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Toxicological effects of cigarette butts for marine organisms

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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*

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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

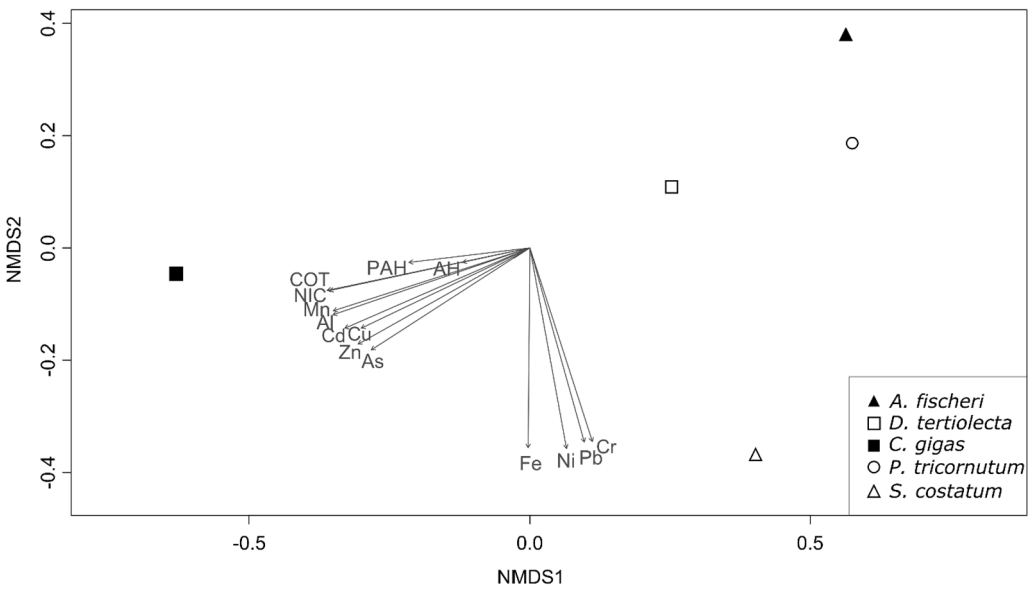

Fig. 1. Non-metric multidimensional scaling plot of bioassays results obtained in *A. fischeri*, *S. costatum*, *P. tricornutum*, *D. tertiolecta* and *Crassostrea gigas* exposed to LEAC_A and LEAC_B. Vectors represent chemicals (concentrations in leachates) correlated to observed endpoints (EC_{50}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

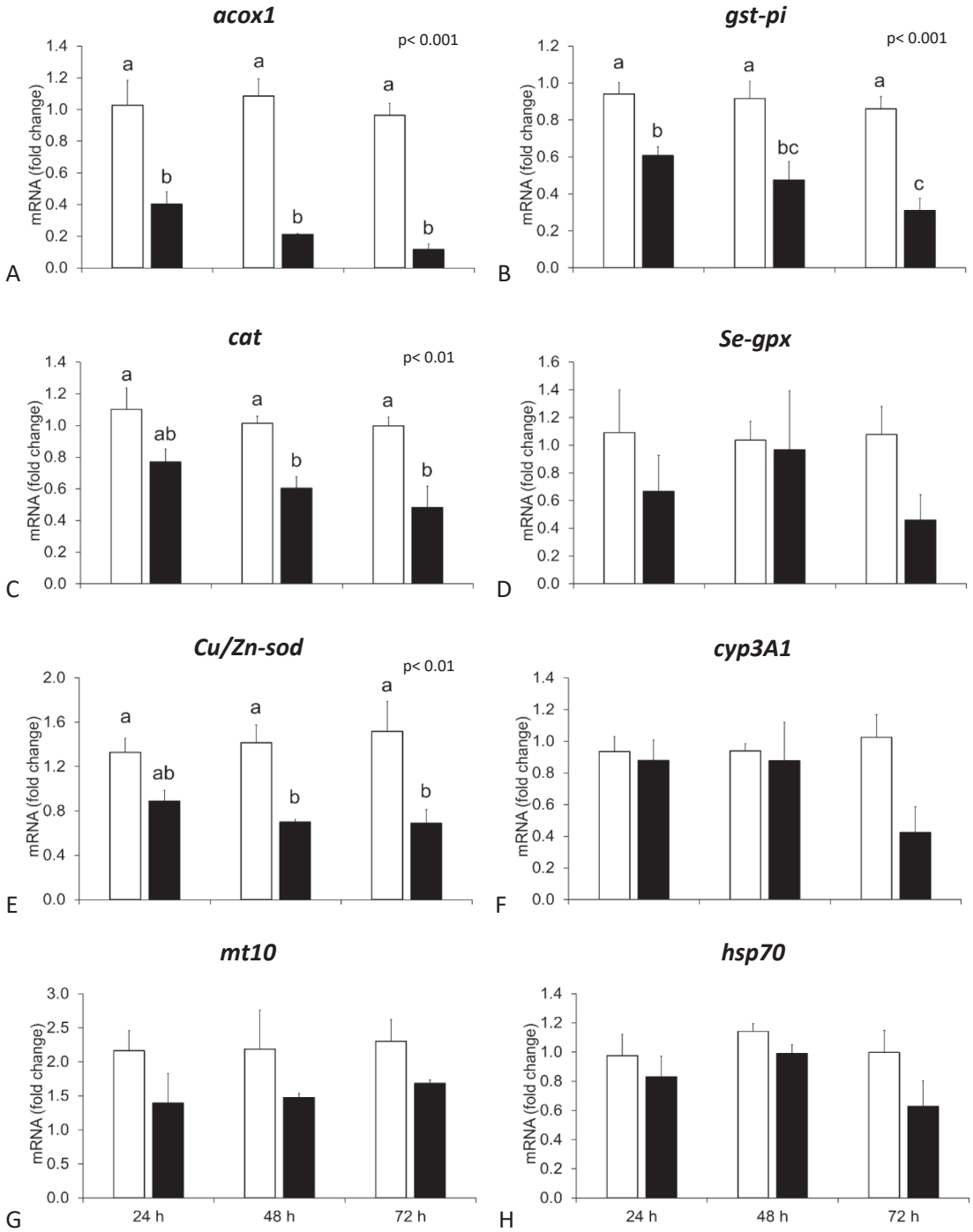

Fig. 2. mRNA levels of *acox1* (A), *gst-pi* (B), *cat* (C), *Se-gpx* (D), *Cu/Zn-sod* (E), *cyp3A1* (F), *mt10* (G) and *hsp70* (H) in PCTS exposed, for 24, 48 and 72 h, to CTRL (white) and LEAC_A diluted 50 % (black). Data are expressed as mean values ± standard errors (n = 3). *p*-values are given 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

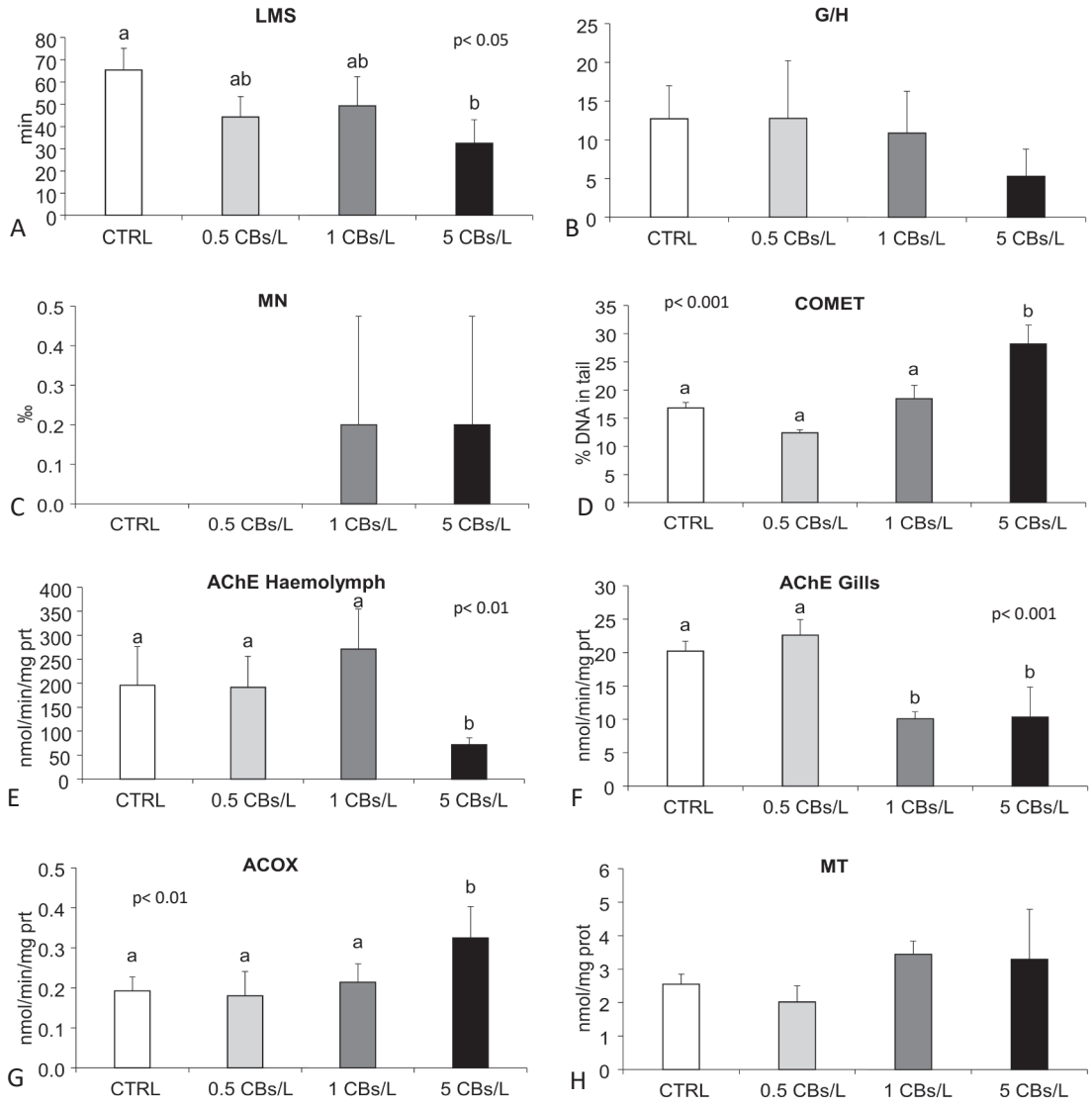

Fig. 3. Lysosomal membrane stability (A), granulocytes/hyalinocytes ratio (B), frequency of micronuclei (C), DNA damage (D) in haemocytes; acetylcholinesterase activity in haemolymph (E) and in gills (F), acyl-CoA oxidase activity (G) and metallothioneins (H) in digestive gland of mussels exposed to 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

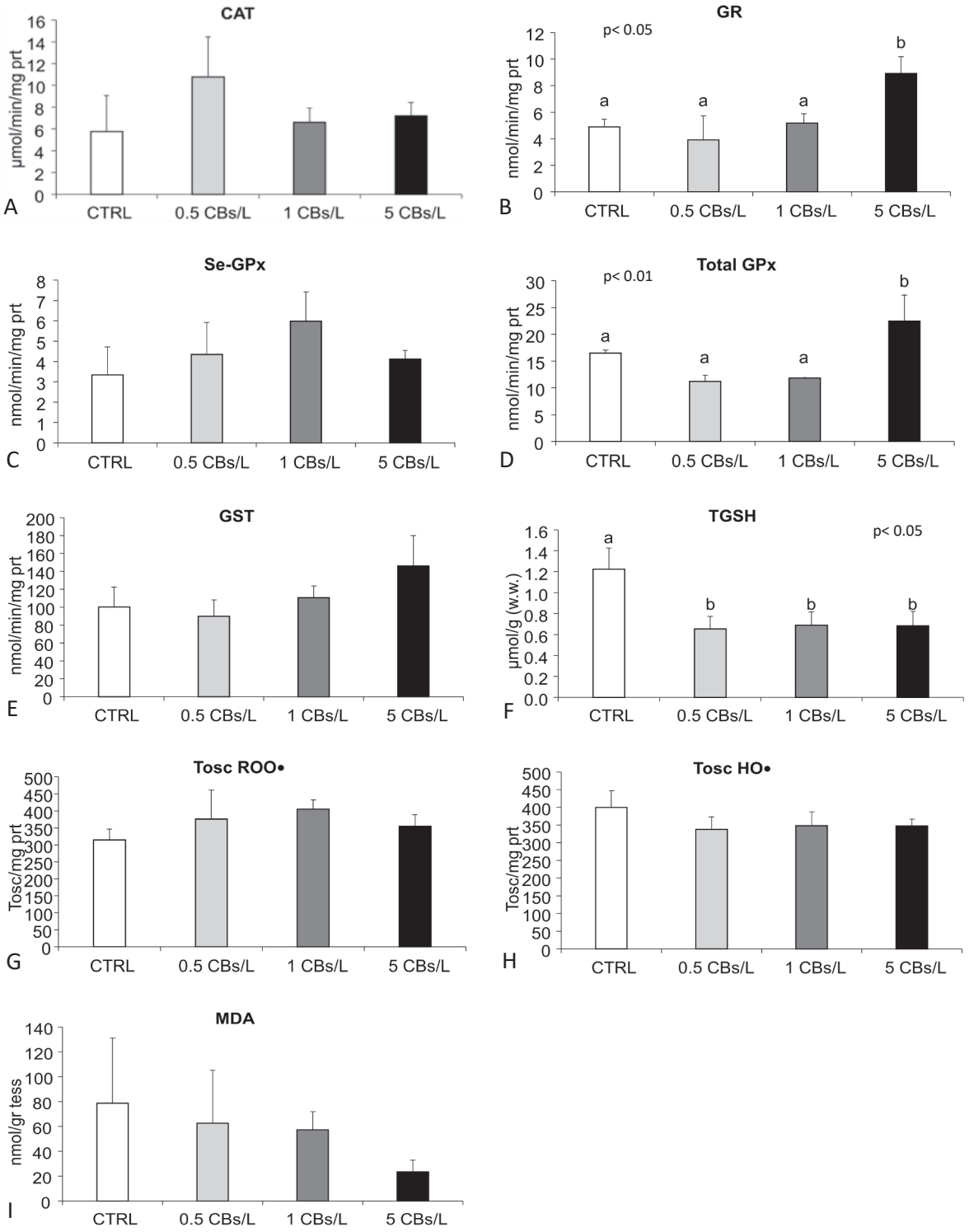

Fig. 4. Catalase (A), glutathione reductase (B), Se-dependent and total glutathione peroxidases (C and D), glutathione S-transferase (E), glutathione (F), total oxyradical scavenging capacity toward peroxyl radical and hydroxyl radical (G and H), malondialdehyde (I) in digestive gland of mussels exposed 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

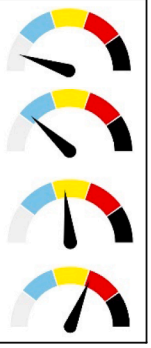

Fig. 5. Elaboration of specific hazards for LOEs on bioavailability, biomarkers, bioassays data and integrated Weight of Evidence (WOE) classification of risk for different concentrations of CBs.

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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