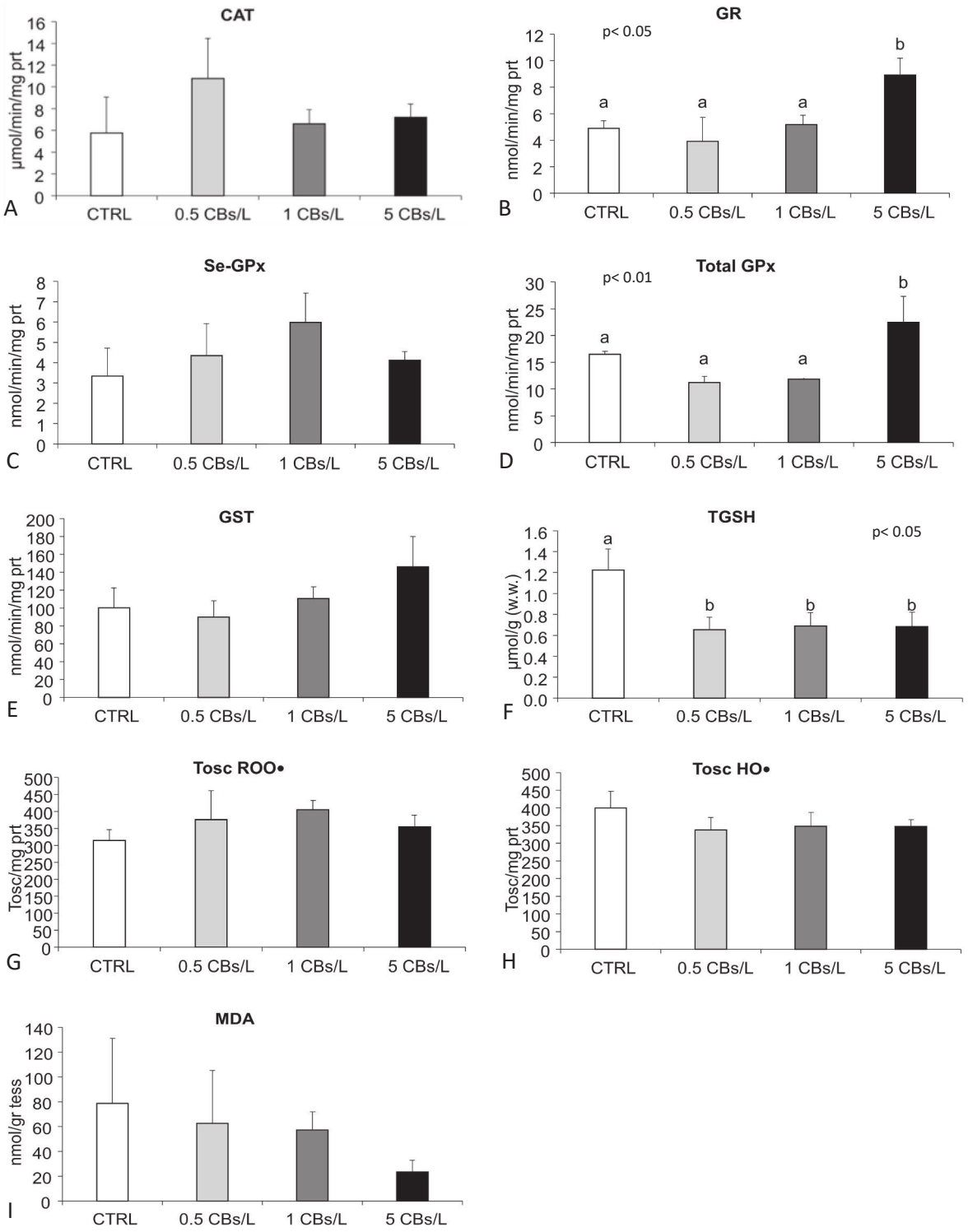

Fig. 4. Catalase (A), glutathione reductase (B), Se-dependent and total glutathione peroxidases (C and D), glutathione S-transferase (E), glutathione (F), total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (G and H), malondialdehyde (I) in digestive gland of mussels exposed to CBs in various experimental conditions. Data are given as mean values ± standard deviations (n = 3). Letters indicate statistical differences between groups (p-value < 0.01 for total glutathione peroxidases and < 0.05 for glutathione reductase and total glutathione). *p*-values are given for statistically significant effects, while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

used as biomarker of exposure to organic pollutants in aquatic organisms such hydrocarbons, phthalates, plasticizers (Cajaraville & Ortiz-Zarragoitia, 2006). At the same time, the lack of metallothioneins variations is also consistent with bioaccumulation results which did not highlight marked variations in tissue levels of trace elements, particularly for Cd.

Among antioxidant defences, glutathione showed a significant decrease in all exposure groups, partly compensated in mussels exposed to 5 CBs/L by the increase of GR, responsible for regenerating the functional form of reduced GSH. Glutathione has a great importance

against prooxidant chemicals and reactive oxygen species (ROS), acting as scavenger of ROS and as cofactor of antioxidant enzymes (Regoli & Giuliani, 2014). Oxidative pressure in mussels exposed to 5 CBs/L was further confirmed by the significant increase of total GPx, which protect cells from oxidative damage caused by organic and inorganic hydroperoxides (H_2O_2): other important antioxidants like GST and catalase did no exhibit significant variations in all exposure groups. The rather constant TOSC values and the decreasing levels of malondialdehyde, a typical marker of oxidative damage, highlighted a limited prooxidant impact of the mix of substances released from the CBs on mussels capability to counteract the oxidative pressure.

The overall results obtained in this study highlight a complex network of effects that can influence the impact of CBs on marine species, confirming the challenge of assessing the risks from emerging pollutants. The importance of integrating chemical analyses with measurement of biological effects has gradually risen in monitoring strategies. Multidisciplinary approaches for the characterization of aquatic environment quality are now recommended by European Directives such as the Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). The lack of standardized procedures for the integration of complex datasets of heterogeneous results, often prevents the adoption of such multidisciplinary approaches in decision-supporting procedures (Dagnino et al., 2008; Linkov et al., 2009; Semenzin et al., 2008; Piva et al., 2011; Benedetti et al., 2012). In Sediqualsoft model, different typologies of data are independently elaborated with specific criteria, which weight typology of chemical pollutants and toxicological relevance of measured endpoints, as well as the number and magnitude of observed variations normalized toward specific thresholds. Synthetic and quantitative hazard indices are calculated, before their overall integration in the WOE assessment, assigning the risk to 1 of 5 classes, from absent to severe (Piva et al., 2011; Regoli et al., 2019). In recent years this approach was validated in several case studies for environmental risk assessment associated with polluted sediments, harbor areas, off-shore platforms, marine incidents, management of complex industrial areas or marine installations, as well toward a better assessment of the impacts of specific classes of pollutants and their interactions with multiple stressors on the marine environment (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al. 2014; Bebianno et al., 2015; Mestre et al., 2017; Pittura et al., 2018; Lehtonen et al., 2019; Regoli et al., 2019; Morroni et al., 2020, Cacciatore et al., 2022; Nardi et al., 2022; Pittura et al., 2022). Sediqualsoft criteria for weighted elaboration of chemical data and ecotoxicological bioassays have been incorporated in the last Italian law dredged marine sediments (DM 173/2016), providing the rationale for different management options associated to the class of environmental risk.

In this study, the Sediqualsoft model allowed to synthesize the biological significance of the results observed in ecotoxicological bioassays and in mussels exposed to CBs, for an easier qualitative and quantitative comparison of different conditions. With such approach, the weighted elaboration of ecotoxicological bioassay, bioaccumulation and biomarker results provided specific hazard indices increasing with exposure dose from "Slight" to "Moderate" for both bioavailability and cellular effects while from "Absent" to "Major" for bioassay (Fig. 5): accordingly, the integrated WOE risk index was Slight for 0.5 CBs/L, Moderate for 1 CBs/L and Major for 5 CBs/L. The evident increase of the overall biological impact at higher CBs exposure levels reinforces the ecological risk caused by these litter items which should be considered as true emerging pollutants.

The presented WOE approach and elaboration procedure, beside the integration of different LOEs for a more complex level of risk assessment, has also a great importance in terms of communication and risk management, still maintaining scientifically robust info derived from the weighted elaboration of various results.

5. Conclusions

This study presented a multidisciplinary assessment of the impact of CBs on marine organisms, demonstrating the capability of the hazardous items to release chemical compounds in seawater, to induce acute effects on a battery of ecotoxicological bioassays, to modulate gene expression in *ex vivo* models (PCTS) of *M. galloprovincialis*, but also a significant accumulation of PAHs and nicotine, paralleled by several alterations of immune system, antioxidant responses, lipid metabolism, neurotoxic and genotoxic responses in *in vivo* exposed mussels. Considering the complexity of results obtained by each typology of data, the results of this study corroborate the importance of an integrative approach based on multiple LOEs and their weighted elaboration to better address and communicate the impact and risks of cigarette butts on marine environment, raising smokers' environmental awareness and a more efficient public management of this typology of waste. The clear evidence of an increasing dose-dependent risk from CBs on marine organism, corroborates their role as emerging pollutants, highlighting the need to prioritize their removal from beach not only as an aesthetic problem but to rather limit a pollution source for the marine environment.

CRediT authorship contribution statement

Giulia Lucia: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Maria Elisa Giuliani:** Conceptualization, Supervision, Validation, Writing – original draft. **Giuseppe d'Errico:** Formal analysis,

Sample	LOE Bioavailability	LOE Biomarkers	LOE Bioassays	WOE
CTRL	Absent	Absent	Absent	ABSENT
0.5 CBs/L	Slight	Slight	Absent	SLIGHT
1 CBs/L	Moderate	Slight	Moderate	MODERATE
5 CBs/L	Moderate	Moderate	Major	MAJOR

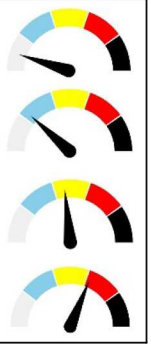

Fig. 5. Elaboration of specific hazards for LOEs on bioavailability, biomarkers, bioassays data and integrated Weight of Evidence (WOE) classification of risk for different concentrations of CBs.

Supervision, Validation, Writing – original draft. **Emily Booms:** Formal analysis, Investigation. **Maura Benedetti:** Supervision, Validation, Writing – original draft, Writing – review & editing. **Marta Di Carlo:** Investigation, Validation. **Daniele Fattorini:** Investigation, Supervision. **Stefania Gorbi:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Francesco Regoli:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107733.

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