UNIVERSITY OF NAPLES, FEDERICO II

PhD in Biology XXXIV Cycle

(co-founding SZN)

Characterization, treatment and valorization of contaminated sediment: the potential role of invertebrate

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Thesis structure

This thesis has been organized as a hybrid-paper-based document including five chapters. Chapter 1 displays the state of art on the major lines and problem formulation of research. In Chapter 2, the issue of contaminated sediment has been investigated applying a model organism, the sea urchin *Paracentrotus lividus* (Lamarck, 1816) from the morphological to the molecular point of view using a mesocosm-based approach. In Chapter 3, the embryotoxicity and genotoxicity of four PAHs (naphthalene, fluoranthene, phenanthrene and benzo(k)fluoranthene) was evaluated on marine crustacean *Artemia franciscana*. In Chapter 4, all available literature on the harmful impact of chemical remediation techniques of contaminated sediment was reviewed. The collected data have been analyzed by species sensitivity distribution (SSD) approach. In Chapter 5, the final objective of the thesis was addressed. Specifically, after the study of all available literature, the differences of common contaminants (PAHs) spiked marine sediment concentration after AC and nZVI treatment were showed. Moreover, their toxic effects on the branciopod crustacean *A. franciscana* Kellogg, 1906 (Anostraca) was evaluated.

In **Table 1**, I briefly summarized my scientific production in relation to the PhD development.

Chapter	Paper	Impact Factor	Citations
1	**Albarano L.**, Costantini M., Zupo V., Lofrano G., Guida M., Libralato G., 2020. Marine sediment toxicity: a focus on micro- and mesocosms towards remediation. Science of Total Environment 708, 134837.	7.963	11
2	**Albarano L.**, Zupo V., Caramiello D., Toscanesi M., Trifuoggi M., Guida M., Libralato G., Costantini M., 2021b. Sub-chronic effects of slight PAH- and PCB-contaminated mesocosms in *Paracentrotus lividus* Lmk: a multi-endpoint approach and de novo transcriptomic. International Journal of Molecular Sciences, 22, 6674.	5.923	1
	Albarano L., Guida M., Zupo, V., Libralato G. Costantini M., PAHs and PCBs affect functionally intercorrelated genes in the sea urchin *Paracentrotus*	5.923	

	lividus embryos. International Journal of Molecular Sciences, 22, 12498.		
3	**Albarano L**., Serafini A., Castaldo F., Costantini M., Zupo V., Guida M., Libralato G. Genotoxicity in *Artemia franciscana* nauplii and adults: the case of phenanthrene, naphthalene, fluoranthene and benzo(k)fluoranthene (*submitted*).		
4	**Albarano L.**, Lofrano G., Costantini M., Zupo V., Carraturo F., Guida M., Libralato G., 2021a. Comparison of in situ sediment remediation amendments: risk perspectives from species sensitivity distribution. Environmental pollution, 115995.	8.07	1
5	**Albarano L.**, Costantini M., Zupo V., Guida M., Libralato G. Comparison between AC and nZVI as restoring methods of common contaminants (PAHs) spiked marine sediment and evaluation of their toxic effects on crustacean *Artemia franciscana* (*in preparation).*		

Table 1. Scientific production of the candidate during the PhD researches, along with impact factors of the journals and number of citations

List of Abbreviations

°C degree Celsius

9-MANT 9 metilAntracene

µL microlitre

A apatite

AC activated carbon

ACE acenaphthene

ANOVA analysis of variance

ANT anthracene

ACY acenaphthylene

As arsenic

BaANT benzo(a)anthracene

BaP benzo(a)pyrene

BeP benzo(e)pyrene

BbF benzo(a)fluoranthene

B(g,h,i)PER Benzo[ghi]perylene

BkF benzo(k)fluoranthene

BLAST basic local alignment search tool

bp base pairs

BP biological process

BR Bioaccumulation reduction

BSAF biota-sediment accumulation factor

CC cellular component

Cd cadmium

cDNA complementary deoxyribonucleic acid

CHR chrysene

cm centimetre

COD chemical oxygen demand

Cr chromium

Cu copper

DB(a,h)ANT Dibenz[a,h]anthracene

DE differentially expressed

DOC dissolved organic carbon

dw dry weight

F fluorene

FDR false discovery rate

FLR fluorine

FLT fluoranthene

FPKM fragments per kilobase of exon per million fragments mapped

FSW filtered sea water

g gram

GC-MS gas chromatography-mass spectrometry

GI gonadal index

GO gene ontology

h hour

H hardness

$H_2 0_2$ hydrogen peroxide

HC hazardous concentration

HCl hydrochloric acid

HNO_3 nitric acid

hpf hours post fertilization

ICP-MS inductively coupled plasma with mass spectrometry

IG inhibition growt

IPYR Indeno[1,2,3-cd]pyrene

Kg kilogram

KOH postassium hydroxide

LOD limits of detection

LOQ limits of quantification

log logarithm

M mortality

MeOH methanol

MF molecular function

mg milligram

min minute

Mix mixture

mL millilitre

mV millivolt

na not available

NAP naphthalene

ne not effects

NCBI national center for biotechnology information

NGS next generation sequencing

NH_3^- ammonia

Ni nickel

NO_2^- nitrites

NO_3^- nitrates

nZVI nano-Zero Valent iron

OC organoclay

OECD Organizzazione per la cooperazione e lo sviluppo economico

PAHs polycyclic aromatic hydrocarbons

PC protective concentration

PCBs polychlorinated biphenyls

PD predicted data

PHE phenanthrene

PCA principal component analysis

PCR polymerase chain reaction

PO_4^{3-} phosphates

ppm parts per million

PYR pyrene

qPCR quantitative polymerase chain reaction

R reproduction

RD raw data

REST relative expression software tool

RIN RNA integrity number

RNA ribonucleic acid

RNAseq RNA sequencing

rpm revolutions per minute

rRNA ribosomal RNA

RT room temperature

S salinity

SD standard deviation

SED sediment

SSD species sensitivity distribution

SSW synthetic seawater

T temperature

TAN total ammonia nitrogen

TOC total organic carbon

Z zeolite

Zn zinc

$ZnSO_4$ Zinc Sulfate

ww wet weight

W water

µg microgram

Abstract

Marine sediments are fundamental and integral parts of water bodies and they are composed of soluble and insoluble compounds. Marine pollution in coastal areas is a major concern, due to the large number of toxic substances discharged and accumulated in the sediment. Natural (*i.e.,* bioturbation) or artificial (*i.e.,* dredging) perturbative events can release accumulated contamination, causing acute toxication to water column organisms and the re-allocation of contaminants within the same aquatic environment. This study investigates the effects of sediment spiked with PAHs, PCBs and Zn at sub-chronic concentration (at 192 µg/L, 0.15 µg/L and 40 mg/Kg, respectively) on the sea urchin *Paracentrotus lividus*. Results demonstrated that Zn was lethal to *P. lividus* adults after two weeks of exposure. Moreover, a toxigenic effect on embryos generated in females exposed for two months to PAHs and PCBs has been shown. (i) Morphological observations under optical microscopy revealed that PAHs and PCBs induced an increase of malformed and/or delayed embryos, and an increase of bioaccumulated compounds, compared to negative control. (ii) Molecular analysis by *de novo* transcriptome and *Real Time qPCR* showed that PAHs and PCBs are able to affect the expression levels of several genes involved in different cellular processes. After acute tests with four PAHs (naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT) and benzo(k)fluoranthene (BkF)), results showed that FLT was the most toxic compound to nauplii and adults of the crustacean branchiopod *Artemia franciscana*. In the third part of this work, all the available literature on the potential effects related to the use of amendments in sediment remediation (activated carbon (AC), nano-Zero-Valent-Iron (nZVI), apatite (A), organoclay (OC) and zeolite (Z)) has been reviewed and analyzed considering the sensitivity of the model species. The HC5 values in descending order were: AC (4.79 g/L) > nZVI (0.02 g/L) > OC, A and Z (1.77E-04 g/L). Moreover, the work investigated the differences in PAHs concentrations removal after AC and nZVI experiments both in sediment and seawater, and on their embryotoxic and genotoxic effects on *A.*

franciscana. Results showed that efficiencies of removal of AC and nZVI were similar both for the sediment and the seawater. nZVI showed higher toxicity and genotoxicity than AC. This study represents the first demonstration of the toxic effects of common pollutants spiked in sediments, on adults and embryos of the sea urchin *P. lividus,* adopting sub-chronic concentrations. Furthermore, the present work exhibits considerable ecological relevance, opening new perspectives on the study of negative impact on the resident biota of remediation methods.

1. Chapter 1: State of the art and thesis objectives

1.1 Contaminated marine sediment and EU directives

Environmental concerns arose in most European countries in the mid-1960s, and national governments started to be more active in their attempts to monitor and control environmental pollution. Major focus was devoted to developing and implementing environmental quality guidelines in policies and regulations (Van Wensem, 1990). Special attention was dedicated to the disposal of sediment, resulting from dredging activities of port channels and seaways to maintain maritime navigation. Marine sediments are fundamental and integrated parts of water bodies and are composed of soluble and insoluble matter, primarily rock and soil particles transported from land areas to the oceans by some natural processes, mainly rivers, glaciers and ice, windblown dust, coastal erosion (Brils, 2008). Recent studies showed that Mediterranean coastal sediment may be heavily contaminated, due to industrial, shipping and other anthropogenic activities (Lofrano et al., 2018; Pougnet et al., 2014). Sediment pollution is associated with potential economic, social and environmental problems (Arizzi Novelli et al., 2006; Eggleton and Thomas, 2004; Libralato et al., 2008; Lofrano et al., 2016). Marine pollution in coastal areas is a major concern, due to the large number of toxic substances discharged and accumulated in sediment, which act as sink and source of pollution (Arizzi Novelli et al., 2006). Especially in harbours and marinas, where exchanges of water masses with the open sea is limited, the accumulation of toxic substances can pose major concerns for human and environmental health such as in presence of recreational waters and maricultural activities (Mamindy-Pajany et al., 2010). Frequently, contaminants occur as mixtures showing combined effects, which are still largely unknown. Dredging activities in industrial and commercial ports tend to remobilize sediment, as well as the associated pollution through the washing out of both short- and long-term contaminant loadings (Arizzi Novelli et al., 2006; Libralato et al., 2008). An effective assessment of sediment quality is compulsory for the management of marine environment as required by

the Water Framework Directive (2000/60/EC) (WFD) and the Marine Strategy Framework Directive (2008/56/EC) (MSFD) that considered sediment as one of the key issues for the proper management of surface water bodies. Sediment toxicity proved to be essential in monitoring studies to characterize the state-of-the-art of aquatic environments and to take decisions about contaminated areas according to the TRIAD approach (Hurel et al., 2017; Libralato et al., 2008; Losso and Ghirardini, 2010). Polluted sediment represents an important issue for fresh, brackish and marine ecosystems, especially coastal ones, due to the high human pressures (i.e. commercial and industrial port activities, human settlements and tourism) and sedimentation rates caused by solid discharges from catchment basins (Lofrano et al., 2016; Nikolaou et al., 2009). Sediment drains and temporarily segregates pollution with potential direct acute and chronic effects for benthic communities. Natural (i.e., bioturbation) or artificial (i.e., dredging) perturbative events can release the accumulated contamination causing acute concerns to water column populations and the re-allocation of contaminants within the same aquatic environment. Thus, contamination can be scattered in vaster areas or sometimes exported outside from confined aquatic ecosystem (e.g., lake or lagoon) due to flooding events or tides (Arizzi Novelli et al., 2006; Mamindy-Pajany et al., 2010; A. Nikolaou et al., 2009). This activity must be carried out in a highly efficient and environmentally safe manner to reduce and keep impacts. Dredged sediment can be treated ex situ “on-site” or “off-site” and, finally, transported to its destination (e.g., landfill) or second life (e.g. construction materials). Sometimes the problem of polluted sediment can be tackled without dredging, keeping them in place especially when it must not be removed for assuring drafting ships and if physical dynamics of sites (i.e., currents and wave actions) are not of concern. Dredging and disposal of dredged material are constantly identified as top priority concern by European seaports since 1996 and they are a top priority independently of the port’s size. As recently estimated, up to 200 million m3 of dredged sediment are produced yearly at EU (SedNET, 2004;

http://www.sednet.org/download/Sednet_booklet_final.pdf). No clear indications on how to manage them, besides landfill disposal, are available.

Anthropogenic activities can change the natural compositions, the rate, the deposition and the transport of sediment causing a radical shift of biological responses (Burton and Johnston, 2010). Since sediment represents the habitat for benthic community, they could negatively affect the marine flora and fauna (Yi et al., 2011). Several biological communities can react differently to contaminant exposure: i) tolerant species will respond differently with respect to sensitive species; ii) the overall community tolerance is often induced by the constant contaminants' exposure (Clements and Rohr, 2009). The distribution of contaminants within sediment and their affinity with sediment particles may change following receipt to sediment disturbance that stimulates the mobilization of contaminants. Desorption, partitioning, bacterial degradation and the oxidation of organic compounds can accelerate changes of the redox potential and pH (Eggleton and Thomas, 2004). Anthropogenic activities, such as maintenance, capital dredging and the disposal of historically contaminated sediment are also able to induce the remobilization of pollutants in the water column (Eggleton and Thomas, 2004). This resuspension can negatively influence the marine environment and human health, because pollutants can accumulate in bottom feeders and magnify through marine food webs, reaching humans through fishery products (Zupo et al., 2019). For these reasons, the European Water Framework Directive 2000/60/EC (WFD) was developed and constantly updated. This directive provided a new approach for preventing the qualitative and quantitative deterioration of water, to improve the state of water and to assure a sustainable use (Borja et al., 2004). This directive does not specifically address sediment, although sediments are essential for the aquatic environmental. Since the sediment management plays an important role in preservation of good marine water status, the European Marine Strategy Framework Directive 2008/56/EC (MSFD) includes 11 descriptors including sediment as well (Rice et al., 2012). Both

Directives indicated what a "good marine environmental status" is, but the WFD classified ecosystems at different levels by comparing their features until combining them; whereas the MSFD focuses on the 11 descriptors, which together represent the function of the entire system. Thus, the WFD could be combined with the MSFD (Borja et al., 2010). Considering the potential great impact of contaminated sediment to the marine environment, the first step to deal with this problem could be the control of the contaminant source and/or its elimination of both in situ and ex situ treatments (Gomes et al., 2013). Nevertheless, these techniques for the remediation of polluted environments could negatively affect the resident biota, especially when applied *in situ* (Libralato et al., 2018; Lofrano et al., 2018).

1.2 Microcosm and sediment toxicity

The first applications of mesocosms in aquatic studies referred to the patterns of productivity and respiration during eutrophication in lower Narragansett Bay (Rhode Island, USA; (Oviatt et al., 1986); see **Figure 1A**). Another study was applied to detect the effects of chemicals in freshwater environment, varying in size, design, construction and costs according to the purpose (Beklioglu and Moss, 1996). Mesocosm experiments were used to study the interaction of sediment influence, fish predation and aquatic plants with the structure of phytoplankton and zooplankton communities (Beklioglu and Moss, 1996).

The Multiscale Experimental Ecosystem Research Center (MEERC) conducted several experiments in mesocosms with the aim of quantifying the effects of scale in terms of time, depth, radius, exchange rate and ecological complexity, on biogeochemical processes and trophic dynamics in different coastal habitats (Peterson et al., 2003; see **Figure 1B**). To this end, a series of experiments were performed, in which time, space and complexity in a variety of estuarine habitat types were systematically manipulated. Firstly, the effects of mesocosm size and shape on the dynamics of planktonic-benthic ecosystems were assessed. Fifteen cylindrical mesocosms were constructed according to five sizes, three volumes and three replicates per size and they were organized into three series: one with a constant depth

(1.0 m), one with a constant shape (radius-to-depth ratio = 0.57) and one with constant volume (10 m^3). Mesocosms received artificial light on a 12:12 light-dark cycle and an exchange of filtered estuarine water at a rate of 10% per day, mixed to mimic turbulence in a tidal environment. Because tidal estuarine marshes play an important role in processing nutrients and organic matter at the land-sea interface, the effects of species diversity on this function was evaluated. Six marsh mesocosms were constructed using fiberglass tanks (6 m long, 1 m wide, 1 m deep) angled at a 20:1 slope. In this system pumps were used to simulate a 12.5-h tidal exchange with filtered water from an adjacent estuary. The experiment was run for five years to evaluate long-term dynamics of three common plant species found in Chesapeake marshes (*Spartina alterniflora*, *Spartina patens* and *Distichlis spicata*) and no macrofauna were added. While the plant species diversity over the temporal extent of the experiment was preserved, unsuccessful maintenance of the additional animals introduced in the high diversity treatment was recorded. More in general, the results indicated that the effects might be categorized in fundamental (evident in both natural and experimental conditions) and artefacts of enclosure (attributable to the artificial environment in mesocosms).

A mesocosm study was also applied to detect the impact of resuspension on the fate and bioaccumulation of mercury and methylmercury in shallow estuarine environment (Kim et al., 2006). Two experiments of four weeks were performed in spring and fall in cylindrical tanks (1 m^2 of surface area and 1 meter deep), demonstrating that the interplay between mercury and methylmercury degradation determined the overall methylmercury pool in sediments. In turn, their resuspension lead to changes in the association to mercury binding phases, influencing its methylation.

Wagenhoff et al., (2012) studied macroinvertebrate responses along broad stressor gradients of deposited fine sediment and dissolved nutrients in a stream mesocosm. Most responses were additive multiple-stressor patterns. Later these authors also studied the composition of

benthic algae and cyanobacteria using the same experimental approach (Wagenhoff et al., 2013; see **Figure 1D**).

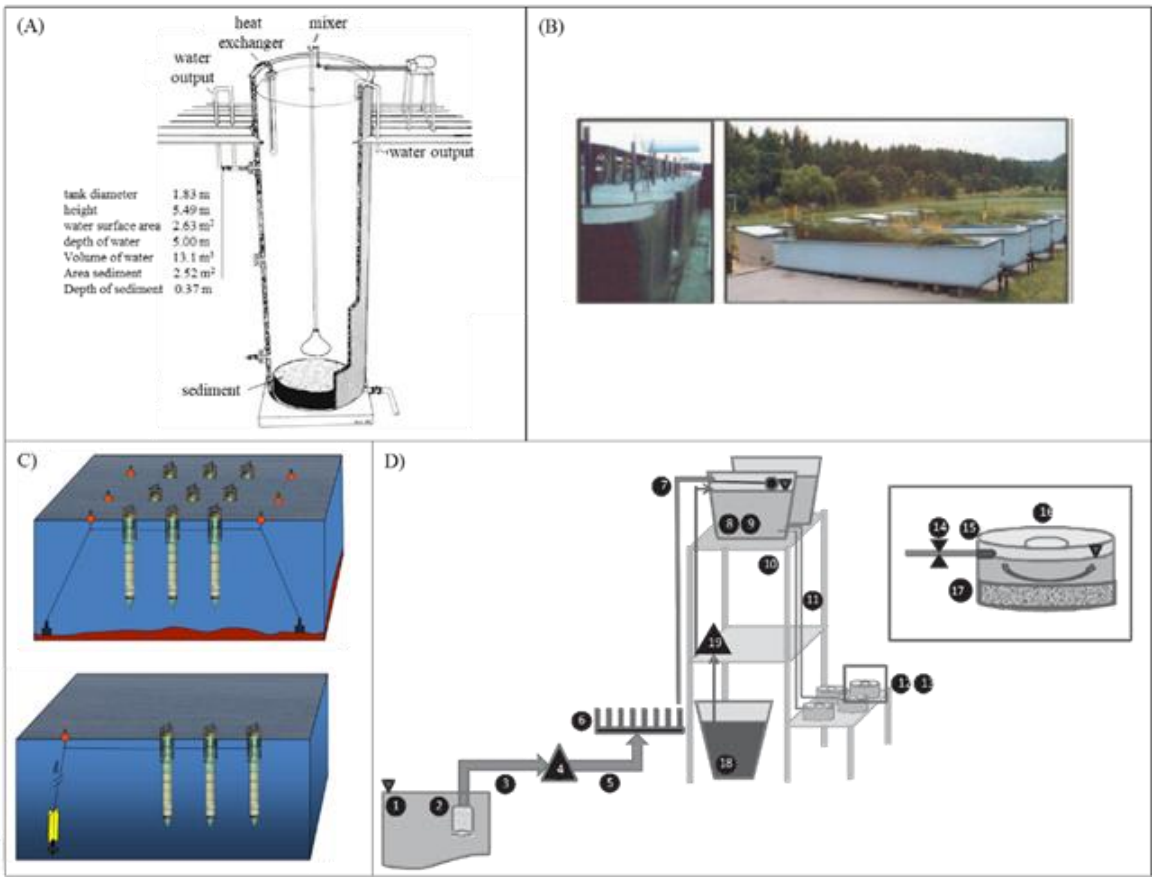

Figure 1: Mesocosms. (A) Cross section of a mesocosm, showing input and output pipes, mixer, sediment container and heat exchanger. The tanks are constructed of fiberglass reinforced resin with interior walls to maximize the reflection of sunlight. Seawater is fed in a pulsed flow of 10 1iters per minutes for 12 minutes period every six hours. Temperature control is accomplished with glass heat exchangers able to heat and cool (modified from (Oviatt et al., 1986). (B) Multiscale, multihabitat experimental ecosystems for planktonic–benthic systems (left panel), marsh (right panel) (modified from (Peterson et al., 2003). (C) Schematic representation of the two modes of mesocosm operation: mesocosms in moored mode in packs of three with anchor weight at each end (upper panel); mesocosms in free-floating mode connected to a weighted drogue hanging from a buoy at 150 m water depth (lower panel) (modified from Biebesell et al., 2013). (D) Experimental set-up. (1) Kauru River, (2) pump inflow covered with 4.5 mm mesh, (3) 20 m of 50-mm polythene piping, (4) two centrifugal pumps, (5) 80 m of 50-mm polythene piping, (6) eightfold manifold, used to split water flow into eight pipes, (7) 8 9 10 m of 25-mm polythene piping, (8) eight header tanks (volume 135 L), each one feeding a batch of 16 mesocosms, (9) water level in header tanks regulated by ball-cock, (10) two-level scaffold, 4.1 m high and 20 m long (11), 128 9 4 m of 13-mm polythene piping supplying individual mesocosms with water, (12) 1-m-high and 1.2-m-wide wooden bench running along scaffold, (13) 128 circular flow-through stream mesocosms, (14) tap regulator, (15) inflow jet, (16) mesocosm water outflow, (17) base layer of substratum, (18) seven barrels (volume 300 L) containing highly concentrated $NaNO_3$ and KH_2PO_4 solution for nutrient treatment levels 2–8, level one received ambient nutrient concentrations, (19) seven fluid metering pumps used to drip highly concentrated nutrient solutions into seven header tanks (mod. from Wagenhoff et al., 2013).

To perform mesocosm experiments in different hydrographic conditions and in areas considered most sensitive to ocean change, Riebesell et al., (2013) developed a mobile sea-going mesocosm facility, the Kiel Off-Shore Mesocosms for Future Ocean Simulations

(KOSMOS). The KOSMOS platform, transported and deployed by mid-sized research vessels, was designed for operation in moored and free-floating mode under low to moderate wave conditions (up to 2.5 meters wave heights; see **Figure 1C**). It consisted of a water column 2 meters in diameter and 15 to 25 m deep (about 50–75 m^3) without disrupting the vertical structure of the planktonic community. This system minimized the differences in starting conditions between mesocosms, extended experimental duration, determined the mesocosm volume and air–sea gas exchange.

Open system mesocosms were mainly used to simulate the ocean acidification and it has been demonstrated that this approach is low-cost and allows for pioneering long-term experiment with continuous intake of seawater ensuring the maintenance of quasi-natural environmental conditions (Jokiel et al., 2014). This approach is based on a low-cost method applicable to small or very large experimental systems and consisted of a gravity-feed seawater supply system with a peristaltic pump to regulate CO_2 injection rates and a power head, cavitating the injected CO_2 into microscopic bubbles that are dissolved immediately. The system permitted long-term experiments under full sunlight with rapid seawater turnover rate with realistic environmental conditions in the experimental chambers.

Moreover, a modular, small size (60 L) and indoor mesocosm was used to detect the environmental risk of engineered nanomaterials (Auffan et al., 2013). Initially, they waited for the settlement of suspended particles, then the stabilization of the physical parameters (O_2, redox potential and pH) and the growth of organisms were measured. This experimental system allowed the simultaneous monitoring of several parameters (e.g. aggregation, biotransformation, oxidative stress, microbial diversity) under environmentally meaningful conditions. This experimental design can accommodate several types of ecosystems such as lotic, lentic, estuarine, or lagoon environments, without the necessity of expensive infrastructures.

Pansch et al., (2016) used open systems to study the global change of temperature, ocean acidification, rising sea level and eutrophication. The system consisted in tanks of 170 x 85 cm connected to a measurement system used to assess different parameters such as temperature, dissolved oxygen and pH. Closed micro- and mesocosm systems are self-sustaining once built-up without intake of nutrients or more water. These systems were mainly used in toxicity experiments because they avoided the environmental dispersion of contaminants. Another interesting study was recently conducted by Lehto et al., (2017) using a closed mesocosm of 15 x 14 x 8 cm to explore possible effects of deposition of particulate organic matter and the release of metals from sediment. The examples showed the high versatility of these systems to different conditions, suggesting their challenging applications for contaminated sediment assessment.

1.3. Pros and cons of m-cosms for sediment quality assessment

At the beginning, the expectancy on the use of microcosms were very high (Draggan, 1976), but three years later (Gillett, 1989) concluded that they needed substantial improvements in the accuracy, sensibility and reliability to fulfil these expectations. Later, Hammons, (1981) reported about the improvements and discussed about advantages and disadvantages of the use of these systems. One of the major points concerned technical questions on the physical, chemical, biotic structure as well as the duration of experimental micro- and mesocosm (**Table 2**).

Advantages	Disadvantages
More sensitive than laboratory experiments	Low number of replicates
More reproducible data and easier to conduct experiments	Small size and short experiment time
Use of more species for defining the interaction between them	Inapplicable to understanding less simple parts of nature

Fills a gap between laboratory and field studies	Not realistic

Table 2. Advantages and disadvantages in the use of micro- and mesocosms.

Moreover, another critical point referred to the relationship of the results with a natural ecosystem, considering if m-cosms were analogous to natural ecosystems and/or are model ecosystems. Ausmus and O'neill, (1978) suggested some criteria for the extrapolation of m-cosms experiments to the field, considering if they reflected a natural ecosystem.

As reported, concerning the different types of m-cosm systems (Anderson et al., 1979; Clarholm et al., 1981; Coleman et al., 1977; Parkinson et al., 1979) the best results for a valuable comparison can be expected from open systems (Huhta et al., 1988; Setälä et al., 1988; Taylor and Parkinson, 1988; Williams and Griffiths, 1989). Some authors reported the results of laboratory microcosm experiments (Teuben and Roelofsma, 1990) and field experiments with mesocosms (Teuben, 1991) comparing them with measurements of process variables from the field, to test the relevance of these micro- and mesocosm studies. These results concluded that m-cosms were reliable to unravel the intrinsic soil ecosystem interactions, but mesocosms in the field were referred to laboratory microcosms. At the beginning microcosm tests are more expensive and time consuming, than other types of traditional tests for environmental issues (Gorsuch et al., 1993).

In addition, it is important to consider that the prevention of the ecological impacts of global environmental changes require scientific evidences, but the temporal and spatial scales of these environmental problems are too extensive and become difficult to gain only by using the traditional experimental manipulation (McCann, 2007). M-cosms might help, even if a 'credibility gap' can arise from within the scientific community, in understanding if insights from these experimental systems could be relevant to study larger scale processes. Benton et al., (2007) considered the key role of m-cosms in the attempt to understand large-scale processes. having two important roles: i) providing ideal systems on which the statistical

approaches can be tested; ii) managing recommendations on systems with limited knowledge of the ecological processes are involved.

Despite their distinct contribution to our understanding of ecology and the environment, however, microcosm approaches have been often criticized for being irrelevant for policy-relevant issues (Carpenter, 1996). An opposite viewpoint was presented by Cadotte et al., (2005), claiming that laboratory models were necessary for investigating complex ecological communities. This may have been pushed by the urgency to explore the fundamental processes of ecology rather than with the aim to use model systems to address real world and global-scale problems.

Ecologists tried to build projects around microcosm experiments, because, in addition to the basic-science insights, *m*-cosms have pragmatic advantages important in the competition between environmental sciences and molecular biology (Carpenter, 1996). They can provide i) results to be rapidly published; ii) modest costs. Ecology is now considered a significant applied science with a responsibility versus the society in contributing to solve environmental problems by using appropriate scaled field studies.

More recently, Drake and Kramer, (2012) evidenced that m-cosms are ecologically simplistic, mainly the subset of species included, such as the exclusion of large-bodied species (Schindler, 1998), meaning that the reduction of complexity decreases the capacity of an experiment to provide reliable predictions about the ecosystem. M-cosms did not consider natural spatial and temporal variation, being small in size and short in duration and so limiting physical environmental heterogeneity and excluding processes at large spatial extents (Ricklefs, 2004). The use of mesocosms, essentially larger than microcosms, often includes at least partially these sources of variation (Relyea, 2005), giving rise to potential artefacts.

The use of m-cosms enables to study the stress response of various species and the effects on the communities at different levels (Crossland and La Point, 1992). Microcosm studies

were more sensitive to detect the toxic impacts when compared to laboratory experiments. Santos et al., (2018) studied the toxicity of pore water of three different sites using two approaches: i) experiments in microcosms, ii) and in laboratory. In both cases, different toxicity between three sites has been established. Microcosm experiments are easy to conduct and give more reproducible data than field experiments. Chen and Edwards, (2001) tested the effects of three different fungicides using a plastic tube as a microcosm, obtaining good results and reproducing the basic features of natural environmental at a small scale.

One of the advantages of using these systems is the great number of organisms that can be used. The use of mesocosms offered a solution to fill a gap between laboratory and field experiments, as in the case of Riebesell et al., (2013), where they made analyses about the effects of ocean challenges on pelagic community.

Hose and Van Den Brink, (2004)compared the species-sensitive distribution (SSD) curves of laboratory with local experiments in mesocosms and field data to evaluate the water quality in Australia. The mesocosm species were less sensitive than the species in laboratory tests, suggesting that the laboratory data can be used to determine the concentrations of mesocosms.

Several studies showed that the combination of the field, mesocosm and *in vitro* results were interesting to define toxicant effects. Cash et al., (2003) evaluated the regional assessment of rivers by using these combined studies establishing the main effects of the discharges and the community responses to these stressors.

Grenni et al., (2012) tested the effects of microbial activity for ecological bioremediation of contaminated ecosystems in mesocosm systems. Using these systems, they assessed the degradation property of microbial activity under different natural conditions and under more stressor factors by providing realistic results. In any case, careful attention must be paid in generalizing the conclusion to natural ecosystems (Petersen et al., 1997).

1.4. Sediment remediation assessment and m-cosms

Marine sediment is a fundamental and integrated part of water bodies accumulating biological, physical and chemical contaminants (Brils, 2008). Organic pollutants in sediment represent a worldwide problem, considering that sediment acts as a sink of hydrophobic, recalcitrant and hazardous compounds (Perelo, 2010). According to the biogeochemical processes, these contaminants are available to benthic organisms as well as to organisms present in the water column and toxic and carcinogenic, entering in the food chain and accumulating in the biological tissue. Among these compounds polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals represent the most abundant pollutants present in some marine sediments (Mamindy-Pajany et al., 2010), being released during perturbation events, absorbed by suspended particles and therefore settle at the bottom with a high impact on marine environment and consequently on human health (Geffard et al., 2001).

Because of the potential toxic effects of these compounds on human health, researchers explored methods to detoxify/remove them from natural marine environments with both *in situ* and *ex situ* treatments, using different chemical, biological and thermal methods (Bamforth and Singleton, 2005; Gomes et al., 2013). These processes were classified as forms of "bioremediation", which refer to a process through which toxic compounds are biologically degraded and/or transformed to an innocuous state (Mueller et al., 1997). This process consists in the removing of pollutants from the natural environment converting them to a less harmful product (Bamforth and Singleton, 2005).

1.4.1 PAH case studies

Polycyclic aromatic hydrocarbons (PAHs) consist of a group of over 100 different organic compounds, formed by two or more fused benzene rings and pentacyclic molecules differing in their physical and chemical properties. Their toxicity depends on the number of benzene rings (Perelo, 2010) and can be generated by: i) natural events such as volcanic eruptions,

fossil fuels or incomplete combustion of organic materials; ii) anthropogenic activities such as industrial processes, oil and diesel spills and waste incineration. Anthropogenically produced substances that are considered the most impacting inputs into the natural environment (Bamforth and Singleton, 2005). These compounds tend to persist in the environment because of their low water solubility and hydrophobicity that permit them to be adsorbed and accumulated in sediment, where their degradation is very slow. The persistence of PAHs in the environment can also be controlled by such environmental factors such as soil type, pH, temperature, oxygen levels (Bamforth and Singleton, 2005). Sixteen PAH compounds were classified as priority pollutants by the United States Environmental Protection Agency (US-EPA; Perelo 2010). Some studies have demonstrated that these organic molecules can have toxic effects for human health with many PAHs showing carcinogenic, teratogenic and mutagenic properties (Ferrarese et al., 2008). For these reasons, several remediation techniques were used for PAHs removal from contaminated sediments (**Table 3**).

Strategy	**Site**	**Operating conditions**	**Efficiency of treatment**	**References**
Biological	Italian harbor in Mediterranean Sea	Mixtures of inorganic nutrients and sand amendments	40% PAHs	(Beolchini et al., 2009)
	Ancona port	Mixtures of inorganic nutrients and switch of temperature, in aerobic and anaerobic conditions	80% PAHs	(Dell'Anno et al., 2012)
	Carteau cove	Combination of marine polychaeta *Nereis diversicolor* and oil contaminated sediments	Increase of bacteria communities to reduce contaminations	(Cuny et al., 2007)
	Dredged sediments from DeGray Reservoir	Inoculation of *Mycobacterium sp.*	25% 2-methylnaphthalene, 74% phenanthrene,	(Heitkamp and Cerniglia, 1989)

			34% pyrene, 5% benzo-α-pyrene	
	Van-veen grab in Nyborg port	Combination of marine polychaeta *Capitella* sp. I and oil contaminated sediments	No effects on PAHs decrease	(Holmer et al., 1997)
	Stanford le Hope, Essex, UK	Mixtures of inorganic nutrients and three hydrocarbon-degradation bacteria	78% PAHs with *Alcanivorax*, 70% *Thalassolituus*, 70% all bacteria	(McKew et al., 2007)
	Kamaishi (Japan)	Inoculation of *Cycloclasticus sp.*	38% PAHs	(Miyasaka et al., 2006)
	Gulf of Fos (France)	Natural develop of hydrocarbon-degradation bacteria	35% PAHs	(Syakti et al., 2006)
	Yiu Lian Floating Dock (France)	PAHs-addition	Increase PAHs-degradation bacteria, 50-98% PAHs	(Wang et al., 2011)
Chemical/ physical	Oxford	AC-addition	80% PAHs	(Bussan et al., 2016)
	River Tyne	AC-addition	94% PAHs	(Hale et al., 2010)
	Oslo harbor	Capping	93% PAHs retained	(Eek et al., 2008)
	Norwey	AC-Capping	93% PAHs retained	(Samuelsson et al., 2015)
	Rivers Tyne and Wear (UK)	MAC-addiction	98% PAHs retained	(Han et al., 2015)

Table 3. Strategy, site, operating conditions, efficiency of treatment for bioremediation by PAHs.

Some researchers suggested bioremediation techniques using the microbial metabolism to remove pollutants from contaminated sediments. Three fundamental methods exist: a) bacterial degradation, b) lignolytic and c) non-lignolytic fungal degradation, which all together on benzene rings (Bamforth and Singleton, 2005). The bacterial degradation may take place aerobically, anoxically, or anaerobically, but PAH-restoring are usually associated

with anoxic/aerobic biodegradation because most of the contaminated sediments are in these conditions (Lu et al., 2011).

The main mechanism for aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the activity of dioxygenase enzymes, which can then be further metabolised via catechol to carbon dioxide and water. The metabolic pathways and enzymatic reactions involved in the microbial degradation of naphthalene have been studied in detail (Cerniglia, 1993). Several bacteria can oxidize naphthalene through dioxygenase enzymes, including organisms from the genus *Pseudomonas* and *Rhodococcus* (Cerniglia, 1993; Mueller et al., 1996; Ravelet et al., 2000).

PAH-restoring also took place thanks to the action of other microorganisms, as cyanobacteria, green algae and diatoms, which can transform naphthalene into different metabolites. Several studies under controlled conditions were performed using the miniaturized ecosystems micro- and mesocosms (Grenni et al., 2012). The first study was performed by Heitkamp and Cerniglia, (1989), using sediment and water collected with a Peterson dredge from DeGray Reservoir (a manmade impoundment which receives no significant exposure to chemical compounds), near Arkadelphia (Arkansas, USA). A microcosm (consisting of a flow-through system, 0.5 L glass tanks containing 20 g of sediment and 180 mL of lake water) was used to conduct biodegradation tests to monitor mineralization and recovery of chemicals. *Mycobacterium* sp. was added to sediment and water from this pristine ecosystem. Microcosms inoculated with the *Mycobacterium* sp. showed enhanced mineralization, alone and as components of a mixture of 2-methylnaphthalene, phenanthrene, pyrene, and benzo[*a*]pyrene. In (Miyasaka et al., 2006), the efficiency of bacteria in PAHs marine sediment restoring has been confirmed. In fact, they investigated three different bioremediation methods using microcosms: biostimulation with fertilized, bioaugmentation with *Cycloclasticus* sp. and mixtures of fertilized and

bacteria. All microcosm experiments resulted efficiently for PAHs restoring after 90 days of exposure, having effects on bacterial abundance.

Considering the important role of the microorganisms in contaminated sediment bioremediation, several studies were performed on the effect of nutrient additions (e.g., nitrates) on the microbial communities of contaminated sediment with PAHs. The structural properties and the recycling processes of these microorganisms were highly enriched after inorganic materials addiction, suggesting that this bioremediation *in situ* can be stimulated by the contaminated sediments for bioremediation were collected from an Italian harbour in the Mediterranean Sea, using microcosms with 20 g of wet sediment samples and 100 mL of pre-filter seawater. Inorganic materials were added to all tanks (nitrogen and phosphorus) and to sand amendments and after thirty days the growth of prokaryotic organisms was detected, and the PAHs decreased of 40%. Dell'Anno et al. (2012) showed that bacterial abundance and biodiversity changed and increased at high temperature in both aerobic and anaerobic conditions. Bioremediation experiments were performed with sediment samples from two sites of the port of Ancona (Italy) characterized by two different redox potentials, using microcosms in anaerobic and aerobic conditions. The nutrients were added together with the increasing of temperature from 20 °C to 35 °C, observing major changes in the bacterial biodiversity and abundance at higher temperature in both conditions (aerobic and anaerobic), with a subsequent decrease of PAHs. No significant effects of nutrient addictions were evidenced. A part from the important role of hydrocarbon-degradation bacteria in restoring sediments, little information is available on their different strategy of bioremediation. McKew et al. (2007) analysed the degradation efficiency of three hydrocarbon-degradation bacteria (*Alcanivorax*, *Thalassolituus*, *Cycloclasticus*) adding inorganic nutrients (i.e. N and P) and using microcosm and seawater collected from a site (Essex, UK) neighbouring the Shell-Haven and Coryton BP oil refineries. Different microcosms were built under several conditions: oil-only microcosm, *Alcanivorax* + N-P

microcosm, *Thalassolituus* + N-P microcosm and *Cycloclasticus* + N-P microcosm, all bacteria + N-P + microcosm. They observed an increase of all bacteria after one month, mainly of *Alcanivorax*. However, in all microcosms with nutrients, they recorded a decrease of PAHs levels, showing a significant correlation between availability of nutrients and bacteria abundance.

Furthermore, it has been demonstrated that bacterial biodiversity and abundance were correlated to contamination. Syakti et al. (2006) recorded an increase of hydrocarbon-degradation and heterotrophic bacteria in the oil contaminated sediments. Specifically, microcosms were used with/without oil-contaminated sediments to assess PAHs-biodegradation and phospholipids fatty acid (PLFA) composition. An increase of hydrocarbon-degradation bacteria was observed after 21 days, due to a composition change in PLFA and consequently the decrease of PAHs. Wang and Tam, (2011) demonstrated an increase of PAHs-degradation bacteria by adding PAHs to hydrocarbon-contaminated sediment. This experiment was performed in a microcosm (consisting of 12 conical flasks of 250 mL, each containing 150 mL sediment slurries prepared by mixing 800 g fresh marine sediments and 1600 mL sterilized) with/without added PAHs to contaminated sediment. A clear enhancement of degradation bacteria was recorded after 60 days, and a reduction in species diversity in all treatments. This change was coupled by a significant depletion of PAHs (50-98%; see also **Table 2**).

Moreover, Cuny et al. (2007) observed that petroleum contaminated sediments inhabited by a marine polychaete *Nereis diversicolor*, allowed the growth of specific bacteria, such as *Psychroserpens burtonensis*, known to degrade PAHs. Then, they investigated on the possible influence of these polychaetes on the development of different bacterial species using microcosms, consisting of twelve 2.5 L aquariums. They established that the presence of *H. diversicolor* in contaminated sediments promoted the degradation of oil and the development of bacterial communities able to reduce pollution after one month of exposure.

However, the possible role of some polychaetes on PAHs-degradation was already evaluated in previous studies. Holmer et al. (1997) conducted a study on the polychaete *Capitella* sp. influence in the biogeochemistry of oil-contaminated sediments. Specifically, they used microcosms inoculating the PAH fluoranthene and worms, and established that the initial rate of PAHs did not decrease but these compounds were transported to deeper sediment layers where microbial degradation activity was less efficient.

Several studies were conducted to assess the potential role of chemical oxidation in PAHs degradation in contaminated sediments. There are various reactive liquids (such as modified Fenton's reagent, hydrogen peroxide, potassium permanganate, activated sodium persulfate) and he residual concentration of PAHs after each type of chemical oxidation has been followed to assess the efficiency of treatments. The best results were achieved with the use of modified Fenton's reagent, hydrogen peroxide and potassium permanganate (Ferrarese et al., 2008).

Another method of chemical remediation is the addition of activated carbon (AC) to contaminated sediment, that can be defined as all substances with high carbon content and porosity enabling to absorb different compounds (Rakowska et al., 2012). PAHs-concentrations in sediment decreased of > 50% after AC treatment. The efficiency of this amendment depends on the quantity of AC added to the sediment per unit of volume/weight. The ratio, then, is influenced by the physical-chemical properties of sediment. Recently Bussan et al. (2016) investigated the role of AC in PAHs bioremediation of polluted sediments using marine microcosm systems before any treatment with AC. Then they set up different experimental designs: i) only sediments without amendments, ii) autoclaved sediments, iii) sediments + biochar; iv) sediments + AC. After two weeks, they observed a considerable mercury-depletion in all treatments. Hale et al. (2010) compared bioremediation and AC amendment for PAHs contaminated sediment from River Tyne. The efficiency of different amendments was followed using microcosm, including: i) an

unamended system of 7 g sediment and 40 mL of River Tyne water and two amended systems with 7 g sediment, ii) 40 mL nutrient solution and iii) 40 mL nutrient solution inoculated with *Pseudomonas putida* (i.e. a strain that can degrade naphthalene and phenanthrene). These three systems showed a significant decrease in total sediment PAH concentrations over one month. Polyethylene passive (PE) samplers were embedded for 21 days in these sediment microcosms in order to measure the available portion of PAHs and accumulated from the unamended, biostimulated, and bioaugmented microcosms, respectively. Higher PAH uptake by PE samplers in biostimulated and bioaugmented microcosms coincided with slower degradation of spiked phenanthrene in sediment-free filtrates from these microcosms compared to filtrates from the unamended microcosms. Microbial community analyses revealed changes in the bacterial community directly following the addition of nutrients, but the added *P. putida* community failed to establish itself.

This amendment was usually associated to another *in situ* treatment, the capping, which is a physical cover of contaminated sediment, made of several materials, such as sand, clay or mixtures of different components. The combination between capping and AC is successful and do not lead to massive AC resuspension (Cornelissen et al., 2006). Eek et al. (2008) tested a mineral cap (crushed limestone) to limit the PAHs spread into the water column using microcosms. During 410 days of treatment, they assessed that the flux of contaminants from capped sediment was low respect the flux from uncapped sediment. Samuelsson et al. (2015) evaluated three different *in situ* AC-capping (AC-only, AC + clay and AC + sand) to assess the cap efficiency. After one year of capping, they collected samples to understand the best method using microcosms, using the worm *H. diversicolor* and the clam *Abra nitida* as test organisms. They measured the PAHs-bioaccumulation of two organisms, after one month, demonstrating that the AC-capping + clay was most efficient. Nevertheless, the AC addiction is an efficient method of bioremediation, and it has been demonstrated that it

causes adverse effects on some organisms, especially on benthic organisms. In fact, one part of the AC added can diffuse in the water column causing negative impacts on various organisms. Another option can be the use of iron-based amendments into "magnetic AC" (MAC) because it has been demonstrated that it has remediation features as also AC, but it enables their magnetic retrieval (Libralato et al., 2018). Han et al., (2015) tested the MAC remediation using microcosm experiments to evaluate the remediation capacity and possible negative or positive effects on microorganisms. After six months, they showed that the MAC use efficiently reduced PAHs concentrations. They recorded the 77% of MAC and the remainder of MAC had fewer negative effects respect to AC on *Lumbriculus variegatus* while maintaining PAH availability low.

1.4.2 PCB case studies

Polychlorinated biphenyls (PCB) are persistent organic compounds and are considered among the most impacting contaminants because of their toxic and carcinogenic properties, vast distribution and high permanence in environmental systems (Perelo, 2010; Richard B Meagher, 2000). These compounds mainly derive from lubricants, plasticizers and adhesives, as well as from the production of, hydraulic and dielectric fluids. The accumulation of PCB-contaminated sediment by biota represents a great problem because they can be introduced into the food webs, constituting a high risk for human health (Gomes et al., 2013). Several studies have been conducted to assess the best method of PCB-contaminated sediment remediation (**Table 4**).

Strategy	Site	Experimental design	Operating conditions	Efficiency of treatment	References
Biological	Strážsky (Slovakia)	Microcosm	Addiction of 15 different bacterial strains	10-20% PCB 153 and 118, 25% PCB 138, 40-45% PCB 101, 30-80% PCB 52, 55-80% PCB 28	(Dudášová et al., 2014)

	Busan coast (South Korea)	Microcosm	Addiction of 2 bacterial strains and plant terpenes	60-70% PCB	(Kwon et al., 2017)
	Lake Hartwell, New Bedford harbor, Roxana Marsh	Microcosm	Addiction of iron to produce hydrogen	Enhancement of PCB-degradation	(Varadhan et al., 2011)
	Brentella canal (Venice, Italy)	Microcosm	Addiction of iron to produce hydrogen	Enhancement of PCB-degradation	(Zanaroli et al., 2012)
	Anacostia River	Microcosm	Mixtures of electron donors, alternate halogenated electron acceptors, tetrachloroben zene, pentachloronit robenzene and bacteria	Enhancement of PCB-degradation	(Krumins et al., 2009)
	Brentella canal (Venice, Italy)	Microcosm	Addiction of PCBs	90% PCB	(Zanaroli et al., 2010)
	Mar Piccolo (Taranto, Italy)	Microcosm	Addiction of PCB-degradation bacteria	Important decrease of PCB	(Matturro et al., 2016)
	Grasse River (Massena, NY)	Microcosm	Addiction of PCB-degradation bacteria	83% PCB	(Kaya et al., 2017)
	Grasse River (NY), Fox River (WI), Baltimore	Microcosm	Addiction of PCB-degradation bacteria	51-88% in Grasse River, 83-88% in Fox River and 70% in Baltimore harbor	(Kaya et al., 2018)

	harbor (MD)				
Chemical/ physical	Grasse River (Massena, NY)	Microcosm	AC-addition	70-90% PCB	(Sun and Ghosh, 2007)
	Grasse River (Massena, NY), Hunters Point Naval Shipyard (San Francisco Bay, CA)	Microcosm	AC-addition	70-90% PCB	(Mcleod et al., 2008)
	Lac du Bourget (Savoie, France)	Microcosm	GAC + biofilm	Efficiently PCBs adsorption	(Mercier et al., 2013)
	Lac du Bourget (Savoie, France)	Microcosm	Three different granular AC-addition	98.3-100% PCBs	(Mercier et al., 2014)
	Baltimore harbor (USA)	Mesocosm	Bioaugmentation with anaerobic halorespiring *Dehalobium chlorocoerci* and aerobic *Burkholdeira xenovorans,* and GAC addition	>80% PCBs	(Payne et al., 2013)
	Oslo harbor	Microcosm	Capping	93-98% PCBs	(Eek et al., 2008)

Table 4. Strategy, site, operating conditions, efficiency of treatment for bioremediation by PCBs.

The use of bacteria to reduce PCBs concentration in marine contaminated sediment was reported. Kwon et al., (2017) demonstrated that the best rate of bacterial degradation can be

obtained by adding plant terpenes. To date, data on the application of plant terpenes in marine sediments contaminated with PCBs remained limited, being mainly used in soil systems. Various microcosms were built only sediment, sediment + plant terpenes and sediment + plant terpenes + bacteria. In this case, the experimental setup was prepared into a 250 mL Erlenmeyer flask. A significant PCBs decrease has been observed after one month in microcosms that contained both plant terpenes and bacteria with respect to other two conditions. Krumins et al., (2009) used microcosm experiments and demonstrated that the enhancement of PCBs-degradation can be obtained with biostimulation and bioaugumention. Microcosms were constructed, using 200 mL site sediment in 250 mL stock bottles with homogenized sediment recovered from the Anacostia River (USA) capping site control plot. Various microcosm conditions were used as the following: only sediment (control), sediment + bacteria, sediment + mixture of electron donors, alternate halogenate acceptors, tetrachlorobenzene, pentachloronitrobenzene and sediment + bacteria + bioaugumentation. After 400 days, they assessed a decrease of PCB-concentrations in microcosms with bioaugumentation and biostimolation. Moreover, the PCB-degradation bacteria, as PAHs-degradation microorganisms, can be isolated from contaminated sediments and can be stimulated by adding pollutants (PCBs). In fact, Zanaroli et al. (2010) demonstrated that the degradation of PCBs was enhanced by the addition of contaminants, because it promoted the development of PCBs-dichlorination bacteria. They showed an important decrease of PCBs after 30 months, caused by the growth of bacteria after the addition of the contaminants. Kaya et al., (2017) conducted similar experiments using microcosms with addition of PCBs. After 30 days of incubation, they assessed a crucial reduction of PCBs in marine sediments with a corresponding increase of dichlorination bacteria. One year later, Kaya et al. (2018) evaluated the depletion of PCB after the addition of pollutants, using three different sediment microcosm samples. After 30 days, they measured a decrease of PCB concentrations, which were recorded at different percentages,

suggesting that there were different dichlorination activities. Certainly, it is not related to different contamination history of three sediments because the sites with the highest percentages of dichlorination had also the lowest initial bacterial concentrations. Probably, individual congeners of PCBs may affect the activity of individual PCBs-degradation bacteria. Dudášová et al., (2014) performed microcosm experiments to assess the bacteria role in PCB-restoring using 15 different bacterial strains, isolated from PCBs-contaminated sediments. After 21 days, five of the 15 bacterial strains were suitable for restoring PCBs-contaminated sediments, because these microorganisms demonstrated high biodegradation ability compared to others.

Varadhan et al., (2011), enhanced the PCBs-degradation by dehalorespiring microorganisms in contaminated sediments by providing hydrogen produced in anaerobic corrosion of iron added to sediments. In that case, three different microcosm sediments were used, consisting of 125-mL borosilicate serum bottles, in which they added different iron concentrations. After 18 months, they observed an improved performance of bacterial degradation, at low levels of iron, suggesting that the bacteria preferred low concentrations of hydrogen. Zanaroli et al., (2012) conducted a similar study using microcosm experiments in which they added iron to stimulate PCBs microbial dichlorination. After 36 weeks, an inhibitor effect of iron on sediment indigenous microbial communities was observed, favoring the growth of PCB microbial dichlorination and their biodegradation activity. The iron, probably, reduced the lag phase of PCB-dichlorination by blocking the sulfate reducing bacteria and by promoting hydrogen release. Matturro et al., (2016) studied the PCBs-degradation in marine sediments (consisting in sterile 250-mL serum), using different microcosm experiments with sediments and sediment with/without addition of *Dehalococcoides mccartyi*, microorganism known able to degrade PCBs. After 320 days, they observed an increase of the PCBs-degradation community and a decrease of PCBs in microcosms with *D. mccartyi.*

Furthermore, different studies were conducted on possible roles of AC in PCBs-degradation. In fact, as reported in Sun and Ghosh, (2007) the use of little AC addition in PCBs-contaminated sediments reduced the bioavailability of these compounds for the oligochaete *Lumbriculus variegatus*. Setting-up different microcosms (only sediment, sediment + AC and sediment + AC + worms), after one month of exposure a decrease of PCB-concentrations was registered together with a reduction of PCB bioavailability in worm's chemical activity. Mcleod et al., (2008) performed microcosm experiments with AC additions to reduce the PCBs bioavailability in contaminated sediments from two different sites using the clams *Corbicula fluminea* and *Macoma balthica* as bioindicators. A considerable reduction of PCBs in microcosms was assessed after 28 days of exposure, and after the addition of AC. In addition, several studies have been conducted on the PCBs absorption of granular activated carbon (GAC) and the biofilms that grow on these particles. Mercier et al., (2013) evaluated the efficiency absorption of three different GAC conditions using different microcosm experiments: raw GAC, GAC particles with and without biofilms. The adsorption capacity was excellent for all conditions after one month, even in the case of GAC particles plus biofilms where the PCBs removal was not hampered by biofilm development. Afterwards Mercier et al., (2014) evaluated the PCBs removal efficiency of three different GACs in contaminated sediments. After 8 months of treatment in microcosms, all granular particles were able to efficiently absorb the PCBs in marine sediments and the bacterial adhesion depended on physical and chemical properties of particles. Later, Payne et al., (2013) demonstrated that the use of GAC with simultaneous application of aerobic and anaerobic microorganisms was able to better reduce the PCB concentrations by about 80%. Different mesocosm conditions were used: only GAC, GAC + sodium lactate, GAC + zerovalent iron, GAC + anaerobic *Dehalobium chlorocoercia* (DF1), GAC+ DF1 + sodium lactate, GAC + DF1 + zerovalent iron, GAC + DF1 + aerobic *Burkholderia xenovorans* (LB400), GAC + DF1 + LB400 + sodium lactate and GAC + DF1 + LB400 + zerovalent

iron. The results demonstrated that i) the best effects were obtained with bioaugmentation using both organisms after one year, and ii) a treatment employing both aerobic and anaerobic microorganisms could be considered a suitable strategy to remove PCBs in contaminated aquatic sediments.

Another strategy used to remove PCBs in contaminated marine environment is the capping, also used for PAHs (see above). Eek et al., (2008) tested a mineral cap (crushed limestone) to prevent PCBs diffusion and to limit bioavailability of these compounds in the marine environment. After 13 months of treatment in microcosm, they showed that the use of capping significantly limited PCBs concentrations respect to uncapped sediment microcosm.

1.4.3 Heavy metals case studies

Heavy metals (zinc, copper, manganese, silver, iron, cadmium, and so on) often dispersed in marine environments derive from anthropogenic activities and represent an issue for marine environments and consequently for human health. These elements can diffuse and accumulate in sediment where they influence physical and chemical factors (Peng et al., 2009). Several studies have been conducted to investigate their removal strategies (**Table 5**).

Strategy	Site	Operating conditions	Efficiency of treatment	References
Biological/ Chemical	Site of US	Injection of ethanol	Increase of bacteria communities able to reduce contaminations	(Cardenas et al., 2008)
	Ancona harbor (Mediterranean Sea)	Mixture of autotrophic and heterotrophic bacteria	>90% Cu, Cd, Hg and Zn	(Beolchini et al., 2009)
	Oak Ridge (Tenessee)	Addition of the electron donors	5-7% with ethanol, 49% with glucose, 93% with methanol	(Madden et al., 2009)
	Three sites in New Caledonia	Addition of glucose in oxic and anoxic conditions	Enhancement of nickel removal	(Pringault et al., 2010)

	Ancona harbor (Mediterranean Sea)	Addition of the electron donors	Change of metal partitioning	(Rocchetti et al., 2012)
	Three sites in New Caledonia	Addition of glucose in oxic and anoxic conditions	Enhancement of zinc removal	(Pringault et al., 2012)
	Ports of Livorno, Piombino and Ancona (Mediterranean Sea)	Mixture of autotrophic and heterotrophic bacteria	40-76% Zn, 0-7% Pb, 13-39% Cd, 1-8% Cr, 20-23% As, 35% Ni	(Fonti et al., 2013)
	Port of Piombino (Mediterranean Sea)	Addition of the electron donors	32-40% Zn, 14-40% Pb, 26% Cd, 15% Cr, 2-20% As	(Fonti et al., 2015)
	Contaminated sediment	Addition of microalgae	Enhancement of copper and zinc removal with RED LEDs	(Kwon et al., 2017)
	Port of Ancona (Mediterranean Sea)	Mixture of different chemical leaching agents and autotrophic and heterotrophic bacteria	30% Zn, 30% Cr, 40% Ni, 35-58% As	(Beolchini et al., 2013)

Table 5. Strategy, site, operating conditions, efficiency of treatment for bioremediation by heavy metals.

As for PAHs and PCBs, the most common applied strategies concerned biological and chemical activities. The potential role of bacteria versus heavy metals degradation was tested in several studies. Beolchini et al. (2009) tested the potential role of different bacteria strains in bioremediation of dredged sediments contaminated by heavy metals using microcosms. Three conditions were set up: i) microcosms with autotrophic bacteria, ii) microcosms with heterotrophic bacteria, iii) and microcosms with mixed culture. In presence of both bacterial types, the efficiency of heavy metal extractions increased after 15 days, suggesting a potential synergistic action of these microorganisms. Fonti et al. (2013) studied three contaminated sediment microcosms in which they added autotrophic Fe/S oxidizing and

heterotrophic Fe-reducing bacteria. The presence of autotrophic Fe/S oxidizing bacteria enhanced after 14 days of treatment, to maintain the acid and oxidative conditions, so influencing metal solubilization in the marine environment. Pringault et al. (2010) evaluated the bacteria role in Ni removal. Different experimental designs were built up: microcosm without metals (control), microcosm with Ni and formaldehyde (to sterilize sediment and seawater), and microcosm with nickel and glucose (to stimulate the bacterial growth). Nickel removal was always significantly higher in non-sterilized microcosms after 8 days, indicating the important role of bacteria. Pringault et al. (2012) evaluated the possible effects of Zn on bacterial structures and if these microorganisms could play a role in adsorption of this metal. They set up oxic and anoxic microcosm experiments using different conditions: one microcosm without Zn (control), one microcosm with Zn, one microcosm with metal and formaldehyde or glucose. The microbial structure changed after 8 days, more in microcosms with glucose than in microcosms with metal. Probably, these different effects were due to the strong adsorption of Zn in presence of bacteria (uptake).

Cardenas et al., (2008) stimulated the bacterial growth in uranium-contaminated sediments injecting ethanol in situ microcosm experiments. After two years of treatments with ethanol addition, they showed that the denitrifying bacteria (especially *Desulfovibrio* spp.) increased with the reduction of U (VI) to U (IV). Madden et al., (2009) added different electron donors (ethanol, methanol, glucose and methanol with humic acids) to improve the bacterial performance in uranium reduction. Microcosm experiments were performed to different exposure time, depending on the type of electron donors: 77-153 days for ethanol, 53 days for glucose and 90 days for methanol. All electron donors stimulated the U-reduction with no difference with humic acids addition. The enhancement of U-reduction was probably due to encouragement of bacterial activities with electron donors' additions. In all microcosms they found different bacteria, known to reducing Rocchetti et al. (2012) added different electron donors, lactose and sodium acetate, to stimulate the prokaryotic development. They

performed anaerobic microcosm experiments at either 20 °C or 35 °C, to investigate the effects of biostimulation activities on sediments contaminated with hydrocarbons and metals. After 60 days of incubation, they determined a significant change of metal partitioning and hydrocarbon concentrations in microcosms with lactose and sodium acetate at 20 °C. These results showed that additions of electron donors and the temperature influenced the prokaryotic metabolism, determining a decrease of PAHs concentrations and a change also on metal partitioning. In fact, the bioremediation strategies on contaminated sediments can influence the mobility and bioavailability of metals found in sediments. As reported in Fonti et al. (2015) during the restoring activities, the use of bacteria and the addition of electron donors to enhance the microbial growth were associated to metal decreases, suggesting a potential role of prokaryotes in metal assembly processes. Specifically, they used microcosms in which lactose and sodium acetate were added, and after 60 days the metal partitioning and the abundance and diversity of bacteria were measured. A considerable increase of abundance and diversity of bacteria was recorded in all microcosms and a decrease of metal concentration, confirming that the bacterial activities play an important role in metal mobility in the sediments. The heavy metal restoration involving microorganisms and algae was not an exception. Kwon et al. (2017) used microcosm experiments with addition of microalgae demonstrating that the use of these microorganisms could be efficient in metals removal. They studied the effects of monochrome and mixed wavelength on absorption of copper and zinc by microalgae *Phaeodactylum tricornutum*, *Nitzschia* sp., *Skeletonema* sp. and *Chlorella vulgaris*, showing in the last one the highest copper and zinc removal.

Another strategy used to remove heavy metals in contaminated marine environment is the chemical treatments. Beolchini et al. (2013) performed microcosm experiments with addition of different chemical leaching agents, oxalic, citric and sulfuric acids, and autotrophic and heterotrophic bacteria to evaluate extraction of toxic metals from

contaminated sediments. After 14 days, they assessed that citric and sulfuric acids were the most appropriate to remove the toxic metals. They also measured similar effects in presence of ferrous iron showing that the role of autotrophic bacteria was the most important in the metal extraction and the heterotrophic bacteria were used as support for the autotrophic bacterial activities.

1.5 An overview of PAHs toxicity: the case of phenanthrene, naphthalene, fluoranthene and benzo(k)fluoranthene

PAHs are an important form of pollution and can cause toxicological effects that occur at all levels of biological organization, compromising the life cycle of aquatic organisms and consequently the human health. These organic compounds have been studied in different compartments of the environment, due to their carcinogenic and mutagenic activities in the life of human organism (Boffetta et al., 1997). Due to their low vapor pressure, some PAHs are present in the air at room temperature, both as a gas and associated with particles. The lighter PAHs, such as phenanthrene, are found almost exclusively in the gas phase while the heavier PAHs, such as benzo [a] pyrene, are almost totally adsorbed on the particles. For these reasons, they are found ubiquitously distributed throughout the aquatic environment, from sediments to aquatic surfaces, impacting all trophic levels from zooplankton to higher organisms (Bellas et al., 2008; Ikenaka et al., 2013).

1.5.1 Phenanthrene

Phenanthrene (PHE) is an aromatic tricyclic hydrocarbon resulting from the fusion of three benzene rings (C14H10) and is among the 129 USEPA priority pollutants (Yan et al., 2004). For these reasons, the PHE toxic impact has been studied in several aquatic organisms such as fish and crustaceans. After 96 h of exposure to different concentrations of PHE, *Clarias gariepinus* (African catfish), showed histopathological alterations of the liver and gills due to a progressive increase in glycogen (Karami et al., 2016). Huang et al., (2013), exposing

Zebrafish embryo to this pollutant, reported various morphological damage, developmental delay, apoptosis and reduction of cell proliferation of the resin. On freshwater fish, *Carassius auratus*, this compound is also able to produce a strong oxidative effect, which is determined by an altered function of the antioxidant enzymes (Sun et al., 2006). A high percentage of immobilization (about 67%) was observed in the freshwater crustacean, *Daphnia magna* (Muñoz and Tarazona, 1993; Zhang et al., 2014). Furthermore, the PHE exposure is also able to impact the adult organism's life. In fact, in a study conducted by Kim et al., (2018), exposing the adults of zebrafish *Danio rerio* to this PAH, an increase in the expression of GST and carboxylesterase, and a decrease in the expression of acetylcholinesterase and the two cytochrome CYP11A1 and CYP17A1 were reported, highlighting that these treatments induce considerable changes in the expression of genes responsible for metabolic processes and defense mechanisms.

1.5.2 Naphthalene

Naphthalene (NAP) is the simplest of the polycyclic aromatic hydrocarbons whit a structure characterized by two condensed benzene rings (Annweiler et al., 2000). It can easily enter in the aquatic environment because it can propagate during the oil extraction or refining processes. Like most PAHs, NAP binds to the hydrophobic sites of macromolecules of aquatic species cells causing damage and disturbances of normal development and vital functioning (Bellas et al., 2008; Yan et al., 2004).

Several studies have reported the toxic effects of this compound at various trophic levels. Kong et al., (2010) showed that the seaweed *Chlorella vulgaris* undergoes oxidative damage following exposure to this compound. Likewise, after a feeding experiment, Saiz et al., (2009) demonstrated that NAP was able to compromise the cellular development of *Oxyrrhis marina* which indirectly impacted the survival of both adults and embryos of *Oithona davisae* copepod. Among the aquatic organisms tested, crustaceans are certainly the most

studied. They are widespread geographically, populating both fresh and salt water environments. They are also important bioindicators and are a source of food for humans.

Following different studies conducted both in fresh and salt water on the *Scylla serrata* crab, an important decrease and protein cessation in the genetic material has emerged. In fact, NAP, by binding to phenylalanine and t-lysine, is able to inhibit their propagation to ribosomes, causing this drastic decrease (Vijayavel et al., 2004; Vijayavel and Balasubramanian, 2006). Similarly, this organic compound caused acute effects on mortality and hemoglobin concentration in *D. magna* (Brausch and Smith, 2009; Crider et al., 1982).

1.5.3 Fluoranthene

Fluoranthene (FLT) with four fused aromatic rings is a member of the most abundant high-molecular-weight PAHs in a variety of environmental contaminants (Jin et al., 2017). The toxic effects of this compound have been reported for various model organisms. Exposing for 72h seven species of marine algae in culture belonging to pico-, nano-, and micro-phytoplankton (*Chaetoceros muelleri*, *Nannochloris sp.*, *Picochlorum sp.*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Alexandrium catenella*) to increasing concentrations of FLT, Othman et al., (2012) demonstrated that FLT caused a dose-dependent decrease in cell density, especially in the dinoflagellate *A. catenella.* Its negative impact has been evaluated conducting a short-term test on copepod *Tisbe battagliai*. By exposing nauplii and reproductive female to FLT, Barata et al., (2002) displayed a reduction of feeding rate and clutch size at concentrations below those affecting survival and population responses. Successively, in a study conducted by Šepič et al., (2003), FLT toxicity was compared to algae (*Scenedesmus subspicatus*), bacteria (*Pseudomonas putida*) and crustaceans (*D. magna* and *Thamnocephalus platyurus*) revealing that crustaceans are the most sensitive organisms. Specifically, they displayed that FLT was toxic only for *D. magna*, but its biodegradation metabolites were toxic for both tested crustaceans.

Over the years, it has been widely demonstrated that the acute toxicity to freshwater or saltwater organisms in both water and sediments of this compound increased in ultraviolet (UV) light conditions (Spehar et al., 1999). For example, in laboratory studies, FLT was 10 to 54 times more acutely toxic to invertebrates and vertebrate in the presence of UV light than when it was tested under fluorescent light (Ankley et al., 1994; Boese et al., 1997; Cho et al., 2003).

1.5.4 Benzo[k]fluoranthene

Benzo[k]fluoranthene (BkF) is one of the most dangerous carcinogenic pollutants to humans (Ferrarese et al., 2008). Recent studies have reported a high amount of B(k)F in sediments as well in water samples around the world (Kilunga et al., 2017; Nam et al., 2008). By sampling different species, such as Mediterranean mussel (*Mitylus galloprovincialis*), Norway lobster (*Nephrops norvegicus*), red mullet (*Mullus barbatus*), European flyingsquid (*Todarodes sagittatus*), Atlantic mackerel (*Scomber scombrus*), blue whiting (*Micromesistius poutassou*) and European hake (*Merluccius merluccius*), Perugini et al., (2007) displayed that B(k)F was the only one of seven cancerogenic PAHs (fluorene, phenanthrene, anthracene, fluoranthene, benz(a)anthracene, benzo(b)fluoranthene and benzo(k)fluoranthene) capable to accumulate in all the species considered. Despite its large presence and danger, very few studies have been conducted to ascertain possible B(k)F effects on aquatic organisms (Bhagat et al., 2017; Ferreira et al., 2015; Kim et al., 2014). As a result of these studies, it has possible to establish that B(k)F influence the metabolic processes in aquatic organisms (Beauchamp and Fridovich, 1971). After 30 days of exposure, an increase of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity in digestive gland of scallop *Chlamys farreri* was registered (Pan et al., 2005; 2006). Moreover, significant histological and metabolic changes in the pale chub *Zacco platypus* liver have been shown as a result of exposure to this compound (Kim et al., 2014). In another study conducted by Bhagat et al., 2017, B(k)F is able to induce oxidative

stress in marine gastropod *Morula granulate*, leading to further genotoxic damage.

1.6 Negative impact of restoring methods and m-cosms

During their applications, all amendments could be dangerous for marine environment considering composition and combinations, its application techniques and rates. Its potential (eco-)toxicity role has been only barely investigated. Particularly, information about their negative impact on aquatic species are scarce, making difficult to choose the best potential technology for *in situ* remediation (Lofrano et al., 2016). The consequences of treatment activities on aquatic environment are generally considered as secondary effects (Libralato et al., 2008; Rakowska et al., 2012). Small-scale experiments in microcosms and/or mesocosms could represent a potential useful method to assess contaminated sediment along with their remediation process. Such kind of approach could strengthen the knowledge about potential side effects of remediation activities (e.g. adverse effects on benthic communities), suggesting the most viable treatment to be administered as well as support technology advancements to reduce the impact of old and new treatment technologies especially those related to the addition of adsorbents (e.g. AC), reactive compounds or capping.

Moreover, current literature still does not describe any potential undesired long-term effect, but it also gives no idea about the different sensitivity of aquatic species after their administration including laboratory and field scale applications is not currently available.

1.7 Species sensitivity distribution

The species sensitivity distribution (SSD) concept was proposed four decades ago as an ecotoxicological tool that is useful for the derivation of environmental quality criteria and ecological risk assessment. The species sensitivity distribution (SSD) approach was used to better understand the taxonomic differences in species sensitivity for each remediation method. For the first time, the SSD analysis was evaluated from an updated toxicity database and shown as a cumulative probability distribution for multiple species. SSD curve describes the variation in sensitivity among a set of species toward a contaminant or mixture of

contaminants by a statistical or empirical distribution function (Posthuma et al., 2002). The use of this method was proposed for the first time by Kooijman, (1987) and later enhanced by further studies (Aldenberg and Jaworska, 2000; Posthuma et al., 2002; Newman et al., 2000; Aldenberg and Slob, 1993; Wagner and Løkke, 1991). Generally, SSDs are generated from laboratory-derived toxicity data offering protection for a wider range of organisms in the field (Hose and Van Den Brink, 2004). The aim of SSDs is to calculate the toxicant concentration affecting a specific number of species usually identified as the hazardous concentration (HC) impairing the 5% (HC5) of organisms, thus the protective concentration (PC) for the 95% of species (PC95) can be calculated as well (Posthuma et al., 2002; Newman et al., 2000).

1.8 Model organisms

1.8.1 The sea urchin *Paracentrotus lividus*

Sea urchins are classified within the large *phylum Echinodermata*, that is divided in five classes: crinoids (feather stars), asteroids (sea stars), ophiuroids (brittle stars), echinoids (sea urchins) and holothurians (Ausich, 1998). *Paracentrotus lividus,* popularly known as the "purple sea urchin", is a rather large sea urchin with a diameter of about 7.5 cm (Lozano et al., 1995). This specie is densely clothed in long and sharply pointed spines that are usually purple but are occasionally also of other five colors, including pink, brown, grey, green and black (CalderÓn et al., 2010). Moreover, it is a widespread species, distributing throughout Mediterranean Sea and in the Eastern Atlantic Ocean from western Scotland and Ireland to the Azores, Canary Islands and Morocco (Boudouresque and Verlaque, 2001).

Sea urchins are deuterostomes as they develop the anus from the blastopore (or near it) and the mouth from the opposite cavity. The adult body has pentamerous symmetry (the body is divided in 5 regions arranged around a central plate) with a size typically ranging from 6 to 12 cm, although large species can reach up to 36 cm. The body is divided equatorially into two hemispheres. The side facing the substrate is known as the oral hemisphere. The mouth

is situated in the middle of this area and can be recognized by the presence of a structure used to chew (the 5 teeth lantern of Aristotle; see **Figure 2**), surrounded by a membranous layer called the peristomal membrane.

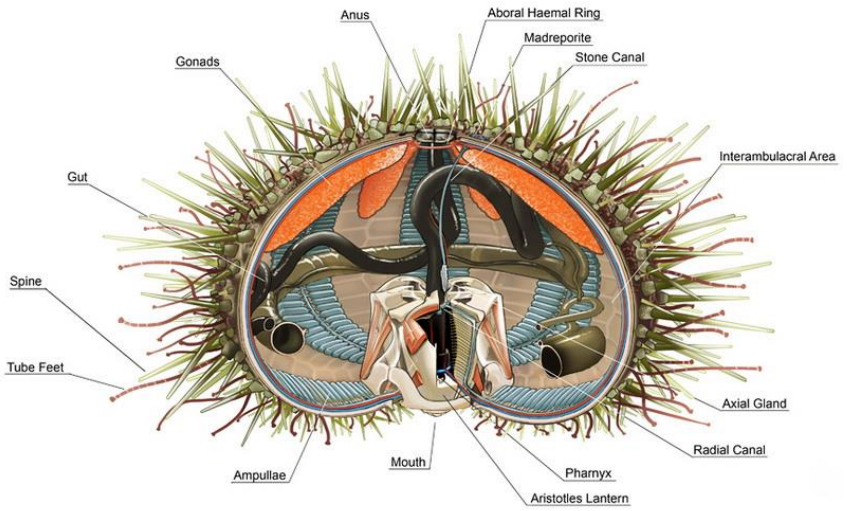

Figure 2. Schematic representation of sea urchin morphology (http://www.staroftheeast.us/2016/02/sea-urchin-facts.html).

The upper part is called aboral hemisphere, where the anal region is localized. A structure known as madreporite or madreporic plate is also present on the aboral surface, through which the liquid of the aquifer system is in connection with the external environment. This is connected to the ring canal, which in turn is joined with the radial canals. Sea water is brought into the tube feet allowing the sea urchin movements on the substrate.

The reproductive system consists of five gonads, which when mature are large and orange, joined together at the inner surface of the inter-ambulacral areas, and extend from the aboral hemisphere, where they communicate with the external environment, almost to the lantern of Aristotle. The spawning season of *P. lividus* starts in January and ends in May. The eggs released by a good mature female are usually ready to be fertilized immediately. Both meiotic divisions and maturation occur in the ovary, after which the polar bodies are lost. Generally, the rate of cleavage and development are temperature-dependent. At 20° C, zygotes of *P. lividus* reach the first division in about an hour. The normal life cycle of sea urchins is shown in **Figure 3**.

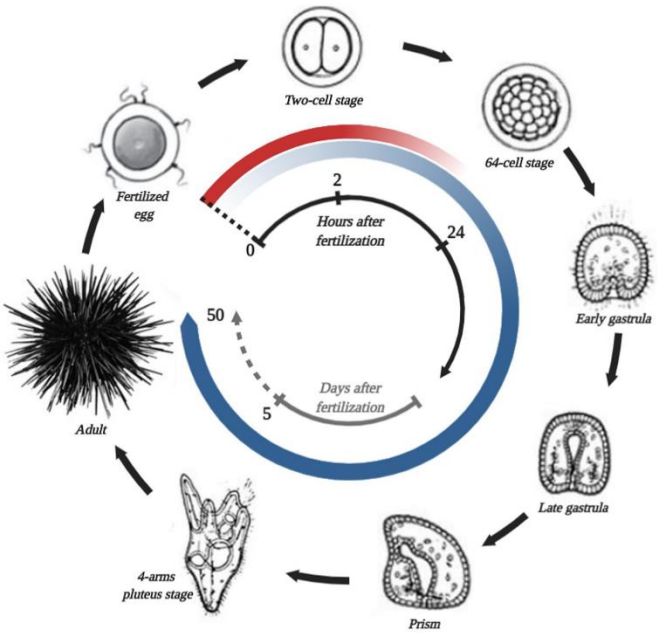

Figure 3. Simplified sea urchin life cycle, stages of which are connected by black arrows (Adonin et al., 2021)

Among the echinoderms, the sea urchin *P. lividus* is considered a suitable organism to study the ecotoxicological response of marine invertebrates to environmental pollutants for several reasons: it is an important component of benthic marine communities in the Mediterranean Sea and Atlantic Ocean; the extraction and maintenance of gametes are easy; the embryos grow rapidly and synchronously (pluteus stage at 48 hours post fertilization); the embryos are transparent and therefore suitable for microscopic detection of sub-lethal effects of pollutants on normal development; they have a long reproductive period (from October to May). For these reasons the sea urchin represents a good model for ecotoxicological studies on the response of marine invertebrates to environmental pollutants, such as physical and chemical xenobiotics (Bellas et al., 2008; Bonaventura et al., 2011; Pinsino et al., 2010; Zito et al., 2005)

1.8.2 The crustacean *Artemia franciscana*

The brine shrimp *Artemia* is classified as Branchiopoda within the phylum Arthropoda, and includes seven species and 50 strains, commonly known as *Artemia franciscana*, *Artemia sinica*, *Artemia parthenogenetica*, *Artemia sp.*, *Artemia tunisiana*, *Artemia urmiana* and *Artemia persimilis* (Santhanam et al., 2018). These crustaceans are widely distributed in salt

pans, inland salt lakes, and hypersaline water bodies throughout the world. *A. franciscana* strains mainly from San Francisco Bay (California), Great Salt Lake (Utah) in the United States, and salt lakes from Saskatchewan in Canada (Vikas et al., 2012).

The total length is usually about 8-10 mm for the adult male and 10-12 mm for the female, but the width of both sexes, including the legs, is about 4 mm (**Figure 4**; (Criel and Macrae, 2002)).

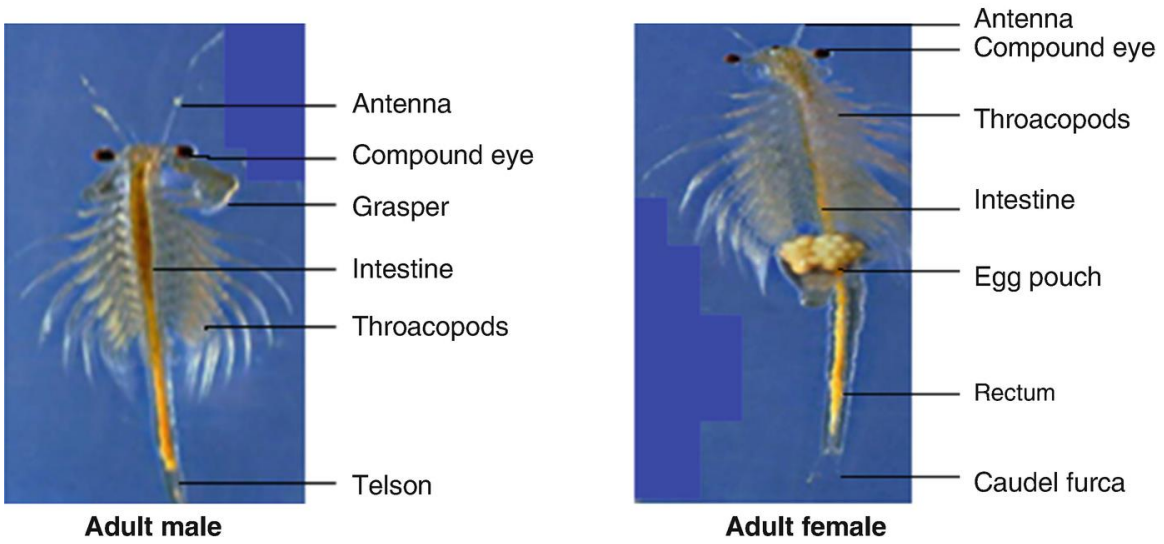

Figure 4. Adult male and female of *Artemia* (Santhanam et al., 2018)

The adults (male and female) *Artemia* have two compound eyes, antenna, thoracopods, intestine, rectum, telson and caudal furca. Moreover, the egg pouch of a single female can hold up to about 200 eggs. Adult females release oocytes from their two oviducts into the ovisac where they become fertilized by males. At this point the reproductive process can follow two completely different paths: i) under favorable environmental conditions the embryos develop directly inside the egg pouch and released from them directly as nauplii; ii) in contrast, under harsh environmental conditions the oviparous sac produces diapausing eggs which dry up after their release (cysts). Only after cysts rehydrating, the hatching can take place and the nauplii can be obtained. The development of nauplii into sub-adults occurs within 1–3 weeks (Hollergschwandtner et al., 2017). The normal life cycle of *Artemia* is shown in **Figure 5**.

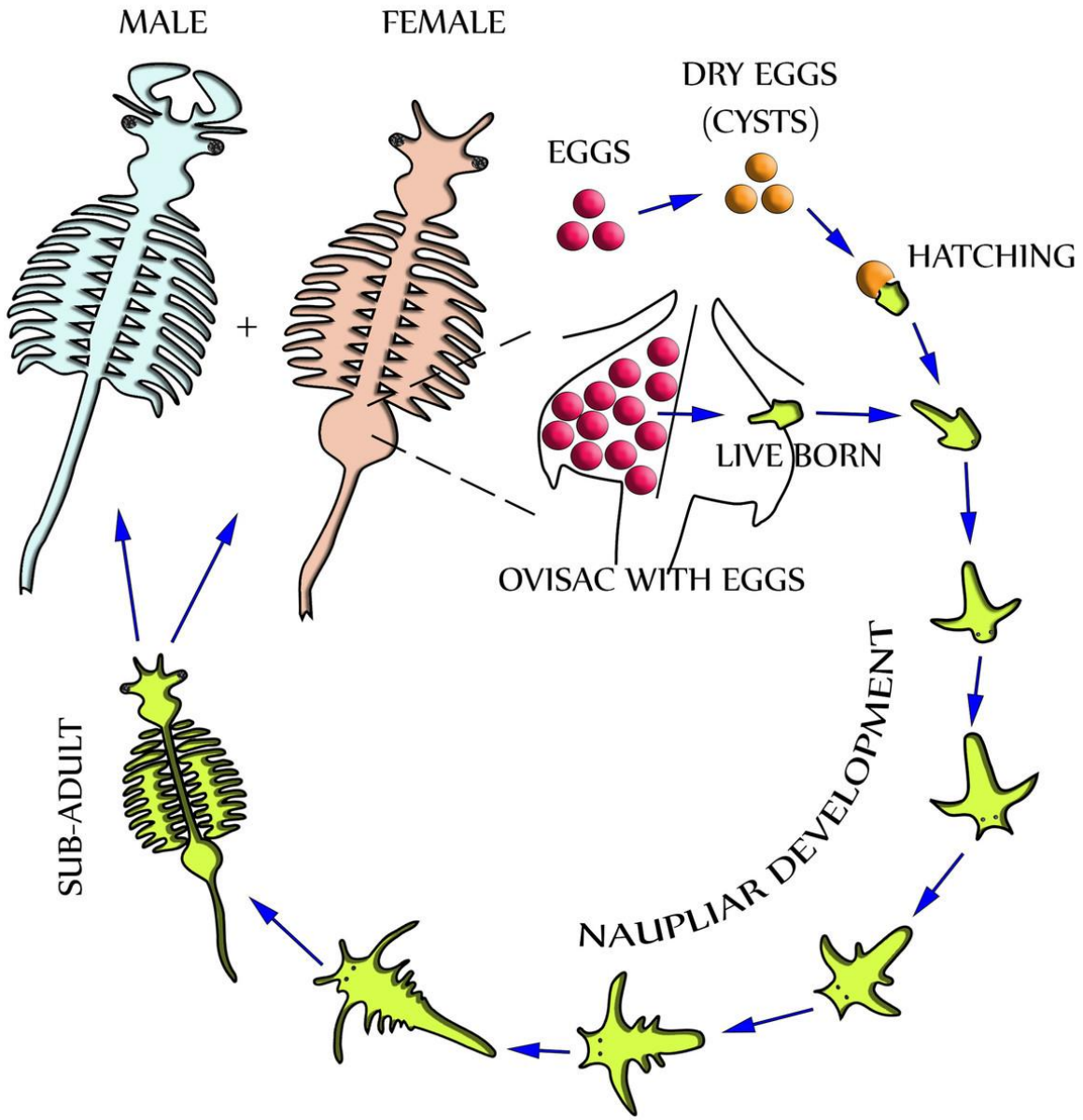

Figure 5. Simplified *Artemia* life cycle, stages of which are connected by blue arrows (Hollergschwandtner et al., 2017)

Among saltwater species crustacean, *A. franciscana* is considered a good model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants (Libralato, 2014; Libralato et al., 2007; Migliore et al., 1993). The use of this model organism is favored by greatest advantage: the nauplii can be hatched as needed from commercially available durable cysts. This avoids the need of maintaining laboratory cultures, contrary to what is required for many species used in ecotoxicity tests. Moreover, the embryo grows rapidly in laboratory conditions (the nauplius stage is reached in 24 h); their small body size allows the conduct of the tests in small beakers or plates; and finally,

they have adaptability to a wide range of salinities (5-300 g/L) and temperatures (6-40 °C; (Manfra et al., 2016)).

Some data reported in this Chapter have been published in:

1) **Albarano L.,** Costantini M., Zupo V., Lofrano G., Guida M., Libralato G., 2020. Marine sediment toxicity: a focus on micro- and mesocosms towards remediation. Science of Total Environment 708, 134837. (Appendix B)

1.9 Aims of the thesis

Marine sediments are fundamental and integrated parts of water bodies and are composed of soluble and insoluble matter, primarily rock and soil particles transported from land areas to the oceans by some natural processes, mainly rivers, glaciers and ice, windblown dust, coastal erosion (Brils 2008). As extensively reported in Paragraph 1.1, polluted sediment represents an important issue for fresh, brackish and marine ecosystems, especially coastal ones, due to the high human pressures (i.e. commercial and industrial port activities, human settlements and tourism) and sedimentation rates caused by solid discharges from catchment basins (Nikolaou et al., 2009a; Lofrano et al., 2016). Anthropogenic activities, such as maintenance, capital dredging and the disposal of historically contaminated sediment are also able to induce the remobilization of pollutants in the water column (Eggleton and Thomas 2004). This resuspension can negatively influence the marine environment and human health, because pollutants can accumulate in bottom feeders and magnify through marine food webs, reaching humans through fishery products (Zupo et al., 2019). For these reasons, the European Water Framework Directive 2000/60/EC (WFD) was developed and constantly updated.

In this context, the present Ph. D. project aims to shed light on characterization, treatment and enhancement of contaminated sediments studying the potential role of invertebrates and other organisms by using multidisciplinary approaches.

In particular, the project has two main objectives, which will be achieved applying the use of micro- and mesocosm approaches:

1) to study the possible toxigenic effects of contaminated sediments on embryonic development of the sea urchin *Paracentrotus lividus*;

2) to study the possible toxigenic effects of common pollutants in spiked sediment on crustacean *Artemia franciscana*;

3) to study the possible restoring of contaminated marine sediments and its negative effects on marine invertebrates.

Specifically, the project focuses on the following issues:

1) Possible toxigenic effects of contaminated sediments on the development of the sea urchin *P. lividus.*

i) Setting up of microcosms for contaminated sediment experiments.

A closed system was set up in the Animal Facility of the Stazione Zoologica Anton Dohrn, in which synthetic sediment (10 litres) contaminated with different pollutants and pre-filtered seawater (55 litres) were added. Contaminated sediment experiments in these microcosms had the aim to bring ecologically relevant components of the natural environment under controlled conditions (Watts and Bigg, 2001).

ii) Definition of morphological effects of contaminated sediment on the sea urchins *P. lividus*.

In each tank, ten adults of the sea urchin *P. lividus* were exposed for two months to contaminated sediment and fed with alga *U. rigida* (1 gram per day per sea urchin), which represent natural dietetic items and are characteristic of environments populated by *P. lividus*. At the end of exposure in the closed system described before, morphological analysis (by microscopic observation) on embryos spawned from adults treated with PAHs + sediment and PCBs + sediment has been performed in comparison with those treated with uncontaminated sediment and with only seawater.

iii) Molecular effects of contaminated sediment on embryos at the pluteus stage of sea urchins *P. lividus.*

Since the genome of the sea urchin *P. lividus* is still not available, high throughput *Next Generation Sequencing* (NGS) studies have been applied to identify target genes affected by these pollutants. Specifically, *RNA sequencing* (RNA-Seq) experiments were performed to monitor the variations of gene expression levels of the Transcriptome after treatment with contaminated sediment. In the RNA-Seq method, complementary DNAs (cDNAs) generated from the RNA of interest are directly sequenced using next-generation sequencing technologies. The high-quality reads obtained have been aligned in order to construct a whole-genome transcriptome map, providing a far more precise measurement of levels of transcripts and their isoforms than other methods. Moreover, this sequencing technology and assembly algorithms have facilitated the reconstruction of the entire transcriptome even without a reference genome. To this aim, eggs and sperms have been collected from sea urchins treated with contaminated sediment and then fertilized. Samples were collected at the pluteus stage (embryos at 48 hours post fertilization) and then *de novo* transcriptome analysis has been performed on these embryos in comparison with the embryo control.

iv) Gene isolation, interactomic analysis and RealTime qPCR

The sequences of twenty-five new genes were retrieved from the transcriptome of the sea urchin *P. lividus* deposited in the SRA database and from NCBI database. For each gene, specific primers were designed on the nucleotide sequences. PCR fragments were purified from agarose gel using the QIAquick Gel Extraction, and the specificity of the PCR product was

checked by DNA sequencing. The network analysis was performed by NetworkAnalyst 3.0.

Moreover, the expression levels of these twenty-five genes plus sixty-two genes, (previously isolated and functionally interconnected among them and involved in stress, skeletogenesis, detoxification, development and differentiation processes) were followed by Real Time qPCR.

2) **Acute toxicological effects of common contaminants spiked sediment on marine organisms**

i) Definition of acute effects of four PAHs (NAP, PHE, FLT and BkF) on the adults and nauplii of crustaceans *A. franciscana*.

Ten nauplii and five adults of *A. franciscana* were exposed to the PHE, NAP, FLT and BkF to define acute toxicity (LC50) both after 24 h- and 48 h using conceivable concentrations in marine environment (from 0.025 to 10 mg/L; from 0.36 to 2.3 E+2 mg/L; from 0.40 to 3.9 E+2 mg/L; and from 0.025 to 94 mg/L for NAP, PHE, FLT and BkF, respectively), detected from polluted sediment associated to various pollution sources (Arienzo et al., 2017).

ii) Genotoxicity of NAP, PHE, FLT and BkF on both *A. franciscana* tested life stages.

After 48 h under sublethal exposure for both nauplii and adults, the expression level of different genes were evaluated to understand the molecular basis of toxic effects of these PAHs. To this aim, changes in expression of several key genes involved in stress response was assessed, and, in addition, only for nauplii the impact on development genes was evaluated by *RealTime qPCR*.

3) **Comparison between AC and nZVI as restoring methods of common contaminants (PAHs) spiked marine sediment and evaluation of their toxic effects on crustacean *Artemia franciscana*.**

i) Setting up of microcosms for comparing two different restoring methods (AC and nZVI)

A closed system was set up in the University of Naples Federico II, in which natural sediments (collected from two sites within Gulf of Naples) and synthetic seawater 1:4 were added. The AC and nZVI (3% of sediment dry wt) were also added to PAHs remediation. Chemical analyses were done on sediment and seawater already after the addition of amendments, 3 h, 6 h, 21 h, 24 h, 72 h and 21 days using GC–MS method.

ii) Toxicity and Genotoxicity of restoring methods on *A. franciscana.*

Ten nauplii of *A. franciscana* were exposed to water solutions collected at seven different time to define acute toxicity (LC50) using increasing percentage concentrations (from 0% to 100%). To evaluate the molecular response, 200 nauplii were exposed for 48h to 100% solutions collected at the end of experiment (21 days). The expression level of nine different genes involved in stress response and development processes, were followed by *Real Time qPCR*.

Acknowledgments

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2. Chapter 2: Setting up of microcosm for contaminated sediment experiments and definition of morphological and molecular effects on the sea urchin *Paracentrotus lividus* development

2.1 Introduction

The first part of this Ph.D. project was focused on experimentally defining the possible negative effect of contaminated sediment on the adults of the sea urchin *Paracentrotus lividus* reared in m-cosms systems. In order to reach this aim, the first step consisted in the setting-up of a microcosm, that had the aim to mimic natural conditions bringing ecologically relevant components of the natural environment under controlled conditions. Specifically, this research assessed the effects of three pollutants, commonly present in contaminated sediment, on adults of sea urchin *P. lividus.* The sea urchin *P. lividus* was used since it is considered a good model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants (Bonaventura et al., 2011; Pagano et al., 2017; Pinsino et al., 2010; Zito et al., 2005). Moreover, sea urchins display a strong ecological relevance taking active part in the formation of coastal marine communities in the Mediterranean Sea and the Atlantic Ocean (Agnetta et al., 2015; Boudouresque and Verlaque, 2001; Shears and Babcock, 2002). In fact, it has a benthic lifestyle, long reproductive period (from October to May), a rapid response and high sensitivity to many types of contaminants. Furthermore, the extraction and the manipulation of gametes is easy; the embryo grow rapidly in laboratory conditions (the pluteus stage is reached 48h post fertilization); and finally, the embryos are transparent and thus making simple microscope observation of possible sub-lethal effects of contaminants on development (Bonaventura et al., 2011; Pagano et al., 2017; Pinsino et al., 2010; Zito et al., 2005). More specifically, in this work sea urchins *P. lividus* were exposed to artificial sediments, enriched with three toxicants, commonly found in Italian sediment previously characterized (Arienzo et al., 2017; Mangoni et al., 2016; Qu et al., 2018; Trifuoggi et al., 2017), PAHs, PCB, Zinc Sulfate

($ZnSO_4$) and mixture of these pollutants, at comparable concentrations to those detected in the control sediment (Picone et al., 2018) for demonstrating that these contaminants are able to induce dangerous effect on development and adult sea urchins even at lower concentrations. In particular, this research analyzed the hypothesis that two months exposure to polluted sediments could induce i) morphological effects on the development of embryos obtained from treated sea urchins and ii) variation on the expression level of several genes (involved in stress response, skeletogenesis, detoxification and development/differentiation) analyzed by *RNA sequencing* and *Real Time qPCR*. Moreover, we isolated 25 new genes, involved in stress response and embryonic developmental processes, from the sea urchin *P. lividus*. These genes were subjected to interactomic analysis, demonstrating that they were functionally interconnected with 10 genes already described in previous studies investigating the response of *P. lividus* embryos to xenobiotic compounds. Chemical analyses were done on sediment and seawater before and after the addition of contaminants, and at the end of the experiment. Moreover, a chemical extraction was also performed from theca + spines, gonads and intestine, dissected at the beginning and at the end of experiment, to detect the possible accumulation of contaminants.

The aim of this work is to demonstrate that the pollutants at low concentrations, found in sediment used as control (Picone et al., 2018), could be able to cause negative effects on *P. lividus* adults. Moreover, this study displayed a great relevance in eco-toxicology because represents the first time in which these effects on adult organisms were analyzed using m-cosm approach.

2.2 Materials and methods

2.2.1 Ethics Statement

The sea urchin *P. lividus* were collected from a site in the Bay of Naples (Italy) that is not privately owned or protected in any way, according to Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected

species. All experimental procedures on animals followed the guidelines of the European Union (Directive 609/86).

2.2.2 Sea urchin collection

Adult sea urchin *P. lividus* were collected in January (corresponding to the beginning of the reproductive cycle) by scuba-divers in the Gulf of Naples, Italy. Adult individuals of an average weight of 40 g were collected and immediately transported to the laboratory, using a thermically insulated box containing seawater. Further, they were transferred to plastic tanks with recirculating seawater prior to starting the feeding experiments. Sea urchins were individually measured using a calliper, to record the maximum horizontal diameter of thecae; adult specimens with a diameter between 4 and 5 cm (excluding the spines), that is a typical diameter for mature adults, were selected for the experiments.

2.2.3 Experimental design and Mesocosms

Our experimental design included six scenarios: (i) negative control—seawater (W) (check filtered seawater background quality); (ii) negative control—not spiked sediment in mesocosm filled with W (W + SED) (check reconstructed sediment background quality); (iii) sediment spiked with PAHs (192 µg/L, nominal) (W + SED + PAHs); (iv) sediment spiked with PCBs (0.15 µg/L, nominal) (W + SED + PCBs); (v) sediment spiked with Zn (40 mg/Kg, nominal) (W + SED + Zn); and (vi) sediment spiked with mixture of three pollutants (PAHs, PCBs and Zn (at 192 µg/L, 0.15 µg/L and 40 mg/Kg, respectively)) (W + SED + Mix). All experiments were carried out in triplicates.

Each of the 18 testing mesocosms, located at the Stazione Zoologica Anton Dohrn, was characterized by an independent and closed seawater recirculation system (**Figure 6**).

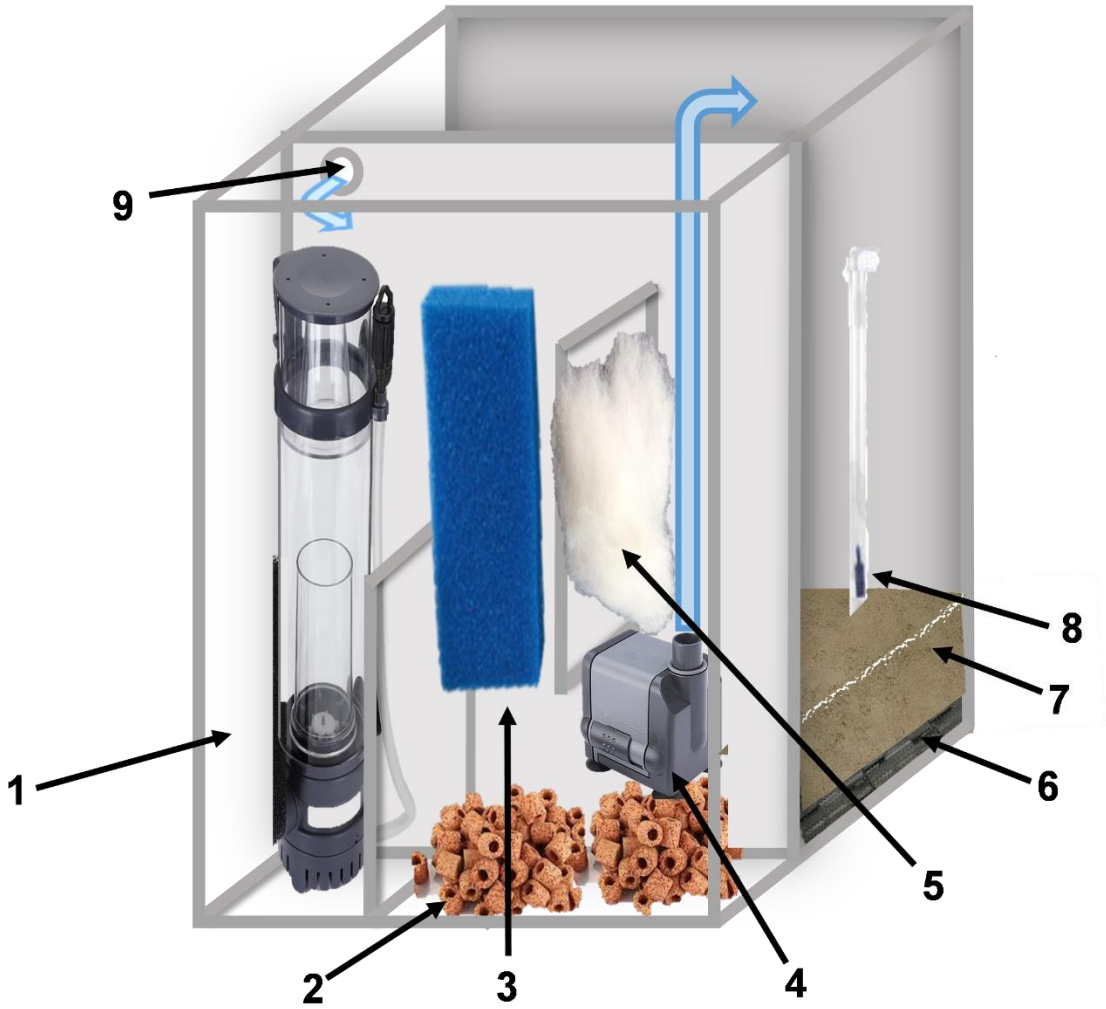

Figure 6. Schematic representation (frontal view) of one of the twelve experimental tanks of the mesocosms, with the description of all the elements: (1) protein skimmer to remove organic matter, such as food particles and faecal pellets, using a pump in which establishes a contact between air and water flow, producing fine bubbles by Venturi effect. The water movement promotes the binding of molecules on the bubbles until their interface was saturated. When the bubbles increase on the top of water skimmer, condensed them into a foam collected in the cup. (2) Porous ceramic rings act as biologic filters, favoring the growth of nitrifying bacteria able to oxidize the ammonia into nitrites and then to nitrates. Bacterial activator is also added to promote the growth of Nitrobacter and Nitrosomonas species that adhere on the porous surface of these filters. (3) Synthetic sponge for a mechanical filtration that together with (5) perlon wool (consisting of thin glass fibres) for trapping particulate matter, such as faecal pellets and sediment suspended particles. (3) Pump for water recirculation. (6) Under-gravel filter to create a constant flow of water through (7) the sediment. (8) Air bubble stone for the aeration and oxygenation the water. (9). Flow of the water from tank to the filter compartment.

A pump (Micra 400 L/h, SICCE, Italy) promoted seawater circulation from the filtration compartment (containing porous ceramic filters, synthetic sponge and Perlon wool) to the other compartment containing the sediment. Each tank (50 × 36 × 48 cm) was filled with 55 litres of natural seawater through a 200-micron mesh sock filter, collected from the Gulf of Naples and treated with zeolite and activated carbon for one week to remove most pollutants

prior to chemical analyses (see Appendix A; **Supplementary Tables S1** and **S2**). The seawater volume was kept constant during the experiment. It was checked daily and, if necessary, topped up with distilled water. The artificial sediment (10 L) present in each mesocosm was produced by mixing 36.5% of quartz sand (0 mm–3 mm, G. Build, s.r.l.) and 62.5% of coarse sand (grain size 0.4–0.8, Arena Silex, Manufacturas Gre, S.A.) and 1% calcium carbonate) (OECD, 2007). Before spiking and prior to adding sea urchins, mesocosms were aged for one week. Spiking occurred by adding contaminants directly to the mesocosm water column (i.e., simulating a discharge event). One week after spiking, organisms were added to the mesocosms: seven females and three males for each tank. To evaluate the compartmentalization of PAHs and PCBs in sediment and seawater, their concentrations were evaluated: (i) before the addition of contaminants (t0), (ii) after the addition of contaminants (t1) and (iii) at the end (tf) of the experiment. PAH spiking (acenaphthene (ACE), acenaphthylene (ACY), anthracene (ANT), benzo(a)anthracene (BaA), chrysene (CHR), fluoranthene (FLT), fluorine (FLR), phenanthrene (PHE), pyrene (PYR)) required the addition (in each 55 L mesocosm) of 330 µL of a solution prepared by weighing 30 mg of each PAH in 10 mL of acetone/n-hexane (1:1 v/v; the nominal concentration of water stock solution is 35.3 g/L). PCB spiking required the addition (in each mesocosm) of 82 µL of a certified standard solution of 100 µg PCB/mL (Ultra Scientifc, Italy). We did not carry out control experiments with PAH diluents (acetone/n-hexane) due to the insignificant volume added (as compared to mesocosm's volume) and their high volatility.

2.2.4 Grain size analysis

After one week of aging, 50 mL samples were collected from each tank and treated with 10% H_2O_2 and distilled water (2:8) for 48 h at room temperature in order to remove salts and organic matter. After drying (24 h at 105 °C), sediment fractions were mechanically separated with multiple vibrating sieves (Ro-Tap Particle Separator, Giuliani, HAVER &

BOECKER Oelde Germany) with a 63 μm mesh to distinguish between sandy and silt–clay fractions (Danovaro, 2010; Danovaro et al., 2008). Each fraction was weighted separately. Gain size data were analysed with GradiStat software (version 8.0, based on Blott and Pye methods descripted in (Blott and Pye, 2001)) and expressed as a percentage of the total dry weight.

2.2.5 Physico-Chemical Analyses

Temperature, dissolved oxygen, redox potential, salinity and pH were checked three times a week (Appendix A; **Supplementary Figures S1-S4**). Temperature and dissolved oxygen were detected by a multiparameter probe (YSI 85, Ohio, US); redox (REDuction-Oxidation) state (270 mV) and pH were evaluated using WTW 197-S (SenTix® 41, Göteborg-Sweden) electrodes (7.8–8.1); salinity was measured by a refractometer (Sper Scientific, Scottsdale, Arizona) and fixed with distilled water when its value exceeded 38 ± 1 PSU. The analysis of nutrients included the detection of nitrites (NO_2^-), nitrates (NO_3^-), phosphates (PO_4^{3-}) and ammonia (NH_3^-), using a colorimetric test (HACH Odyssey DR/2500 spectrophotometer, HACH Company Loveland, Colorado, United States) (Appendix A; **Supplementary Figures S5-S8**).

For PAHs and PCBs analyses, seawater samples were extracted by a solid-phase extraction (SPE): 1.0 l of water was filtered and preconcentrated on a C18 disk (ENVI, -18 DSK SPE Disk, diam. 47 mm). The analytes were eluted with a solution of 1:1 dichloromethane and n-hexane.

The determination of PAHs and PCBs in the sediment was performed by considering 5 g of dry sediment extracted with acetone/n-hexane 1:1 v/v (10 mL), using an ultrasonic disruptor (Brason, US). The extract was concentrated to 1 mL in Multivap under nitrogen flow (Multivap, LabTech, Italy). A total of 10 μL of a 1 mg/L solution of internal standard (mixture of deuterated PAHs) was added to the extract and injected to a gas chromatography–mass spectrometry (GC-MS) (MS-TQ8030-Shimadzu, Japan). The limits

of detection (LOD) and quantification (LOQ) were calculated, and the average values for the seawater samples were 0.02 µg/L and 0.05 µg/L for PCBs and 0.004 µg/L and 0.01 µg/L for PAHs, respectively. For sediment samples, LOD and LOQ values were 0.03 µg/kg and 0.1 µg/kg for PCB and 0.16 µg/kg and 0.1 µg/kg for PAHs, respectively. Data quality was ensured by certified reference materials (ERM-CA100 (European Commission) for PAHs and QC1033 (Supelco) for PCBs). The recovery percentage was 70%–110% for PAHs and 65%–120% for PCBs (Arienzo et al., 2017; Carotenuto et al., 2020).

For the determination of PAHs and PCBs in sea urchin tissues (thecae, spines, gonads and guts), approximately 3 g of tissues were homogenated and placed in an automatic extractor, under reflux, at 80 ◦C for 2 h with a 2 M KOH solution in methanol. After extracting with 20 mL of cyclohexane three times, the extract was purified on sodium sulphate, dried in a rotary evaporator and recovered with a 1 mL mixture of hexane/acetone (1:1 v/v). The extract was analysed by GC–MS.

For Zn analyses, seawater was filtered using 0.45 µm regenerated cellulose membrane filters and acidified with 3% v/v HNO_3. Samples were analysed with inductively coupled plasma with mass spectrometry (ICP-MS, Aurora M90, Bruker, USA). The quantitative analysis was performed using an external calibration curve built with five concentrations for each of the analysed elements using multi-element standard solutions for ICP TraceCERT® in 5% nitric acid (Sigma-Aldrich, Milan, Italy) and ultrapure deionized water with conductivity < 0.06 µS/cm. The calculated average values of LOD and LOQ were 0.9 and 10 µg/L, respectively. For Zn determination in sediment, samples of 0.5 g of ≤2 µm dry sediments were weighed in ceramic vessels and analysed by a total organic carbon analyser, Skalar (The Netherlands). The ≤2000 µm fraction was used for analyses of Zn by digesting about 0.5 g of sediment in 12 mL of H_2O_2–HCl-HNO_3, in Teflon vessels in an Ethos Plus Microwave Lab Station (Milestone) for 15 min; the obtained solution was taken to a final volume of 100 mL with 5% HCl and then filtered by 0.45 µm (Trifuoggi et al., 2017).

Samples were analysed by ICP-MS (Aurora M90 Bruker, US). The calculated average values of LOD and LOQ were 0.06 and 0.16 μg/g, respectively. The quality of the analytical results ranged from a minimum of 85% to a maximum of 97% (Carotenuto et al., 2020; Trifuoggi et al., 2017).

For the determination of Zn bioaccumulation, 0.5 g of tissues were mineralized with 9 mL of 65% HNO_3 and 1 mL 30% H_2O_2 and heated at 95 °C for 15 min. If needed, the mineralized was passed on a 0.45 μm filter and brought a volume of 10 mL with ultrapure water and analysed by an ICP-MS. The limit of quantification (LOQ) was of 0.4 μg/kg wet weight (w.w.) for PAHs and 2 μg/kg w.w. for PCBs and Zn. The average recoveries of PAHs and PCBs were >70% and 80–110% for metal (Arienzo et al., 2019).

2.2.6 Sea urchin collection and exposure, gamete collection for morphological analysis

Methods for sea urchin collection (according to Italian laws, DPR 1639/68, 09/19/1980 confirmed on 01/10/2000) and the conditions of their exposure in the mesocosms are reported in details in Ruocco et al., (2020). Animals were fed with *Ulva rigida* according to Ruocco et al., (2018). After two months of exposure, sea urchins were collected and their gametes were obtained. Fertilization, embryonic growth until the pluteus stage (48 hpf) and morphological observations were carried out according to Romano et al., (2011). In particular, the percentage of embryos still at the gastrula stage, as well as normal and malformed plutei, were determined 48 h post-fertilization by counting at least 200 embryos for each sample under light microscopy (Zeiss Axiovert 135TV). Pictures were taken using a Zeiss Axiocam connected to the microscope.

Gonadal indices (GIs) were initially evaluated on gonads from five adult sea urchins (t0) (representing the starting point), and evaluations were repeated on five specimens after two months of exposure to PAHs and PCBs as well as in control sediments (W + SED). Animals were weighed, sacrificed and dissected; gonads were weighted for the GI determined (where

GI indicates gonadal wet weight (g)/sea urchin wet weight (g) x 100 according to (Fabbrocini and Adamo, 2011)).

2.2.7 De novo Transcriptome Assembly and Data Analysis

To confirm the possible toxic effects of contaminants on *P. lividus* embryos showed by morphological observations, molecular analyses were conducted on embryos deriving from adult sea urchins treated with pollutant.

After two months of exposure, about 5000 eggs (in 50 mL of filtered sea water, FSW) from each female treated with contaminants and from controls were collected and fertilized. Samples at 48 hpf were collected by centrifugation at 4000***g*** 4 °C for 10 minutes in a swing out rotor. The embryos were suspended in10 volumes of RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at -80 °C. Total RNA was extracted with Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to Ruocco et al., (2017). For RNA-seq the amount of total RNA extracted and RNA integrity were measured according paragraph (Fertilization, sample collection and RNA extraction).

The sequencing was carried out in Genomix4Life S.r.l. (Baronissi, Salerno, Italy) using Illumina Truseq mRNA stranded 2 × 150—NextSeq500. De novo transcriptome assembly and annotation of 9 samples (1–3 triplicates for the control condition, embryos at the pluteus stage deriving from adult sea urchin reared in the mesocosm in tanks with seawater plus sediment (W + SED) without contaminants, indicated with “Control”; 4–6 triplicates for embryos at the pluteus stage deriving from adult sea urchins exposed to PAHs, indicated as “Treated_1”; and 7–9 triplicates for embryos at the pluteus stage deriving from adult sea urchins exposed to PCBs, indicated as “Treated_2”) were carried out to discover differentially expressed genes between the two treatments and to perform functional analysis (**Supplementary Table S3**).

RNA sequencing was performed in paired-end mode. Fastq underwent quality control using the FastQC tool (Brils, 2008). The tool Trinity (Trinity Release v2.10.0; (Grabherr et al., 2011)) was used to perform transcriptome assembly. Expression analysis was performed by RSEM (version 1.1.21) using default parameters, and expression values were converted to FPKM (fragments per kilobase of exon per million fragments mapped; (Roberts et al., 2011)). DESeq2 (Love et al., 2014) was used to perform the normalization matrix and differentially expressed genes of all samples were considered. OmicsBox (version1.2.4) uses the Basic Local Alignment Search Tool (BLAST) to find sequences similar to the query set in FASTA format. The Gene Ontology (GO) terms were assigned based on annotation with an E-value of 10-5. The full dataset of raw data is deposited in the Sequence Read Archive (SRA database; available at https://www.ncbi.nlm.nih.gov/sra; accession number: SUB6701449; accessed on 15 February 2021).

2.2.8 Gene isolation, interactomic analysis and *Real Time qPCR*

To identify the relationships on the basis of associated functions and data mining from experimental studies reported in the literature, sixty-two genes (Esposito et al., 2020; Ruocco et al., 2017b; Varrella et al., 2016, 2014); see also **Supplementary Table S4**) were analyzed by NetworkAnalyst 3.0 software (https://www.networkanalyst.ca/; (Zhou et al., 2019)), using STRING interactome of protein–protein interactions (Szklarczyk et al., 2019). Human orthologs of selected genes were used to compute the network analysis. The relations among genes (confidence score cut-off = 400), displaying experimental evidence were highlighted. Twenty-five mostly connected genes were then chosen and analyzed. The sequences were retrieved from the transcriptome of the sea urchin *P. lividus* deposited in the SRA database (Sequence Read Archive, available at https://www.ncbi.nlm.nih.gov/sra, accession number PRJNA495004, (Ruocco et al., 2019); accession number SUB2817153, (Ruocco et al., 2018a); accession number SUB6701449, (Luisa Albarano et al., 2021) and from NCBI

(available at https://www.ncbi.nlm.nih.gov). For each gene, specific primers were designed on the nucleotide sequences (see **Table 6**).

Gene names	Acronym	Accession Number	Primer	Sequence (5'→3')	(bp)
Calcium/calmodulin-dependent protein kinase type 1D	*CM-K*	PRJNA495004	Pl_CM_F1	GTTATCCTCCATTTTACGATGAG	168
			Pl_CM_R1	GCAGATATACGTGTGAGGAAG	
Camp-responsive element	*CREB*	PRJNA495004	Pl_CREB_F2	GTAACTAAAGCATCTGGGAGAC	158
			Pl_CREB_R2	GGTTCAGATATTAGTGGATGC	
Cholinesterase	*ChE*	SUB6701449	Pl_ChE_F2	CGAGATGGCGTATGTTTTGAG	160
			Pl_ChE_R2	GACTATGTTCCCGCTGACTG	
Citochrome P450 2UI isoform X2	*CYP-2UI*	SUB6701449	Pl_CYP-2UI_F1	GCGCCTCTTCGTTCTATTCC	174
			Pl_CUP-2UI_R1	CGGCATAGTAGTAGACTAGC	
Epidermal growth factor	*EGF*	PRJNA495004	Pl_EGF_F1	CGGCGGTGTGTGTATCGATG	189
			Pl_EGF_R1	CAGTAGCCATCCTAGTGTTCC	
Frizzled7	*FZ-7*	PRJNA495004	Pl_FZ_F1	GATCGTGAGCGTAGCATATAC	175
			Pl_FZ_R1	CATGGTCTTTTTGGGCACTA	
Glutathione-S-transferase	*GST*	SUB6701449	Pl_GST_F4	GCCCGACTTACCTACTTTGC	165
			Pl_GST_R4	CTTGCAGCTCATCACTGATGG	
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	PRJNA495004	Pl_GAPDH_F1	GTACTACTTCTCATTCACCTTG	213
			Pl_GAPDH_R1	CATAGCTCTGACACCGCCAC	
heat shock protein 75	*hsp75*	PRJNA495004	Pl_hsp75_F2	GGACTGGTGGAACAACTATATC	173
			Pl_hsp75_R2	CGATCACCACTCTCTGTCAC	
heat shock protein 90	*hsp90*	SUB6701449	Pl_hsp90_F1	GGGTGTGGTAGATTCTGATG	148
			Pl_hsp90_R1	GCTCTCCATGTATTCATCAG	
Hedgehog	*HH*	PRJNA495004	Pl_HH_F1	GGTACATGAGGCACAAGCTAG	193
			Pl_HH_R1	CCACTTCACATCACTTGACC	
Janus kinase	*JAK*	XM_030985987	Pl_JAK_F2	CACCTTACCCATACTAGACAG	192

			Pl_JAK_R2	CTTGCCAGAGCCTCCGCTGAC	
Lefty	*Lefty*	SUB6701449	Pl_Lefty_F2	CAGTCCAGACATGGGTGGCAG	182
			Pl_Lefty_R2	CATTTCGTCGACCACCTGCTG	
maternal Vg1	*M-Vg1*	SUB6701449	Pl_M-Vg1_F1	GCACCTGCACCTAGAGACTC	145
			Pl_M-Vg1_R1	GCATGACCTTTTCCGGCCTG	
NADH dehydrogenase	*NADH*	SUB2817153	Pl_NADH_F1	GTCTCCGTCGGATAAATCAAAG	194
			Pl_NADH_R1	CCGAAAAGGAAATAACGAAGC	
Nemo-like kinase protein	*NLK*	AY442297	Pl_NLK_F1	CCTCTACCAGATTCTCAGAG	192
			Pl_NLK_R1	GTGACACAGTACTACCGCGC	
Notchless protein	*NOTCHLESS*	PRJNA495004	Pl_Notchless_F1	GGGAAGCTAAGGCATCAGAC	145
			Pl_Notchless_R1	CGATCCTCTCAAGCACTTTAG	
Patched	*Ptc*	SUB6701449	Pl_Ptc_F1	CGGTCGTCAGTATCATCATG	135
			Pl_Ptc_R1	GCAACCACGACTCCGTAAGC	
Phospholipase C	*PLC*	AJ012336	Pl_PLC_F1	GAGACATTCACAGTGCCCAC	139
			Pl_PLC_R1	CTGACCGATACCAAGCTGTAC	
PLAUF 3 RNA-binding protein AUF1 mRNA	*PLAUF 3*	AY682309.1	Pl_PLAUF3_F2	GGAGGATACGGCGGTGGCGG	182
			Pl_PLAUF3_R2	GTGTTGACTCCACAGGAGTG	
Polyketide synthase	*PKS*	SUB6701449	Pl_PKS_F1	GCTTCCTCGACCAGTCTGTC	142
			Pl_PKS_R1	CCTCCGAAGACAGTCATCTG	
signal transducer and activator of transcription	*STAT1*	PRJNA495004	Pl_STAT_F1	GTGTGTCAATCAGCCAGTGC	196
			Pl_STAT_R1	GTACATCATGAGCTTACCATTTC	
Smoothened	*Smo*	SUB6701449	Pl_Smo_F1	CACGATCCATTACGGCGTTG	217
			Pl_Smo_R1	GCCCAACTCACACCCATGAC	
sulfotransferase 1C2-like	*SULT1*	SUB6701449	Pl_SULT1_F2	CAGGCACTCACTGGCTCATG	140
			Pl_SULT1_R2	CTCTTCAGCTCTCGTCTTCG	
Tumor necrosis factor alpha	*TNF*	SUB2817153	Pl_TNF_F1	CCTGATGTGTATGCCTCTATC	144

Pl_TNF_R 1	CAAGATCCTCATGTCA GGAAG	

Table 6. Gene name, acronym, accession numbers, primer names, primer sequences and amplicon lengths of PCR fragments.

PCR fragments were purified from agarose gel using the QIAquick Gel Extraction kit (Qiagen, Milan, Italy), and the specificity of the PCR product was checked by DNA sequencing. PCR products were aligned to the original sequences by MultAlin Software ((Corpet, 1988); see **Figure S9**). Five serial dilutions were set up to determine Ct values and PCR efficiencies for all primer pairs (for RT-qPCR conditions see below). Standard curves were generated for each oligonucleotide pair using Ct values versus the logarithm of each dilution factor (**Figure S10**). The biological functions for twenty-five genes are reported in **Table 7**.

Gene name	Acronym	Function	References
Calcium/calmodulin-dependent protein kinase type 1D	*CM-K*	Calcium-binding protein that is present in eggs and involved in the control of nuclear envelope breakdown (NEB) during mitotic division	(Baitinger et al., 1990; Floyd et al., 1986)
Camp-responsive element	*CREB*	Transcription factor that binds certain DNA sequences, named cAMP response elements (CRE), increasing or decreasing the	(Ingham, 1998)

		expression of target genes	
Cholinesterase	*ChE*	Protein belonging to a group of esterases expressed in response to environmental stresses	(Cunha et al., 2005; Gambardella et al., 2013)
Citochrome P450 2UI isoform X2	*CYP-2UI*	This gene encodes for heme-thiolate monooxygenase enzymes, which are involved in stress response	(Goldstone et al., 2007, 2006)
Epidermal growth factor	*EGF*	Growth factor that regulates various aspects of cell growth, differentiation and morphogenesis during embryo development	(Bisgrove et al., 1991; Hursh et al., 1987; Yamasu et al., 2000)
Frizzled7	*FZ-7*	Binding to Wnt6, this receptor is responsible for initiating β-catenin nuclearisation in macromeres at the 5th cleavage, which is necessary for	(Lhomond et al., 2012)

		endoderm specification	
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	A glycolytic enzyme that plays a major role in the production of energy required for sperm cell movement	(Miki et al., 2004; Mita and Yasumasu, 1983; Muronetz et al., 2015)
Glutathione-S-transferase	*GST*	This enzyme is expressed in the intestine tissue and it is involved in the response of environmental stresses	(Cunha et al., 2005)
heat shock protein 75	*hsp75*	*hsp75* was first described, together with *hsp70*, in relation to the heat shock stress. This protein is currently known to be also expressed in response to cold or heavy metals exposure, during oogenesis and embryo development of the sea	(Geraci et al., 2004; Sconzo et al., 1997)

		urchin *P. lividus*	
heat shock protein 90	*hsp90*	A family of proteins that are expressed in response to stressful conditions. *Hsp90* plays important roles in embryogenesis	(Bédard and Brandhorst, 1986)
Hedgehog	*HH*	Protein expressed downstream to Brachyury and FoxA in the endomesoderm gene regulatory network during gastrulation that participates to the mesoderm organization	(Walton et al., 2009)
Janus kinase	*JAK*	Transcription factor that, binding to the STAT1, plays a kay role in the developmental processes	(Hou et al., 2002; Ito et al., 2004)

Lefty	*Lefty*	This protein, together with *Delta* and *Notch*, is involved in left-right establishment in sea urchins	(Warner and Mcclay, 2014)
maternal Vg1	*M-Vg1*	This gene, together with *Nodal*, is involved in ventral-dorsal establishment in sea urchins	(Range and Lepage, 2011)
NADH dehydrogenase	*NADH*	This enzyme is involved in the maintenance of cellular redox homeostasis for modulating numerous biological events. Changing its phosphorylation state, *NADH* modulates sperm motility	(Fujiwara, Akiko, Yasumasu, 1997; Loza-Huerta et al., 2013)
Nemo-like kinase protein	*NLK*	This protein, positively regulated by Delta-Notch signal, induces the specification of mesodermal cells by downregulating TCF.	(Röttinger et al., 2006)

Notchless protein	*NOTCHLESS*	Protein involved in the *NOTCH* pathway implicated in the differentiation of secondary mesenchyme cells, which are fated to produce mesodermal cells	(Lossie et al., 2012; Royet et al., 1998)
Phospholipase C	*PLC*	An enzyme positively regulated by the *CM* signal and involved in egg activation and during embryo development in sea urchins	(Coward et al., 2004; Sidhu et al., 2005)
PLAUF 3 RNA-binding protein AUF1 mRNA	*PLAUF3*	This protein, binding to *H3.3 histone* 3`UTR, is probably implicated in mRNA instability	(Pulcrano et al., 2005)
Polyketide synthase	*PKS*	The polyketide compound synthesized by PKS gene possibly has a role in the immune defense of the developing embryo	(Beeble and Calestani, 2012)

Patched	*Ptc*	Co-receptor of HH expressed within the neighboring skeletogenic and non-skeletogenic mesoderm	(Walton et al., 2009)
Smoothened	*Smo*	Developmental regulator that actives HH signaling	(Nachtergaele et al., 2013)
signal transducer and activator of transcription	*STAT1*	Transcription factor that, binding to the *Jak* factor, plays a kay role in the developmental processes	(Brown et al., 2001; Darnell, 1997; Hou et al., 2002)
sulfotransferase 1C2-like	*SULT1*	Enzyme that catalyzes the bioactivation of arylamines	(Barrett et al., 2016)
Tumor necrosis factor alpha	*TNF*	Protein that restricts and terminates inflammatory responses through the modulation of the ubiquitination status of central components in *NF-κB*, *IRF3* and apoptosis signaling cascades	(Vereecke et al., 2011)

Table 7. Gene name, acronym, function and reference of new fourteen genes.

Collection of embryos at the pluteus stage (about 5000 sea urchin plutei) for *Realtime qPCR* was performed according to (Ruocco et al., 2020). After total RNA extraction was performed using Aurum™ Total RNA Mini kit (BioRad), its amount and integrity were estimated according to Ruocco et al. 2017. Real-time qPCR experimental protocols as reported in Ruocco et al. 2018 and Ruocco et al. 2019 were followed (see Appendix A, **Supplementary Figure S11** reported all the analysed genes). In particular, about 1 µg of RNA was used for cDNAs synthesis by iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions without dilution in a reaction containing a final concentration of 0.3 mM for each primer and 1 x FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were conducted in a ViiATM7 *Real Time PCR* System (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: one cycle for cDNA denaturation at 95° C for 10 min; 40 cycles for amplification at 95° C for 15 s and 60° C for 1 min; one cycle for final elongation at 72° C for 5 min; one cycle for melting curve analysis, from 60° C to 95° C, to verify the presence of a single product. The expression of each gene was analyzed and normalized against the housekeeping genes Ubiquitin and 18S rRNA, using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method (Pfaffl, 2001a; Pfaffl et al., 2002). Relative expression ratios greater than ±1.5 were considered significant. Each real-time qPCR plate was repeated at least twice.

Fold change values were represented through an Heatmap generated by Heatmapper Software (http://www.heatmapper.ca/; (Babicki et al., 2016)). Values were scaled by column and hierarchical clustering was applied on rows by Average Linkage and Euclidean Distance Measurement algorithms.

2.2.10 Statistical analysis

Morphological data were reported as means ± standard deviations (SD). These data were analysed by the Shapiro–Wilk normality test and F-test. The statistical significance between

groups was performed by one-way ANOVA followed by the Holm–Sidak test (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com, accessed on 15 February 2021) for multiple comparisons, indicating ** $p < 0.01$, *** $p < 0.001$. Statistical differences of GI values between t0 and after two months were evaluated by the Mann–Whitney U test (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com, accessed on 15 February 2021). P values > 0.05 were considered not significant.

2.3 Results

2.3.1 Sediment Grain Size and Water Features and Spiking Levels

The sediment showed a typical sandy profile. Sandy fraction represented 99.9%, where the coarse sand (0.5 mm–1 mm, representing 41.1%) was the dominant component (**Figure 7**).

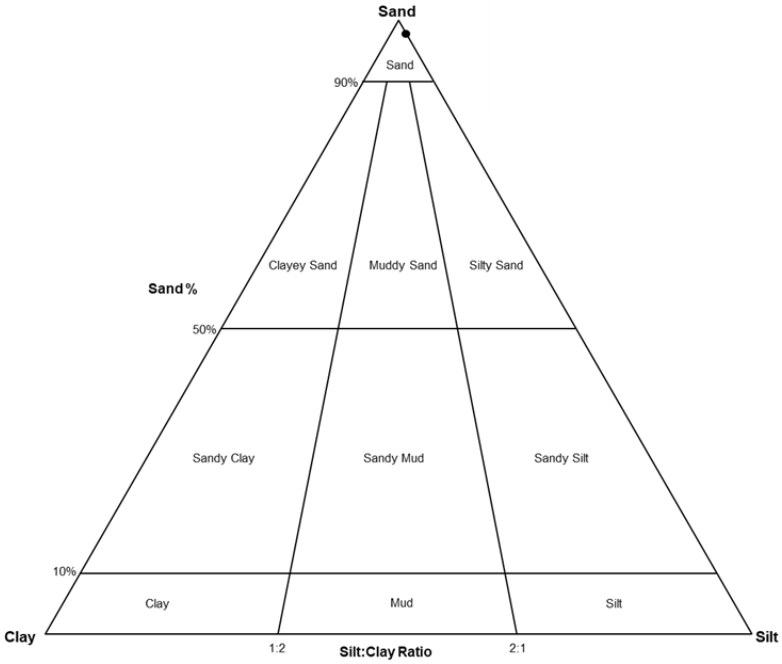

Figure 7. Data of the sediment grain size analyzed with GradiStat software (version 8.0 based) to calculate particle size statistics for sieve or laser granulometric data.

The fine and medium sands (from 0.25 mm to 0.5 mm) were 2.1% and 15.7%, respectively, whereas the mud fraction represented a small percentage (about 0.1%).

During two months of exposure to PAH-, PCB-, Zn- and Mix-contaminated sediments, the physical and chemical values of the seawater in the mesocosm were almost constant. PAHs

and PCBs detected in all sediment and water samples from all mesocosms were below the relative detection limit values considering all investigation times (t0, t1 and tf). Only for Zn, a change of distribution in sediment and in seawater was reported (**Tables 8** and **9;** see also Appendix A, **Supplementary Table S5–S10** for more details).

	t0			t1			tf		
	ΣPAHs	**ΣPCBs**	**Zn**	**ΣPAHs**	**ΣPCBs**	**Zn**	**ΣPAHs**	**ΣPCBs**	**Zn**
W	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED+PAHs	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED+PCBs	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED+Zn	< 0.005	< 0.005	< 1	< 0.005	< 0.005	2310.3±2001	< 0.005	< 0.005	129.3±29.1
W+SED+Mix	< 0.005	< 0.005	< 1	< 0.005	< 0.005	1167.8±87	< 0.005	< 0.005	117±12.5

Table 8. Chemical analyses of Total PAHs, PCBs and Zn on seawater collected at time zero (t0, corresponding at the beginning of the experiments, before adding compounds), time one (t1, corresponding at just after adding compounds) and at the end of experiments (tf). Data are expressed as µg/L. W = Water; W+SED = Water+ sediment; W+SED+PAHs = Water + sediment + PAHs; W+SED+PCBs = Water + sediment + PCBs; W+SED+Zn = Water + sediment + Zn; W+SED+Mix = Water + sediment + Mixture of PAHs, PCBs and Zn . Data are reported as mean ± SD, N=3.

	t0			t1			tf		
	ΣPAHs	**ΣPCBs**	**Zn**	**ΣPAHs**	**ΣPCBs**	**Zn**	**ΣPAHs**	**ΣPCBs**	**Zn**
W+SED	< 0.005	< 0.005	4.5±1.8	< 0.005	< 0.005	19.4±1.8	< 0.005	< 0.005	16.0
W+SED+PAHs	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED+PCBs	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1	< 0.005	< 0.005	< 1
W+SED+Zn	< 0.005	< 0.005	4.5±0.3	< 0.005	< 0.005	64.9±14.8	< 0.005	< 0.005	83.2±14.4
W+SED+Mix	< 0.005	< 0.005	12.1±4.2	< 0.005	< 0.005	66.4±13.6	< 0.005	< 0.005	93.6±25.7

Table 9. Chemical analyses of Total PAHs, PCBs and Zn on sediment collected at time zero (t0, corresponding at the beginning of the experiments, before adding compounds), time one (t1, corresponding at just after adding compounds) and at the end of experiments (tf). Data are expressed as µg/L. W+SED = Water+ sediment; W+SED+PAHs = Water + sediment + PAHs; W+SED+PCBs = Water + sediment + PCBs; W+SED+Zn = Water + sediment + Zn; W+SED+Mix = Water + sediment + Mixture of PAHs, PCBs and Zn . Data are reported as mean ± SD, N=3

2.3.2 Effects of contaminated sediment on adult growth, gonadal index and sea urchin development

After 2 months of exposure, the gametes were collected only from sea urchin treated with PAHs, PCBs and from controls (with/without sediment) because Zn treatments resulted

strongly toxic for sea urchins. In fact, after 2 weeks of exposure, an increase of the mortality was registered in tanks with Zn. After 4 weeks, this increase of mortality was also registered in tanks with Mix, but was not reported for other conditions. These results showed that the presence of Zn at this concentration was strongly toxic not only on development, but also on adults (**Figure 8**).

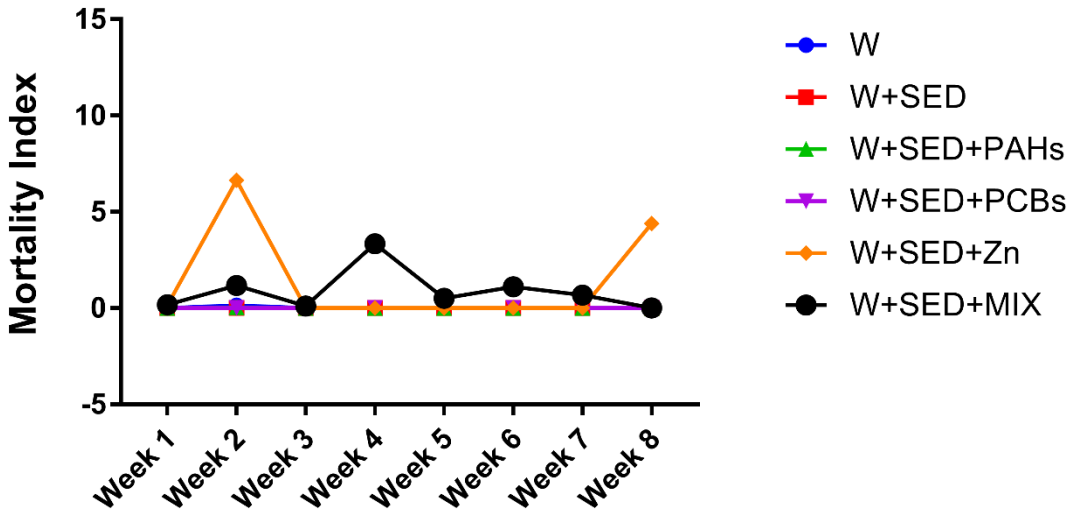

Figure 8. Mortality index (calculated as the number of individuals died in a given time period/total number of individuals for the three experimental conditions) of the adult sea urchin *P. lividus* during the two months of exposure to the sediment contaminated with PAHs, PCBs, Zn and Mixture. The values were reported in Water (W, blue line), Water + sediment (W + SED, red line), Water + sediment + PAHs (W + SED + PAHs, green line), Water + sediment + PCBs (W + SED + PCBs, purple line), Water + sediment + Zn (W + SED + Zn, orange line) and Water + sediment + mixture (W + SED + MIX, black line).

None of the conditions imposed in the negative controls (W and W + SED) negatively affected sea urchins, suggesting that all the subsequently observed effects could be attributed to the treatments. After the exposure period (two months), a mortality rate of 1% was detected in all experimental conditions (W, W + SED, W + SED + PAHs and W + SED + PCBs), revealing good health conditions of the sea urchins after two months of exposure (**Figure 8**). After two months of exposure, no significant differences in growth rates were found between adults exposed to PAHs and PCBs as compared to organisms collected in the field at the beginning of the experiment ($p > 0.05$), similar to GI values (p value > 0.05; Appendix A, **Supplementary Table S11**).

After gamete collections, three important endpoints of sea urchin embryonic development

were detected: (i) fertilization success; (ii) first mitotic division; and (iii) the pluteus stage, occurring at 48 hpf. Exposure to both contaminants, PAHs and PCBs, did not show significant effects on the percentages of fertilization success and first mitotic cleavage with respect to the controls (in tanks with seawater (W) and in tanks with seawater + sediment (W + SED) without contaminants; $p > 0.05$). Observation of the embryos at the pluteus stage revealed that PAH and PCB treatments induced malformations, mainly affecting arms, spicules, apices and the entire body shape as compared to control embryos (**Figure 9**).

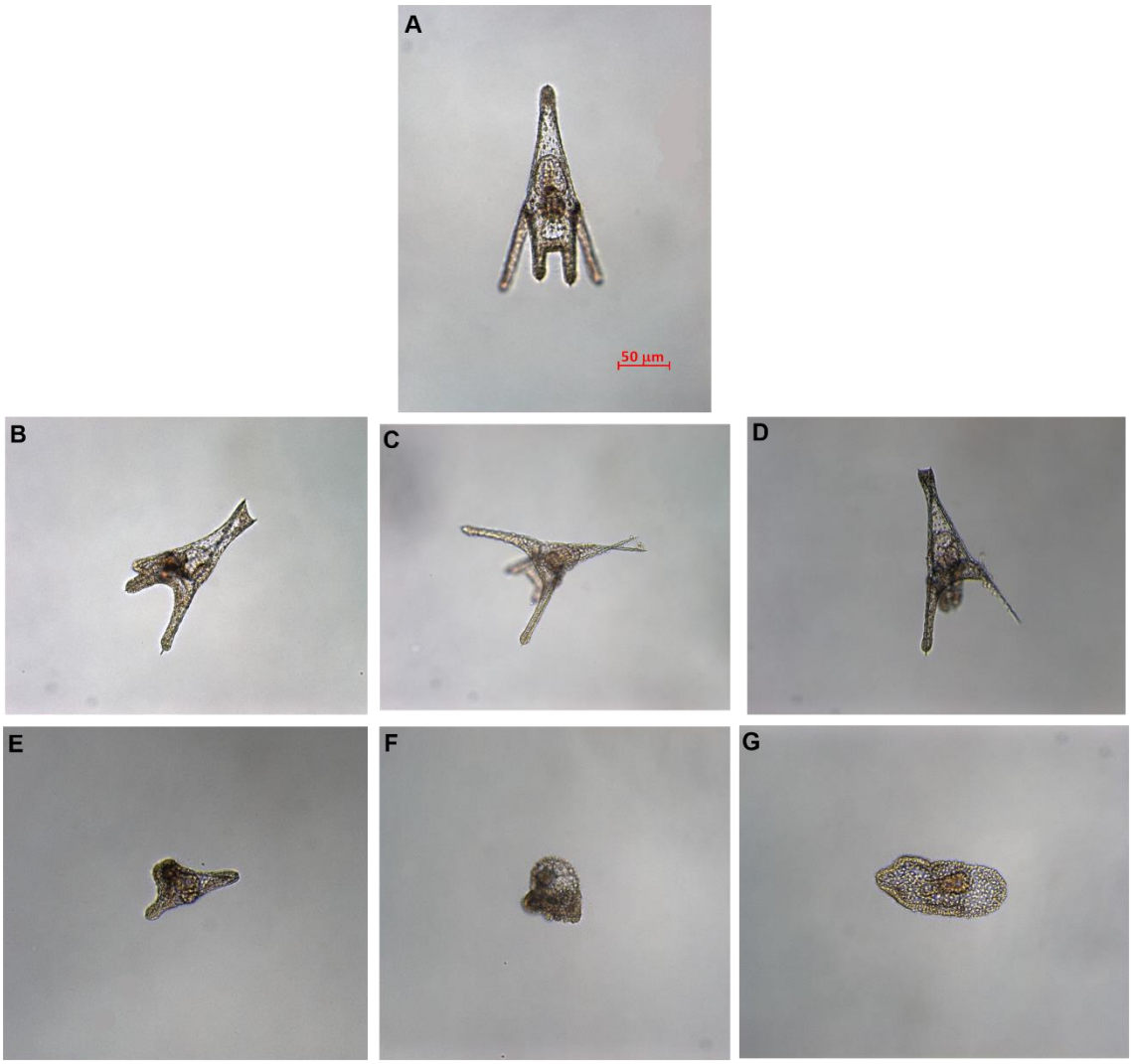

Figure 9. Examples of malformations observed in *P. lividus* plutei spawned from adults treated for two months with PAHs and PCBs. (A) control embryos spawned from adults collected from W / W + SED. Such plutei showed (B) a poorly-formed apex with (C-D) spicules that appeared crossed at the apex or disjoined at the tip, or with poorly-formed and degraded arms. Moreover, some embryos treated with PCBs showed (E-F) a delayed (gastrula stage) and (G) abnormal development. Scale bar = 50 µm.

In particular, at the pluteus stage, an increase in malformed embryos was observed in larvae deriving from sea urchins exposed to contaminated sediments with respect to the controls, represented by water and water + sediment without contaminants (**Figure 10**).

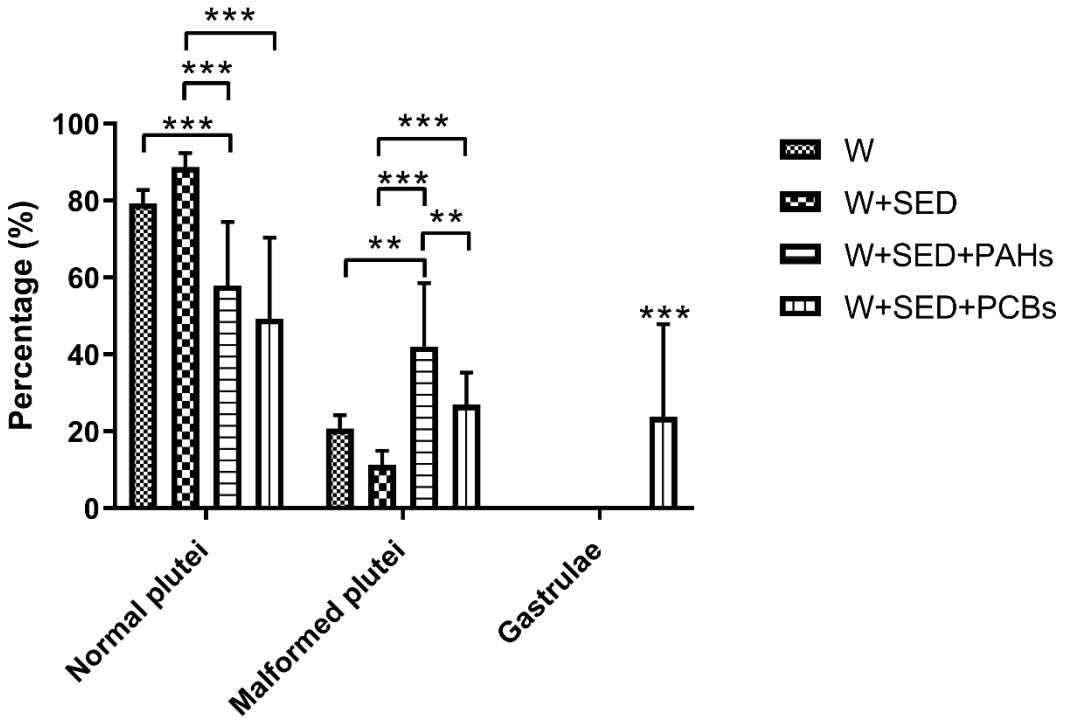

Figure 10. Percentage of normal plutei, malformed embryos at the pluteus and gastrula stage from sea urchins, deriving from adult sea urchins exposed to sediment contaminated with with PAHs (Water + sediment + PAHs) and PCBs (Water + sediment + PCBs) and in control condition represented by adults reared in control tanks (Water and Water + sediment). Data are reported as mean ± standard deviation One-Way ANOVA by Holm Sidak's test (**p<0.01, ***p<0.001)

PAHs induced an increased percentage of malformed embryos (about 42%) with respect to control water + sediment (about 10%, $p < 0.001$). The exposure to PCBs generated approximately 27% ($p < 0.001$) of malformed plutei and developmental delays, with some embryos still at the gastrula stage (about 24%; $p < 0.001$), which were also malformed. These results, valid for the above-mentioned doses applied to *P. lividus*, demonstrated that PAHs are more harmful than PCBs, being supported also by chemical analyses of the contaminant's bioaccumulation.

After two months of exposure, the bioaccumulation of PAHs and PCBs was also detected in three sea urchin tissues: thecae (including spines), gonads and guts. Chemical results showed that (i) 12.4 µg/kg of PAHs (including acenaphthylene, acenaphthene, fluorene, anthracene,

phenanthrene, 9-methylanthracene and benzo[a]anthracene) were accumulated in the theca, including the spine (**Table 10**); (ii) 16.3 µg/kg of total PAHs (including acenaphthylene, acenaphthene, fluorine, anthracene, phenanthrene, fluoranthene, pyrene and benzo[a]anthracene) were accumulated in the gonads; and (iii) no PAH accumulation was found in the guts. The target body compartments in sea urchins were the body wall and the spines when individuals were exposed to contaminated water and the guts when they were exposed to contaminated foods (Danis et al., 2005).

	Theca + spines			**Gonads**			**Intestine**	
	W+SED+PAHs	**W**	**W+SED**	**W+SED+PAHs**	**W**	**W+SED**	**W+SED+PAHs**	**W+SED**
NAP	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
ACY	**1.0**	< 0.4	< 0.4	**2.2**	< 0.4	< 0.4	< 2	< 2
ACE	**0.5**	< 0.4	< 0.4	**0.7**	< 0.4	< 0.4	< 2	< 2
F	**5.8**	< 0.4	< 0.4	**1.1**	< 0.4	< 0.4	< 2	< 2
ANT	**1.1**	< 0.4	< 0.4	**3.2**	< 0.4	< 0.4	< 2	< 2
PHE	**1.2**	< 0.4	< 0.4	**0.8**	< 0.4	< 0.4	< 2	< 2
9-MANT	**2.2**	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
FLT	< 0.4	< 0.4	< 0.4	**3.0**	< 0.4	< 0.4	< 2	< 2
PYR	< 0.4	< 0.4	< 0.4	**4.4**	< 0.4	< 0.4	< 2	< 2
B(a)ANT	**0.7**	< 0.4	< 0.4	**0.9**	< 0.4	< 0.4	< 2	< 2
B(b)FLT	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
B(k)FLT	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
B(e)PYR	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
B(a)PYR	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
IPYR	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
DB(a,h)ANT	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
B(g,h,i)PER	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
Coronene	< 0.5	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2

Retene	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 2	< 2
Total PAHs	**12.4**	< 0.4	< 0.4	**16.3**	< 0.4	< 0.4	< 2	< 2

Table 10. Concentrations in microgram (µg)/ kilogram (kg) of PAHs in three different tissues: theca + spines, gonads and intestine**.** The values of Water + sediment + PAHs (W + SED + PAHs) accumulation were compared to controls, Water (W) and Water + sediment (W + SED).

However, the accumulation in these marine organisms was more efficient when exposed via water than via the food. No detectable events of PCB bioaccumulation were observed in the analysed tissues (**Table 11**).

	Theca + spines			**Gonads**			**Intestine**	
	W+SED+PCBs	**W**	**W+SED**	**W+SED+PCBs**	**W**	**W+SED**	**W+SED+PCBs**	**W+SED**
PCB1	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB5	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB18	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB31	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB44	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB52	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB66	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB87	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB101	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB110	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB138	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB141	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB151	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB153	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB170	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB180	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB183	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB187	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2
PCB206	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2

Total PCBs	< 0.5	< 0.5	< 0.5	< 2	< 2	< 2	< 2	< 2

Table 11. Concentrations in microgram (µg)/ kilogram (kg) of PCBs in three different tissues: theca + spines, gonads and intestine**.** The values of Water + sediment + PCBs (W + SED + PCBs) accumulation were compared to controls, Water (W) and Water + sediment (W + SED).

2.3.3 Transcriptomic assembly and differentially expressed genes in plutei from adults Exposed to PAHs and PCBs (*RNA-seq*)

Another interesting result was the large-scale genomic information herein reported, which greatly improved the few molecular tools available for the sea urchin *P. lividus*, despite its importance as a marine model organism. For this reason, the de novo transcriptome obtained in this work represents a promising tool to identify new *P. lividus* genes, which can be considered general biomarkers placed in motion from the sea urchin to deal with environmental pollution.

All the results obtained by RNA sequencing are summarized below.

BLASTx top-hit species distribution of matches for all the transcriptomes with known sequences indicated (**Supplementary Figure S12**, Appendix A) that the majority of *P. lividus* contigs (reads) showed the highest similarity with *Strongylocentrotus purpuratus* (BLAST hits = 1000).

The other most represented species included Apostichopus japonicas (BLAST hits 50) and Acanthaster planci (BLAST hits 45). All alignments were carried out setting the E-value thresholds at a value of ≤1 e−5.

To perform the RNA-seq assembly de novo, Trinity was used (Grabherr et al., 2011). We obtained the trinity assembly with the statistics reported: Counts of transcripts: Total "trinity genes": 216864, Total "trinity transcripts": 611356, Percent GC: 38.26%. Then, we performed a differentially expression analysis in Trinity, selecting Deseq2 R package (Love et al., 2014), and we obtained the genes differentially expressed with respect to the several conditions (less than 3000 genes and 8000 isoforms). Of the isoforms differentially expressed, we performed a BLASTx alignment with respect to the nucleotide non-redundant

database in NCBIi, using OmicsBox (version1.2.4) (Conesa and Götz, 2008). Differentially expressed genes were identified between the three conditions: embryos at the pluteus stage spawned by adults exposed for two months to sediment contaminated with (i) PAHs or (ii) PCBs, with comparisons made with (iii) those exposed in tanks with sediment without contaminants as the control, including three biological replicates for each treatment.

The score plot showed that the replicates for the controls were very similar, with a clear separation from the treated samples, suggesting a greater number of down- and up-regulated genes in the treated samples compared to that of the controls (**Supplementary Figure S13**). As reported in **Supplementary Table S14**, (i) 1898 genes were differentially expressed (DE) genes with a false discovery rate (FDR) of ≤ 0.05, of which 993 genes were upregulated (FC ≥ 1.5) and 965 were down-regulated (FC ≤ 1.5) in plutei deriving from adult *P. lividus* exposed to sediment contaminated with PAHs (indicated as Treated_1); (ii) 2396 genes were DE with a false discovery rate (FDR) of ≤ 0.05, of which 1079 genes were up-regulated (FC ≥ 1.5) and 1317 were down-regulated (FC ≤ 1.5) in plutei deriving from adult *P. lividus* exposed to sediment contaminated with PCBs (indicated as Treated_2) compared to the control; (iii) 1356 genes were DE with a false discovery rate (FDR) of ≤ 0.05, of which 755 genes were up-regulated (FC ≥ 1.5) and 601 were down-regulated (FC ≤ 1.5), considering Treated_1 compared to Treated_2. After the annotation, (i) 488 genes were found up-regulated (with a FC range between 1.6 and 99) and 271 genes down-regulated (with a FC range between 1.7 and 95) for Treated_1 vs. Control, and, of these, some genes showed very high values of fold changes, such as the four up-regulated genes (*RNA-directed DNA polymerase from mobile element jockey-like, calmodulin-like protein 4, arylsulfatase A and fibropellin-1-like isoform X6*) and the three down-regulated genes (*beta1,3-galactosyltransferase 1-like, isocitrate dehydrogenase (NADP) cytoplasmic isoform X2 and fibrillin-1-like*); (ii) 311 genes were found up-regulated (with a FC range between 1.7 and 95) and 420 genes down-regulated (with a FC range between 1.6 and 95) for Treated_2 vs.

Control, and, of these, some genes showed very high values of fold changes, such as the three up-regulated genes (*dnaJ homolog subfamily B member 13, rho guanine nucleotide exchange and factor 39ATP-dependent RNA helicase DHX8-like*) and the two down-regulated genes (*betaine-aldehyde dehydrogenase and serine/threonine-protein kinase TNNI3K*); (iii) 177 genes were found up-regulated (with a FC range between 1.9 and 90) and 239 genes down-regulated (with a FC range between 1.5 and 98) for Treated_1 vs. Treated_2, and, of these, some genes showed very high values of fold changes, such as the three up-regulated genes (*actin-related protein 2/3 complex subunit 3, acyl-CoA dehydrogenase and arylsulfatase I*) and the two down-regulated genes (*cyclin-dependent kinase 11B and glucose-6-phosphate 1-dehydrogenase isoform X1*).

This large-scale genomic information represents a significant finding, being the first molecular attempt to define PAH and PCB effects on sea urchin *P. lividus* by molecular approaches. PAHs and PCBs targeted different genes and had several common targets, as shown in the Venn diagrams considering up-regulated genes and down-regulated genes, comparing the groups "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control (plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants)", "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2" (**Figure 11** and **Supplementary Tables S13** and **S14**).

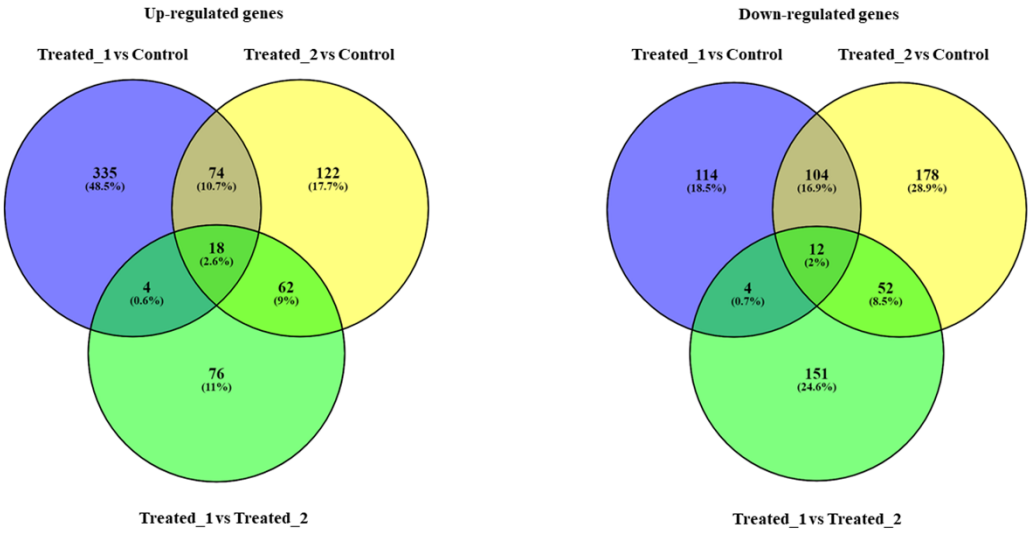

Figure 11. Venn diagrams considering up-regulated genes and down-regulated genes, comparing the groups "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control (plutei from adults sea urchin P. lividus reared for two months in tanks with sediment without contaminants)", "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2". PAHs (Treated_1) and PCBs (Treated_2) induced an increase in the expression of 335 (48.5%) and 122 (17.7%) genes, respectively, compared to the Control; they also induced the down-regulation of 114 (18.5%) and 178 (28.9%) genes, respectively. The two contaminants had several common targets (see also **Supplementary Tables S13** and **S14** for the names of the common genes): (i) for up-regulated genes, 74 common genes (10.7%) comparing the groups "Treated_1 versus Control" and "Treated_2 versus Control"; 18 common genes (2.6%) comparing the groups "Treated_1 versus Control", "Treated_2 versus Control" and "Treated_1 versus Treated_2"; 4 common genes (0.6%) comparing "Treated_1 versus Control" and "Treated_1 versus Treated_2"; 62 common genes (9.0%) comparing "Treated_2 versus Control" and "Treated_1 versus Treated_2". (ii) for down-regulated genes, 104 common genes (16.9%) comparing the groups "Treated_1 versus Control" and "Treated_2 versus Control"; 12 common genes (2.0%) comparing the groups "Treated_1 versus Control", "Treated_2 versus Control" and "Treated_1 versus Treated_2"; 4 common genes (0.7%) comparing "Treated_1 versus Control" and "Treated_1 versus Treated_2"; 52 common genes (8.5%) comparing "Treated_2 versus Control" and "Treated_1 versus Treated_2"

Transcriptome is generally dynamic, and it is a good indicator of the cell's state. In addition, in this case, the ease of genome-wide profiling made the transcriptome analysis an integral part of understanding the biological processes affected by PAHs and PCBs. In fact, to identify the pathways in which the genes affected by these two contaminants were involved, a Gene Ontology (GO) term enrichment analysis was performed using DE genes (**Figure 12**). Seventy-seven GO terms were enriched, including 20 in "Biological Process" followed by 23 in "Molecular Function" and 24 in "Cellular Component" ($p < 0.05$). Over-represented GO categories included the oxidation–reduction process, regulation of transcription, DNA integration, cytoskeleton organization, nucleic acid binding, metal ion binding, DNA

binding, zinc ion binding and DNA-binding transcription factor activity. Moreover, these genes are integral components of the membrane and were mainly localized in the cytoplasm, nucleus, extracellular region and microtubule.

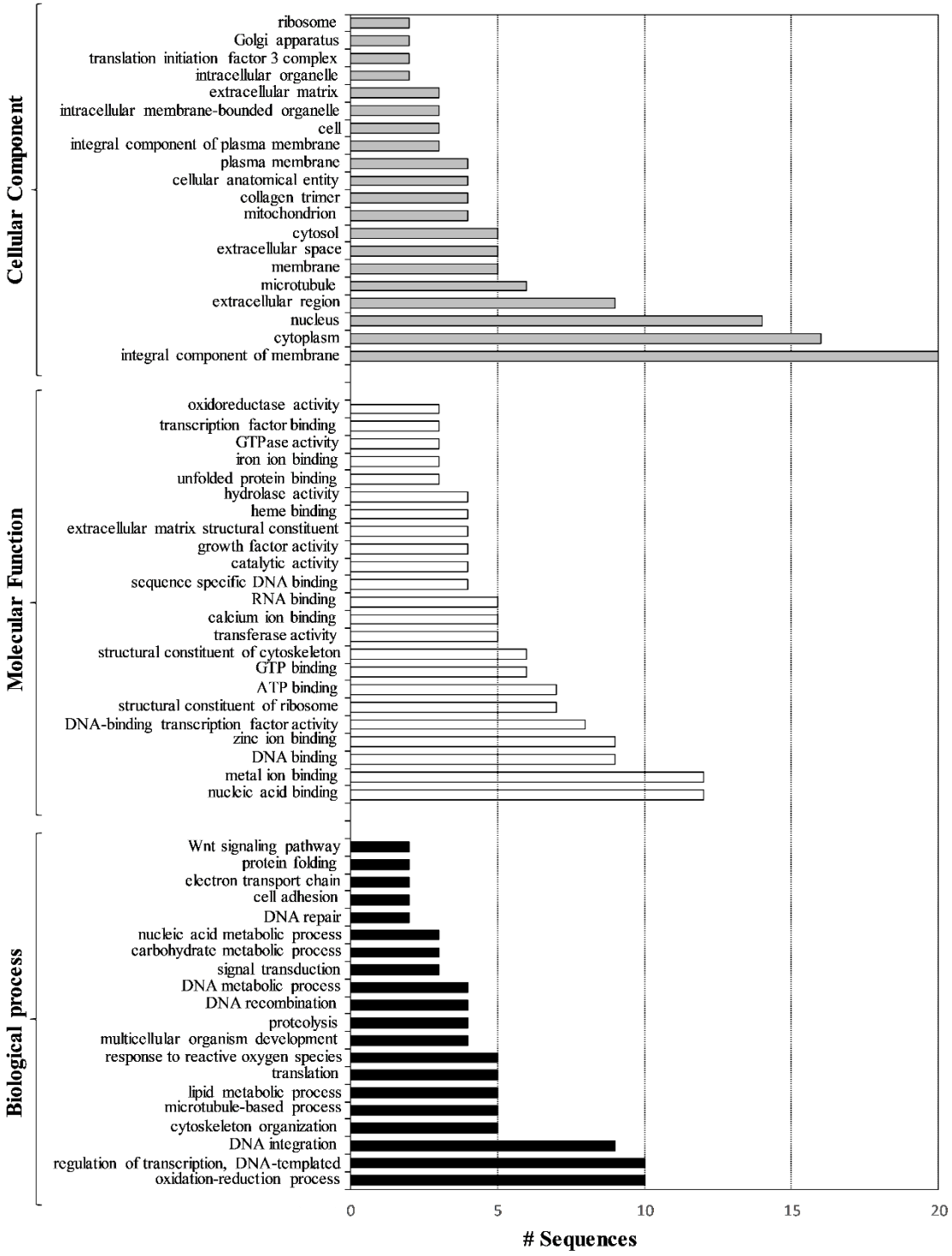

Figure 12. Overrepresented GO terms of sea urchin plutei after artificial contaminated experiments with PAHs and PCBs in the three major functional categories: Biological Process (black bars), Molecular Function (white bars) and Cellular Component (grey bars).

2.3.4 Network analysis

The twenty-five genes analysed through RT-qPCR were found correlated to several genes previously isolated from *P. lividus*. Depending on gene functions, they were divided into two functional networks reporting the highest correlations among stress/detoxification and developmental genes.

Among the ten genes involved in stress response, *hsp90, hsp75* and *GAPDH* were revealed the most correlated to the other ones subjected to network analysis (**Figure 13**).

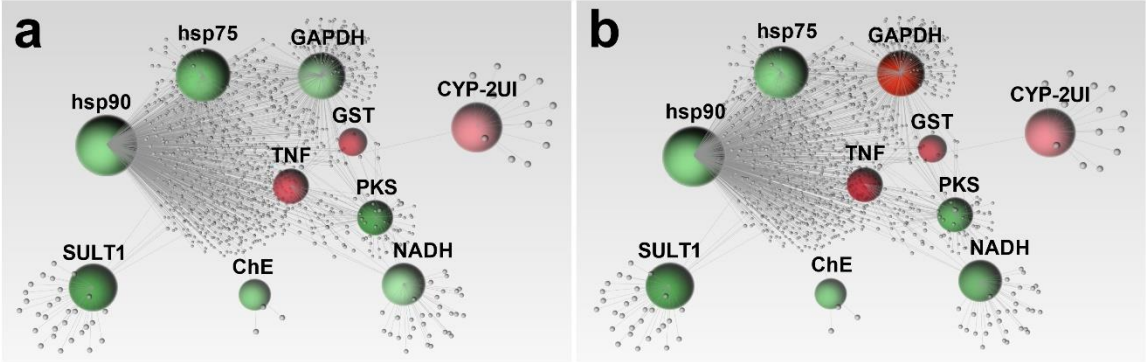

Figure 13. Gene network performed by STRING interactome of ten genes involved in stress response and detoxification. Correlations confidence score cut-off of 400 were reported. Among functionally correlated genes, those with up (red) and down (green) expression affected by PAHs (a) and PCBs (b) were reported. Color shading depends on fold-change values. Gray spheres represent additional connections.

The heat shock protein *hsp90* clearly showed a key role in numerous biological processes since a huge number of additional connections (grey spheres) were observed (**Figure 13**).

Concerning gene expression results, no significant differences were observed between PAHs (**Figure 13a**) and PCBs (**Figure 13b**), suggesting that a similar biological pathway was activated in response to PAHs and PCBs exposure. The sole exception was *GAPDH*, whose expression increased only in the case of PAHs treatment (**Figure 13a**).

Among development and differentiation genes, a huge functional correlation was appreciable, except for *PLAUF3*, whose biological role was found principally correlated to *CM-K* and *STAT1* (**Figure 14**).

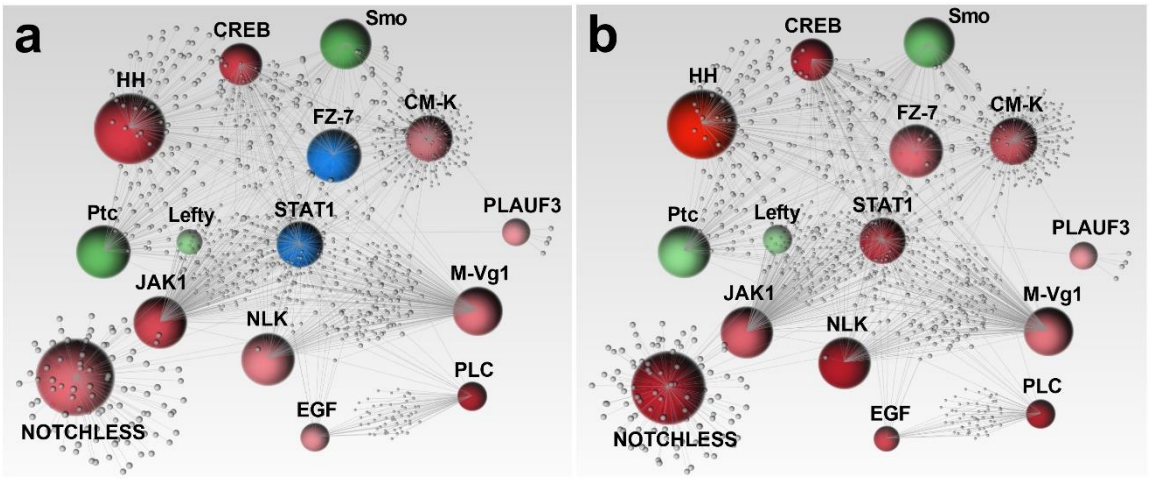

Figure 14. Gene network performed by STRING interactome of fifteen genes involved in develop-mental processes. Correlations confidence score cut-off of 400 were reported. Among functionally correlated genes, those with up (red), down (green) and unchanged (blue) expression affected by PAHs (a) and PCBs (b) were reported. Color shading depends on fold-change values. Gray spheres represent additional connections.

A strong up-regulation of these genes was induced by sea urchin exposure with PAHs and PCBs (**Figure 14**). As reported for stress genes, no striking variations were detected. Ten functionally connected genes, *HH, CREB, NOTCHLESS, JAK, NLK, EGF, PLC, M-Vg1, PLAUF3* and *CM-K*, were all up-regulated in the same biological pathway (**Figure 14**), thus corroborating previous results reporting several aberrations in sea urchin progenies (Luisa Albarano et al., 2021). *FZ-7* and *STAT1* were not affected by PAHs treatment (**Figure 14a**), implying that the biological cascade altered by PAHs and PCBs might be slightly different.

2.3.5 Effects of PAHs and PCBs on Gene Expression by *Real-Time qPCR*

The expression levels of eighty-seven genes (Romano et al., 2011; Varrella et al., 2014, 2016a; Ruocco et al., 2016, 2017b, 2019a; Esposito et al. 2020), involved in different physiological processes, were followed by *Real Time qPCR* (**Figure 15**).

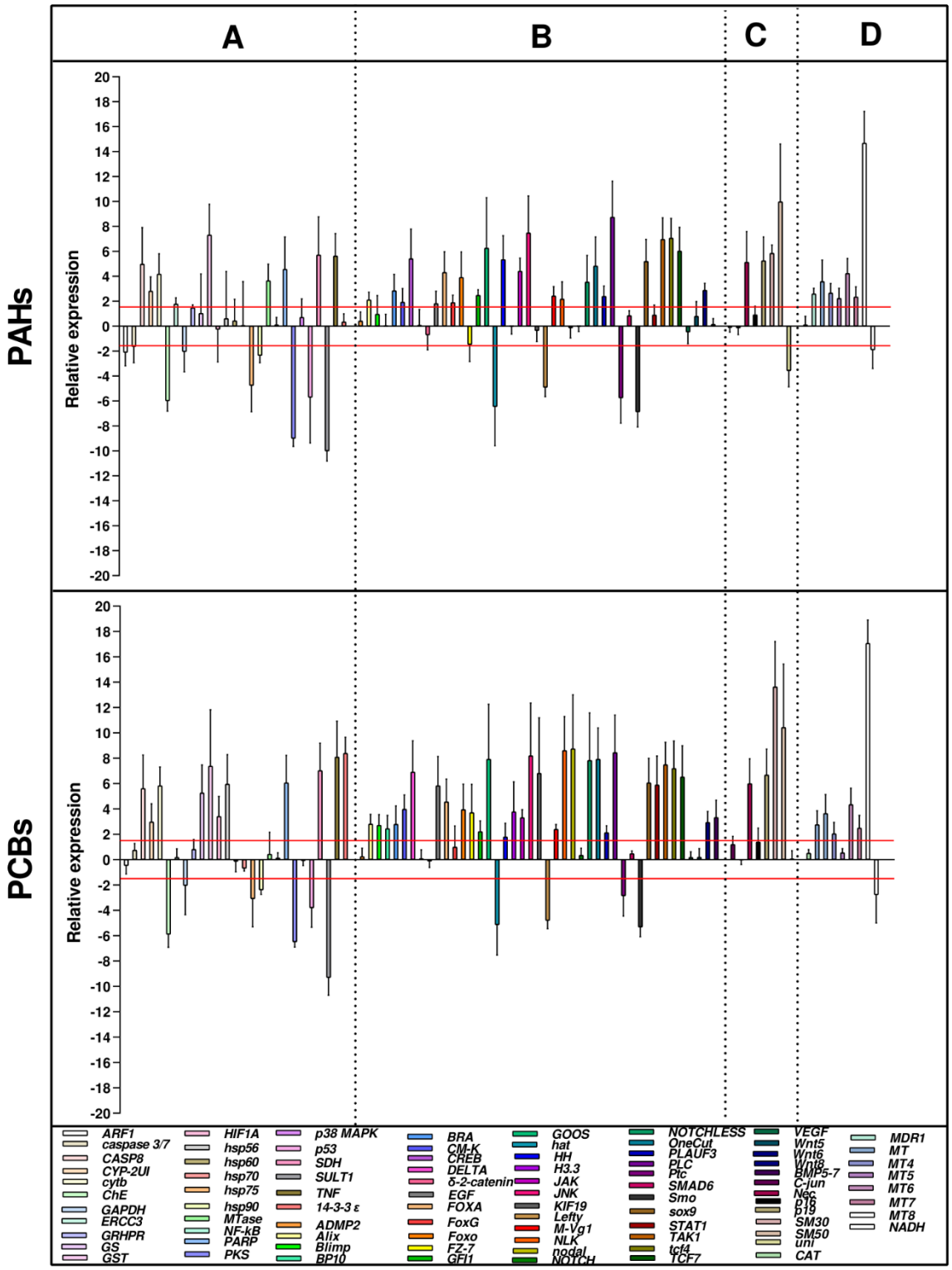

Figure 15. *Real-Time qPCR* at pluteus (48 hpf) stages. Histograms show the differences in expression levels of 87 genes involved different functional processes: stress, skeletogenesis, development/differentiation and detoxification. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant.

These genes were previously selected in (Romano et al., 2011; Varrella et al., 2014, 2016a; Ruocco et al., 2016, 2017b, 2019a; Esposito et al. 2020), and their expression levels were

studied in response to natural toxins produced by marine diatoms. We proposed these genes as possible biomarkers to detect the consequences of the exposure of marine invertebrates to different environmental pollutants (Ruocco et al., 2017). In particular, these genes were defined as a part of the defensome, which was placed in motion by the sea urchin to protect themselves from environmental toxicants (Marrone et al., 2012).

At the pluteus stage at 48 hpf (**Figure 15**, for the numerical values see also Appendix A, **Supplementary Table S15**), PAHs and PCBs had several common targets.

-Stress genes:

All twenty-seven genes were targeted by contaminants treatment with the exception of *GRHPR, hsp60, hsp70, NF-κB* and *p38*. In particular, fifteen genes were equally affected by all contaminants with a down-regulation of *ChE*, *p53, GAPDH, hsp75, hsp90, PKS* and *SULT1* and an up-regulation of *Casp-8, CYP-2UI, Cytb, MTase, Parp, GST, TNF* and *SDH.* Moreover, treatment with PAHs also down-regulated *ARF1*, *Cas 3-7* and up-regulated *ERCC3*; whereas, the exposure of PCBs up-regulated *GS, HIF1A, hsp56* and *14-3-3 ε.*

-Skeletogenic genes:

All eight genes were switched on by PAHs and PCBs exposure with the exception of three genes: *BMP5-7, C-jun* and *p16*. The two contaminants up-regulated *Nec, p19, SM30* and *SM50.* Furthermore, *uni* gene was targeted only after PAHs treatment with a down-regulation.

-Genes involved in development/differentiation:

Gene expression analysis of genes involved in development/differentiation showed that, among forty-three genes analysed by *Real Time qPCR*, only six genes were not targeted by treatments: *ADMP2, DELTA, δ-2-catenin, NOTCH, VEGF* and *Wnt5.* In fact, several genes were significantly affected by both contaminants. In particular, common molecular targets for two contaminants were *Alix, BRA, CM-K, CREB, EGF, FOXA, FOXO, GFI1, Gooscoid, HH, JAK, JNK, M-Vg1, NLK, NOTCHLESS, OneCut, PLAUF3, PLC, sox9, TAK1, tcf4, tcf7,*

Wnt6 (all up-regulated genes) and *hat, Lefty, Ptc, Smo* (down-regulated genes). Moreover, PCBs targeted *Blimp, BP10, FZ-7, H3.3, KIF19, nodal, STAT1* and *Wnt8* increasing their expression levels. However, *FOXG* gene was only up-regulated after PAHs treatment.

-Genes involved in detoxification:

All nine genes analysed were targeted by contaminants with exception of *CAT* gene. *MDR1, MT, MT4, MT6, MT7, MT8* and *NADH* represented common target for two treatments: PAHs and PCBs increased the expression levels of all these genes with exception of *NADH* gene, that was down-redulated. However, *MT5* gene was only target of PAH: in fact, this treatment caused an increase of expression levels.

2.4. Discussion

Experiment of contaminated sediment revealed different dangerous effect of single three pollutants (PAHs, PCBs and Zn) and mixture of compounds. In fact, the Zn and mixture conditions were able to induce an increase of sea urchin death after 2 weeks of exposure, whereas the survival of *P. lividus* was unaffected by exposure to PAHs and PCBs. These results showed that the presence of Zn at this concentration was strongly toxic not only on development, but also on adults.

Embryos spawned from adult sea urchins treated for two months with PAHs and PCBs showed morphological malformations affecting the arms, spicules and apex. In addition, PCBs were also able to induce delay on embryonic development, with some embryos still at the gastrula stage when observed at 48 hpf. The PAHs and PCBs have been shown to affect embryonic development in several marine invertebrates, including sea urchin *P. lividus.* In fact, these compounds were able to induce different dangerous and toxicological effects on marine invertebrate development, such as axial development, suppression of spicules formation, decrease of grown rate (Bellas et al., 2008; Geffard et al., 2001; Pillai et al., 2003; Steevens et al., 1999; Suzuki et al., 2015). Moreover, the PCBs exposure has also been shown

to effects of delay of larval (Anselmo et al., 2011; Coteur et al., 2003; Savriama et al., 2015; Schweitzer et al., 1997).

Contaminated sediment experiments also increased the levels of PAHs and Zn in three tissues of *P. lividus*. In particular, it has been observed a PAHs increase in spines + theca and in gonads of treated (W + SED +PAHs) sea urchins respect to sea urchin tissues collected from control conditions (W/ W + SED). As widely described in **Paragraph 1.4.1**, these compounds have low hydrophobicity and consequently a high oils solubility. Probably, for this feature they have been more adsorbed and accumulated in gonads then other tissues. Moreover, an equal bioaccumulation of Zn was found in both treated (W + SED + Zn) and control (W/ W + SED) sea urchin tissues. Probably, these results could depend on the environmental site from which sea urchins were collected. In fact, they were taken from the Gulf of Naples where this element was ready found in previous investigation (Trifuoggi et al., 2017). However, an increase of Zn concentration was registered in spines + theca and intestine in treated sea urchins respect to control. These results confirmed the role of sea urchin *P. lividus* as indicator of heavy metals pollution. In fact, several studies showed the capability to incorporate greater quantities of these elements into calcareous skeletons of these organisms collected from polluted sites than those that live in uncontaminated zones (Auernheimer and Chinchon, 1997; Salvo et al., 2014). Furthermore, no bioaccumulation of PCBs was detected in three tissues collected from sea urchins treated with PCBs (W + SED + PCBs). Probably, the low concentration of PCBs makes these compounds less capable and instrumentally hard to find to bioaccumulate than PAHs.

As shown in **Paragraph 2.3.1,** chemical analyses on samples of seawater and sediment revealed a change of distribution in sediment and in seawater only for Zn at both T0 and Tf; whereas for PAHs and PCBs conditions, the compounds didn`t registered at both T0 and Tf. Probably, also in this case, the low concentration of PCBs and PAHs makes these compounds less capable and instrumentally hard to find in the analysed samples. PCB bioaccumulation

data on marine organisms are scarce, impeding an effective assessment of their toxicity. Zeng et al., (1996, 2003)studied the uptake patterns of PCB congeners in the sea urchin *Lytechinus pictus*. More than 66 days are necessary for some congeners to attain steady state concentration in *L. pictus* gonad, whereas 28–42 days are required (Boese et al., 1995) in such marine organisms as bivalves, polychaetes and amphipods. Evidence of toxicity with changes in total or gonad weight was only detected at 647 mg/g. Studies on fish indicated that embryos and developing larvae were negatively affected by PCBs at 0.12 mg/g–12 mg/g (Monosson et al., 1994; von Westernhagen et al., 1981). Monosson et al. (1994) observed that PCB effects were due to the congener 3,30,4,40 tetrachlorobiphenyl, which has a greater toxicity than that of the congeners' mixture (Danis et al., 2005), and exposed adult *P. lividus* to 14C-labelled PCB#153 via seawater and food, observing that the bioaccumulation efficiency was similar in the body wall, spines, gut and gonads.

Another interesting result is the large-scale genomic information on *P. lividus* generated in this study. Transcriptomic results indicate that PAHs and PCBs affected genes differently, mainly increasing their gene expressions, supporting those differences observed at the morphological level. In fact, the highest percentage of malformed plutei caused by exposure to PAHs can be linked to the up-regulation of the majority of the studied genes. An example was represented by nodal and nectin genes (data also confirmed by real-time qPCR experiments, see below). The nectin gene is involved in cellular adhesion (Lebesgue et al., 2016; Zito et al., 2010), whereas nodal gene controls the left–right asymmetry in the sea urchins, regulating the expression level of the BMP2 gene (Duboc et al., 2010, 2005, 2004; Yaguchi et al., 2008). The exposure to PCBs caused not only the up-regulation of the nodal gene, but also the up-regulation of the frizzled gene and the down-regulation of the PLC gene. The function of the frizzled gene is similar to that of the nodal gene. Binding to the Wnt6, this receptor is responsible for endoderm specification (McCauley et al., 2013; Robert et al., 2014). Instead, the PLC gene is involved in egg activation in the events immediately

following fertilization and during embryo development in sea urchins (Coward et al., 2004; Rongish et al., 1999; Shearer et al., 1999). Its down-regulation can be one of the causes of the delay effect shown after PCB treatment. Our study also provides new information on the molecular effects of contaminated sediment on sea urchin embryos. The genome of *P. lividus*, is still not available. Hence, the availability of 25 new genes provides a better understanding on the molecular mechanisms of response in sea urchins to toxic substances. These new genes are not only functionally intercorrelated with one another, but also with another 10 genes previously analysed in response to natural toxins.

The interpretation of *de novo* transcriptomic results was also improved with the analysis by *Real Time qPCR* of a set of sixty-two genes previously used to study the response of *P. lividus* embryos to natural toxicants (Romano et al., 2011; Varrella et al., 2014, 2016a; Ruocco et al., 2016, 2017b, Esposito et al. 2020) plus 25 genes isolated in this study. These genes have key roles in different functional processes such as stress response, skeletogenesis, embryonic development, cell differentiation, morphogenesis and detoxification processes (see **Supplementary Figure S12**).

Firstly, all 87 genes were molecular targets of both pollutants, with the only exception of *GRHPR, hsp60, hsp70, NF-κB, p38MAPK*, *ADMP2*, *DELTA, δ-2-catenin, NOTCH, SMAD6, VEGF, Wnt5, BMP5-7, C-jun, p16* and *CAT* (**Figure 15**). Summarizing the *Real Time qPCR* experiments (**Figure 16**): i) fifty-three genes were targeted by all two pollutants; ii) six genes were only targets for PAHs treatment; iii) twelve genes were specifically affected only by PCBs treatment.

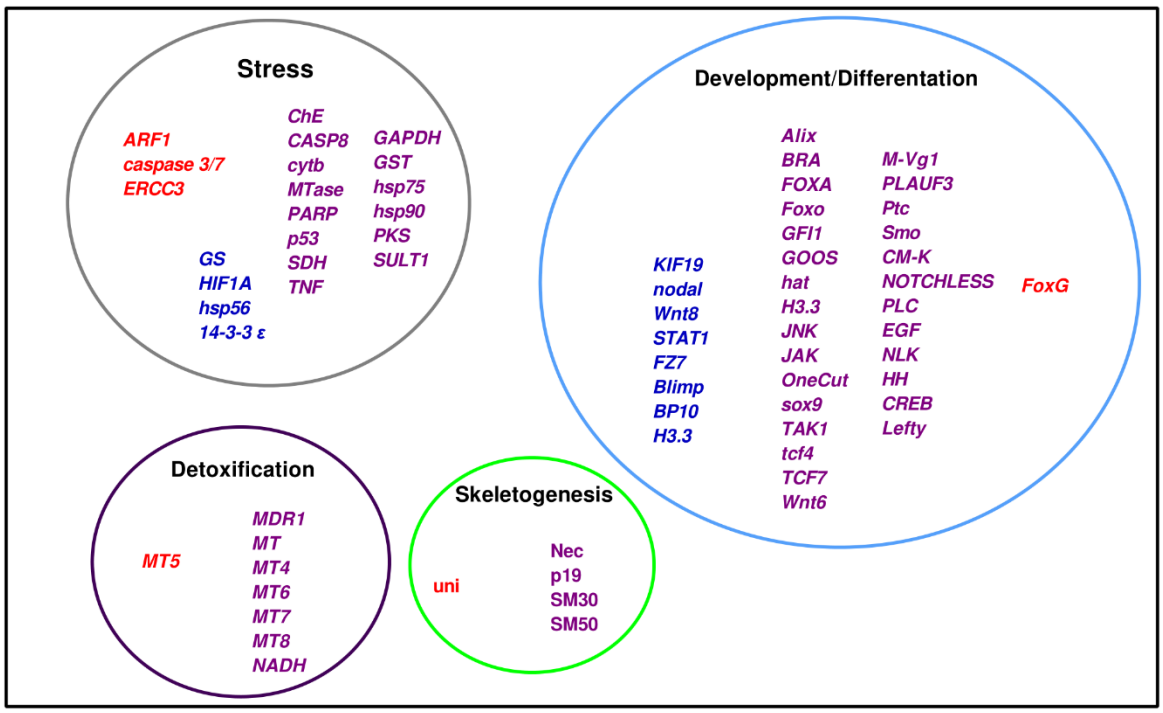

Figure 16. Schematic overview of *P. lividus* genes affected by contaminated sediment under analysis. The red colour represents the genes with changing expression levels (up-regulated and down-regulated genes) by PAHs. The blue colour indicates the genes affected by PCBs; and the purple colour highlights the genes targeted by both treatments.

These data suggest that the plutei deriving from adults treated with PAHs and PCBs were very similar, as also shown in the Heatmap reported in **Figure 17**. In fact, the molecular response to PAHs appeared little different in comparison with PCBs: PCBs mainly up-regulated genes (53 up-regulated genes versus 12 down-regulated genes; see also Appendix A, **Supplementary Table S15**) compared to PAHs (44 up-regulated genes versus 15 down-regulated genes). Even if there were no great differences observed between the two pollutants at the morphological level, the molecular results (and mainly the *de novo* transcriptome) suggest that the toxic effect of PCBs treatment on *P. lividus* was higher than that of PAHs.

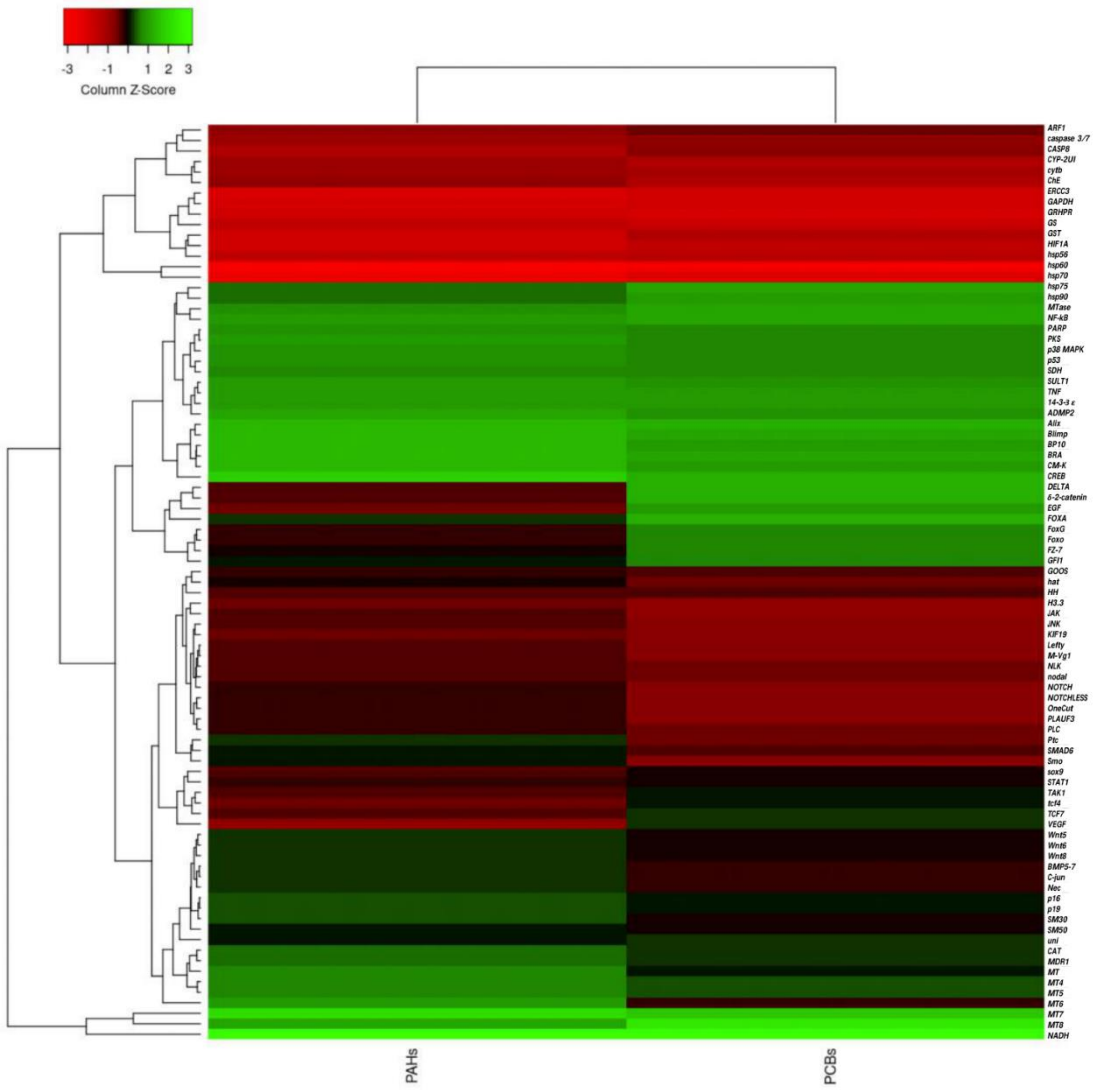

Figure 17. Heatmaps showing the expression profiles and hierarchical clustering of 87 genes analysed through *Real Time qPCR* in embryos deriving from sea urchins treated with PAHs and PCBs. Colour code: red, up-regulated genes with respect to the control; green, down-regulated genes with respect to the control; black, genes for which there was no variation in expression with respect to the control.

All together these molecular results, supported by the morphological observations, revealed that the majority of malformations affected the skeleton, the developmental plan and differentiation of sea urchin embryos. In fact, several genes belonging to the skeletogenic, developmental and differentiation classes were affected by the two toxicants. *PLC* gene is typically regulated by *CM-K*, and together are involved in the events following fertilization and embryo development in sea urchins (Coward et al., 2004; Rongish et al., 1999; Shearer et al., 1999; Sidhu et al., 2005). *M-Vg* gene together with nodal are involved into left-right axis formation, regulating some down-stream effectors, including *BMP2* gene (Duboc et al.,

2004). *NOTCHLESS* gene is involved in the *NOTCH-DELTA* pathway, which, in turn, regulates the expression of *NLK* and *Lefty* genes (Esposito et al., 2020; Royet et al., 1998; Warner and Mcclay, 2014). This pathway has a key role during the induction and differentiation of secondary mesenchyme cells (Materna and Davidson, 2012; Röttinger et al., 2006; Warner and Mcclay, 2014) and could be implicated in the formation of malformed gastrulae. *Ptc* and *Smo* genes showed a down-regulation after both PAHs and PCBs treatments. The *Smo* and *Ptc* proteins are co-receptors of HH ligand and are expressed by the skeletogenic and non-skeletogenic mesoderm (Ingham et al., 1991; Nachtergaele et al., 2013; Walton et al., 2009). Probably, the defects in embryo development were caused by perturbation of this pathway. Moreover, Fz-7 receptor binding to Wtn6 controls the β-catenin signal during the specification of the endomesoderm (Lhomond et al., 2012). The *STAT1* gene together with Jak gene constitute the *JAK/STAT* pathway, that plays a fundamental role into regulation of cellular complex (Brown et al., 2001; Darnell, 1997; Hou et al., 2002).

In conclusion, this study is the first demonstration of the toxic effects of sediment considered as control due to its low concentrations of pollutants on embryos and larvae of the sea urchin *P. lividus*.

All the results reported in this Chapter have been published in:

1) **Albarano L.**, Zupo V., Caramiello D., Toscanesi M., Trifuoggi M., Guida M., Libralato G., Costantini M., 2021b. Sub-chronic effects of slight PAH- and PCB-contaminated mesocosms in *Paracentrotus lividus* Lmk: a multi-endpoint approach and de novo transcriptomic. International Journal of Molecular Sciences, 22, 6674. (Appendix B)

2) **Albarano L.**, Guida M., Zupo, V., Libralato G. Costantini M., PAHs and PCBs affect functionally intercorrelated genes in the sea urchin *Paracentrotus lividus* embryos. International Journal of Molecular Sciences, 22, 12498. (Appendix B)

Acknowledgments

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3. Chapter 3: Genotoxicity in *Artemia franciscana* nauplii and adults: the case of phenanthrene, naphthalene, fluoranthene and benzo(k)fluoranthene

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) consist of a group of over 100 different organic compounds, generated by natural events or anthropogenic activities. PAHs predominantly originate from anthropogenic processes, especially from incomplete combustions of organic fuels. Certain naturally occurring processes, such as volcanic eruptions and forest fires, contribute to the increasing the number of this organic compounds in the environment. In Santos Bay and Estuary, the anthropogenic contributions to PAHs in sediments resulted of about 99% (the concentrations varied from 79.6 for uninhabited area to 15389.1 ng/g for area located in the proximity of industries; (Medeiros and Caruso Bícego, 2004)).

They are formed by two or more fused benzene rings and their toxicity depends on the number of benzene rings. Because of their low water solubility and hydrophobicity, these compounds tend to adsorb onto environmental particles and accumulate in sediment, where their degradation is very slow (Ghosal et al., 2016). The half-lives of low molecular weight PAHs (naphthalene, acenaphthene, fluorene and phenanthrene) ranged from approximately 3-8 days; whereas half-lives of high molecular weight PAHs (pyrene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene) ranged from 73·1780 days (MacRae and Hall, 1998; Shi et al., 2020; Tansel et al., 2011).

Some PAHs are present in the air at room temperature, both as gas (e.g., phenanthrene) and associated with particles (e.g., benzo[a]pyrene — B[a]P) (Perelo, 2010).

These organic molecules can have several toxic effects, such as immunotoxicity, embryonic abnormalities, and cardiotoxicity for aquatic environment, especially impacting fish, benthic organisms, and marine vertebrates (Bellas et al., 2008; Honda and Suzuki, 2020; Ruocco et al., 2020; Albarano et al., 2021). By toxicity bioassays with embryos-larval stages of the sea

urchin *Paracentrotus lividus*, of the ascidian *Ciona intestinalis* and the mussel *Mytilus galloprovincialis*, Bellas and coworkers (2008) demonstrated that 5 different PAHs (naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT), fluorene (FLR), pyrene (PYR) and hydroxypyrene), known to be potentially toxic, inhibited and reduced the larval development and growth of both *M. galloprovincialis* and *P. lividus*. The embryos and larval stages of *C. intestinalis* were sensitive only to treatment with NAP. Interesting was a work conducted by Ikenaka et al., 2013, which estimated the acute toxicity of benzo[a]antracene (BaA), one of the most toxic PAHs, on crustaceans *Daphnia magna* and *Ceriodaphnia reticulata.* Using conceivable concentrations in the environment (from 1 to 32µg/L), they showed that the LC50 for *D. magna* and *C. reticulata* was 4.3 and 4.7 µg/L. This work was following the study conducted by Verrhiest et al., 2001 that established the LC50 for *D. magna*, *Hyalella Azteca* and *Chiromonus riparius* was 50, 15 and 20 mg/kg dry weight sediment for PHE, respectively; and 10, 5 and 15 mg/kg dry weight sediment for FLT, respectively. When these three PAHs have been tested in mixture, a synergic effect has been shown. After PYR, FLT and anthracene (ANT) exposure, *D. magna* and *A. salina* crustaceans displayed higher sensibility than those registered for the mosquito *Aedes aegypti*, the amphibian *Rana pipiens* and the fish *Pimephales promelas* (Kagan et al., 1985). Moreover, *D. magna* and *Caenorhabditis elegans* showed more sensitivity to BaP than *A. salina* (Sese et al., 2009).

In this study we tested the PHE, NAP, FLT and benzo(k)fluoranthene (B(k)F) toxicity on embryos and adults of *Artemia franciscana* using conceivable concentrations in marine environment (from 0.025 to 10 mg/L, from 0.36 to 2.3 E+2 mg/L, from 0.41 to 3.9 E+2 mg/L, and from 0.025 to 9.4 E+1 mg/L for NAP, PHE, FLT and B(k)F, respectively), detected from polluted sediment associated to various pollution sources (Arienzo et al., 2017). To the best of our knowledge, no studies have been carried out so far to investigate direct effects of PAHs on *A. franciscana*. Only, Rojo-Nieto et al., 2012 established that

mixture of ten PAHs (naphthalene, acenaphthene, phenanthrene, fluoranthene, fluorene, pyrene, anthracene, benzo(a)pyrene, benzo(a)anthracene and chrysene) at real concentrations (detected from sediment of the Bay of Algeciras) have not impact on survival of *A. franciscana* using passive dosing.

The crustacean *A. franciscana* was used since it is considered a good model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants (Libralato, 2014; Libralato et al., 2007; Migliore et al., 1993). The greatest advantage of the species is that nauplii can be hatched as needed from commercially available durable cysts. This avoids the need of maintaining laboratory cultures, contrary to what is required for many species used in ecotoxicity tests. In any case, these tests (namely "Toxkit") employing dormant stages ("cryptobiotic eggs") have the same efficacy and sensitivity as tests with cultured animals (Persoone et al., 2009). Moreover, the embryo grows rapidly in laboratory conditions (the nauplius stage is reached in 24 h); their small body size allows the conduct of the tests in small beakers or plates; and finally, they have adaptability to a wide range of salinities (5-300 g/L) and temperatures (6-40 °C; Manfra et al., 2016). Due to their limited sensitivity versus apical endpoints, the use of this crustaceans in ecotoxicology has become increasingly rare (Libralato et al., 2016). To use *A. franciscana* for evaluating the molecular aspects that are on the base of the toxicological effects could give a new life to crustacean as biological model.

More specifically, in this work as well as evaluating PHE, NAP, FLT and B(k)F acute (24h - 48h-LC50) toxicity on nauplii and adults by measuring survival, we defined the molecular basis of these PAHs toxicity. After 48 h under sublethal exposure for both tested life stages, the effect on several key genes involved in stress response (*hsp26, hsp60, hsp70, COXI* and *COXIII*) was assessed. In addition, the impact on development genes (*HAD-like, tcp, UCP2* and *CDC48*) was evaluated for nauplii.

3.2 Materials and methods

3.2.1 Ecotoxicity test

Acute toxicity test using both *A. franciscana* larvae and adults has been developed as standard methods (CNR, 2003) for assessing the lethality of contaminants both in the first stages of development (up to Instar III nauplius, because of 48 h old specimens is considered the most sensitive larval end-point) and in adults. Certified dehydrated cysts of brine shrimp *A. franciscana* (AF/F2005) were purchased from the company ECOTOX LDS (Gallarate, Italy). Hatching of the cysts was obtained by incubating 100 mg of cysts in glass Petri dishes containing seawater prepared by dissolving appropriate amount of Instant Ocean® salt in deionized water, stirred for 24 h under aeration and then filtered through 0.45-μm Millipore cellulose filters. Newly hatched brine shrimp larvae (Instar I nauplius stage) were separated from unhatched cysts and transferred based on-phototaxis into new glass Petri dishes with synthetic seawater (SSW, prepared in according to ISO 10253/16).

3.2.2 Chemicals

The naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene used in the toxicity tests were kindly provided by Sigma-Aldrich. The purity was greater than 97%. Stock solutions of NAP, PHE, FLT and B(k)F were prepared by dissolving in DMSO. Maximum DMSO in test solutions did not exceed 1%. DMSO, which is nontoxic to *A. franciscana* (Barahona-Gomariz et al., 1994). Stock solutions (XX mg/L) of each PAH in DMSO were kept in the dark at room temperature. The exposure solutions were sampled and analyzed for four PAHs by method described in **Paragraph 2.2.5**.

3.2.3 Acute toxicity test

Acute toxicity test on embryos was performed according to standard APAT IRSA CNR 8060 method (CNR, 2003), by adding 10 nauplii to each well of 24-well plates, containing 2 mL of solutions at increasing concentrations of NAP (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, 10 mg/L), PHE (0, 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, 230 mg/L), FLT (0, 0.41, 1, 2.5, 5, 12.5,

25, 50, 97.5, 195, 390 mg/L) and B(k)F (0, 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, 94 mg/L) tested in SSW.

Toxicity on adults was evaluated by adding 5 crustaceans to each well of 6-well plates, containing 10 ml with solutions of different concentrations of NAP, PHE, FLT and B(k)F mentioned above. The plates and beaker were kept at 25±1 °C with salinity 35 ppm for 48 h in a light regime of 16:8 h light: dark, without providing food. At 24 and 48 h, the number of dead nauplii and adults (which were motionless for 10 s) was counted under a stereomicroscope (Leica EZ4 HD), in order to calculate the mortality. The validity of the test was guaranteed by the control group showing < 10% of mortality at 48 h. All the experiments were performed in triplicates.

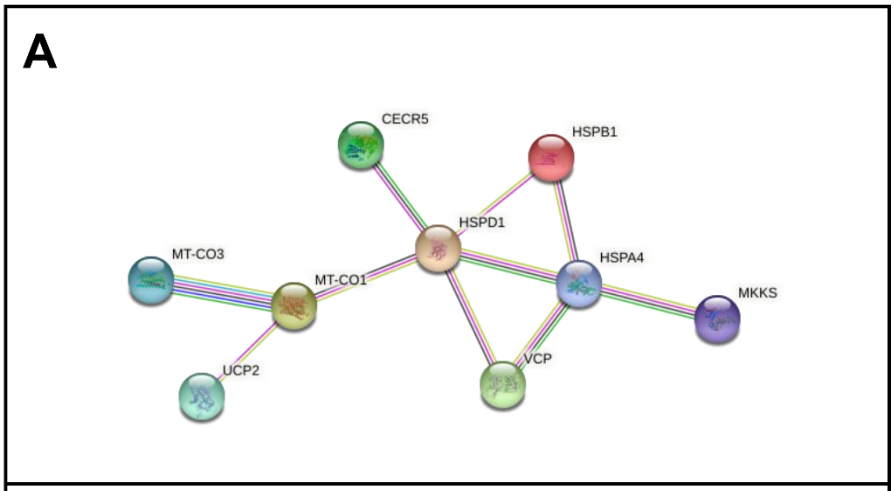
3.2.4 Organisms exposures for RNA extraction

Two hundred nauplii of *A. franciscana* were exposed to NAP, PHE, FLT and BkF at 0.4 mg/L, 2 mg/L, 1 mg/L and 94 mg/L respectively; whereas 10 *A. franciscana* adults were exposed to NAP, PHE, FLT and BkF at 2.5 mg/L, 2 mg/L, 1 mg/L and 94 mg/L, respectively. These concentrations were chosen because the death effect was too strong at higher concentrations while they were lost or not apparent at lower concentrations. All the experiments were performed in triplicates.

Samples were collected after 48 h of exposure by centrifugation at 4000 g for 15 minutes in a swing out rotor at 4° C in a 2 mL tube, kept on ice and were further homogenized in TRIzol (Invitrogen, Paisley, UK) using a TissueLyser II (Qiagen, Valencia, CA, USA) and steal beads of 7 mm diameter (Qiagen, Valencia, CA, USA). Total RNA was extracted and purified using Direct-zolTM RNA Miniprep Plus Kit (ZYMO RESEARCH) The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer 2000 (Thermo Scientific Inc., Waltham, MA USA), to exclude the presence of proteins, phenol and other contaminants (Riesgo et al., 2012).

3.2.5 cDNA synthesis and *Real Time q-PCR*

For each sample, 1000 ng of total RNA was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. The variations in the expression of five genes involved in stress response (*hsp26, hsp60, hsp70, COXI* and *COXIII*, and normalizer *GAPDH* — (Chen et al., 2009); see **Supplementary Figure S14**) were evaluated for adults. For nauplii, the variations in gene expression of another four genes, involved in developmental and differentiation processes, *HAD-like*, *tcp*, *UCP2* and *CDC48* (Chen et al., 2009), were also tested (**Figure 18**).



B

Gene name	*A. franciscana*	*H. sapiens*
heat shock protein 26	*hsp26*	*HSPB1*
haloacid dehalogenase-like hydrolase	*HAD-like*	*CECR5*
heat shock protein 70	*hsp70*	*HSPA4*
chaperonin-containing TCP	*tcp*	*MKKS*
mitochondrial uncoupling protein 2	*UCP2*	*UCP2*
heat shock protein 60	*hsp60*	*HSPD1*
Cytochrome c oxidase subunit I	*COXI*	*MT-CO1*
Cytochrome c oxidase subunit III	*COXIII*	*MT-CO3*
Cell division control protein 48	*CDC48*	*VCP*

Figure 18. A) Interactomic analysis by STRING (https://string-db.org/). The network graphically displays the relationship between genes. The biological relationships between genes are indicated by different colours. Known interactions: reported by database = light blue and determined experimentally = pink. Expected interactions: gene proximity= green; gene fusion = red; genes with similar pattern = light blue. B) Human gene names and the corresponding *A. franciscana* orthologous genes.

Undiluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1× SensiFAST^{TM} SYBR Green master mix (total volume of 10 µL) (Meridiana Bioline). PCR amplifications were performed in a AriaMx Real-Time PCR

instrument (Agilent Technologies, Inc.), according to the manufacturer's instructions System thermal cycler using the following thermal profile: 95 ºC for 10 min, one cycle for cDNA denaturation; 95 ◦C for 15 s and 60 ◦C for 1 min, 40 cycles for amplification; 95 ◦C for 15 s, one cycle for final elongation; one cycle for melting curve analysis (from 60 ◦C to 95 ◦C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were carried out in triplicate. Fluorescence was measured using Agilent Aria 1.7 software (Agilent Technologies, Inc.). The expression of each gene was analyzed and normalized against *GAPDH* gene using REST software (Relative Expression Software Tool, Weihenstephan, Germany, version 1.9.12) on the basis of the Pfaffl method (Pfaffl, 2001b; Pfaffl et al., 2002). Relative expression ratios greater than ± 1.5 were considered significant.

3.2.6 LC50 calculation and statistical analysis

Toxicity data were reported as 'mean ± one standard deviation (SD)'. Data were checked for normality using the Shapiro-Wilk's (S-W) test (p-value <0.05). The statistical significance of differences among different treatments and control was checked by two-way ANOVA followed by Tukey's test for multiple comparisons (GraphPad Prism Software version 8.02 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). P values <0.05 were considered statistically significant. The calculation of LC50 values was done by GraphPad Software through the four parameters logistic equation, which corresponds to the dose-response curve with the slope of the variable slope.

3.3 Results

3.3.1 Nominal PAHs concentrations

In order to verify the correspondence of nominal concentrations, a set of GC-MS analyses (shown in **Table 12**) were conducted on seawater solutions of NAP, PHE, FLT, and BkF at nominal concentrations ranging from 0.025 to 10 mg/L, 0.36 to 2.3E+2 mg/L, 0.41 to 3.9E+2 mg/L and 0.025 to 94 mg/L, respectively.

Compounds	Nominal concentration	Analytical concentration	Nominal/analytical concentration ratio
NAP	0.025	0.015	1.67
	0.05	0.032	1.56
	0.1	0.078	1.28
	0.2	0.11	1.82
	0.4	0.26	1.54
	0.5	0.41	1.22
	1	0.76	1.32
	2.5	1.45	1.72
	5	4.23	1.18
	10	10.1	0.99
PHE	0.36	0.21	1.71
	1	0.71	1.41
	2	1.15	1.74
	3	2.26	1.33
	4	3.45	1.16
	5	4.23	1.18
	10	7.48	1.34
	57.5	48.7	1.18
	115	98.6	1.17
	230	223.4	1.03
FLT	0.41	0.29	1.41
	1	0.81	1.23
	2.5	2.14	1.17
	5	4.41	1.13
	12.5	9.91	1.26
	25	20.4	1.23
	50	45.6	1.10
	97.5	91.6	1.06
	195	179	1.09
	390	325	1.20
BkF	0.025	0.016	1.56
	0.5	0.41	1.22
	1	0.78	1.28
	1.5	0.98	1.53
	3	2.4	1.25
	6	5.3	1.13
	12	10.4	1.15
	23.5	19.5	1.21
	47	41.7	1.13
	94	84.6	1.11

Table 12. Comparisons of nominal vs. analytical concentrations of naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT) and benzo(k)fluoranthene (BkF) in seawater.

The GC-MS determinations provided nominal/analytical ratios close to 2. Altogether, these analyses corroborated the plausible closeness of nominal PAHs concentrations vs. analytical determinations.

3.3.2 Naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene toxicity on nauplii

As reported in **Figure 19**, after 24 h of exposure to NAP, an increase of toxicity was observed at higher tested concentrations, represented by 5 mg/L and 10 mg/L where produced about 38% ($p < 0.0001$) and 43% ($p < 0.0001$) of dead nauplii, respectively (**Figure 19A**). After 48 h, at 0.2 and 0.4 mg/L, a significant increase of toxicity (about 30%) has been displayed respect to 0, 0.025 and 0.05 mg/L ($p < 0.01$). However, the toxicity increases of about 80% (5 and 10 mg/L). These data were statistically significant respect to the control and others used concentrations ($p < 0.0001$).

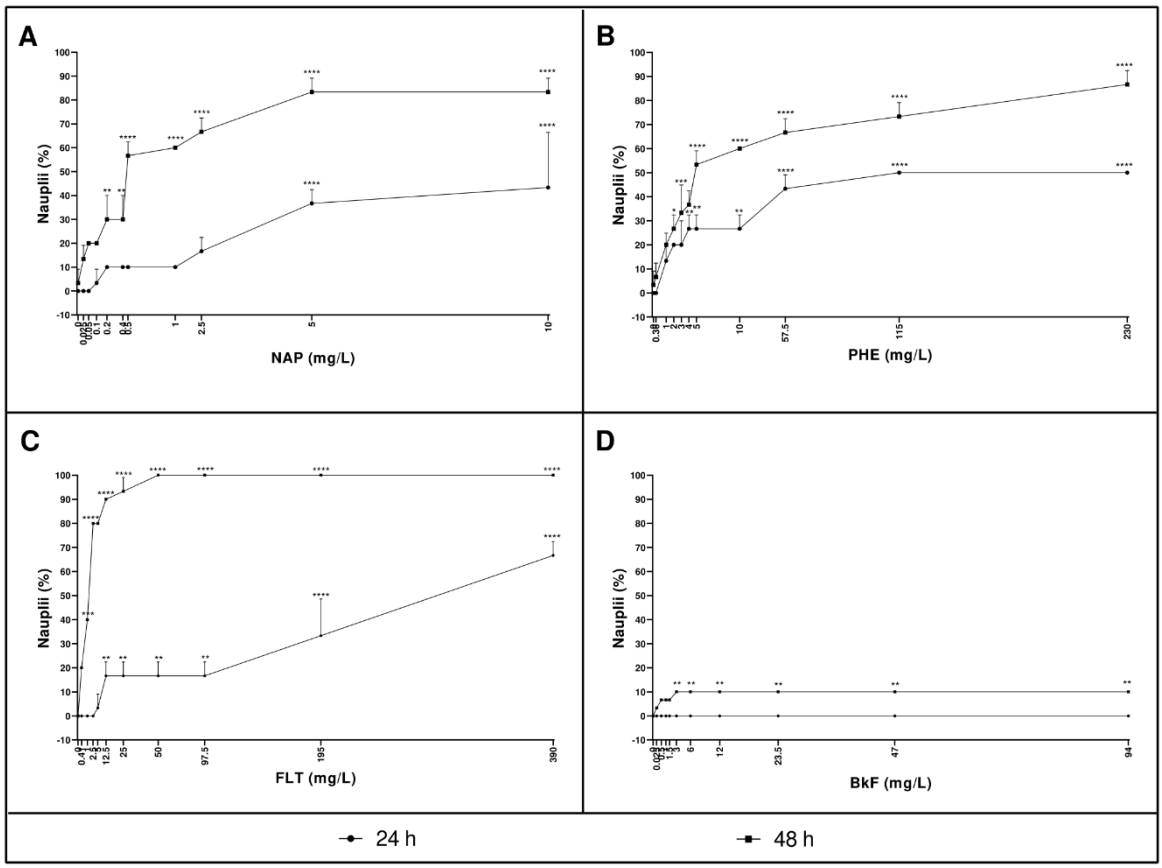


Figure 19. After 24 h and 48 h, the percentage of dead nauplii in control (0 mg/L) and treated samples with A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5 and 10 mg/L; B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115 and 230 mg/L; C) FLT at the concentrations of FLT 0.41, 1, 2.5, 5, 12.5, 25, 50, 97.5, 195 and 390 mg/L; and D) B(k)F at the concentrations of 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, and 94

mg/L was regarded. Data are reported as mean ± standard deviation Two-Way ANOVA by Tukey's test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

After 24 h of exposure, as shown in **Figure 19B**, PHE induced an increase of the percentage of dead (about 26%) with respect to control already from 4, 5 and 10 mg/L. The data reported at these concentrations were statistically significant compared to the two lowest (0 and 0.36 mg/L; p <0.01) and highest (115 and 230 mg/L; p <0.01) concentrations. At 57.5, 115 and 230 mg/L, significant increase of toxicity (about 43.3 %, 50% and 50%, respectively) respect lower tested concentrations (0, 0.36, 1, 2 and 3 mg/L; p <0.0001) has been shown. The scenario after 48 h of exposure was similar than this described after 24 h. About 26% of dead nauplii was registered at 2 mg/L (p <0.05). At 3 and 4 mg/L, the toxicity (about 34%) was statistically significant compared to 0, 0.36, 10, 57.5, 115 and 230 mg/L (p <0.0001). At 10,57.5 and 115 mg/L, about 70% of mortality has been registered; whereas percentage of about 90% of dead has been displayed at 230 mg/L.

Taking into the consideration FLT exposure (**Figure 19C**), an increase of the percentage of dead (about 18%) with respect to control was already recorded from 12.5, 25, 50 and 97.5 mg/L. The data reported at these concentrations were statistically significant compared to the four lowest (0, 0.41, 1 and 2.5 mg/L; p <0.01) and the two highest (195 and 390 mg/L; p <0.0001) concentrations. At 195 and 390 mg/L, significant increase of toxicity (about 35 % and 70%, respectively) respect lower tested concentrations (5, 12.5, 25 and 50 mg/L; p <0.0001) has been shown. After 48 h, at 1 mg/L, a significant increase of toxicity (about 40%) has been displayed respect to 0 (p <0.0001) and 0.41 mg/L (p <0.001). However, the toxicity increases of about 80% (2.5 and 5 mg/L). These data were statistically significant respect to compared to the three lowest (0, 0.41 and 1 mg/L; p <0.0001) and the highest (25, 50, 97.5, 195 and 390 mg/L; p <0.001).

When considered BkF toxicity (**Figure 19D**), after 24h, no effect has been recorded. Only after 48h, at 3, 6, 12, 23.5, 47 and 94 mg/L, a significant increase in toxicity (about 10%) has been shown respect to the control ($p < 0.01$).

3.3.3 Naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene toxicity on adults

After 24 h of exposure, both NAP and PHE and BkF did not affect the survival of crustaceans at all tested concentrations (**Figure 20**).

After 48 h, at 2.5 mg/L, a significant increase of toxicity (about 30%) has been displayed respect to 0, 0.025, 0.05, 0.1, 0.2 and 0.4 mg/L ($p < 0.05$). However, the toxicity increased from about 40% (at 0.5, 1 and 2.5 mg/L) to 60% (at 5 and 10 mg/L). These data were statistically significant respect to the control and others used concentrations ($p < 0.0001$; **Figure 20A**).

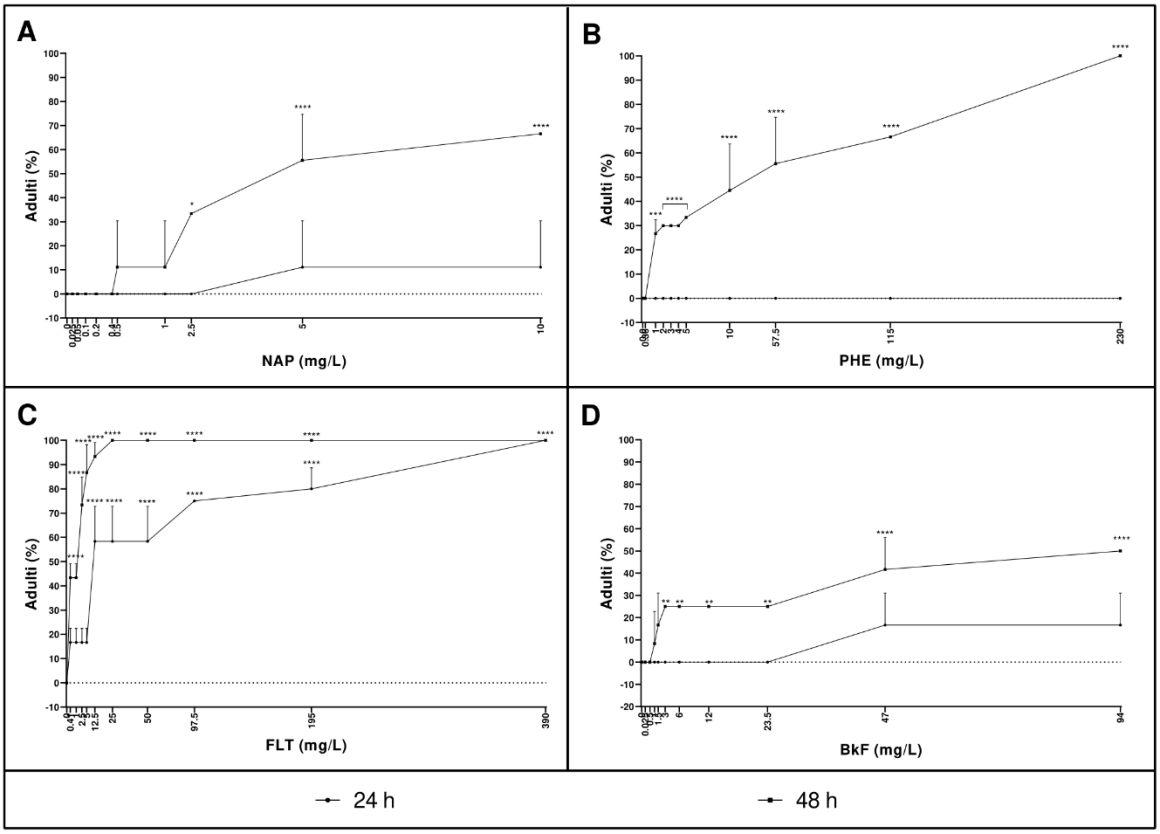


Figure 20. After 24 h and 48 h, the percentage of dead adults in control (0 mg/L) and treated samples with A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5 and 10 mg/L; B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115 and 230 mg/L; C) FLT at the concentrations of FLT 0.41, 1, 2.5, 5, 12.5, 25, 50, 97.5, 195 and 390 mg/L; and D) B(k)F at the concentrations of 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, and 94 mg/L was regarded. Data are reported as mean ± standard deviation Two-Way ANOVA by Tukey's test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

After 48 h of exposure, as shown in **Figure 20B**, PHE induced an increase of the percentage of dead (about 26.6%) with respect to control already from 1 mg/L. The data reported at this concentration were statistically significant compared to the two lowest (0 and 0.36 mg/L; $p < 0.001$) and other concentrations ($p < 0.0001$). At 2, 3, 4, 5 and 10 mg/L, significant increase of toxicity (about 30%, 30%, 30%, 33% and 45%, respectively) respect lower (0, 0.36 and 1 mg/L; $p < 0.0001$) and higher (57.5, 115 and 230 mg/L; $p < 0.0001$) tested concentrations has been shown. At 57.5 and 115 mg/L, the toxicity (about 66%) was statistically significant compared to other used concentrations ($p < 0.0001$). At 230 mg/L, 100% of mortality was registered ($p < 0.0001$).

However, only FLT showed toxic effects already after 24 h of exposure (**Figure 20C**). An increase of the percentage of dead (about 60%) with respect to control was already recorded from 12.5, 25, 50 and 97.5 mg/L. The data reported at these concentrations were statistically significant compared to the four lowest (0, 0.41, 1 and 2.5 mg/L; $p < 0.0001$) and the two highest (195 and 390 mg/L; $p < 0.0001$) concentrations. At 195 and 390 mg/L, significant increase of toxicity (about 95 % and 100%, respectively) respect all others concentrations ($p < 0.0001$) has been shown. After 48 h, at 0.41 and 1 mg/L, a significant increase of toxicity (about 45%) has been displayed respect to 0 ($p < 0.0001$). However, the toxicity increases of about 66% (2.5 and 5 mg/L). These data were statistically significant respect to compared to the three lowest (0, 0.41 and 1 mg/L; $p < 0.0001$) and the highest (25, 50, 97.5, 195 and 390 mg/L; $p < 0.05$), where a percentage of about 100% was registered.

When considered BkF toxicity (**Figure 20D**), after 24 h, no effect has been recorded. Only after 48 h, at 3, 6, 12 and 23.5 mg/L, a significant increase in toxicity (about 25%) has been shown respect to three lowest concentrations (0, 0.025, 0.5 mg/L; $p < 0.01$) and the highest concentration (94 mg/L; $p < 0.01$), where a percentage of about 50% was registered.

3.3.4 Lethal concentrations after 24 h and 48 h of exposure

Considering nauplii exposure, the NAP solution has a LC50 value of 20.0 (1.4 - 40.8 mg/L) and 0.6 mg/L (0.2 – 1.3 mg/L) after 24 h and 48 h, respectively; PHE solution has a LC50 value of 5.3 (2.2 – 13.2 mg/L) and 3.8 mg/L (2.7 – 5.5 mg/L) after 24 h and 48 h, respectively; FLT solution has a LC50 value of 5.7 (3.3 – 54.3E+4 mg/L) and 0.1 mg/L (0.1 – 99.1 mg/L) after 24 h and 48h, respectively; and BkF solution has a LC50 value of 47.8 mg/L (2.7 – 50.6 mg/L) after 48 h (**Table 13**).

	Nauplii				**Adults**			
	24 h		**48 h**		**24 h**		**48 h**	
	LC50	**IC**	**LC50**	**IC**	**LC50**	**IC**	**LC50**	**IC**
NAP	20.0	1.4-40.8	0.6	0.2-1.3	3.5	0-∞	2.9	1.5-5.6
PHE	5.3	2.2-13.2	3.8	2.7-5.5	n.a.	n.a.	1.6	0-∞
FLT	5.7	3.3-54.3E+4	0.1	0.1-99.1	33.7	0.2-117.7	0.9	0.8-103.5
BkF	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	47.8	2.7-50.6

Table 13. LC50 and 95% confidence intervals calculated after 24 h and 48 h of naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT) and benzo(k)fluoranthene (BkF) exposure on both nauplii and adults. The data were expressed in mg/L. n.a.= not available.

When considered adults, the NAP solution has a LC50 value of 3.5 (0 - ∞) and 2.9 mg/L (1.5 – 5.6 mg/L) after 24 h and 48 h, respectively; PHE solution has a LC50 value of 1.6 mg/L (0 - ∞) after 48 h; FLT solution has a LC50 value of 33.7 mg/L (0.2 – 117.7 mg/L) and 0.9 mg/L (0.8 – 103.5 mg/L) after 24 h and 48 h, respectively; and BkF solution has a LC50 value of 47.8 mg/L (2.7 – 50.6 mg/L) after 48 h (**Table 13**).

3.3.5 Gene response to NAP, PHE, FLT and BkF exposure in nauplii and adults

Five genes were analysed for adults, and all were targeted by four PAHs with the exception of *hsp70, COXI* and *COXIII* (**Figure 21**; see also **Supplementary Table S16** for the values). In fact, *hsp70* was targeted only by PHE and FLT, whereas *COXI* and *COXIII* were not

targeted only by FLT.

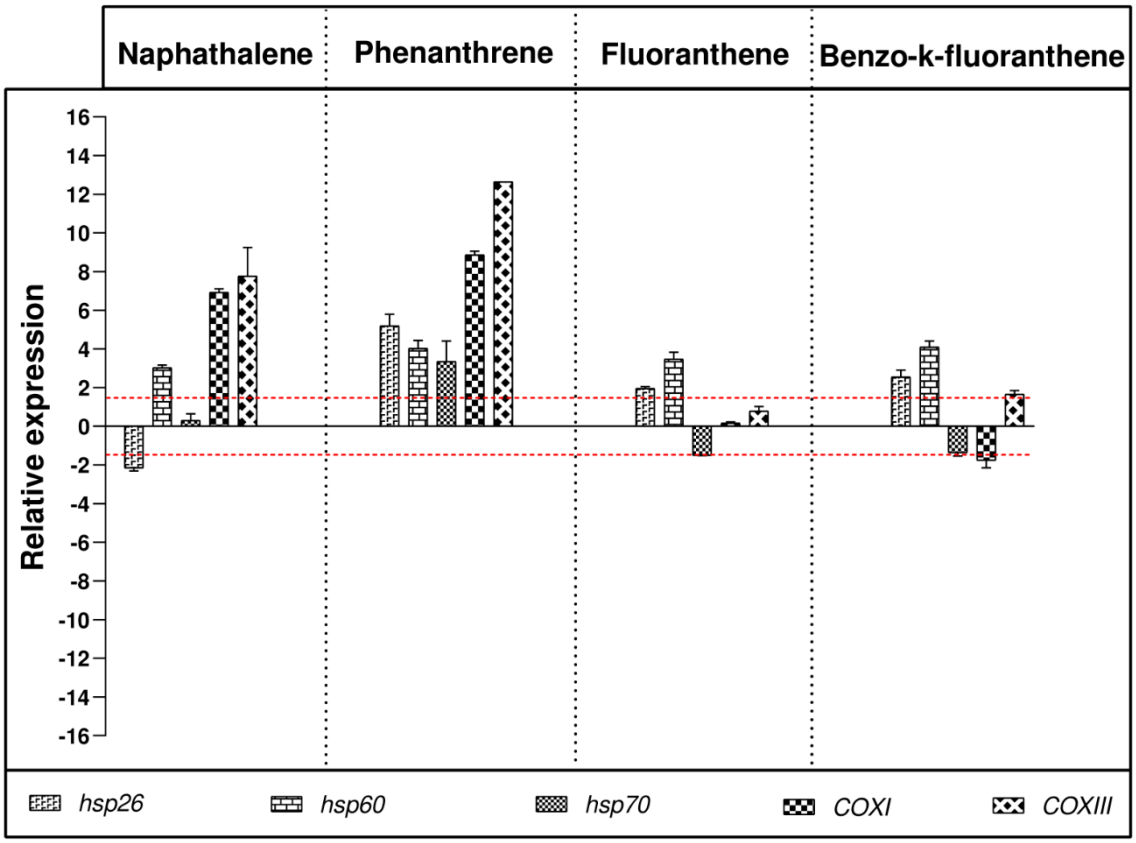


Figure 21. Histograms show the differences in expression levels of five genes involved in stress response. *A. franciscana* adults were exposed to naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene at 2.5 mg/L, 2 mg/L, 1 mg/L and 94 mg/L, respectively. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see **Supplementary Table S16** for the values).

Both contaminants, NAP, PHE and BkF, increased the expression levels of three genes (*hsp60, COXI* and *COXIII*). Moreover, treatment with NAP also down-regulated *hsp26*; the exposure to PHE up-regulated *hsp26* and *hsp70;* FLT is able to *hsp26* and *hsp60*, and down-regulated *hsp70*; whereas the exposure to BkF up-regulated *hsp26* and down-regulated *hsp70* (see also **Supplementary Table S16**)

As shown in **Figure 22**, among the nine genes analyzed, only one gene (*hsp70*) was not targeted by NAP, PHE and FLT. In fact, *hsp70* was target only of BkF. Common molecular targets for four contaminants were *HAD-like*, *tcp*, *UCP2*, *CDC48*, of which *UCP2* was only up-regulated by all treatment; whereas *tcp* and *CDC48* were up-regulated by NAP, PHE and

BkF, and down-regulated by FLT; and *HAD-like* was up-regulated by PHE, FLT and BkF, and down-regulated by NAP.

Moreover, *hsp60* was up-regulated by all PAHs with exception of NAP; *hsp26* resulted up-regulated and down-regulated only after PHE and NAP treatment, respectively; and *COX1* and *COXIII* were dawn-regulated by PHE and upregulated by BkF (see also **Table S17** for the values).

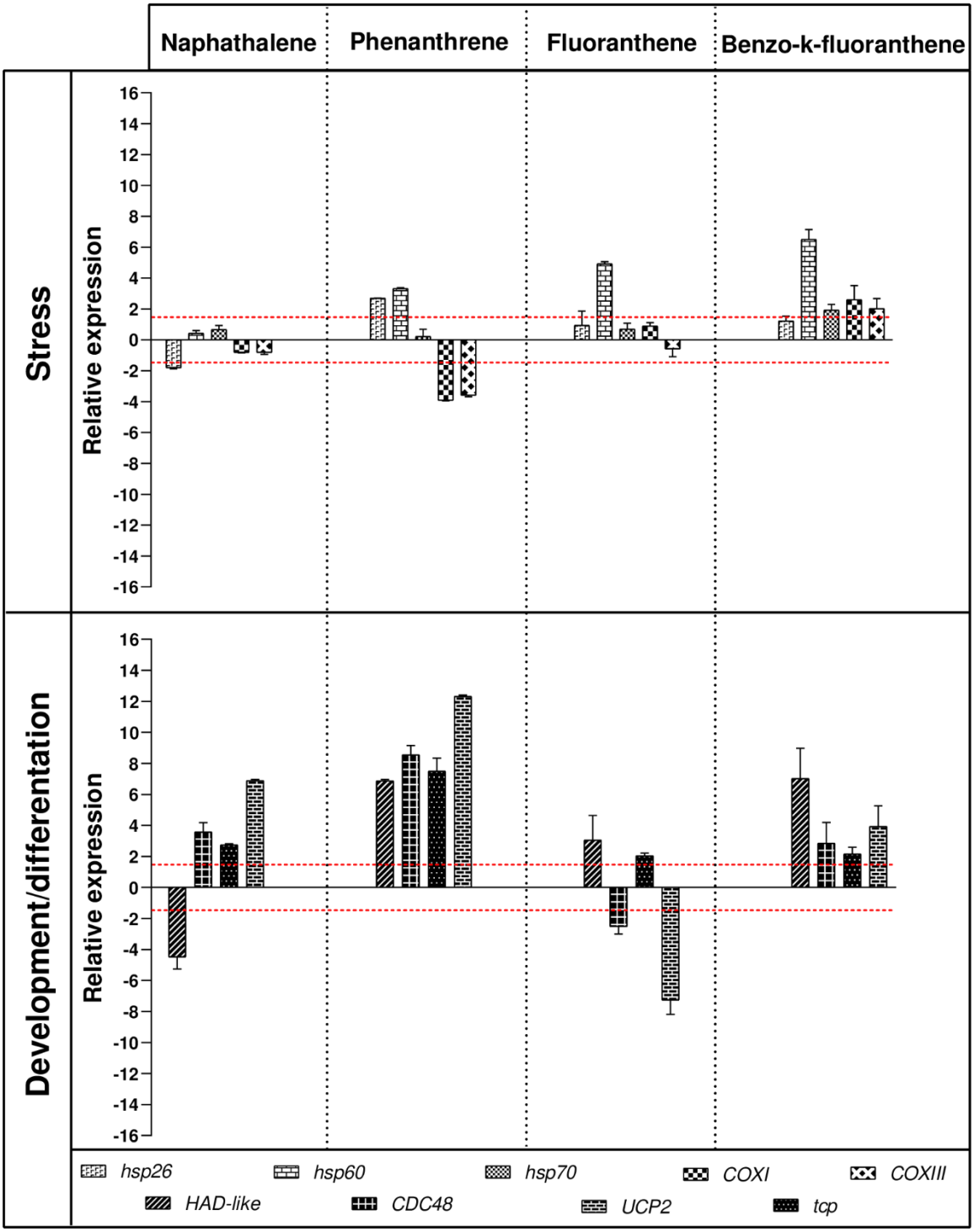


Figure 21. Histograms show the differences in expression levels of five genes involved in stress response and in development/differentiation. *A. franciscana* nauplii were exposed to naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene 0.4 mg/L, 2 mg/L, 1 mg/L and 94 mg/L respectively. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see **Supplementary Table S17** for the values).

3.4 Discussion

Experiment of PAHs showed toxic effects of single four pollutants (NAP, PHE, FLT and BkF). In fact, the NAP, PHE and FLT were able to induce an increase of nauplii death already

after 24 h of exposure, whereas the survival of *A. franciscana* was unaffected by exposure to BkF both after 24 h and 48 h. On the basis of lethal concentrations, the PHE (5.3 mg/L) and FLT (5.7 mg/L) appear to be more toxic than NAP (20.0 mg/L) to 24 h. Ha et al., (2019) showed the similar results in both *H. azteca* and *D. magna* embryos. In fact, exposing these two crustaceans to seven PAHs for 24 h, they revealed that PHE were seven and twenty times more toxic than NAP for *H. azteca* and *D. magna*, respectively. At the same manner, in a study conducted by Barata et al., (2005) the NAP and PHE resulted respectively the less and the most toxic among ten PAHs tested on copepods *Oithona davisae*. Moreover, Bellas et al. (2008), showed that FLT was one of the least toxic among the five tested PAHs (NAP, PHE, PYR, FLR and hydroxypyrene) both for *M. galloprovincialis* and *P. lividus*. Instead, after 48 h of exposure, the NAP (0.6 mg/L) and FLT (0.1 mg/L) showed a toxicity 6- and 38-times higher than this established for PHE (3.8 mg/L). In comparison with PHE results from this study, the 48-h LC50 was similar to that of *C. elegans* (4.7 mg/L), but lower than those of *D. magna* (0.8 mg/L), *Chironomus tentans* (0.4 mg/L) and *Eisenia fetida* (0.1 mg/L) reported in a previous study (Honda and Suzuki, 2020). When considered adults exposure, after 48 h the FLT and PHE showed higher toxicity than these of NAP and BkF. Our results suggest that there is a direct relationship between toxicity and aromatic ring number of the tested compounds. Millemann et al., (2014) showed the same relationship for the number of aromatic rings and their toxicity. In fact, they revealed that the PAHs with three or four aromatic rings was always more toxic than those with two aromatic rings for each of the nine exposed species (*Selenastrum capricornutum, Nitzschia palea, Physa gyrina, D. magna, Chironomus tentans, Gaintaurus minus, Pimephales promelas, Salmo gairdneri and Micropterus salmonid*).

Another interesting result is the large-scale genotoxicity information on *A. franciscana* generated in this study. In fact, the highest percentage of mortality in adults caused by exposure to four PAHs can be linked to the up-regulation of the majority of the studied genes.

Firstly, all 5 genes were molecular targets of four pollutants, with the only exception of *hsp70, COXI* and *COXIII* (**Figure 21**). Summarizing the *Real Time qPCR* experiments: i) two genes were targeted by all four pollutants; ii) two genes were only targets for NAP, PHE and BkF treatment; iii) only one gene was specifically affected only by PHE and FLT treatment. The molecular response to PHE appeared little different in comparison with other PAHs: PHE up-regulated all 5 genes (see also Appendix A, **Supplementary Table S16**) compared to NAP and BkF (3 up-regulated genes versus 1 down-regulated gene); and to FLT (2 up-regulated genes versus 1 down-regulated gene). In particular the three *heat shock proteins* and *COXs* were targeted by all PAHs (with exception of *hsp70* that were not targeted by NAP; and *COXs* that were not targeted by FLT) showing a strong condition of stress for these crustaceans. PHE caused high stress conditions in amphipods *Eulimnogammarus verrucosus, Eulimnogammarus vittatus, Eulimnogammarus cyaneus, Eulimnogammarus marituj Gmelinoides fasciatus* and Gammarus lacustris up- and down-regulating *hsp70* gene (Pavlichenko et al., 2015). After NAP exposure, small and insignificant changes in the *heat shock protein* gene levels have been reported also in the crustacean *Calanus finmarchicus* by Hansen et al., 2008 indicating a small degree of protein damage. A significant increase of *COXI* transcript level has been already reported in *Oncorhynchus mykiss* and in *Oryzias latipes* exposed to others PAHs (Barjhoux et al., 2014; Krasnov et al., 2005).

When considered the *Real Time qPCR* experiments on nauplii, all 9 genes were molecular targets of four pollutants, with the only exception of *hsp26, hsp60*, *hsp70, COXI* and *COXIII* (**Figure 22**). Summarizing: i) 4 genes were targeted by all PAHs; ii) only one gene was target for FLT, PHE and BkF treatment; iii) one gene was specifically affected only by PHE and NAP treatment; iv) and 2 genes were affected by PHE and BkF. These data suggest that the adults treated with NAP, PHE FLT and BkF were very similar, as also shown in the Heatmap reported in **Figure 23**.

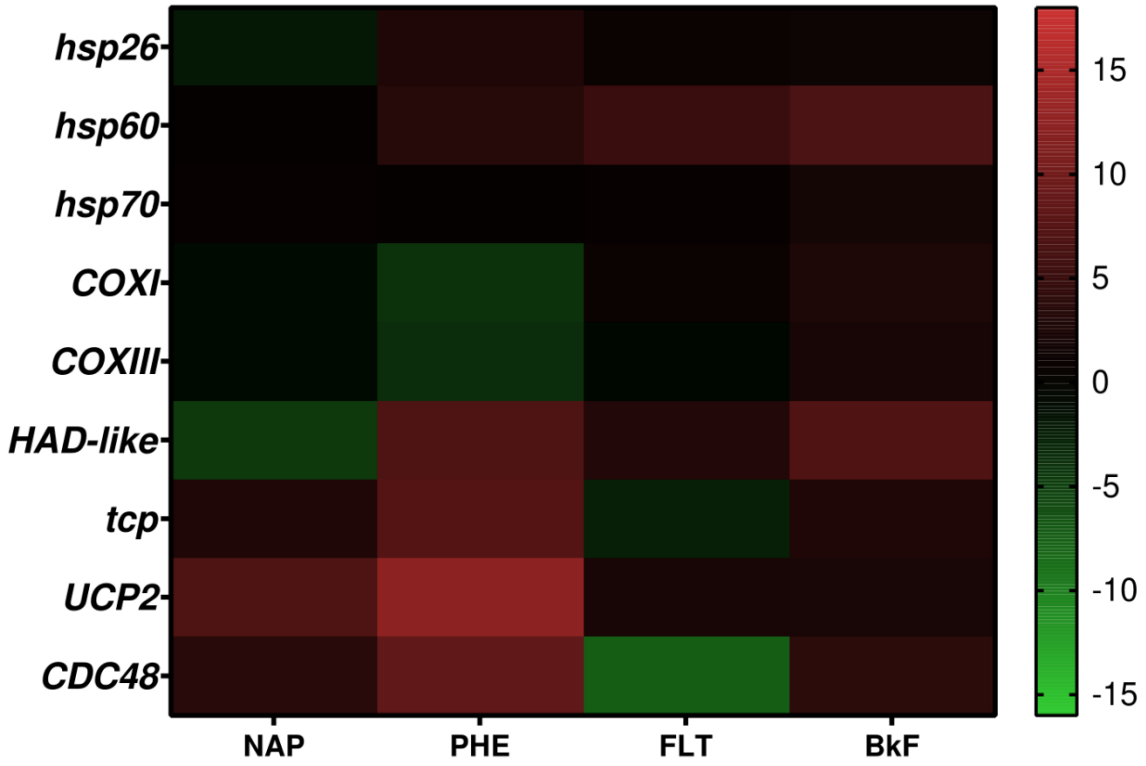


Figure 23. Heatmaps showing the expression profiles and hierarchical clustering of 9 genes analysed through *Real Time qPCR* in nauplii treated with naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT) and benzo(k)fluoranthene (BkF). Colour code: red, up-regulated genes with respect to the control; green, down-regulated genes with respect to the control; black, genes for which there was no variation in expression with respect to the control.

All together these molecular results revealed that the majority of affected genes in *A. franciscana* were involved in the development processes. In fact, all genes belonging to these classes were affected by the four toxicants. *HAD-like*, *tcp*, *UCP2* and *CDC48* in *A. franciscana* are involved into molecular mechanisms underlying post-diapause, common event in diverse taxa, from plants to animals (Chen et al., 2009; Wang et al., 2007).

In conclusion, this study is the first demonstration of the genotoxic effects of PAHs both on nauplii and ad adults of *A. franciscana*.

All the results reported in this Chapter have been published in:

1) **Albarano L.**, Serafini A., Castaldo F., Costantini M., Zupo V., Guida M., Libralato G. Genotoxicity in Artemia franciscana nauplii and adults: the case of phenanthrene, naphthalene, fluoranthene and benzo(k)fluoranthene (*submitted*). (Appendix B)

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4. Chapter 4: Comparison of *in situ* sediment remediation amendments: risk perspectives from species sensitivity distribution

4.1 Introduction

Sediment is a fundamental and integrated part of water bodies. It is composed of soluble and insoluble matter, which can be naturally transported from land to ocean, due to inland soil and coastal erosion and windblown dust (Brils, 2008). Pollution is the greater ecological issue due to various discharged toxic substances that can be accumulated in sediment acting as a source of contamination (Arizzi Novelli et al., 2006; G. Lofrano et al., 2016; Pougnet et al., 2014). Contaminated sediment can strongly impact on aquatic ecosystems, especially in presence of harbors and marinas, embayment and off coastal areas where commercial and industrial port activities, human settlements and tourism are increasingly widespread (Lofrano et al., 2016; Nikolaou et al., 2009). During dredging activities or natural resuspension phenomena (i.e. adverse weather conditions), accumulated contaminants could be released from sediment to the water column influencing the survival and fitness of aquatic biota and potentially human health (Arizzi Novelli et al., 2006; Mamindy-Pajany et al., 2010). Thus, the first step to deal with this issue must be the reduction/removal of toxic compounds considering both in situ and ex situ treatments using different chemical, thermal and biological methods (Gomes et al., 2013; G. Lofrano et al., 2016; Mueller et al., 1996). Nevertheless, these techniques for the remediation of polluted environments could negatively affect the resident biota, especially when applied in situ (Albarano et al., 2020; Libralato et al., 2018; Lofrano et al., 2016). Information about their (eco-)toxicity role impacting on aquatic environment are scarce, making difficult to choose the best potential technology for in situ remediation (Lofrano et al., 2016). The consequences of treatment activities on aquatic environment are generally considered as secondary effects (Libralato et al., 2008; Rakowska et al., 2012), while current literature still does not describe any potential undesired long-term effect, and to the best of our knowledge an overview about the different

sensitivity of aquatic species after their administration including laboratory and field scale applications is not currently available.

This review investigated the potential effects related to the use of amendments in sediment remediation considering the sensitivity of model species from both freshwater and saltwater environments. The species sensitivity distribution (SSD) approach was used to better understand the taxonomic differences in species sensitivity for each remediation method. For the first time, the SSD analysis was evaluated from an updated toxicity database and shown as a cumulative probability distribution for multiple species. SSD curve describes the variation in sensitivity among a set of species toward a contaminant or mixture of contaminants by a statistical or empirical distribution function (Posthuma et al., 2002) . The use of this method was proposed for the first time by (Kooijman, 1987) and later enhanced by further studies (Aldenberg and Slob, 1993; Aldenberg and Jaworska, 2000; Newman et al., 2000; Posthuma et al., 2002; Wagner and Løkke, 1991). Generally, SSDs are generated from laboratory-derived toxicity data offering protection for a wider range of organisms in the field (Hose and Van Den Brink, 2004). The aim of SSDs is to calculate the toxicant concentration affecting a specific number of species usually identified as the hazardous concentration (HC) impairing the 5% (HC5) of organisms, thus the protective concentration (PC) for the 95% of species (PC95) can be calculated as well (Posthuma et al., 2002; Newman et al., 2000). Amendments were selected from previous review papers (Libralato et al., 2018; Lofrano et al., 2018) and were namely activated carbon (AC), nano Zero Valent Iron (nZVI), organoclay (OC), apatite (A) and zeolite (Z).

4.2 Toxicity data identification, collection and management

Toxicity data about AC, nZVI, OC, A and Z were collected from various sources: Google scholar, National Center for Biotechnology Information (NCBI), and Scopus (last update July 30, 2020). We identified and reviewed 56 papers, but only 39 were selected concerning freshwater and saltwater sediment up to the end of July 2020. The other 17 were eliminated

for two main reasons: either i) the tested species were of non-aquatic origin; or ii) the amendments didn`t show toxicity on aquatic species concerned **Table S18**.

Among the investigated papers 17 (43%) focused on freshwater species, 3 (9%) both freshwater and saltwater environment and approximately 48% only seawater species. Literature was reviewed in order to extract several information as summarized in **Table 14** including: taxonomy, endpoints (i.e. mortality (M), reproduction (R), growth inhibition (IG), biota-sediment accumulation factor (BSAF) and bioaccumulation reduction (BR)), exposure time, concentrations, effects of amendments (direct contact) or elutriates (%) and experimental parameters (i.e. temperature (T), pH and salinity (S)). Units of measures for all amendments (i.e. AC -% sediment dry weight; nZVI -mg/L; OC, A, and Z – g/L) were changed in g/L For AC, a density of 480 g/L activated carbon was considered (ASTM D2854-89).

1	2	3	4	5	6	7	8	9
AC	Bacteria	*Escherichia coli*	0.5*	4.8	M	79.5	pH = 6.8	(Van Der Mei et al., 2008)
		Raoultella terrigena	0.5*	4.8	M	65.5	pH = 6.9	(Van der Mei et al., 2008)
	Annelids	*Nereis diversicolor*	28	9.6	BSAF	2	T = 22	(Cornelissen et al., 2006)
		Limnodrilus spp	28	7.2	BR	94	T = 20	(Jonker et al., 2004)
		Neanthes arenaceodentata	28	16.3	BR and IG	50 and 73	T = 20	(Millward et al., 2005)
		Arenicola marina	10	36	M	70	T = 20, pH = 6.15 - 8.61	(Lillicrap et al., 2015)
	Molluscs	*Macoma balthica*	28	16.3	BR	76	T = 13	(McLeod et al., 2007)
		Lymnaea stagnalis	41	8.2	BR	37.3	pH = 5.6 - 6.5, RT, DOC	(Lewis et al., 2016)

							= 35-40	
		Corbicula fluminea	28	12	BR	95	T = 13	(Mcleod et al., 2008)
		Potamopyrgus antipodarum	28	9.6	R	9.7	T = 16, pH = 8.0, DOC = 9.4, COD = 26.5, TAN = 5.35	(Stalter et al., 2010)
		Meretrix meretrix	28	24	IG	36.4	T = 20, pH = 8.05	(Zheng et al., 2018)
	Crustaceans	*Daphnia Magna*	4	19.2	BR	4	T = 20	(Jonker et al., 2009)
		Asellus aquaticus	28	19.2	IG	36	T = 20	(Kupryianchyk et al., 2013)
		Corophium volutator	28	19.2	IG	50	T = 20	(Jonker et al., 2009)
		Leptocheirus plumulosus	10*	16.3	BR	70	T = 20	(Millward et al., 2005)
		Tisbe battagliai	72*	36	M	60	T = 20, pH = 6.15 - 8.61	(Lillicrap et al., 2015)
	Fishes	*Pimephales promelas*	10	24	M	43.8	T = 25, pH = 8.2	(He et al., 2012)
		Dicentrarchus labrax	28	4.8	M	76.4	T = 21.8, pH = 8.0 S = 37, TAN = 0.18	(Aly et al., 2016)
		Gambusia affinis	10	36	IG	62.2	T = 20	(Casini et al., 2006)
nZVI	Bacteria	*Escherichia coli*	0.5*	0.008	M	70	T = 32,	(Lee et al., 2008)

		1*			80	pH = 7.2 T = 30, pH = 5 - 7.4	(Auffan et al., 2008)
	Bacillus subtilis var. niger	5+	0.0002	M	20	T = 20, pH = 9	(Diao and Yao, 2009)
	Pseudomonas fluorescens	5+	0.0002	M	100		
	Vibrio fischeri	2	0.0002	IG	87.2	T = 20, pH = 5 - 9, DOC = 0.01	(Qiu et al., 2013)
	Dehalococcoides	2	0.5	IG	98.2	T = 22, pH = 8.1	(Xiu et al., 2010)
	Microcystis aeruginosa	30	0.5	IG	92	T = 30, TOC = 18.75, TAN = 1.73	(Su et al., 2018)
Algae	*Isochrysis galbana*	4	0.0002	IG	50 and 60		
	Dunaliella tertiolecta	4	0.0013	IG	53	pH = 7.5, RT	(Keller et al., 2012; Othman, 2018)
	Thalassiosira pseudonana	4	0.0004	IG	51		
	Pseudokirchneriella subcapitata	4	0.008	IG	47		
Molluscs	*Lymnaea stagnalis*	41	0.0002	R	59.4	pH = 5.6 - 6.5, DOC = 35-40	(Lewis et al., 2016)
	Mytilus galloprovincialis	28	0.05	IG	14	T = 18, pH = 8.0, S = 30	(Coppola et al., 2019)

		Mytilus galloprovincialis	2	0.008	IG	60	T = 16, pH = 6 - 7, DOC = 0.08	(Kadar et al., 2010)
	Crustaceans	*Daphnia Magna*	28	0.0004	M, IG and R	60, 58 and n.e.	RT	(Jaafar and Yasid, 2018; Keller et al., 2012)
	Fishes	*Oryzias latipes*	10	0.0002	IG	30	T = 25, pH = 7 - 7.6, H = 200	(Li et al., 2009)
				0.05	M	90	T = 26, pH = 7	(Chen et al., 2011)
A, Z and OC	Bacteria	*Escherichia coli*	0.5*	0.004	M and IG	52.9 and 60	T = 37, pH = 7.2	(Rieger et al., 2016)
	Crustaceans	*Hyalella azteca*		0.1	M	67.5	T = 25	(Paller and Knox, 2010)
		Leptocheirus plumulosus		0.1	M	21.3		
		Americamysis bahia	2	0.5	M	47	T = 15	(Burgess et al., 2004)
		Ampelisca abdita	2	0.5	M	48		
		Paranephrops planifrons	10	350	IG	33.3	T = 15, pH = 7, TAN = 2	(Parkyn et al., 2011)
	Fishes	*Danio rerio*	3	0.0001	IG and M	96.5 and 66	pH = 7, RT	(Palčić et al., 2020)
		Oncorhynchus mykiss	0.5	1	BR	86.6	T = 14, pH = 8	(Alak et al., 2016)
			28	0.1	M	83.3	T = 14, pH = 7.4	(Ucar et al., 2017)
		Dicentrarchus labrax	28	0.1	IG	42.2	T = 21.8, pH = 8.0	(Aly et al., 2016)

					S = 37, TAN = 0.18	
Gambusia affinis	10	0.005	M	42.8	T = 20	(Casini et al., 2006)
Oreochromis mossambicus	28	2	BR	41.7	T = 29, pH = 7.8, H = 58	(James et al., 2000)
Oryzias latipes	10	0.005	IG	30	T = 26, pH = 7	(Chen et al., 2011)

Table 14. Sediment treatment (1), group of organisms (2), species (3), time of exposure (days; *h; +min) (4), concentrations (g/L) (5), endpoints (6), effects (%) (7), water quality parameters (8) and references (9) of negative impact of contaminated sediment restoring. Abbreviations: M = Mortality, BSAF = biota-sediment accumulation factor, BR = Bioaccumulation reduction, IG = inhibition growt and R= reproduction, n.e. = not effect, T = Temperature (°C), S = Salinity (ppt), DOC = dissolved organic carbon (mg/L), COD = chemical oxygen demand (mg/L), TOC = total organic carbon (mg/L), TAN = total ammonia nitrogen (mg/L), H = hardness (mg/L), n.a. = not available

According to **Table 14**, we detected nine concentrations (4.8, 7.2, 8.2, 9.6, 12.0, 16.3, 19.2, 24.0 and 36.0 g/L) for AC, six (0.0002, 0.0004, 0.0013, 0.008, 0.050 and 0.5 g/L) for nZVI and eight (0.0001, 0.004, 0.005, 0.1, 0.5, 1, 2 and 350 g/L) for OC, A and Z.

Focusing on toxic effects of AC, five taxonomic groups have been tested, for a total of 19 species. Specifically, Bacteria, Annelids, Crustaceans, Molluscs and Fishes were represented with 2, 4, 5, 5 and 3 species, respectively. About nZVI, tested species were 15 including five taxonomic groups (Bacteria, n = 6); Algae, n = 4), Molluscs (n = 3), Crustaceans (n = 1) and Fishes (n = 1)). About OC, A and Z, we identified twelve testing species belonging to three taxonomic groups (Bacteria (n = 1), Crustaceans (n = 5) and Fishes (n = 6)). Toxicity data for AC, nZVI, OC, A and Z were comparable in size and more than 50% of organisms were from Molluscs and Crustaceans for AC, Bacteria and Algae for nZVI, Crustaceans and Fishes for OC, A and Z.

Besides the values to generate SSD curves, data about temperature (T), pH, salinity (S), total organic carbon (TOC), dissolved organic carbon (DOC), chemical oxygen demand (COD), total ammonia nitrogen (TAN) and hardness (H) were collected. Their amount was highly

insufficient to go further with data analysis like quantitative structure activity relationship or character-activity relationships (Xu et al., 2017; Zhao and Liu, 2018). AC tests were mainly carried out at room temperature (62.5%) with pH value ranging from 5.6 to 8.6. nZVI experiments were performed between 16 °C - 25 °C and pH 5-9. OC, A and Z toxicity were analyzed at increasing temperatures (15 °C - 37 °C) and with pH of 7-8.

4.2.1 Data organization

When more than one toxicity data was registered for the same species (*N. arenaceodentata*, *I. galbana, D. magna*, *E. coli* and *D. rerio*), the geometric mean was calculated and used as the estimate for this species (Kooijman, 1987). Compiled data were elaborated considering two approaches (**Figure 24**): i) only raw data (method 1); and ii) predicted data (method 2).

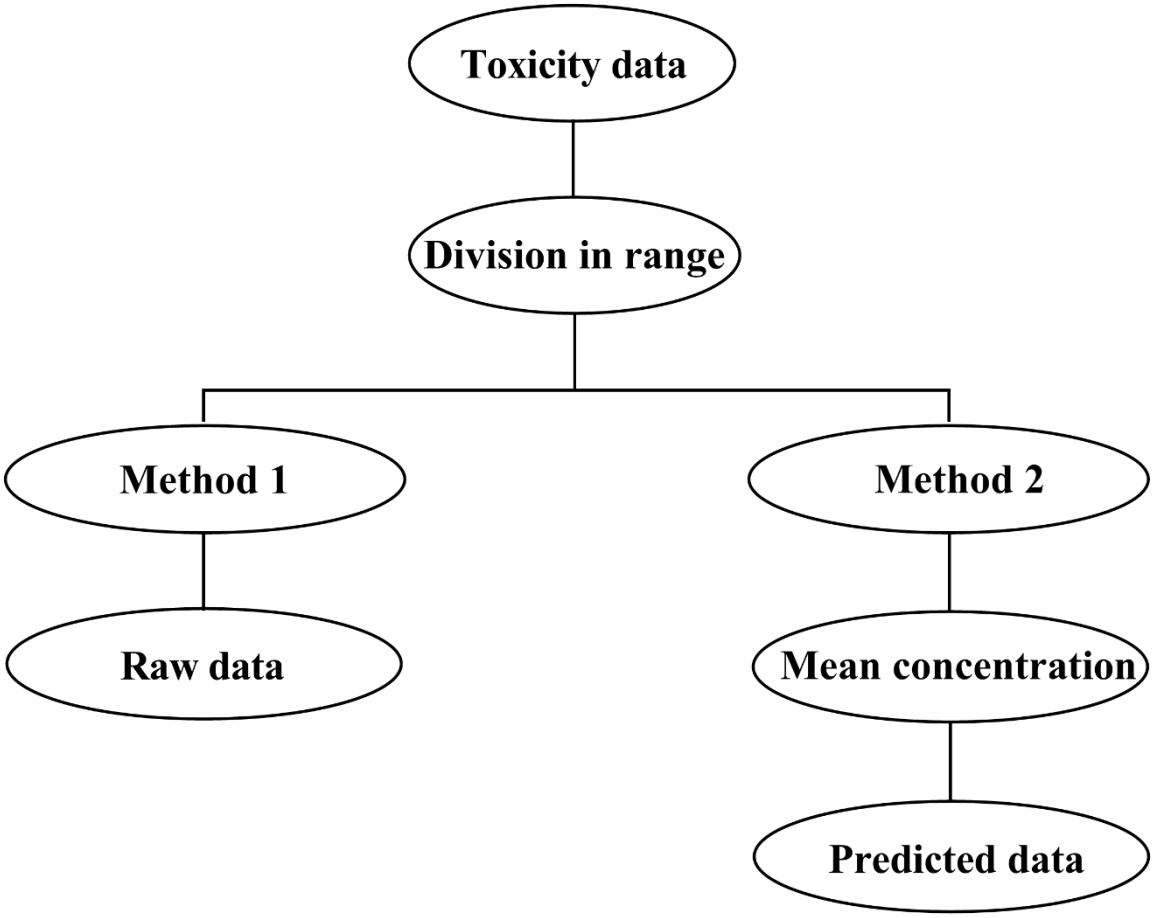

Figure 24. Flowchart of data-processing

About method 1, toxicity data have been shown as raw data without any further processing.

About method 2, average concentrations have been established for each amendment: To improve data presentation, administered concentrations and the respective effects have been divided by range. Reviewed toxicity data have been organized as reported in **Table 15**:

	Concentrations (g/l)	**Concentration range (g/l)**			**Whole range (g/l)**
AC	4.8, 7.2, 8.2, 9.6, 12.0, 16.3, 19.2, 24.0 and 36.0	4.8 - 9.6	12.0 - 16.3	19.2 - 36.0	4.8 - 36.0
nZVI	0.0002, 0.0004, 0.0013, 0.008, 0.050 and 0.5	0.0002 - 0.0013	0.008 - 0.5		0.0002 - 0.5
OC, A and Z	0.0001, 0.004, 0.005, 0.1, 0.5, 1, 2 and 350	0.0001 - 0.1	0.5 - 350		0.0001 - 350

Table 15. Concentrations, concentration range and whole range of three different remediation methods.

i) the nine concentrations of AC have been divided and shown into four range considering also the entire selection (4.8 – 36.0 g/L, Table S2); ii) the values for nZVI collected at six concentrations have been analyzed in the three-range given also the whole range (0.0002 – 0.5 g/L); and iii) finally, also the findings obtained at eight levels of OC, A and Z have been developed in three range taking always into consideration the full variety of values (0.0001-350 g/L, see also **Table 15**). – For each concentration range of AC, equal number of mean concentrations have been established (7.2 g/L, 13.4 g/L, 26.4 g/L and 14.9 g/L). For three selection of nZVI, OC, A and Z same number of average concentrations have been calculated, namely as 0.0006 g/L, 0.186 g/L and 0.0933 g/L for nZVI and 0.03 g/L, 88.4 g/L and 44.2 g/L for OC, A and Z.

The RD and PD were used as the effect metrics adapted to SSD (Van Vlaardingen and Verbruggen, 2007) and the species sensitivity was measured accordingly.

4.2.2 Data treatment and statistical analysis

The toxicity values were log-transformed according to (Burmaster and Hull, 1997; Leo Posthuma, Glenn W. Suter II, 2002; Newman et al., 2000) using Equation 1:

$$\chi = log_{10}(RD \text{ or } PD) \qquad \textit{Equation 1}$$

The associated risk was visualized as cumulative distribution function as defined in

Equation $2y = \sum_{i=1}^{\kappa} n_i$ *Equation 2*

where *y* is the cumulative probability of species and n_i is the absolute frequency of single RD or PD value.

The distribution model was fitted to toxicity data points and evaluated using the χ^2 goodness of fit with the adjusted coefficient of determination R^2 (Adj-R^2). The median hazard concentration (HC50) and the HC affecting the 5% of species were calculated according to Aldenberg and Slob 1993, using Equation 3:

$Log\ (HCp) = \mu - \mathcal{K}p * \sigma$ *Equation 3*

where *HC*p is hazardous concentration for percentage of the species population, $\mathcal{K}$p is Aldenberg extrapolation factor that directly depends of the number of the studied species, μ and σ are the mean and the standard deviation of distribution, respectively.

Data were analysed by Shapiro-Wilk's (S-W) test for normality and F-test for homoscedasticity (p-value <0.05). For each amendment, statistical significance between different groups of organisms was performed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons ($p < 0.05$). Two fixed factors (groups of organisms *vs* remediation methods) were crossed by a two-way ANOVA. All statistical analyses were performed using GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, La Jolla, California, USA, www.gra phpad.com).

4.3 Results and Discussion

As reported in **Table 16**, RD and PD are normally distributed for AC, nZVI, OC, A, and Z.

Sediment treatment	Concentrations (g/L)	Adj-R2	S-W	*F*	*p*
AC	4.8 - 9.6	0.98	0.27	0.05	<0.05
	7.2	0.93	0.20	0.37	<0.05
	12.0 - 16.3	0.97	0.07	0.99	<0.05
	13.4	0.96	0.64	0.12	<0.05
	19.2 - 36	0.93	0.50	0.11	<0.05
	26.4	0.97	0.17	1.00	<0.05
	4.8 - 36.0 (WR)	0.97	>0.9999	0.11	<0.05

	14.9 (MC)	0.97	0.12	0.06	<0.05
nZVI	0.0002 - 0.0013	0.95	0.20	0.86	<0.05
	0.0006	0.90	0.49	0.16	<0.05
	0.008 - 0.5	0.92	0.05	1.00	<0.05
	0.186	0.79	0.08	0.83	<0.05
	0.0002 - 0.5 (WR)	0.93	0.34	0.98	<0.05
	93.3 (MC)	0.96	0.18	0.49	<0.05
OC, A and Z	0.0001 - 0.1	0.97	0.69	0.21	<0.05
	0.03	0.95	0.57	0.47	<0.05
	0.5 - 350	0.94	0.16	0.13	<0.05
	88.4	0.93	0.35	0.17	<0.05
	0.0001 - 350 (WR)	0.97	0.14	0.26	<0.05
	44.2 (MC)	0.97	0.08	0.13	<0.05

Table 16. Goodness-fit, Shapiro-Wilk's (S-W) test for normality and F-test for homoscedasticity of total species for three remediation methods. p is the significance level. WR = whole range, MC = mean concentration.

The value of RD and PD show variance homogeneity (homoscedasticity) for AC (p values = 0.05- 0.99 and 0.06-1.00, respectively), for nZVI (p values = 0.86-1.00 and 0.16-0.83, respectively) and for OC, A and Z (p values = 0.13-0.26 and 0.13-0.47). The results indicated that the lognormal distribution fits with most of the groups data points, with Adj-R^2 ranging from 0.79 to 0.98 (p-value <0.01) (as shown in **Table 16**).

4.3.1 The SSDs of AC

Taking into consideration both the entire selection (4.8 – 36.0 g/L, **Figure 25**) and its mean concentration (14.9 g/L, **Figure 26**), Bacteria were largely susceptible than other organisms. Specifically, at the 14.9 g/L concentration (**Figure 26**), Bacteria group has shown a significant increase of sensitivity to AC respect to Annelids ($p < 0.05$), Molluscs ($p < 0.05$) and Crustaceans ($p < 0.01$) (see also **Table S19**). Crustaceans, in particular *D. magna*, exhibited the lowest susceptibility.

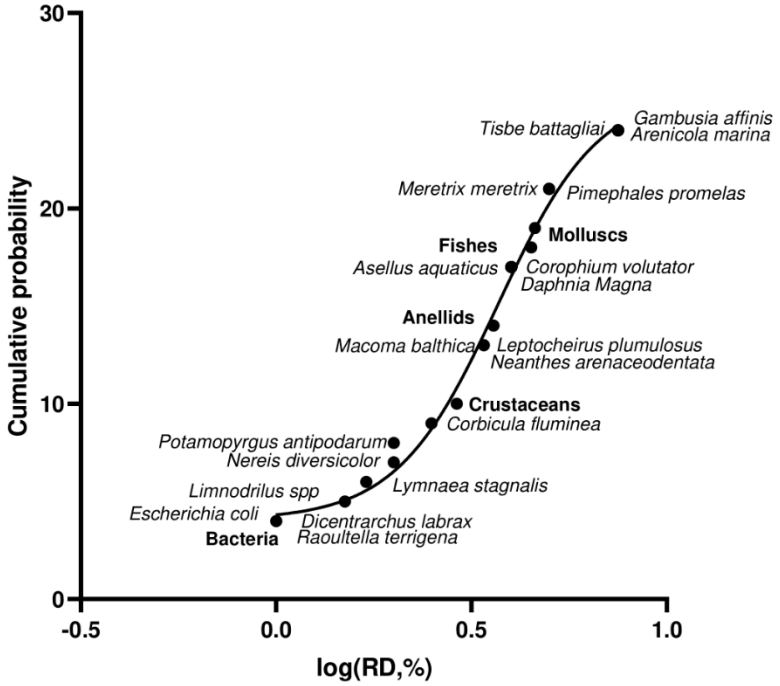

Figure 25. Species sensitivity distribution of different groups species to AC. The data are represented as raw data (RD) collected from 4.8 – 36.0 g/l.

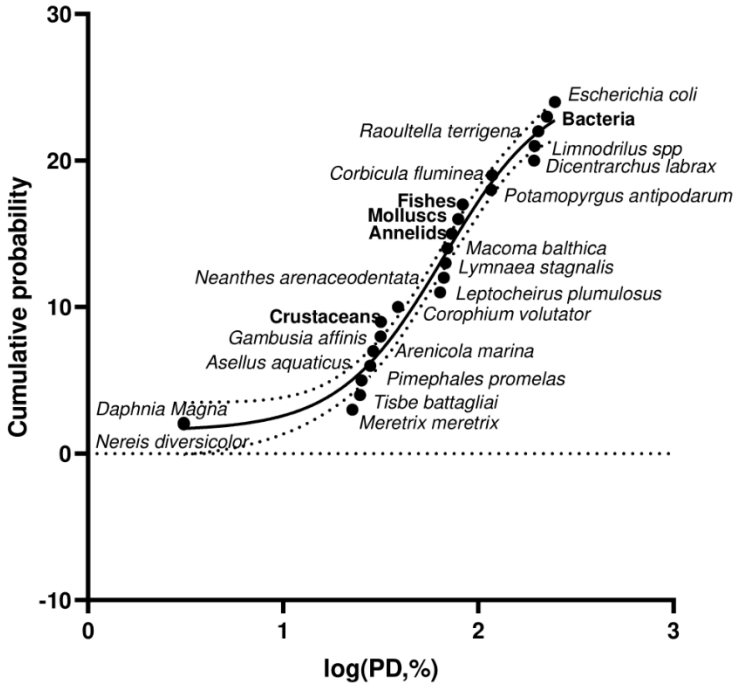

Figure 26. Species sensitivity distribution of different groups species to AC. The data are represented as predicted data (PD) collected from 14.8 g/l.

Considering the range 4.8 – 9.6 g/L (**Figure 27**), Bacteria, especially with *E. coli,* the Annelid *Limnodrillus spp.* and the Mollusc *C. fulminea* were the most sensitive species.

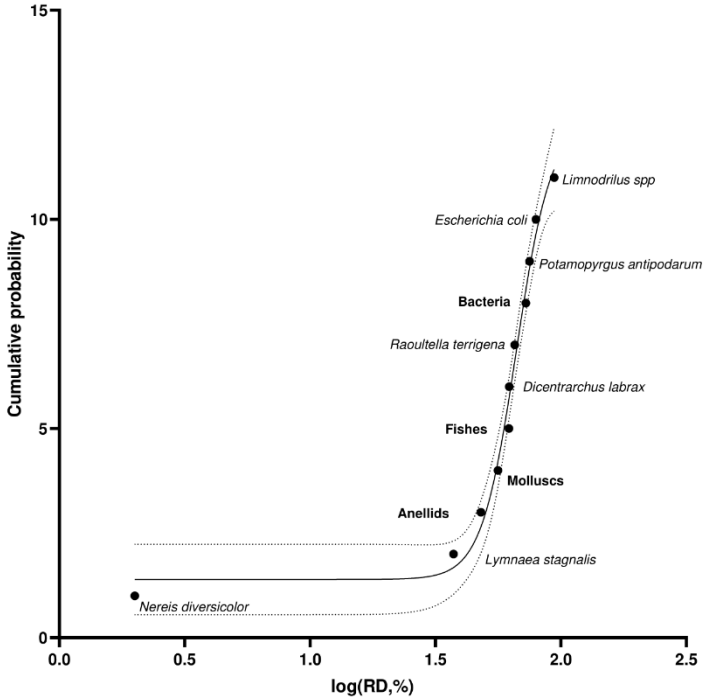

Figure 27. Species sensitivity distribution of different groups species to AC. The data are represented as raw data (RD) collected from 4.8 – 9.6 g/l.

Furthermore, regarding the respective mean concentrations (7.2 g/L, **Figure 28**) Bacteria, with *E. coli* and *R. terrigena*, showed the highest significant sensitivity to AC respect to Molluscs ($p < 0.05$) (**Table S19**).

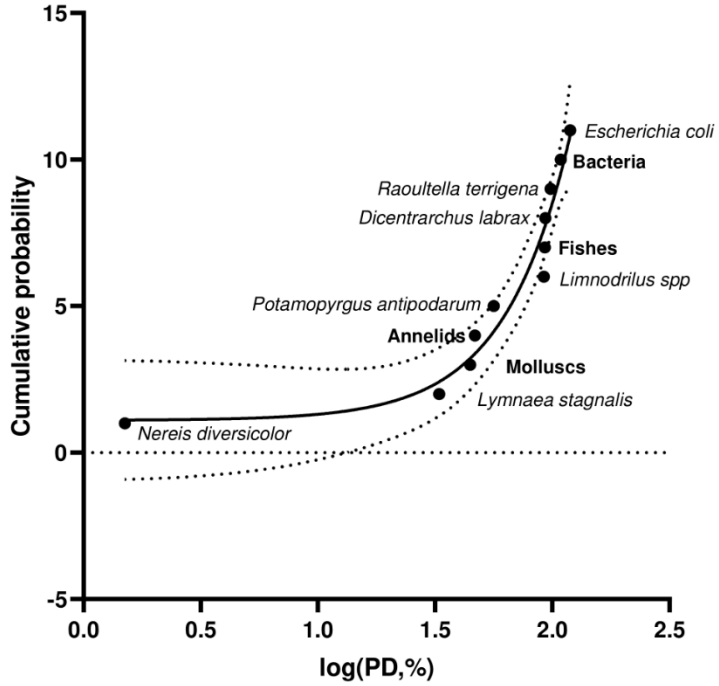

Figure 28. Species sensitivity distribution of different groups species to AC. The data are represented as predicted data (PD) collected from 7.2 g/l.

Given the range 12.0 – 16.3 g/L (**Figura 29**), Annelids group, as reported in **Table S19***,* has displayed a significant sensitivity respect to Molluscs ($p < 0.05$), that in turn were statistically significant respect to Crustaceans ($p < 0.01$).

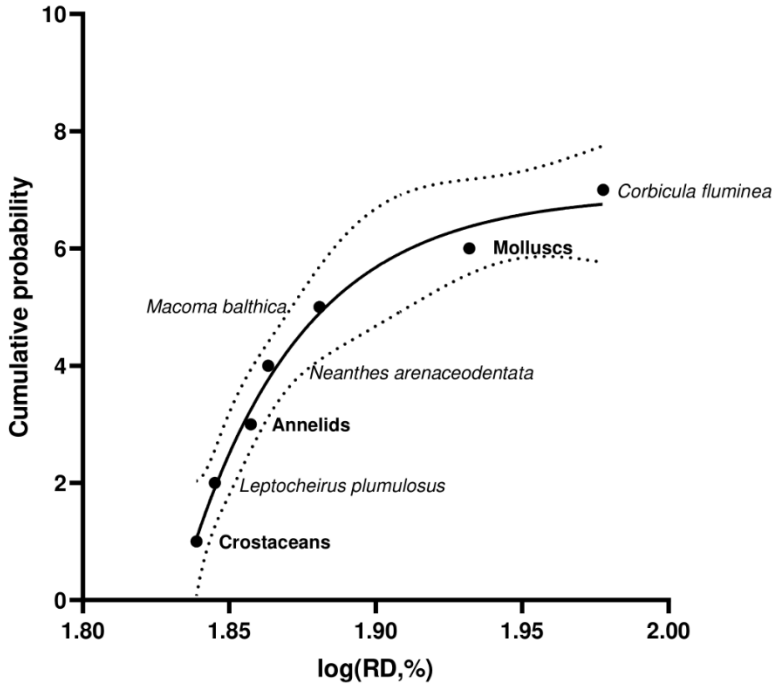

Figure 29. Species sensitivity distribution of different groups species to AC. The data are represented as raw data (RD) collected from 12.0 – 16.3 g/l.

However, when the 13.4 g/L mean concentration has been viewed, Molluscs group were the highest susceptible respect to other groups of species, but not statistically significant (**Figure 30**).

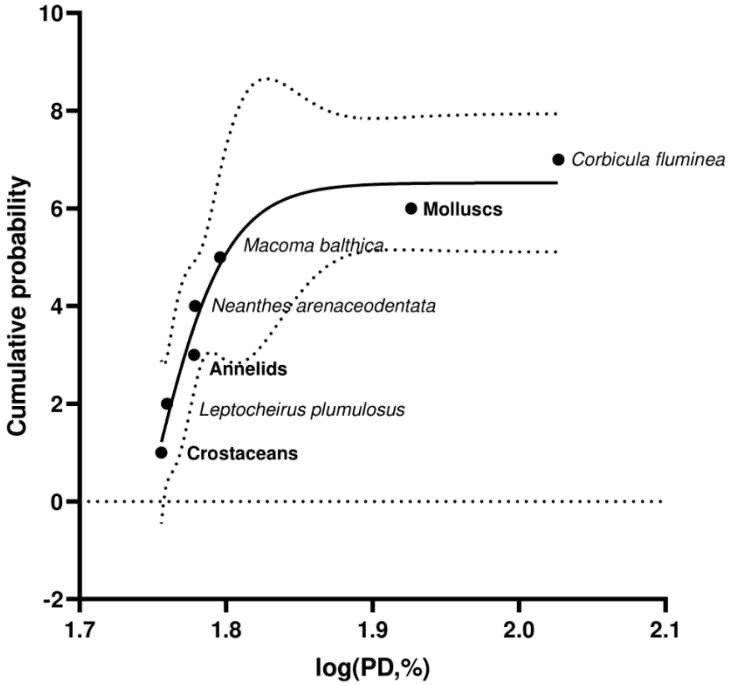

Figure 30. Species sensitivity distribution of different groups species to AC. The data are represented as predicted data (PD) collected from 13.4 g/l.

Analyzing both the range 19.2 – 36.0 g/L (**Figure 31**) and its respective mean concentrations (26.4 g/L, **Figure 32**) the Fish *G. affinis* has shown a high sensitivity despite the p-values were greater than 0.05.

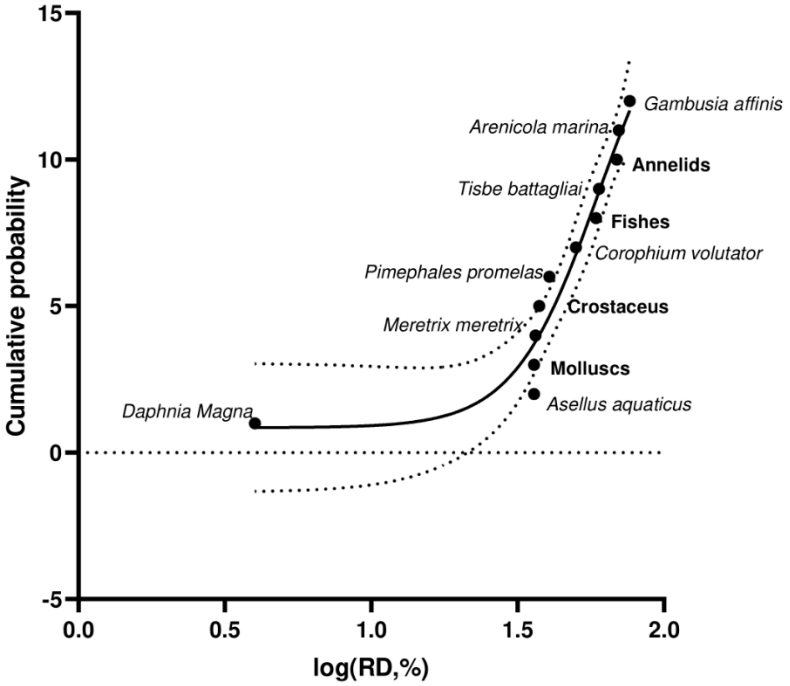

Figure 31. Species sensitivity distribution of different groups species to AC. The data are represented as raw data (RD) collected from 19.2 – 36.0 g/l.

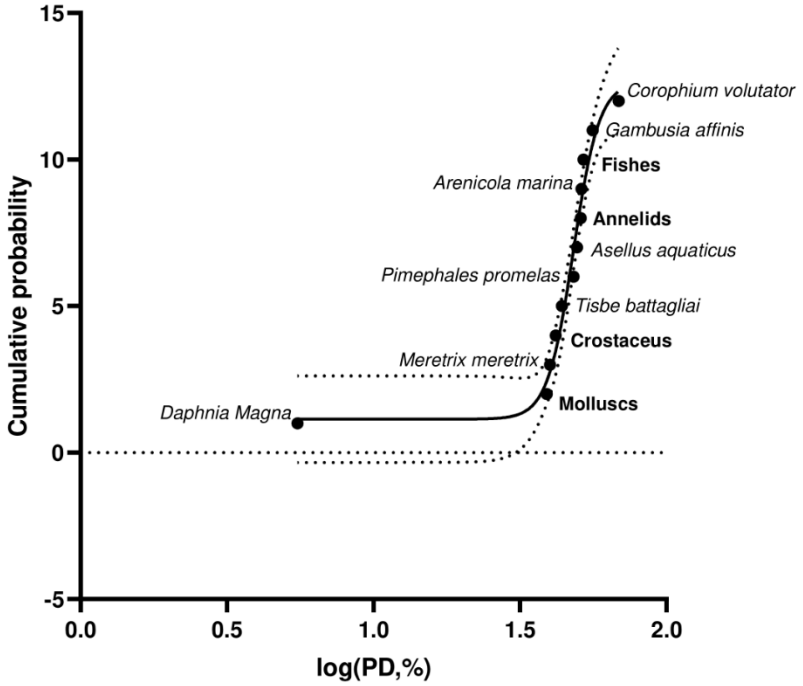

Figure 32. Species sensitivity distribution of different groups species to AC. The data are represented as predicted data (PD) collected from 32 g/l.

As displayed in **Figure 25**, some species within the same taxonomic group responded differently showing a variable sensitivity. The toxicity data of AC showed that the polychaetae *N. diversicolor* and *Limnodrillus spp.* were the least and most sensitive species among the Annelids, respectively. In particular, cumulative probability of *Limnodrillus spp.* exceeded 90% in SSD curves, whereas that of *N. diversicolor* resulted to be of 2%. Among Molluscs group, *M. meretrix* and *L. stagnalis* were the least sensitive species (with a cumulative probability of about ~37%), whereas *C. fluminea* with a cumulative probability of 95% resulted the most sensitive species. Furthermore, *D. magna* and *T. battagliai* were respectively the least and most sensitive species among the Crustaceans group. Specifically, cumulative probability of *T. battagliai* was 60% in SSD curves, whereas that of *D. magna* resulted to be of 4%. Probably, considering that the diameter of the feeding chaetoceros was generally 7 – 9 mm, this substantial variability was due to possible differences in digestive biology of species (Cornelissen et al., 2006; Jonker et al., 2009, 2004; Millward et al., 2005; Zheng et al., 2018).

4.3.2 The SSDs of nZVI

Considering the raw data, also for the nZVI methods the Bacteria, specifically *P. fluorescens*, were largely susceptible than others organisms **(Figures 33-34)**.

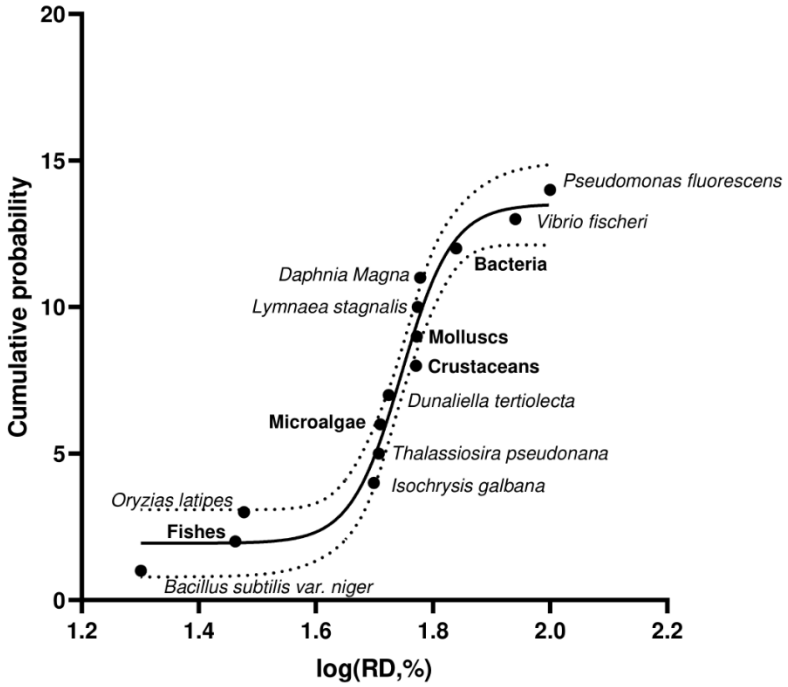

Figure 33. Species sensitivity distribution of different groups species to nZVI. The data are represented as raw data (RD) collected from 0.0002 – 0.0013 g/l.

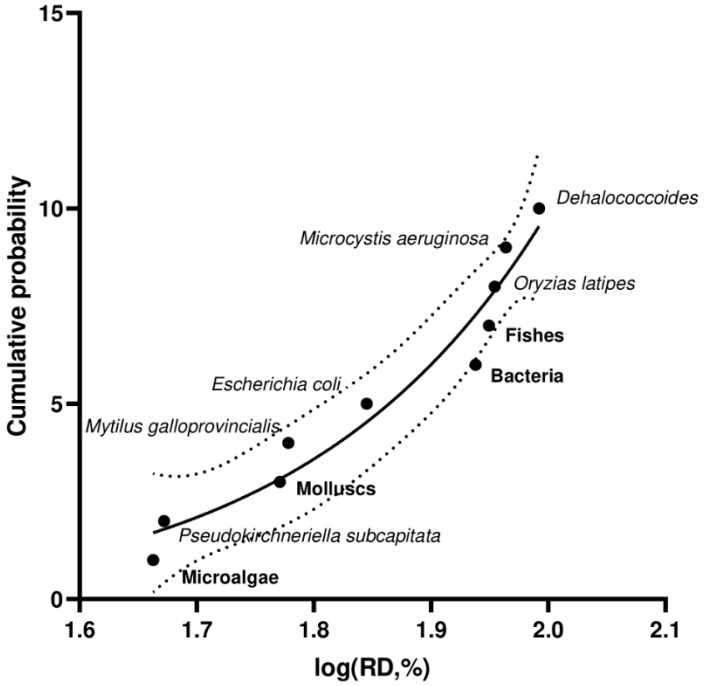

Figure 34. Species sensitivity distribution of different groups species to nZVI. The data are represented as raw data (RD) collected from 0.008 – 0.5 g/l.

Given the whole range (0.0002 – 0.5 g/L, **Figure 35**), Bacteria, specifically *P. fluorescens* and *V. fischeri*, were statistically significant compared to Algae ($p<0.0001$), Custaceans ($p < 0.001$), Molluscs ($p < 0.001$) and Fishes ($p < 0.001$) (**Table S20**).

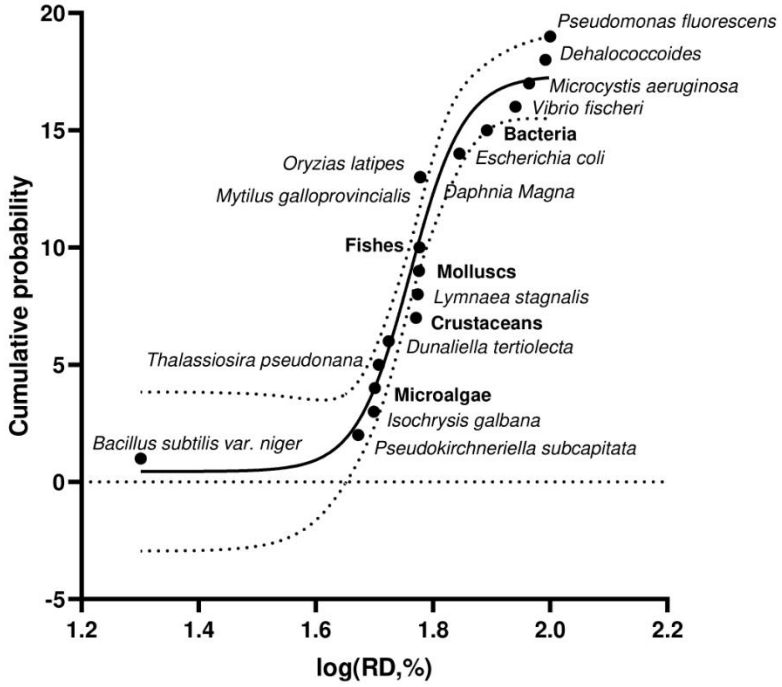

Figure 35. Species sensitivity distribution of different groups species to nZVI. The data are represented as raw data (RD) collected from 0.0002 – 0.5 g/l.

When taking into consideration the predicted data (**Figures 36-38**), Bacteria, with *E. coli*, *P. fluorescens* and *V. fischeri,* and the Molluscs, in particular *L. stagnalis* species, were among the most affected species despite the p-values were > 0.05; whereas the Fishes and Algae were the less impacted species.

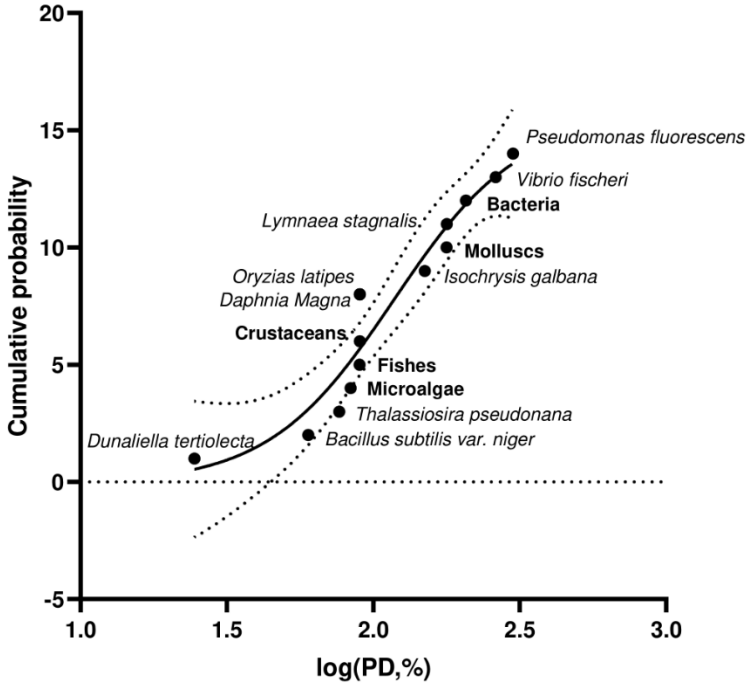

Figure 36. Species sensitivity distribution of different groups species to nZVI. The data are represented as predicted data (PD) collected from 0.0006 g/l.

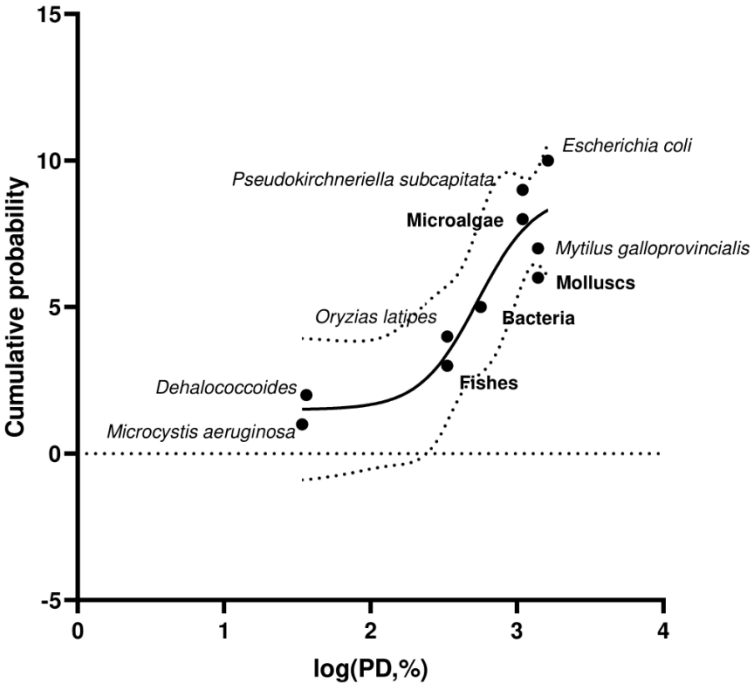

Figure 37. Species sensitivity distribution of different groups species to nZVI. The data are represented as predicted data (PD) collected from 0.186 g/l.

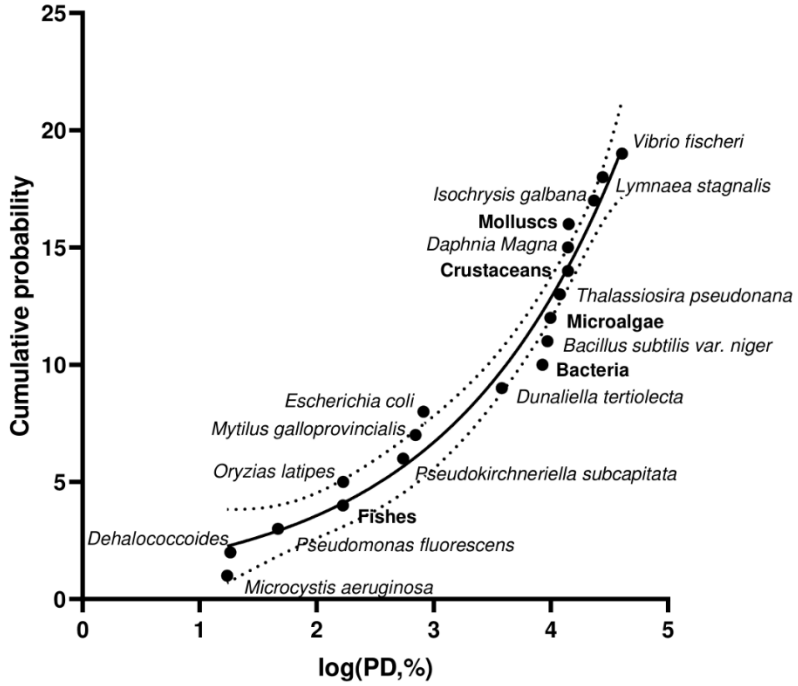

Figure 38. Species sensitivity distribution of different groups species to nZVI. The data are represented as predicted data (PD) collected from 0.0933 g/l.

Furthermore, Bacteria group showed a variable sensitivity. *Bacillus subtilis var. niger* was the least sensitive, whereas *P. fluorescens* and *V. fischeri* were the most sensitive species among Bacteria. Their cumulative probability in SSD curves were 20%, 100% and 87.2%, respectively. *P. fluorescens* and *V. fischeri* are gram-negative bacteria, which are more sensitive to environmental stress respect to *B. subtilis,* which is a gram-positive bacterium (Diao and Yao, 2009). Moreover, probably *A. fischeri* and *P. fluorescens* showed similar effects because they were equally sensitive to metal ions (Abbas et al., 2018; Abbondanzi et al., 2003).

Analysing the range 0.008 – 0.5 g/L (**Figure 34**), Bacteria group showed the highest significant sensitivity to nZVI when compared to Algae ($p < 0.001$) and Molluscs ($p < 0.05$) (see **Table S20**). Algae species were statistically significant respect to Fishes ($p < 0.001$), that in turn displayed high susceptibility respect to Molluscs ($p < 0.01$, **Table S20**).

4.3.3 The SSDs of OC, A and Z

Finally, for the OC, A and Z methods the Fishes, in particular *D. rerio* and *O. mykiss* species, were resulted the most susceptible considering the raw data (**Figures 39-40**), but not statistically significant (**Table S21**).

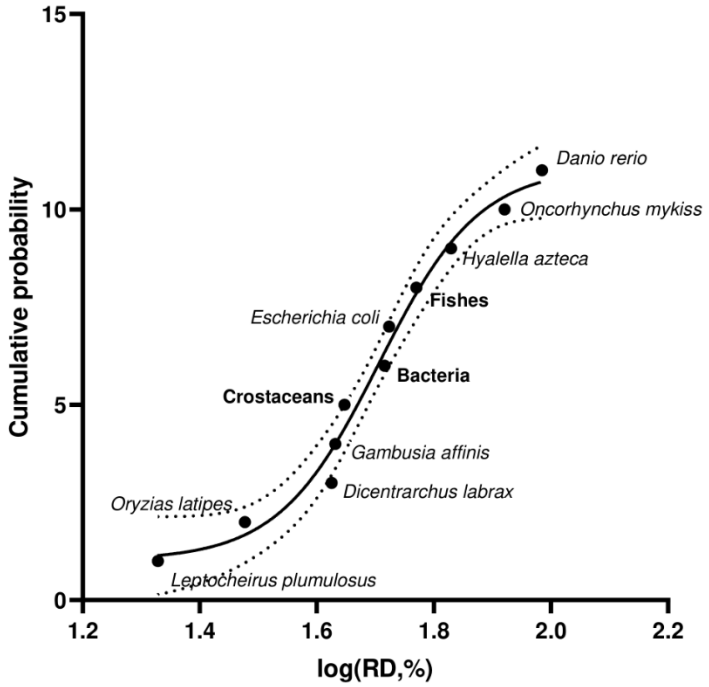

Figure 39. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as raw data (RD) collected from 0.0001 – 0.1 g/l.

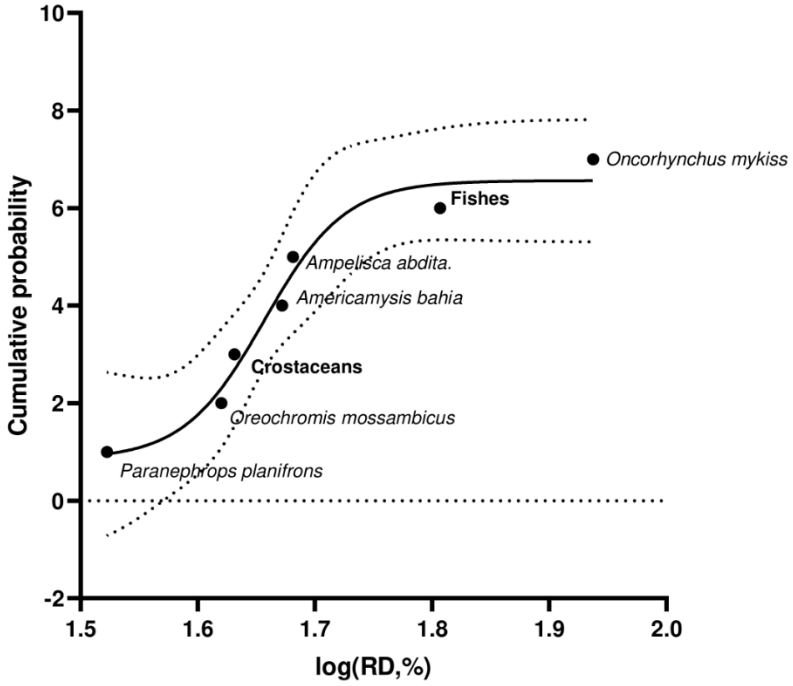

Figure 40. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as raw data (RD) collected from 0.5 – 350 g/l.

When also given the predicted data (**Figures 41-42**), the Fish species, especially *D. rerio,* displayed a highest sensitivity to OC, A and Z remediation methods.

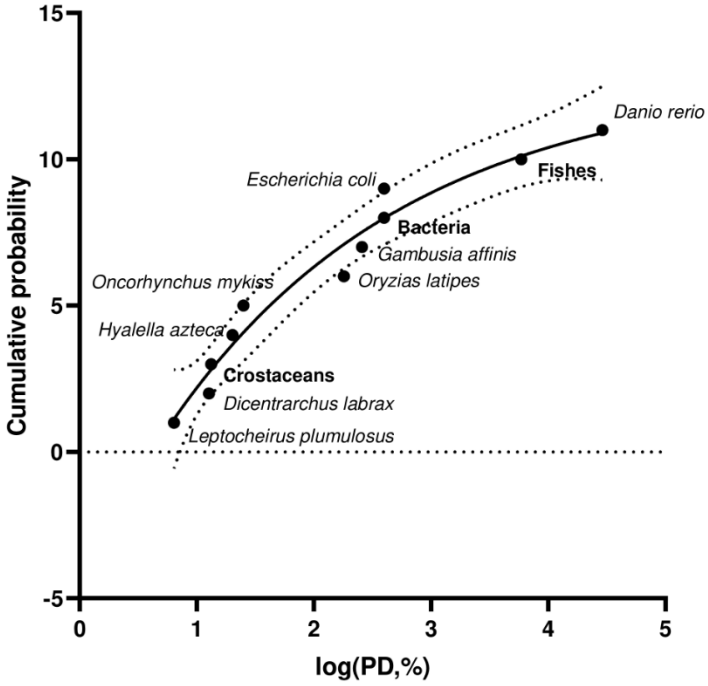

Figure 41. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as predicted data (PD) collected from 0.03 g/l.

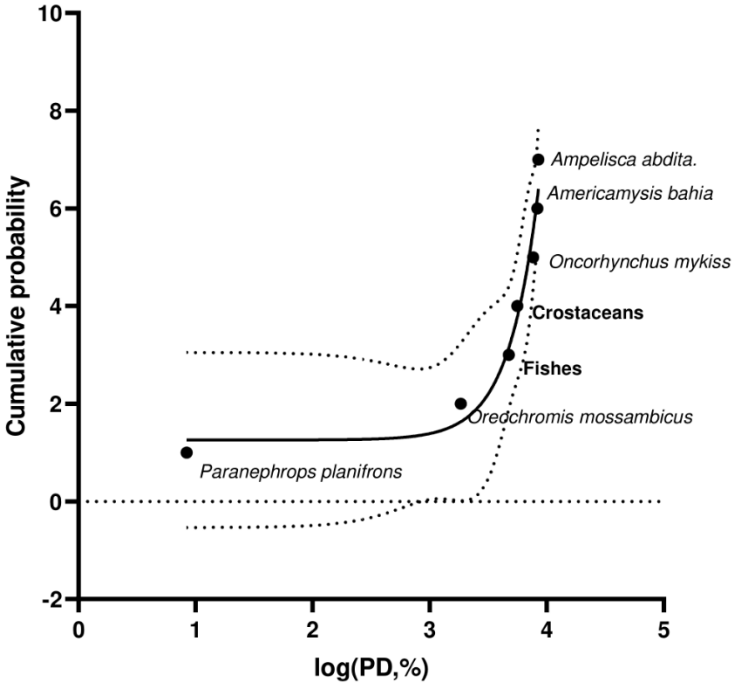

Figure 42. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as predicted data (PD) collected from 88.4 g/l.

Moreover, as shown in **Table S21**, when analyzing 44.2 g/L mean concentration (**Figure 43**) Fishes group were statistically significant respect to Crustaceans ($p < 0.05$) and Bacteria

($p < 0.01$), that in turn displayed high susceptibility respect to Crustaceans ($p < 0.0001$) (see also **Table S21**).

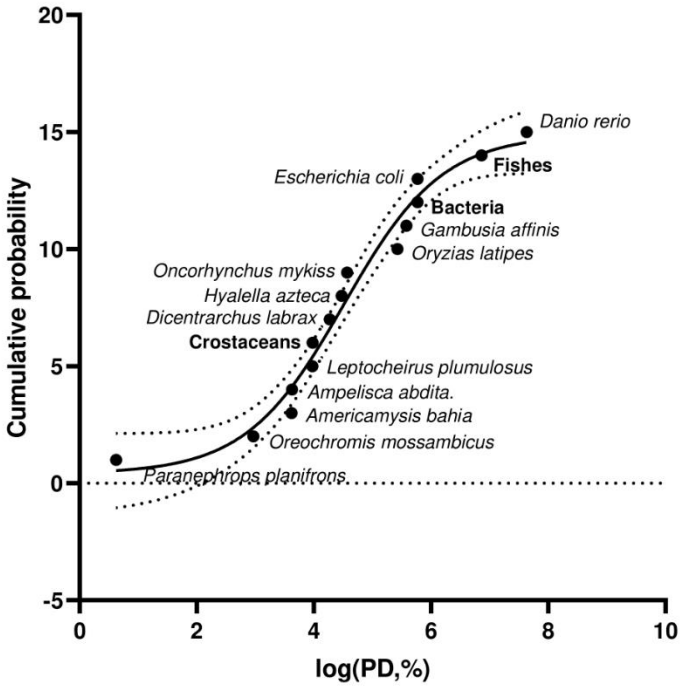

Figure 43. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as predicted data (PD) collected from 44.2 g/l.

Moreover, Fishes group showed a variable sensitivity. *O. latipes* was the least sensitive species (with a cumulative probability of about ~30%), but *D. rerio* displayed the highest sensitivity with a cumulative probability of 96.5%. Probably, the triazoles leaching from the zeolite channels causes the toxicity impact of Z. De La Paz et al., 2017 demonstrated that triazoles inhibit hatching through affecting the hatching enzyme or impairing the release of ZHE1 enzyme.

Furthermore, taking into consideration both raw and predicted data, Crustaceans, in particular *L. plumulosus* and *P. planifrons*, have proven to be the less sensitive species (**Figures 43-44**).

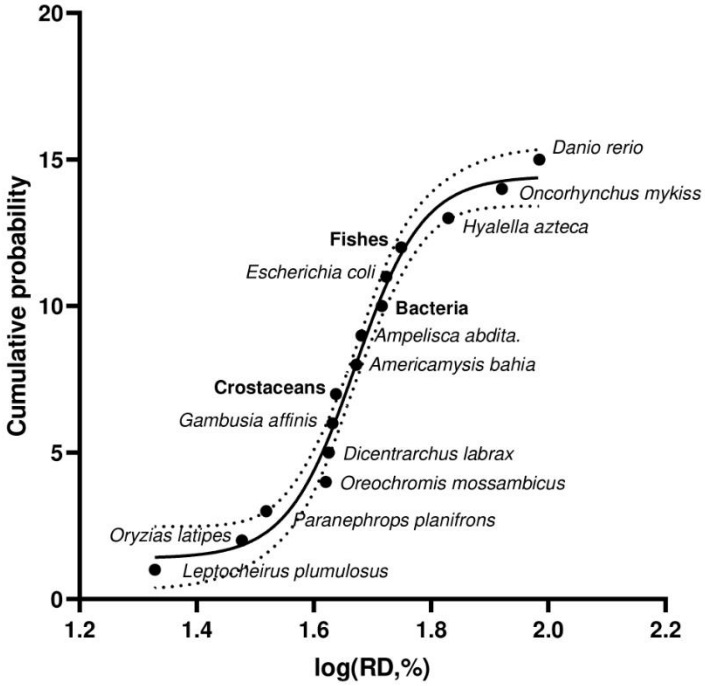

Figure 44. Species sensitivity distribution of different groups species to OC, A and Z. The data are represented as raw data (RD) collected from 0.0001 – 350 g/l.

4.3.4 Whole comparison of SSDs

As shown in **Figures 45-46**, SSDs of different remediation methods based on the total species were constructed and the relationship of sensitivity between individual group species and total species was investigated for all amendments considering both raw and predicted data (**Figures 45-46**).

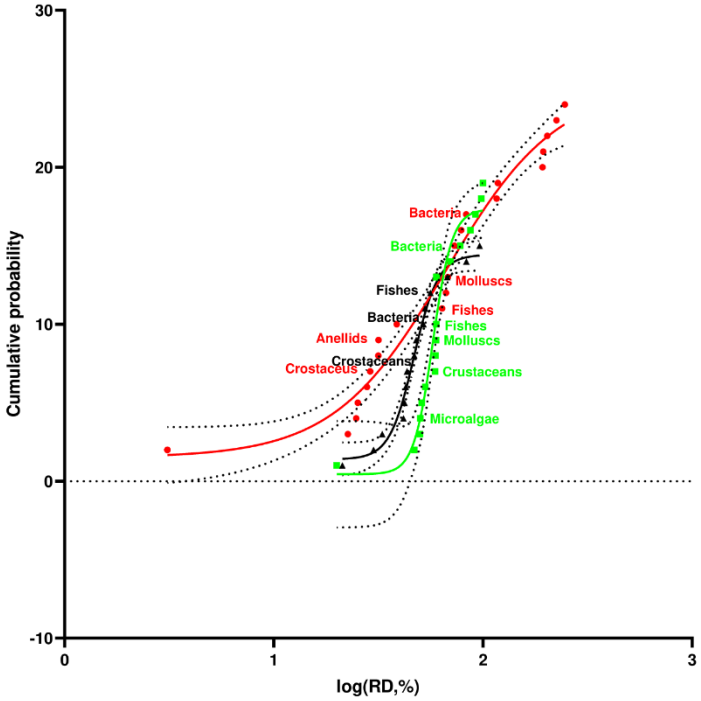

Figure 45. SSD curves for total species exposed to different remediation methods. The data are represented as raw data (RD) for AC (red line), for nZVI (green line) and for OC, A and Z (black line).

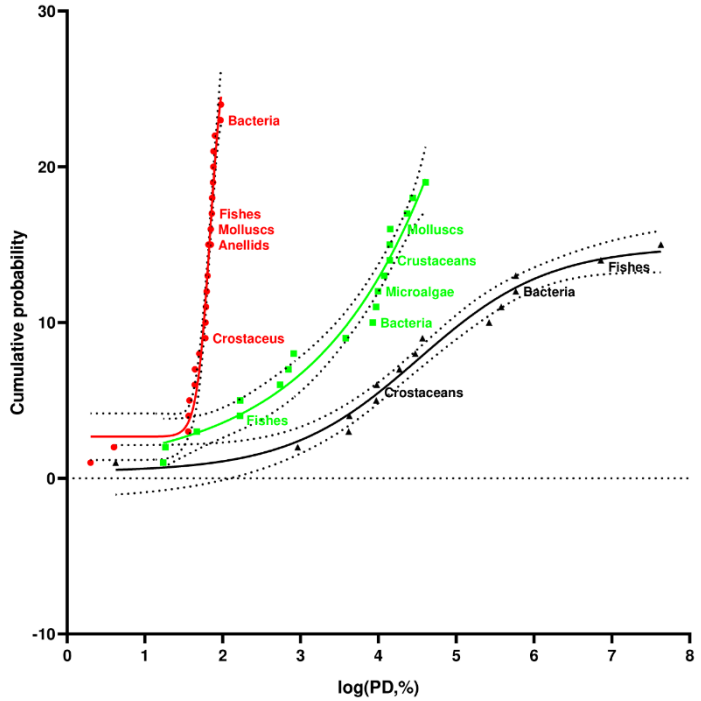

Figure 46. SSD curves for total species exposed to different remediation methods. The data are represented as predicted data (PD) for AC (red line), for nZVI (green line) and for OC, A and Z (black line).

Considering the raw data (**Figure 45**), the curves of remediation methods were almost overlapping with exception of Bacteria that showed the significant increase of sensitivity to nZVI respect to OC, A and Z ($p < 0.05$) and Crustaceans group that were statistically significant to AC respect to nZVI ($p < 0.01$) (**see Table S22**).

At intermediate concentrations and considering predicted data, the AC curve method shifted to the left (**Figure 46**). The adverse effect of nZVI was intermediary between all amendments, whereas the OC, A and Z curve shifted on the right showing higher toxicity for all studied species. Only Fishes group displayed a significant increase of sensitivity to OC, A and Z respect to AC ($p < 0.05$) and nZVI ($p < 0.05$) (**Table S22**).

The AC is more toxic to Fishes than the other four taxonomic groups of analyzed species (with the HC5 calculated at 3.44g/L, CI = 0.18 - 8.97; Table 3), followed by Annelids, Molluscs and Crustaceans. The HC5 value of Crustaceans was found to be more than 3 times higher (11.16 g/L, CI = 5.92 – 14.81, **Table 17**) than that measured in Fishes.

		HC5 (g/L)	CI	HC50 (g/L)	CI
AC	Bacteria	n.a.	n.a.	n.a.	n.a.
	Annelids	4.88	1.09 - 8.82	14.59	8.11 - 26.23
	Molluscs	6.56	2.76 - 9.64	14.19	9.87 - 20.39
	Crustaceans	11.16	5.92 - 14.81	19.66	15.06 - 25.65
	Fishes	3.44	0.18 - 8.97	17.30	6.12 - 48.87
	Total	4.74	2.96 - 6.53	14.00	10.89 - 17.98
nZVI	Bacteria	1.3E-02	2.43E-05 - 0.26	8.29	0.53 - 130.1
	Algae	8.58E-02	2.49E-03 - 0.35	1.15	0.29 - 4.65
	Molluscs	4.15E-02	5.46E-07 - 0.53	1.87	0.07 - 51.44
	Crustaceans	n.a.	n.a.	n.a.	n.a.
	Fishes	n.a.	n.a.	n.a.	n.a.
	Total	0.02	2.9E-03 - 0.08	1.92	0.67 - 5.46
OC, A and Z	Bacteria	n.a.	n.a.	n.a.	n.a.
	Crustaceans	5.59E-03	1.18E-05 - 0.08	1.38	0.10 - 18.44
	Fishes	9.46E-05	3.78E-05 - 1.4E-03	2.93E-02	2.6E-03 - 0.33
	Total	1.77E-04	5.69E-06 - 1.6E-03	1.21E-01	0.02 - 0.71

Table 17. The calculated HC5 and HC50 values (including their CI (Confidence interval)) of Bacteria, Annelids, Molluscs, Crustaceans, Algae, Fishes and total species for three remediation methods. n.a. = not available.

Similarly, it occurred for HC50. The decreasing sensitivity is: Fishes > Annelids > Molluscs > Crustaceans. Only for Bacteria, the HC5 and HC50 values were not calculated because just one concentration was available. nZVI method has a highest impact on Algae (8.58E-02 g/L, CI = 2.49E-03 - 0.35, Table 3) respect to Molluscs and Bacteria. The HC5 value of

Molluscs was found to be more than 2 times higher than that of Algae. For Crustaceans and Fishes, HC5 and HC50 were not calculated because just one concentration was available. The decreasing sensitivity is: Algae > Bacteria > Molluscs.

About OC, A and Z methods, Fishes were the most affected species with HC5 of 9.46E-05 (CI = 3.78E-05 - 1.4E-03, **Table 17**). HC5 of Crustaceans group results to be more than 59 times higher than that of Fishes (5.59E-03, CI = 1.18E-05 - 0.08, see **Table 17**). Moreover, also in this case, for Bacteria, the HC5 and HC50 values were not calculated because just one concentration was available. As reported in **Table 17**, when considered the impact of both AC, nZVI, OC, A and Z methods on total species, OC, A and Z was the most toxic with the HC5 value of 1.77E-04 (CI = 5.69E-06 - 1.6E-03), followed by nZVI and AC. Specifically, the HC5 value of AC is much greater than that measured for OC, A and Z. In this case, the decreasing sensitivity is: OC, A and Z> nZVI > AC (**Table 17**).

Focusing on HC5 and HC50 of remediation methods for each taxonomic group of species and total species, the toxicity profiles have been established basing on criteria of OECD (2006). Basing on GHS criteria, AC and nZVI methods have been identified as "no-hazardous" to aquatic environment. However, OC, A and Z remediation display highly toxicity for species of both saltwater and freshwater environment. These methods show both acute and chronic toxicity of category three representing harmful risk to aquatic life.

4.4 Conclusion

The present study investigated the toxicity of different remediation methods towards saltwater and freshwater species. In general, when the RD were considered, Bacteria group showed the higher sensitivity to nZVI respect to OC, A and Z and Crustaceans to AC respect to nZVI. Taking into consideration the PD, Fishes group were deemed to be more vulnerable to OC, A and Z respect to AC and nZVI. Overall, basing on HC5 and HC50, AC, OC, A and Z should have the most adverse effect on Fishes. The toxicity of nZVI should be the most dangerous to Algae, followed by Bacteria and Molluscs. Result more interesting was that

both AC and nZVI are considered safe for health and environmental hazards, basing on GHS criteria. OC, A and Z proved to have both acute and chronic toxicity unlike other activities. The toxicities of considered methods were listed in a descending order: OC, A and Z>nZVI>AC.

All the results reported in this Chapter have been published in:

1) **Albarano L.**, Lofrano G., Costantini M., Zupo V., Carraturo F., Guida M., Libralato G., 2021a. Comparison of *in situ* sediment remediation amendments: risk perspectives from species sensitivity distribution. Environmental pollution, 115995. (Appendix B).

Acknowledgments

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5. Chapter 5: Comparison between AC and nZVI as restoring methods of common contaminants (PAHs) spiked marine sediment and evaluation of their toxic effects on crustacean *Artemia franciscana*

5.1 Introduction

Gulf of Naples extends along the coasts of the central to southern and Tyrrhenian Sea. Over the past few years, high concentrations of heavy metals (especially Cd, Cu, Zn, Cr, Ni, As), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and total hydrocarbons (HC) both in the sediments and water from the stretch of sea were reported by different studies (Arienzo et al., 2019, 2017; Montuori et al., 2013; Montuori and Triassi, 2012; Trifuoggi et al., 2017). In the northern sector site, the Gulf is characterized by the presence of petroleum industries. The greater contribution of pollutants (especially PAHs) is given by the activity of ILVA of Bagnoli (the second largest integrated steel plant in Italy; Arienzo et al., 2017; 2019; Trifuoggi et al., 2017). In the south-western sector site, the Gulf is mainly affected by the Sarno River (defined as "the most polluted river in Europe"). The main causes of pollution of the Sarno River are the massive use of fertilizers and pesticides in agriculture and the industrial development. Among the substances mentioned above, the PAHs are certainly the pollutants most drained from the river in the Gulf (Montuori et al., 2012; 2013).

Polycyclic aromatic hydrocarbons (PAHs) are a well-known group of environmental pollutants predominantly generated by anthropogenic activities (about 99% of PAHs in the sediment are originated by anthropogenic contributions; (Medeiros and Caruso Bícego, 2004). The evidence of their genotoxicity and carcinogenicity for animal species is already available, and epidemiological studies have demonstrated a correlation between PAHs exposure and cancer incidence. Benzo(a)pyrene (BaP), which is one of these compounds, has been classified by IARC as the prototypical carcinogenic and positively genotoxic PAH

due to its adverse and toxic effects on various cells and tissues, reproduction, development and immune system of animals (Singh et al., 1998). Take into consideration the high risk, in recent years have been implemented various remediation activities to reduce or removal toxic compounds using different chemical, thermal and biological methods both in situ and ex situ treatments (Gomes et al., 2013; Lofrano et al., 2016; Mueller et al., 1996). Among the most used methods there are definitely chemical techniques (such as activated carbon (AC), nano-Zero-Valent-Iron (nZVI), apatite (A), organoclay (OC) and zeolite (Z)) which could negatively affect the resident biota, especially when applied in situ (Libralato et al., 2018; Lofrano et al., 2016). Albarano et al., (2021) reviewed all literature available on possible toxigenic effects of remediation methods both on freshwater and saltwater species, and using model of species sensitivity distribution evaluated that nZVI was more toxic than AC for all considered species. Activated carbon (AC) can be defined as all substances with high carbon content and porosity enabling to absorb different compounds (Rakowska et al., 2012). The PAHs concentrations decrease in sediment after AC treatment ranged from 50 to 100% and depends on the quantity of amendments added (Bussan et al., 2016; Hale et al., 2010). The nano zero valent iron (nZVI) is characterized by structure where the nucleus consists of zero valent and a mixed valent oxide shell of Fe^{2+} and Fe^{3+} that is formed as a result of oxidation of the core shell. nZVI is fairly reactive in water and possess excellent electron donating properties which makes it a versatile remediation material (Mukherjee et al., 2016). The removal efficiency of organic compounds ranged from 90 to 99.8% in aqueous solutions after nZVI addition (Kim et al., 2010; Liu et al., 2012).

The aim of this third part of my PhD project was to study restoring of PAHs contaminated seawater and marine sediments with nZVI and AC using microcosm experiments. We have sampled two different sediment, one less polluted and one more polluted, following a previous environmental characterization (Arienzo et al., 2019, 2017; Montuori et al., 2013; Montuori and Triassi, 2012; Morroni et al., 2020). The PAHs concentrations in aqueous

solution were chosen in according to total PAHs concentration detected in sediment more polluted. Specifically, we added AC and nZVI (3% wt in sediment dry) to all experimental conditions. Just after amendments adding (T0), 3 hours (T1), 6 hours (T2), 21 hours (T3), 24 hours (T4), 72 hours (T5) and 21 days (T6), chemical analysis were conducted on water and sediment samples to evaluated the efficiency of removal of AC and nZVI.

Moreover, at all sampling times we have evaluated the negative impact of these treatments on *Artemia franciscana* following two viewpoints, embryotoxicity and genotoxicity. The crustacean *A. franciscana* was used since it is considered a good model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants (Libralato, 2014; Libralato et al., 2007; Migliore et al., 1993). The greatest advantage of the species is that nauplii can be hatched as needed from commercially available durable cysts. Moreover, their small body size allows the conduct of the tests in small beakers or plates; the embryo grows rapidly in laboratory conditions; and finally, they have adaptability to a wide range of salinities (5-300 g/L) and temperatures (6-40 °C; Manfra et al., 2016). To best understand the molecular basis, after 48 h to 100% of aqueous solutions collected from all conditions at the end of experiment (T6), the expression levels of several key genes involved in stress response (*hsp26, hsp60, hsp70, COXI* and *COXIII*) and in development processes (*HAD-like, tcp, UCP2* and *CDC48*) was followed by *RealTime qPCR*.

5.2 Materials and methods

5.2.1 Sediment collection and chemical characterization

Sediments were sampled with a Van Veen bucket between January 2020 and February 2020, at two sites within Gulf of Naples (Tyrrhenian Sea, Italy; **Figure 47**).

Figure 47. Map of the Gulf of Naples with location of sediment collection sites SED1 and SED2.

In particular, sediments were collected from Sarno River Mouth at 1500mt South (namely SED1; 40°42'41.38"N and 14°28'45.14"E) and from Bagnoli Bay (namely SED2; 40°48'54.8"N and 14°09'44.2"E). These samples were chosen, following a previous environmental characterization of the Bagnoli Bay (Arienzo et al., 2017; 2019; Morroni et al., 2020) and Sarno River (Montuori et al., 2012; 2013). After collection, sediment samples for chemical analyses were stored at -20°C, until analysed for grain-size distribution (gravel, sand, silt, and clay) and polycyclic aromatic polyaromatic hydrocarbons (PAHs). Measurements were carried out through validated methods described in **Paragraphs 2.2.4** and **2.2.5**. The characteristics of the sediments and initial concentrations of PAHs are summarized in **Figure S15** and **Table 18**, respectively.

	SED 1	**SED 2**
Acenaphthylene	< 0.05	18.5
Anthracene	21.9	115.1
Phenanthrene	< 0.05	58.4
Fluoranthene	23.6	323.0
Pyrene	22.5	282.9
Benzo(a)Antracene	< 0.05	122.7
Chrysene	11.9	159.0
Benzo(b)Fluorantene	< 0.05	201.6
Benzo[k]fluoranthene	< 0.05	74.7
Benzo(e)Pirene	< 0.05	119.4

Benzo[a]pyrene	< 0.05	112.1
Indeno[1,2,3-cd]pyrene	< 0.05	104.3
Benzo[ghi]perylene	< 0.05	101.9
Total PAHs	80	1793.5

Table 18. Initial concentrations of PAHs in sediments. The data were reported in µg/Kg.

5.2.2. Amendments

The remediation methods evaluated in the present study were nano-Zero-Valent (nZVI) and activated carbon (AC). NANOFER-Star-Zero-Valent-iron (air-stable powder of FeNPs stabilized by inorganic stabilizers; nZVI) was purchased from NANOIRON Future Technology (Židlochovice Czech Republic), with particle sizes smaller than 100 nm. Activated carbon (air-stable powder; AC) was purchased from J.T. Baker® (Deventer, Holland) with particle sizes smaller than 100 nm.

5.2.3. Experimental Design

Two experiments were performed to determine changes in sediment and seawater PAHs concentrations and the concentration of available PAHs after a period of remediation with activated carbon and nano-Zero Valent-iron. The conditions of experiments included five scenarios: (i) negative control—synthetic seawater (SSW, prepared in according to ISO 10253/16) plus amendments (W + AC or W + nZVI); (ii) positive control—seawater spiked with PAHs (1800 µg/L, nominal; in according to total PAHs concentration in Bagnoli sediment (SED1)) (W + PAHs) (check the loss of PAHs by evaporation); (iii) seawater spiked with PAHs + amendments (W + PAHs + AC or W + PAHs + nZVI); (iv) seawater + sediment collected from Sarno River (SED1) with amendments (W + SED1 + AC or W + SED1 + nZVI); and (v) seawater + sediment collected from Bacoli Bay (SED2) plus amendments (W + SED2 + AC or nZVI).

Each of the 15 testing microcosms, located at the University of Naples, Federico II, was characterized by an independent and closed seawater system (glass bottle).

Each bottle (500 mL; the size was chosen according to amount of sediment necessary for the specific test run) was filled with 50 g of sediment and topped with 150 mL of synthetic seawater respecting a ratio of 1:4 (sediment:seawater). 1.7 g of AC and nZVI (3% of sediment dry wt in according to (Brändli et al., 2009; Choi et al., 2009)) were added to all experimental conditions with exception of positive control (W + PAHs). These microcosms were shaken on orbital shaker (120 rpm) at 20±1°C in darkness condition for 21 days. All experiments were carried out in triplicates.

5.2.4. Extraction and Analysis of PAHs

To evaluate changes in sediment and seawater of PAHs concentrations, 20 mL of liquid sample and 5 g of sediment were withdrawn using a glass pipet already after the adding of amendments (T0), 3 h (T1), 6 h (T2), 21 h (T3), 24 h (T4), 72 h (T5) and 21 days (T6). For PAHs concentration after nZVI remediation, seawater and sediment samples were extracted in according to methods reported in **Paragraphs 2.2.5.**

For PAHs analyses after AC restoring, seawater samples were extracted by a solid-phase extraction (SPE): 1.0 l of water was filtered and preconcentrated on a C18 disk (ENVI, -18 DSK SPE Disk, diam. 47 mm). The analytes were eluted with a solution of carbon sulfide. The determination in the sediment was performed by considering 5 g of dry sediment extracted with acetone/n-hexane 1:1 v/v (10 mL), using an ultrasonic disruptor (Brason, US). The extract was concentrated to 1 mL in Multivap under nitrogen flow (Multivap, LabTech, Italy). A total of 10 µL of a 1 mg/L solution of internal standard (mixture of deuterated PAHs) was added to the extract and injected to a gas chromatography–mass spectrometry (GC-MS) (MS-TQ8030-Shimadzu, Japan). The limits of detection (LOD) and quantification (LOQ) were reported in **Paragraph 2.2.5**.

5.2.5. Acute toxicity test and genotoxicity

Acute toxicity test on embryos was performed according to **Paragraph 3.2.3** by exposing ten nauplii to increasing percentage concentrations (0%, 6.25%, 12.5%, 25%, 50% and

100%) of aqueous solutions, collected from all experimental conditions using a glass pipet at T0, T1, T2, T3, T4, T5 and T6. After 48 h, the number of dead nauplii (which were motionless for 10 s) was counted. All the experiments were performed in triplicates.

To evaluate the molecular basis of amendments toxicity, 200 nauplii of *A. franciscana* were exposed to 100% of aqueous solutions collected from all conditions at the end of experiment (T6). These concentrations were chosen because the death effect was lost or not apparent at lower concentrations. All the experiments were performed in triplicates.

RNA extraction, cDNA synthesis and *Real Time qPCR* were performed as reported in **Paragraphs 3.2.4** and **3.2.5.**

5.2.6 Statistical analysis

Toxicity data were reported as 'mean ± one standard deviation (SD)'. Data were checked for normality using the Shapiro-Wilk's (S-W) test (p-value <0.05). The statistical significance of differences among different percentage of treatments and control was checked by two-way ANOVA followed by Tukey's test for multiple comparisons (GraphPad Prism Software version 8.02 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). P values <0.05 were considered statistically significant.

5.3 Results

5.3.1 Effect of nZVI and AC dosage on PAHs removal

To evaluate the effect of nZVI and AC on the removal efficiency of PAHs, experiments were conducted by adding amendments dose of 3% of sediment dry wt.

As reported in **Figure 48**, results demonstrated that the nZVI addition yielded high PAHs degradation both in sediment and seawater. When considered W + PAHs + nZVI condition, already after 21 hours (T3) of treatment, the degradation of pollutants was total (**Figure 48**). Since the loss of total PAHs by evaporation at T3 was only 20%, this result can be considered completely due to the treatment with nZVI.

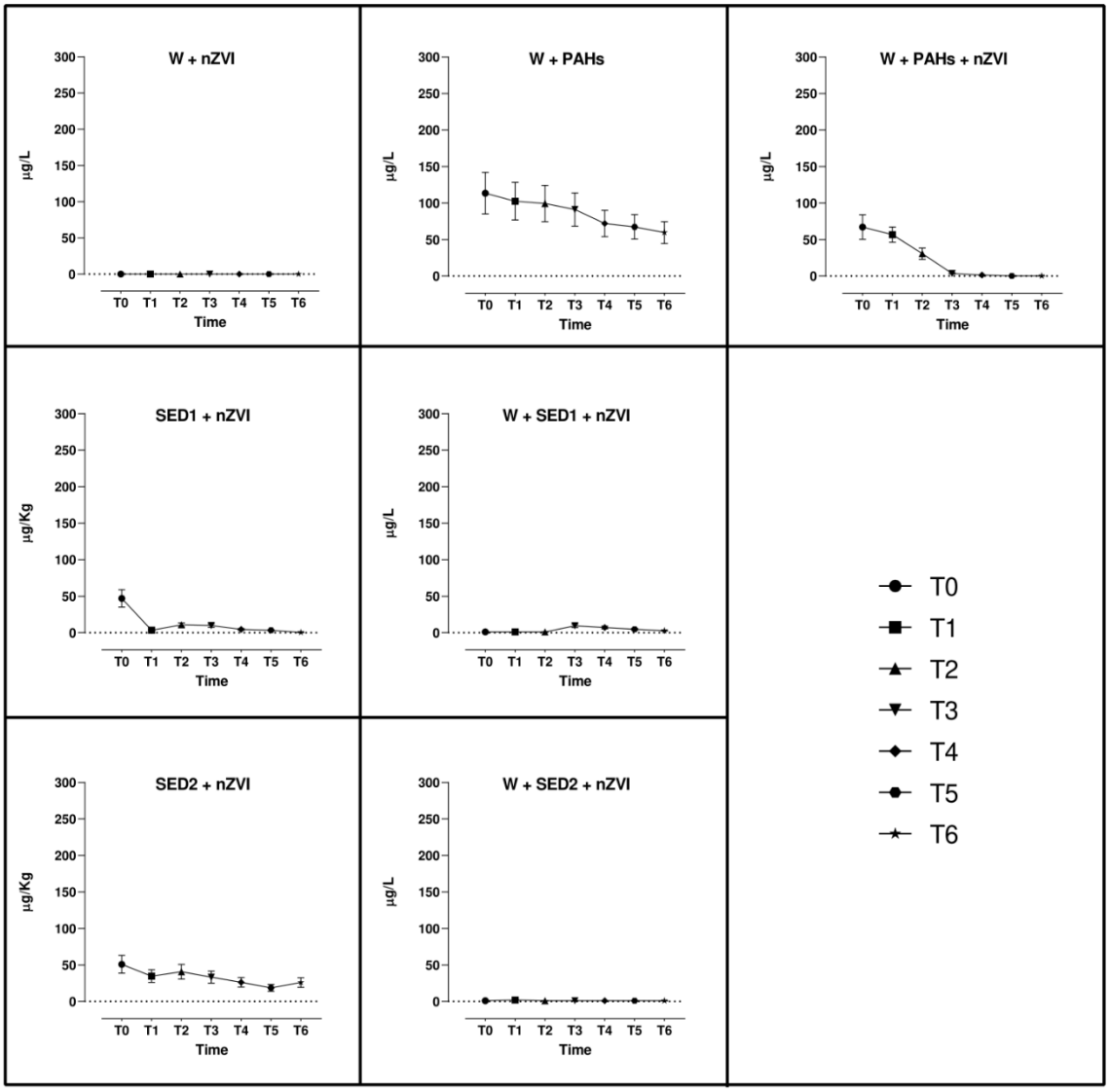

Figure 48. Effect of nZVI dosage on PAHs removal. Experimental conditions: negative control (W + nZVI); positive control (W + PAHs); sediment spiked with PAHs + amendments (W + PAHs + nZVI); seawater + sediment1 (SED1) with amendments (W + SED1 nZVI); and seawater + sediment2 (SED2) plus amendments (W + SED2 + nZVI). Data points represent averages of three samples from triplicate reactors. (For more details regarding the removal of individual PAHs, see also **Figure S16**).

Considering the aqueous solution of SED1 and SED2 (W + SED1+ nZVI and W + SED2 + nZVI, respectively), no PAHs have been detected at all studied time (from T0 to T6) with only T3 and T4 exception of W + SED1+ nZVI (see also **Figure 48**), where little concentrations of 9 and 8 µg/L have been shown, respectively.

In the case of sediment of Sarno (SED1), the removal of hydrocarbons was total already after 3 hours of treatment (T1); whereas taking in the consideration the sediment of Bagnoli (SED2), at the end of experiment (after 21 days, T6) a residue of about 26 µg/Kg is still measured in the sediment (**Figure 48**). In assessing the removal of individual compounds

from SED2 (**Figure S16**), the results show that this amendment is not able to completely remove the fluoranthene, pyrene, benzo(b)fluoranthene, benzo(a)anthracene, Indeno(1,2,3-cd)pyrene and benzo(a)pyrene.

The addition of 3% AC reduced the aqueous concentration of PAHs, with pollutants concentrations below detection limits already after 21 h (T3) of amendment (**Figure 49**). Also in this case, since the loss of total PAHs by evaporation at T3 was only 20%, this result can be considered completely due to the treatment with AC. Moreover, considering the aqueous solution of SED1 and SED2 (W + SED1+ AC and W + SED2 + AC, respectively), no PAHs have been detected at all studied time (from T0 to T6; see also **Figure 49**).

The results obtained from sediment treatments with AC were almost similar to those obtained for nZVI. In fact, total removal of PAHs has been shown for SED1; whereas a residue of about 25.7 μg/Kg is still measured in the sediment of Bagnoli (SED2) from T1 to the end of experiment (after 21 days, T6) (**Figure 49**). In assessing the removal of individual compounds from SED2 (**Figure S17**), the results show that this amendment is not able to completely remove the fluoranthene, benzo(a)anthracene, dibenzo(a,h)anthracene, Indeno(1,2,3-cd)pyrene and benzo(a)pyrene.

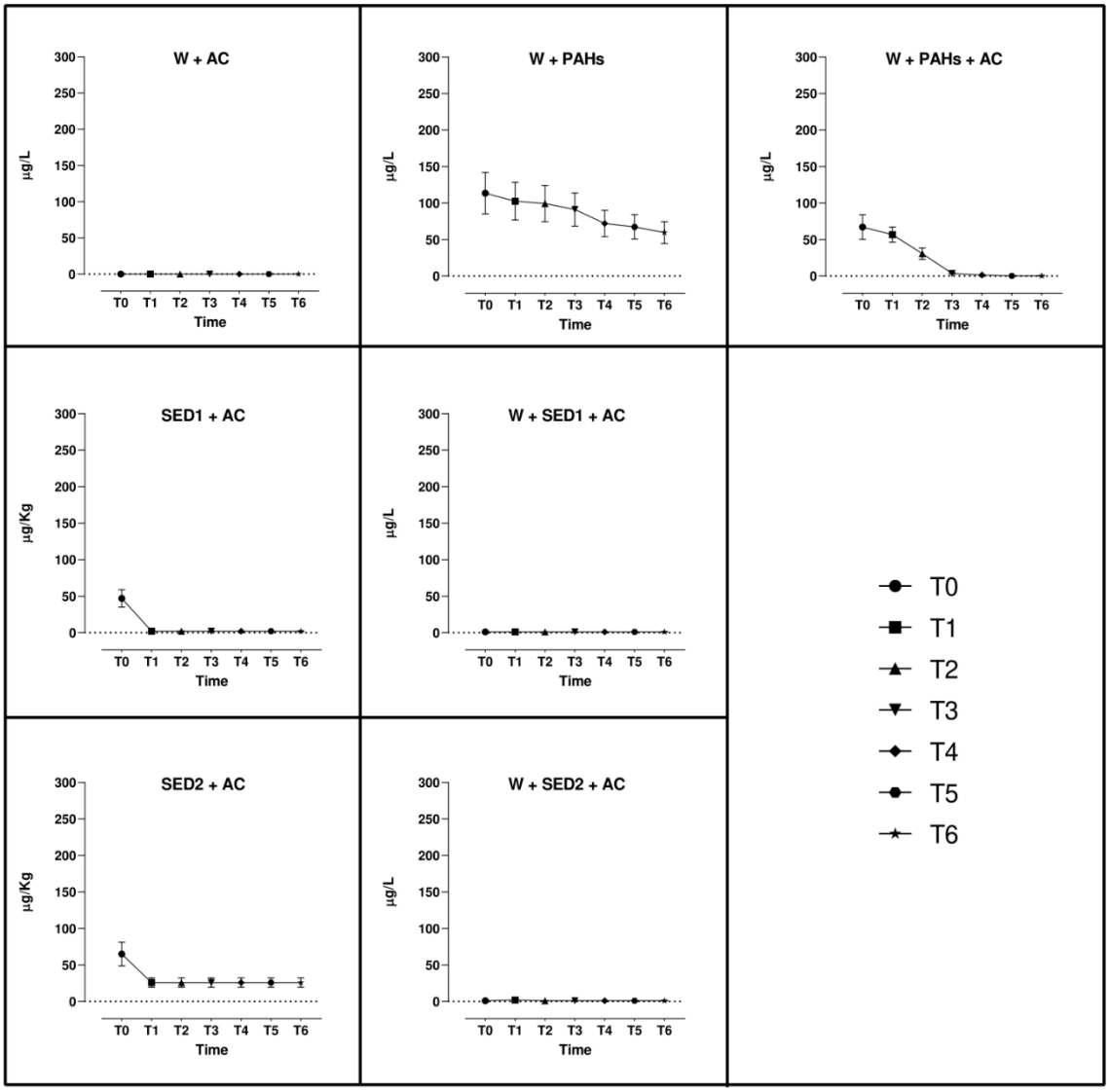

Figure 49. Effect of AC dosage on PAHs removal. Experimental conditions: negative control (W + AC); positive control (W + PAHs); sediment spiked with PAHs + amendments (W + PAHs + AC); seawater + sediment1 (SED1) with amendments (W + SED1 + AC); and seawater + sediment2 (SED2) plus amendments (W + SED2 + AC). Data points represent averages of three samples from triplicate reactors. (For more details regarding the removal of individual PAHs, see also **Figure S17**).

The removal efficiencies of two remediation methods demonstrated that they are much more efficient for PAHs removal from aqueous solutions than sediments (**Table 19**).

	AC W + PAHs + AC (µg/L)			**nZVI** W + PAHs + nZVI (µg/L)		
	T0	**T6**	**Removal efficiency (%)**	**T0**	**T6**	**Removal efficiency (%)**
Acenaphthylene	0	0	n.a	0	0	n.a
Acenaphthene	0.1	0.1	0	157.7	0.25	99.8
Fluorene	10.6	0.1	99.1	176	0.25	99.9
Anthracene	6.35	0.1	98.4	13	0.25	98.1
Phenanthrene	0	0	n.a	0	0	n.a
Fluoranthene	6.89	0.1	98.5	88	0.25	99.7

Pyrene	7.63	0.1	98.7	32	0.25	99.2
Benzo(a)Antracene	0	0	n.a	0	0	n.a
Chrysene	5.24	0.1	98.1	2.3	0.25	89.1
Benzo(b)Fluorantene	0	0	n.a	0	0	n.a
Benzo[k]fluoranthene	3.68	0.1	97.3	1	0.25	75
Benzo[a]pyrene	0	0	n.a	0	0	n.a
Indeno[1,2,3-cd]pyrene	0	0	n.a	0	0	n.a
Dibenz[a,h]anthracene	0	0	n.a	0	0	n.a
Benzo[ghi]perylene	0	0	n.a	0	0	n.a
Total PAHs	**2.70**	**0.05**	**98.3**	**31.3**	**0.1**	**99.6**
	SED1 + AC (µg/Kg)			**SED1 + nZVI (µg/Kg)**		
Acenaphthylene	4.18	0.5	88.0	4.18	0.5	88.0
Acenaphthene	0	0	n.a	0	0	n.a
Fluorene	0	0	n.a	0	0	n.a
Anthracene	11.75	0.5	95.7	11.75	0.5	95.7
Phenanthrene	0	0	n.a	0	0	n.a
Fluoranthene	74.5	0.5	99.3	74.5	0.5	99.3
Pyrene	63.15	0.5	99.2	63.15	0.5	99.2
Benzo(a)Antracene	56.97	0.5	99.1	56.97	0.5	99.1
Chrysene	59.96	0.5	99.2	59.96	0.5	99.2
Benzo(b)Fluorantene	85.26	0.5	99.4	85.26	0.5	99.4
Benzo[k]fluoranthene	35.46	0.5	98.6	35.46	0.5	98.6
Benzo[a]pyrene	67.13	0.5	99.3	67.13	0.5	99.3
Indeno[1,2,3-cd]pyrene	51.59	0.5	99.0	51.59	0.5	99.0
Dibenz[a,h]anthracene	12.55	0.5	96.0	12.55	0.5	96.0
Benzo[ghi]perylene	44.82	0.5	98.9	44.82	0.5	98.9
Total PAHs	**37.8**	**0.4**	**98.9**	**37.8**	**0.4**	**98.9**
	SED2 + AC (µg/Kg)			**SED2 + nZVI (µg/Kg)**		
Acenaphthylene	6.5	2	69.2	15.6	8.39	46.2
Acenaphthene	2	2	0.0	2	0.47	76.5
Fluorene	2	2	0.0	2	0.96	52.0
Anthracene	85.2	11.2	86.9	19.7	15.04	23.7
Phenanthrene	35.4	10.4	70.6	11.4	4.87	57.3
Fluoranthene	125	54.6	56.3	130.8	77.61	40.7
Pyrene	146	35.6	75.6	101.2	60.75	40.0
Benzo(a)Antracene	85.6	54.6	36.2	57.8	30.62	47.0
Chrysene	64.7	10.3	84.1	47.7	26.87	43.7
Benzo(b)Fluorantene	32.4	5.6	82.7	112.6	45.45	59.6
Benzo[k]fluoranthene	78.9	26.8	66.0	42.4	21.92	48.3
Benzo[a]pyrene	65.4	35.6	45.6	81.1	35.73	55.9
Indeno[1,2,3-cd]pyrene	74.5	45.3	39.2	70.4	30.65	56.5
Dibenz[a,h]anthracene	104.3	85.6	17.9	13.4	4.53	66.2
Benzo[ghi]perylene	68.9	3.6	94.8	60.7	25.93	57.3
Total PAHs	**65.1**	**25.7**	**60.6**	**51.3**	**26.0**	**49.3**

Table 19. Removal efficiencies achieved with different remediation methods. n.a. = not available.

The degradation efficiencies were 98.3% and 99.6% for total PAHs removal from aqueous solutions (W + PAHs + nZVI or AC; see **Table 19**), and 98.9% from Sarno sediment (SED1) plus amendments (SED1 + nZVI or AC). The percentage of total PAHs removal from Bagnoli sediment was 60.6% and 49.3% for AC and nZVI methods, respectively (**Table 19**).

5.3.2 Toxicity effect of nZVI and AC on *Artemia franciscana*

As reported in **Figure 50**, after 48 h of exposure to different percentage of aqueous solutions of all experimental conditions of nZVI, an increase of toxicity was observed at higher tested percentage, represented by 50% and 100%.

Considering W + nZVI condition at T0 (**Figure 50A**), a little percentage of dead nauplii (about 6.6%) has been shown already at the lowest percentage (6.25%). These data were statistically significant respect to the control ($p < 0.05$) and others used concentrations ($p < 0.0001$; see also **Table S23**). At 100%, significant increase of toxicity (about 30%) respect lower (0% and 12.5%; $p < 0.0001$ and $p < 0.001$, respectively) and higher (25% and 50%; $p < 0.001$) tested concentrations has been shown. Taking into consideration T1, at 25%, 50% and 100% a significant decrease of survival (about 13%, 20% and 23%, respectively) respect lower concentrations (0%, 6.25% and 12.5%; $p < 0.0001$ **(Table S23**) has been detected. Moreover, the data reported at 25% were statistically significant respect to 50% ($p < 0.05$) and 100% ($p < 0.001$). Considering T2, T3 and T4 only at 50% and 100% a significant decrease of survival (about 10% and 20%, respectively) respect all lower concentrations ($p < 0.0001$) and among them ($p < 0.001$; **Table S23**).

As reported **Figure 50B**, W + PAHs condition at T0 and T1 caused a little survival decrease (about 10%) already at 6.25% that was statistically significant respect to control ($p < 0.0001$) and others concentrations (($p < 0.0001$; **Table S23**). At 25%, a significant decrease of about 50% respect lower concentrations ($p < 0.0001$ **(Table S23**) and higher concentrations (50% and 100%; $p < 0.0001$) has been detected. From T2 to T6, the results were similar (**Figure 50B**). In fact, at 25%, a significant decrease of about 30% respect lower concentrations (p

<0.0001 **(Table S23)** and higher concentrations (50% and 100%; p <0.0001) has been shown.

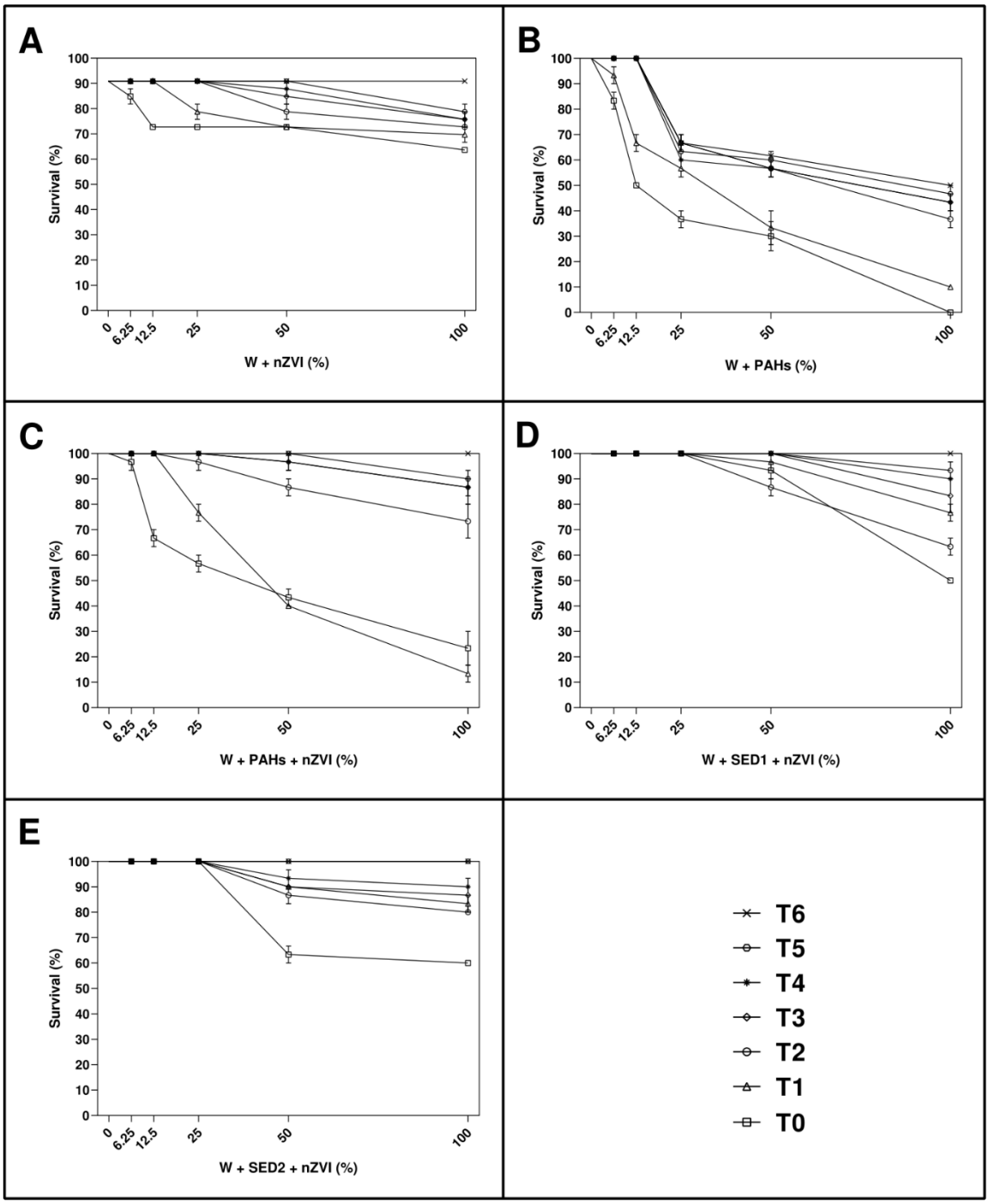

Figure 50. After 48 h, the percentage of surviving nauplii detected at seven time (T0 = after adding amendment; T1 = 3 h; T2 = 6 h; T3 = 21 h; T4 = 24 h; T5 = 72 h; and T6 = 21 days) both in control (0 %) and treated samples with 6.25%, 12.5%, 25%, 50% and 100% of A) W + nZVI; B) W + PAHs; C) W + PAHs + nZVI; D) W + SED1 + nZVI; and E) W + SED1 + nZVI. Data are reported as mean ± standard deviation.

Considering W + PAHs + nZVI condition at T0 and T1 (**Figure 50C**), a little percentage of dead nauplii (about 25%) has been shown at 12.5%. At 100%, significant increase of toxicity (about 80%) respect lower (0%, 6.25% and 12.5%; p <0.0001) and higher (25% and 50%; p

<0.0001) tested concentrations has been shown (**Table S23**). Considering T2, T3, T4 and T5 only at 50% and 100% a significant decrease of survival (about 10%) respect all lower concentrations (p <0.0001) and among them (p <0.0001; **Table S23**). At T6, no toxicity has been displayed.

Take into the consideration W + SED1 + nZVI condition at T0, T1 and T3 (**Figure 50D**), a little percentage of dead nauplii (about 10%) has been shown at 50%. At 100%, significant increase of toxicity (about 20-30%) respect lower (0%, 6.25% and 12.5%; p <0.0001) and higher (25% and 50%; p <0.0001) tested concentrations has been shown (**Table S23**). At T4, T5 and T6, no toxicity has been displayed.

Similar scenario can be described for W + SED2 + nZVI condition (**Figure 50E**). In fact, from time 0 to time 4 only at 50% and 100% a significant decrease of survival (about 10% and 20%, respectively) respect all lower concentrations (p <0.0001). At T5 and T6, no toxicity has been shown.

As reported in **Figure 51**, after 48 h of exposure to different percentage of aqueous solutions of all experimental conditions of AC, the scenario was little different.

Considering W + AC condition at T0 and T1 (**Figure 51A**), at 100%, significant percentage of dead nauplii (about 10-20%) has been shown respect others concentrations (0%, 6.25%, 12.5%, 25% and 50%; p <0.0001, see also **Table S24**). Taking into consideration T2, T3, T4, T5 and T6 no toxicity has been detected. At the same manner, take into consideration W + PAHs + AC and W + SED1 + PAHs (**Figure 51C-D**), only at 100%, significant percentage of dead nauplii (about 10-20%) has been shown respect others concentrations (0%, 6.25%, 12.5%, 25% and 50%; p <0.0001, see also **Table S24**).

Only when considered W + SED2 + AC (**Figure 51E**), from T0 to T3, at 100%, significant percentage of dead nauplii (from about 10 to 30%) has been displeyed respect others concentrations (p <0.0001, see also **Table S24**). At T4, T5 and T6, no decrease of survival nauplii has been observed.

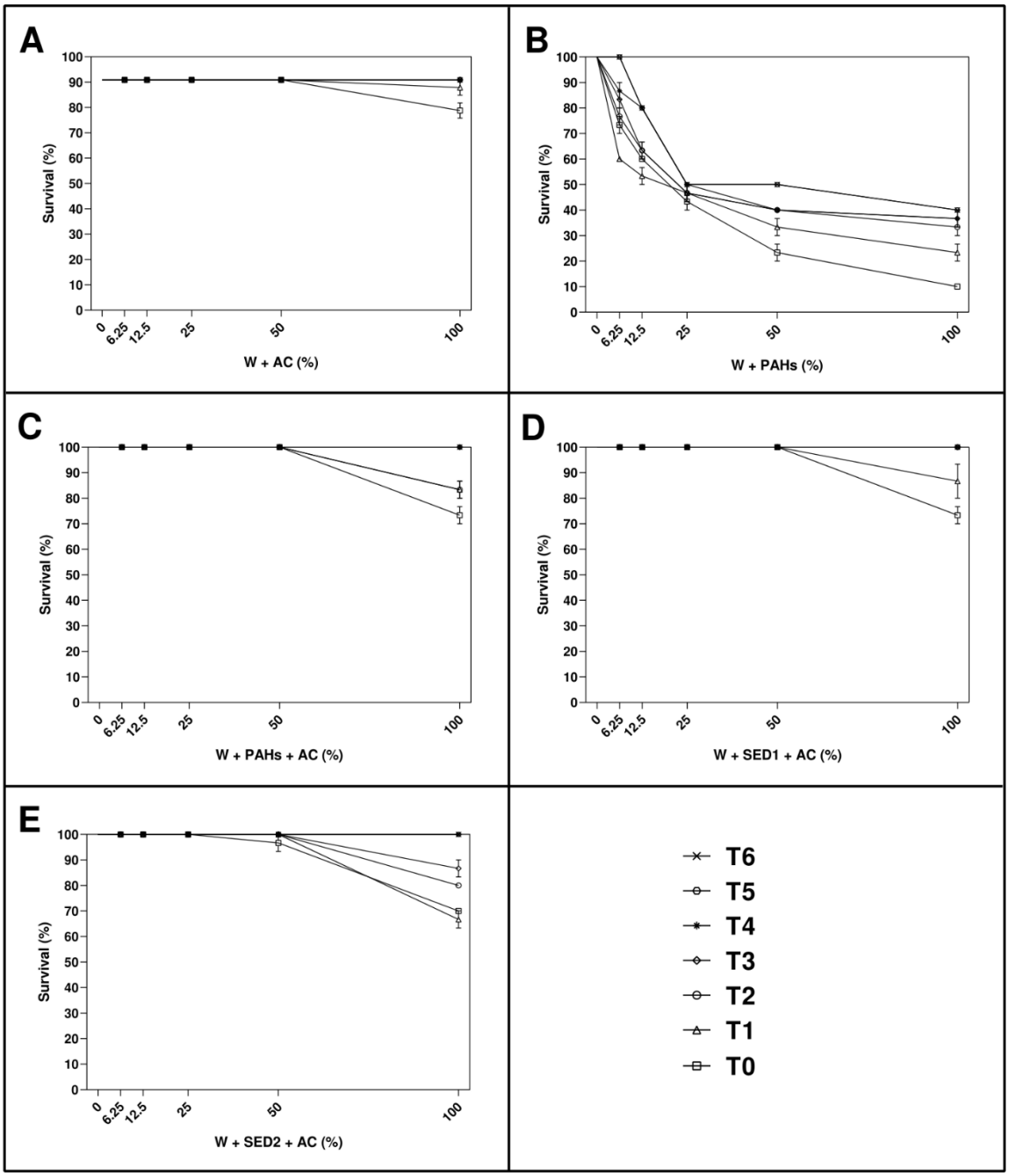

Figure 51. After 48 h, the percentage of surviving nauplii detected at seven time (T0 = after adding amendment; T1 = 3 h; T2 = 6 h; T3 = 21 h; T4 = 24 h; T5 = 72 h; and T6 = 21 days) both in control (0 %) and treated samples with 6.25%, 12.5%, 25%, 50% and 100% of A) W + AC; B) W + PAHs; C) W + PAHs + AC; D) W + SED1 + AC; and E) W + SED1 + AC. Data are reported as mean ± standard deviation.

5.3.3 Effects of nZVI and AC on Gene Expression by *Real-Time qPCR*

The expression levels of nine genes (Chen et al., 2009), involved in different physiological processes, were followed by *Real Time qPCR* after nZVI remediation experiment (**Figure 52;** see also **Supplementary Table S25** for the values).

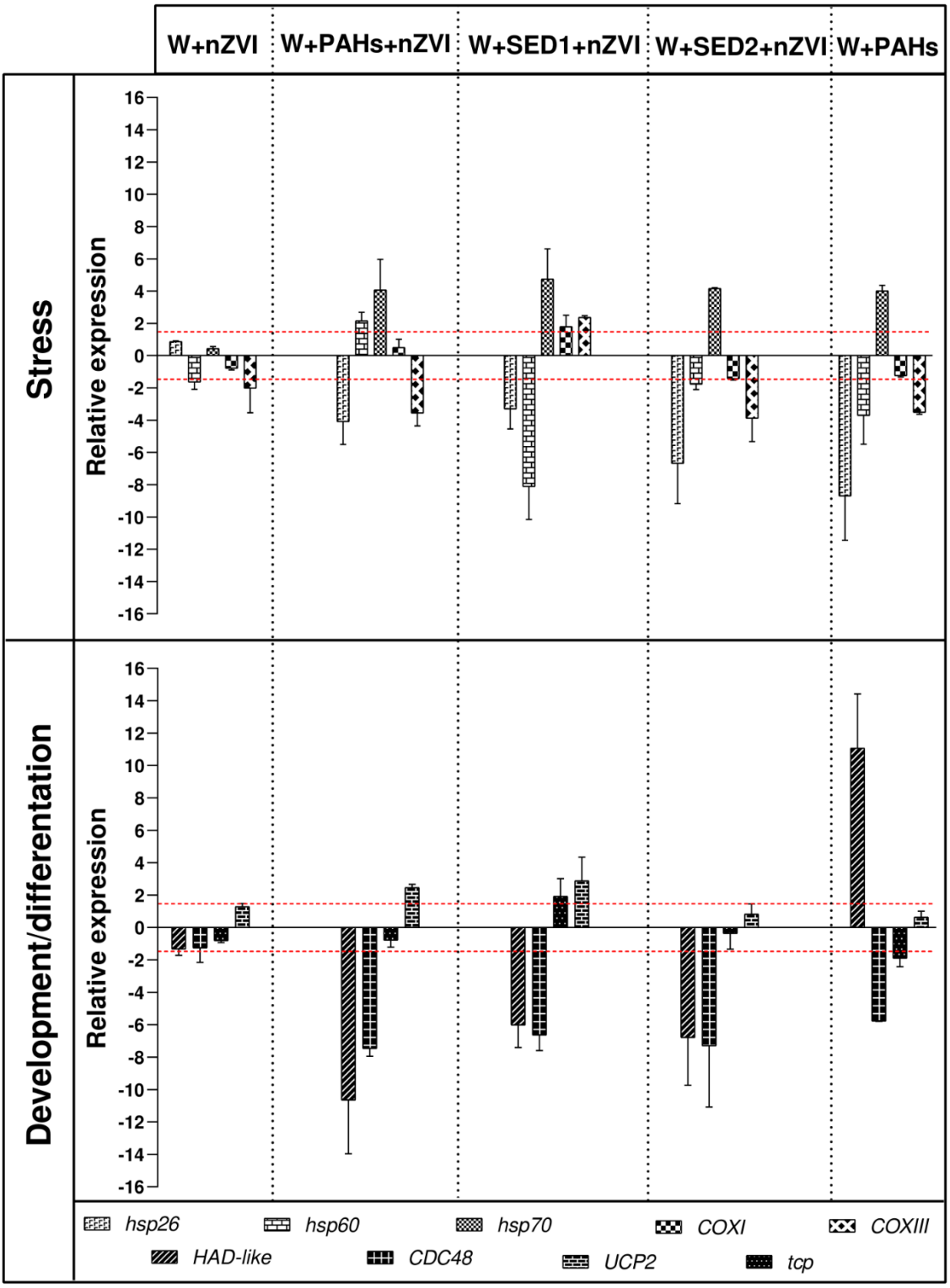

Figure 52. Histograms show the differences in expression levels of nine genes involved in stress response and in developmental processes. *A. franciscana* nauplii were exposed to W + nZVI, W + PAHs, W + PAHs + nZVI, W + SED1 + nZVI and W + SED2 + nZVI at 100%. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see **Supplementary Table S25** for the values).

Considering the stress response (**Figure 52**), *hsp 60* and *COXIII* were targeted by all experimental conditions. Specifically, *hsp 60* was down-regulated by W + nZVI, W + PAHs, W + SED1 + nZVI and W + SED2 + nZVI, but was up-regulated by W + PAHs + nZVI.

COXIII was down-regulated by W + nZVI, W + PAHs, W + PAHs + nZVI and W + SED2 + nZVI, but was up-regulated by W + SED1 + nZVI. Moreover, *hsp 26* and *hsp70* were down-regulated and up-regulated, respectively, by all conditions with exception of W + nZVI. Finally, *COXI* was up-regulated only by W + SED1 + nZVI (see **Table S25**).

Take into the consideration the genes involved in developmental processes (**Figure 52**), four genes were targeted by all conditions with exception of W + nZVI. Common molecular targets for all conditions were *HAD-like* and *CDC48*, of which *HAD-like* was down-regulated by W + PAHs + nZVI, W + SED1 + nZVI and W + SED2 + nZVI and up-regulated only by W + PAHs; whereas *CDC48* was down-regulated by all conditions. *tcp* was up-regulated by W + SED1 + nZVI, and down-regulated by W + PAHs; and *UCP2* was up-regulated by W + PAHs + nZVI and W + SED1 + nZVI (see **Table S25**).

Also, after AC remediation experiment, these nine genes expression levels were evaluated (**Figure 53;** see also **Supplementary Table S26** for the values).

Evaluating the stress response, three *heat shock protein*s were gene target for almost all test conditions. In particular, *hsp 60* was down-regulated by W + PAHs, and up-regulated by W + AC, W + SED1 + AC and W + SED2 + AC; whereas *hsp 70* was down-regulated by W + SED1 + AC, and up-regulated by W + PAHs, W + PAHs + AC and W + SED2 + AC. *hsp26* was molecular target only for W + PAHs showing a down-regulation. Moreover, *COXI* and *COXIII* were up-regulated by W + PAHs + AC, W + SED1 + AC and W + SED2 + AC; and only *COXIII* was down-regulated by W + PAHs (**Figure 53**).

Considering the impact on developmental processes (**Figure 53**), also in this case, four genes were targeted by all conditions with exception of W + AC.

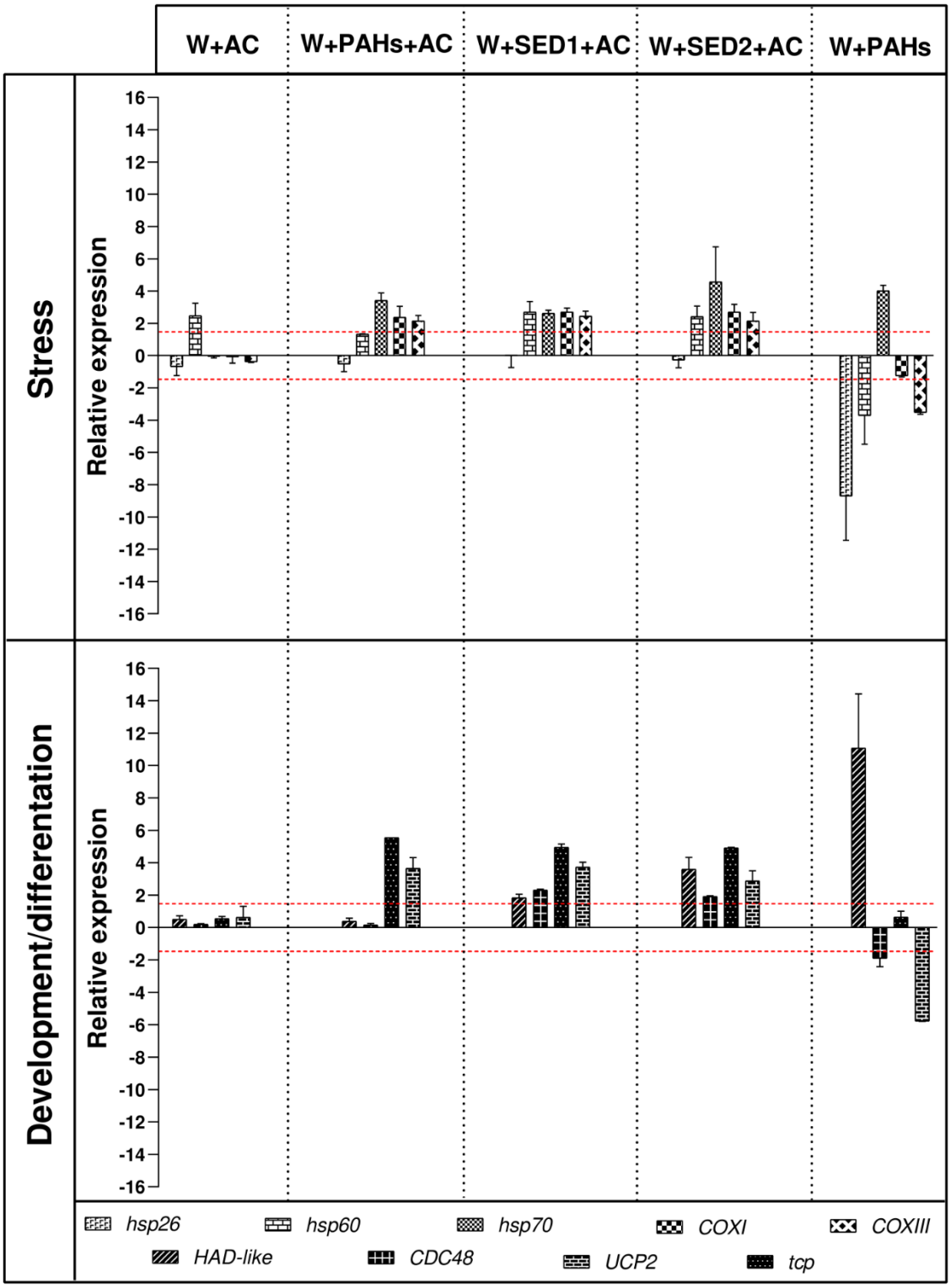

Figure 53. Histograms show the differences in expression levels of nine genes involved in stress response and in developmental processes. *A. franciscana* nauplii were exposed to W + AC, W + PAHs, W + PAHs + AC, W + SED1 + AC and W + SED2 + AC at 100%. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see **Supplementary Table S26** for the values).

Common molecular target for all conditions was *tcp* that showed an up-regulation after W + PAHs + AC, W + SED1 + AC and W + SED2 + AC treatment, and down-regulation after W + PAHs exposure. *HAD-like* was up-regulated by W + PAHs, W + SED1 + AC and W +

SED2 + AC; whereas *CDC48* was down-regulated by W + PAHs and up-regulated by W + SED1 + AC and W + SED2 + AC. Finally, *UCP2* was up-regulated by W + PAHs + AC, W + SED1 + nZVI and W + SED2 + AC (see **Table S26**).

5.4 Discussion

Experiments of remediation with nZVI and AC were able to drastically decrease PAHs concentration in aqueous solutions. In fact, nZVI and AC efficiency of removal were 99.6% and 98.3%, respectively. These results are in according to available literature. In fact, similar data have been showed after remediation experiment on aqueous solutions spiked both with trichloroethylene (TCE) and PAHs using a quantity of nZVI and AC corresponding to those used in the following study (Kim et al., 2010; Kumar et al., 2019).

When considered the removal of PAHs from sediment, we observed different results for the two sediment. In the case of sediment of Sarno (less polluted), both after AC and nZVI treatment, the PAHs were totally removal already after T3 (21 h). In fact, we observed the same percentage of removal, 98.9% (see **Table 19**). However, considering remediation of Bagnoli sediment (more polluted), nZVI and AC showed a percentage of removal of about 49.3% and 60.6%, respectively. Moreover, the results displayed that, for both nZVI and AC, PAHs concentrations of the less molecular weight are reduced to a greater extent than that for the more molecular weight. In fact, as reported in **Figures S16-S17**, these amendments is not able to completely remove the benzo(a)anthracene, Indeno(1,2,3-cd)pyrene and benzo(a)pyrene. Previous studies found that for PAH-spiked sediments, activated carbon and nZVI addition reduced significantly reduces the availability of low molecular weight products (Lebo et al., 2003; Zimmerman et al., 2005).

Significant mortality of *A. franciscana* was observed upon exposure to aqueous solutions containing up to 3% of AC and nZVI (see **Figures 50** and **51**). Specifically, considering the negative controls at T0, nZVI showed a little percentage of dead nauplii (about 6.6%) already at the lowest percentage (6.25%), which was of about 30% at the maximum concentration

(100%). Instead, AC displayed a small toxicity of about 20% only at the highest concentration. These results are in line with expectations (see **Chapter 4**; Albarano et al., 2021). Also, the data agree with previously reported observations of about 100% survival of different organisms, including *Daphnia magna*, on AC-enriched sediments (Cornelissen et al., 2006; Jonker et al., 2009; Lewis et al., 2016). This simple, first tier response thus suggests the absence of harmful effects of AC addition. However, a closer look at the exposure systems revealed an indication for toxic effects of nZVI. Keller et al., 2012 and Jaafar et al., 2018 showed similar results (about 60% of mortality) exposing *D. magna* to nZVI for 28 days.

Another interesting result is the large-scale genotoxicity information on *A. franciscana* generated in this study. In fact, the highest percentage of mortality caused by exposure to nZVI can be linked to the down-regulation of the majority of the studied genes. Firstly, all 9 genes were molecular targets of this amendment, with the only exception of *hsp26, hsp70, COXI, HAD-like, CDC48, UCP2* and *tcp* which were not molecular target of negative control (**Figure 52**). When considered the AC experiments, only *hsp60* was gene target of W + AC emphasizing the low toxicity of this amendments (**Figure 53**).

Summarizing the *Real Time qPCR* experiments of nZVI: i) two genes were targeted by all five experimental conditions; ii) four genes were targeted by all experimental conditions with exception of negative control; iii) one genes was only targets for W + PAHs and W + SED1 + nZVI; iv) only one gene was specifically affected only by W + PAHs + nZVI and W + SED1 + nZVI; v) one gene was targeted by W + SED1 + nZVI.

Take into consideration experiment with AC, we could observe: i) only one gene was gene target of negative control; iii) three genes were targeted by W+ PAHs, W + SED1 + AC and W + SED2 + AC; iv) two genes were targeted by W+ PAHs + AC, W + SED1 + AC and W + SED2 + AC; v) two genes were targeted by W+ PAHs, W + PAHs + AC, W + SED1 + AC and W + SED2 + AC; vi) one gene was gene target only of W+ PAHs.

The molecular response to nZVI appeared little different in comparison with AC: negative control of nZVI (W + nZVI) down-regulated two gene involved in stress response, *hsp60* and *COXIII* (see also Appendix A, **Supplementary Table S25**) compared to negative control of AC that up-regulated only 1 gene (*hsp60*). In previous studies, nZVI genotoxicity has been amply demonstrated. In fact, this amendment was able to cause high oxidative stress in plants *Allium bulbs*, in Bacterial strains *Erwinia amylovora, Xantomonas oryzae, Bacillus cereus* and *Streptomyces spp*., and also in *Artemia salina* (Barzan et al., 2014; Ghosh et al., 2017; Kumar et al., 2017).

All together these molecular results revealed that the affected genes in *A. franciscana* were involved both in the stress response and development processes. In fact, all genes belonging to these classes were affected by all experimental conditions.

All the results reported in this Chapter have been published in:

1) **Albarano L.**, Costantini M., Zupo V., Guida M., Libralato G. Comparison between AC and nZVI as restoring methods of common contaminants (PAHs) spiked marine sediment and evaluation of their toxic effects on crustacean *Artemia franciscana* (*in preparation*). (Appendix B)

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Appendix A

Supplementary Table S1. Chemical analyses of heavy metals on sea water collected a time zero (to, corresponding at the beginning of the experiments) and after one week of treatement with zeolite and activated carbon. Data are expressed as μg/L and the standard deviation is also reported for the values that are higher than the reference values.

Heavy metals		t0	1 week
Al		< 10	< 10
	SD		
Sb		< 2	< 3
	SD		0.6
As		24.6	21.5
	SD	5.2	0.7
Ba		< 30	90
	SD		16.9
Be		< 5	< 5
	SD		
B		4179.1	4802
	SD	919.4	771.8
Cd		< 5	< 5
	SD		
Co		< 2	< 2
	SD		
Cr		10	7
	SD	2.3	2.3
Fe		< 50	<50
	SD		
Mn		2.1	< 1
	SD	0.4	
Hg		< 0.5	< 0.5
	SD		
Mo		17.7	24.9
	SD	4.1	2
Ni		< 10	< 10
	SD		

Pb		< 1	< 1
	SD		
Cu		< 10	< 10
	SD		
Se		99.9	69.5
	SD	22	37.5
V		194.3	205
	SD	41	6.4
Zn		13.2	13.8
	SD	2.6	0.2

Supplementary Table S2. Chemical analyses of ammonia, nitrates, nitrites an phosphates on sea water collected a time zero (to, corresponding at the beginning of the experiments) and after one week (1 week) of treatement with zeolite and activated carbon. Data are expressed as mg/L and the standard deviation is also reported for the values that are higher than the reference values.

Sample	Ammonia	Nitrite	Nitrate	Phosphates	
					SD
t0	< 0.05	< 0.05	< 1	< 0.15	
1 week	< 0.05	< 0.05	< 1	0.3	0.3

Supplementary Table S3. Sample names, condition and paired reads for each sample. Control: samples deriving from adults reared in tanks with water and sediment without contaminants; Treated 1, samples deriving from adults reared in tanks with sediment contaminated with PAHs; Treated 2, samples deriving from adults reared in tanks with sediment contaminated with PCBs.

Samples	Sample_Name	Condition	#Paired Reads
1	Control_1	Control	21,023,491
2	Control_2		18,633,056
3	Control_3		20,875,354
4	Treated1_1	Treated1	18,430,344
5	Treated1_2		18,277,648
6	Treated1_3		20,716,663
7	Treated2_1	Treated2	20,751,933
8	Treated2_2		19,621,258
9	Treated2_3		20,793,011

Supplementary Table S4. Gene name, acronym and reference of 62 genes.

	Gene name	Acronym	Reference
Stress	*ADP-ribosylation factor 1*	*ARF1*	Esposito et al., 2020
	caspase-8	*CASP8*	Romano et al., 2011
	Sp-Cspe3/7L	*caspase 3/7*	Ruocco et al., 2016
	Cytochrome b	*cytb*	Marrone et al., 2012
	DNA-methyltransferase 1	*MTase*	Marrone et al., 2012
	ERCC excision repair 3	*ERCC3*	Ruocco et al., 2019a
	Glutamine synthetase	*GS*	Marrone et al., 2012
	Glyoxylate reductase/hydroxypyruvate reductase	*GRHPR*	Esposito et al., 2020
	Heat Shock Protein 56	*hsp56*	Marrone et al., 2012
	Heat Shock Protein 60	*hsp60*	Marrone et al., 2012
	Heat Shock Protein 70	*hsp70*	Marrone et al., 2012
	Hypoxia inducible factor 1-alpha	*HIF1A*	Varrella et al., 2016a
	Nuclear factor kappalight-chain-enhancer of activated B cells	*NF-kB*	Russo et al., 2014
	Poly(ADP-ribose) polymerase 1	*PARP-1*	Esposito et al., 2020
	p38 mitogen-activated protein kinase		

		p38 MAPK	Marrone et al., 2012
	Succinate dehydrogenase	*SDH*	Esposito et al., 2020
	Tumor protein p53	*p53*	Varrella et al., 2016
	14-3-3 epsilon protein	*14-3-3 ε*	Marrone et al., 2012
Development/ Differentiation	*ALG-2 interacting protein X/1*	*Alix*	Varrella et al., 2014
-			
	Antidorsalizing morphogenetic protein 2	*ADMP2*	Esposito et al., 2020
	Blastula protease 10	*BP10*	Marrone et al., 2012
	Blimp	*Blimp*	Varrella et al., 2014
	Brachyury	*Bra*	Esposito et al., 2020
	Cadherin-associated protein (catenin) delta 2	*δ-2-catenin*	Varrella et al., 2016a
	c-Jun N-terminal kinase	*JNK*	Ruocco et al., 2017
	Delta	*Delta*	Esposito et al., 2020
	Forkhead box protein A	*FOXA*	Ruocco et al., 2017
	Forkhead box protein G	*FoxG*	Ruocco et al., 2017
	Forkhead box protein O	*Foxo*	Ruocco et al., 2017
	Goosecoid	*Goosecoid*	

		Esposito et al., 2020
Growth factor indipendent 1	*GFI1*	Ruocco et al., 2017
Hatching enzyme	*hat*	Marrone et al., 2012
Histone H3.3	*H3.3*	Esposito et al., 2020
Kinesin-19	*KIF19*	Esposito et al., 2020
Nodal	*nodal*	Ruocco et al., 2017
Notch	*Notch*	Esposito et al., 2020
One Cut Homeobox 1	*OneCut*	Ruocco et al., 2017
Smad6	*Smad6*	Esposito et al., 2020
SRY (sex determining region Y)-box9	*sox9*	Marrone et al., 2012
TGF beta-activated kinase	*TAK1*	Ruocco et al., 2017
Transcription factor 4	*tcf4*	Ruocco et al., 2017
Transcription factor 7	*TCF7*	Ruocco et al., 2017
Vascular endothelial growth factor	*VEGF*	Ruocco et al., 2017
Wnt5	*Wnt5*	Varrella et al., 2014
Wnt6	*Wnt6*	Varrella et al., 2014
Wnt8	*Wnt8*	Varrella et al., 2014

Skeletogenesis	*Bone morphogenetic protein 5-7*	*BMP5-7*	Marrone et al., 2012
	Jun	*C-jun*	Russo et al., 2014
	Nectin	*Nec*	Marrone et al., 2012
	Pl-p16	*p16*	Costa et al., 2012
	Pl-p19	*p19*	Costa et al., 2012
	Spicule matrix protein 30	*SM30*	Marrone et al., 2012
	Spicule matrix protein 50	*SM50*	Marrone et al., 2012
	Univin	*uni*	Marrone et al., 2012
Detoxification	*Catalase*	*CAT*	Varrella et al., 2014
	Multi drug resistance protein 1	*MDR1*	Varrella et al., 2014
	Metallothionein	*MT*	Marrone et al., 2012
	Metallothionein 4	*MT4*	Ragusa et al., 2013
	Metallothionein 5	*MT5*	Ragusa et al., 2013
	Metallothionein 6	*MT6*	Ragusa et al., 2013
	Metallothionein 7	*MT7*	Ragusa et al., 2013
	Metallothionein 8	*MT8*	

Supplementary Table S5. Chemical analyses of Total PAHs, Total PCBs and Zn on sediment collected at time zero (t0), corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED.

	t0													
(µg/L)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
NAP	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACY	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PHRE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
CHR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(b)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(k)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
IPYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005

DB(a,h)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(ghi)PER	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PCB1	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB5	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB18	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB31	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB44	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB52	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB66	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB87	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB101	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB110	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB138	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB141	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB151	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB153	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB170	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

PCB180	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB183	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB187	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB206	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Zn	-	-	-	-	-	-	< 2	3.2	5.8	12.4	11.9	< 2	1.5	7.4

Supplementary Table S6. Chemical analyses of Total PAHs, Total PCBs and Zn on seawater collected at time zero (t0), corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED, 15-16=W.

	t0															
(µg/L)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
NAP	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACY	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PHRE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PYR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
CHR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(b)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(k)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)PYR	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
IPYR	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

DB(a,h)ANT	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(ghi)PER	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB1	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB5	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB18	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB31	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB44	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB52	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB66	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB87	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB101	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB110	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB138	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB141	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB151	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB153	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB170	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

PCB180	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB183	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB187	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB206	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Zn	-	-	-	-	-	-	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1

Supplementary Table S7. Chemical analyses of Total PAHs, Total PCBs and Zn on sediment collected at time one (t1), corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED.

	t1													
(µg/L)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
NAP	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACY	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PHRE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
CHR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(b)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(k)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
IPYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005

DB(a,h)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(ghi)PER	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PCB1	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB5	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB18	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB31	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB44	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB52	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB66	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB87	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB101	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB110	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB138	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB141	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB151	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB153	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB170	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

PCB180	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB183	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB187	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB206	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Zn	-	-	-	-	-	-	81.1	61.6	52.0	78.0	51.5	69.9	18.1	20.6

Supplementary Table S8. Chemical analyses of Total PAHs, Total PCBs and Zn on seawater collected at time one (t1), corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED, 15-16=W.

	t1														
(µg/L)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**14**	**15**	**16**
NAP	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACY	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PHRE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PYR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
CHR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(b)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(k)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)PYR	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
IPYR	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

DB(a,h)ANT	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(ghi)PER	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB1	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB5	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB18	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB31	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB44	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB52	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB66	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB87	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB101	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB110	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB138	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB141	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB151	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB153	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB170	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

PCB180	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB183	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB187	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB206	-	-	-				-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Zn	-	-	-	-	-	-	4620	1095	1215	1092	1263	1148	< 1	< 1	< 1

Supplementary Table S9. Chemical analyses of Total PAHs, Total PCBs and Zn on sediment collected at tf, corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED.

	tf													
(µg/L)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NAP	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACY	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ACE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PHRE	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
CHR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(b)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(k)FLT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(a)PYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
IPYR	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005

DB(a,h)ANT	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
B(ghi)PER	< 0.005	< 0.005	< 0.005	-	-	-	-	-	-	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
PCB1	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB5	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB18	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB31	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB44	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB52	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB66	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB87	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB101	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB110	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB138	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB141	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB151	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB153	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB170	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

PCB180	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB183	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB187	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PCB206	-	-	-	< 0.001	< 0.001	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Zn	-	-	-	-	-	-	74.5	99.8	75.3	118.4	67.1	95.4	14.2	17.7

Supplementary Table S10. Chemical analyses of Total PAHs, Total PCBs and Zn on seawater collected at tf, corresponding at the beginning of the experiments, before adding compounds. The samples are numbered as 1-2-3=W+SED+PAHs, 4-5-6=W+SED+PCBs, 7-8-9=W+SED+Zn, 10-11-12= W+SED+Mix; 13-14= W+SED; 15-16=W.

	tf															
(µg/L)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
NAP	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACY	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ACE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PHRE	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PYR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
CHR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(b)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(k)FLT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(a)PYR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
IPYR	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

DB(a,h)ANT	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
B(ghi)PER	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB1	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB5	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB18	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB31	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB44	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB52	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB66	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB87	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB101	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB110	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB138	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB141	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB151	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB153	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB170	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

PCB180	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB183	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB187	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
PCB206	-	-	-	< 0.05	< 0.05	< 0.05	-	-	-	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Zn	-	-	-	-	-	-	110	115	163	103	127	121	< 1	< 1	< 1	< 1	

Supplementary Table S11. Adult sizes (in millimetres) and gonadal index (GI ± SD, n = 5/group) of adults of sea urchin *P. lividus* collected in the field at the beginning (t0) and after two months in the three experimental conditions: W, W+SED, W+SED+PAHs and W+SED+PCBs (p value > 0.05). W = water; W+SED = Water+ sediment; W+SED+PAHs = Water + sediment + PAHs; W+SED+PCBs = Water + sediment + PCBs.

		W	W+SED	W+SED+ PAHs	W+SED+PCBs
Adult size,	50.5 ± 4.36				
t_0		44.5 ± 1.1	51.9 ± 5.6	58.8 ± 5.3	56.6 ± 3.7
2 months		37.2 ± 9.6	52.2 ± 5.1	57.6 ± 5.9	56.1 ± 3.9
GI					
t_0	3.68 ± 0.01				
2 months		0.88 ± 0.01	3.78 ± 0.02	2.32 ± 0.02	2.22 ± 0.004

Supplementary Table S12. Number of differentially expressed (DE) genes and isoforms, identified with a False Discovery Rate (FDR) ≤ 0.05, and with a Fold Change (FC) ≥ 1.5 (upregulated genes/isoforms) and ≤ −1.5 (down-regulated genes/isoforms).Control: plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants; Treated1: plutei deriving from adults exposed for two months to sediment contaminated with PAHs; Treated2: plutei deriving from adults exposed for two months to sediment contaminated with PCBs.

	FDR ≤ 0.05	**FC ≥ 1.5 FDR ≤ 0.05**	**FC ≤ -1.5 FDR ≤ 0.05**
Genes			
Treated1 vs Control	1898	933	965
Treated2_ vs Control	2396	1079	1317
Treated2 vs Treared1	1356	755	601
Isoforms			
Treated1 vs Control	5591	2200	3388
Treated2_ vs Control	7703	3715	3985
Treated2 vs Treared1	4762	2911	1850

Supplementary Table S13. Common up-regulated genes in the Venn diagrams (for more details see also **Figure 12** and legend to this figure) among the three experimental groups: "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control" (plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants, "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2".

Up-regulated genes
"Treated_1 vs Control" and "Treated_2 vs Control"
DNA replication licensing factor mcm7-A isoform X1
unnamed protein product
diamine acetyltransferase 2
arylsulfatase
PREDICTED: uncharacterized protein LOC578221
high-affinity choline transporter 1
PREDICTED: uncharacterized protein LOC100889675
PREDICTED: fibropellin-3
PREDICTED: uncharacterized protein LOC100891625
nucleolin isoform X3
PREDICTED: titin
spectrin alpha chain, non-erythrocytic 1 isoform X6
185/333
tethering factor for nuclear proteasome STS1 isoform X2
tryptophan 5-hydroxylase 1 isoform X1
high-affinity choline transporter 1-like
DYI2_HELCRRecName: Full=Dynein intermediate chain 2, ciliary
hypothetical protein B7P43_G03953
putative nuclease HARBI1
dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
nodal
putative TBC1 domain family member 2A
PREDICTED: uncharacterized protein LOC105436430
threonine synthase-like 1
E3 ubiquitin-protein ligase RNF8 isoform X1
PREDICTED: trypsin-1-like
uncharacterized protein LOC111859709
PREDICTED: uncharacterized protein LOC575796

MFS-type transporter SLC18B1

antivin/lefty

deleted in malignant brain tumors 1 protein

PREDICTED: uncharacterized protein LOC105441695

PREDICTED: uncharacterized protein LOC100888967

hypothetical protein pdam_00008083

cell wall-associated hydrolase

alpha-5 collagen

retinol dehydrogenase 8

MYCBP-associated protein isoform X4

heparan sulfate 2-O-sulfotransferase 1

PREDICTED: uncharacterized protein LOC100889612

PREDICTED: uncharacterized protein LOC754545

PREDICTED: uncharacterized protein LOC756027

uncharacterized protein LOC113054557

condensin complex subunit 3 isoform X1

PREDICTED: uncharacterized protein LOC105444166

transmembrane 9 superfamily member 4

PREDICTED: uncharacterized protein LOC105439689

PREDICTED: uncharacterized protein LOC764367

DNMT1_PARLIRecName: (cytosine-5)-methyltransferase PliMCI

NFU1 iron-sulfur cluster scaffold homolog, mitochondrial

hypothetical protein BSL78_07137, partial

PREDICTED: uncharacterized protein LOC108671849

forkhead transcription factor Q2

ubiquitin carboxyl-terminal hydrolase CYLD

uncharacterized protein K02A2.6-like

hypothetical protein X975_19031, partial

PREDICTED: uncharacterized protein LOC105447528

putative RNA-directed DNA polymerase from transposon BS

PREDICTED: uncharacterized protein LOC105439906

PREDICTED: uncharacterized protein LOC105442605

PREDICTED: uncharacterized protein LOC100890383

reverse transcriptase family protein

dnaJ homolog subfamily B member 13

laminin subunit alpha

meiosis-specific protein MEI4-like

uncharacterized protein LOC114575677

PREDICTED: uncharacterized protein LOC100892571

hypothetical protein BBROOKSOX_704

PREDICTED: uncharacterized protein LOC105444429

PREDICTED: uncharacterized protein LOC105326080

long-chain-fatty-acid--CoA ligase 4

coiled-coil domain-containing protein 40

PREDICTED: uncharacterized protein LOC590579

PREDICTED: uncharacterized protein LOC105335220

"Treated_1 vs Control", "Treated_2 vs Control" and "Treated_1 vs Treated_2"
putative RNA-directed DNA polymerase from transposon X-element
PREDICTED: uncharacterized protein LOC100890353
hypothetical protein
PREDICTED: uncharacterized protein LOC105439418
RNA-directed DNA polymerase from mobile element jockey-like
PREDICTED: uncharacterized protein LOC105444767
protein FAM166B
uncharacterized protein LOC110990766
PREDICTED: uncharacterized protein K02A2.6-like
PREDICTED: uncharacterized protein LOC105440140
PREDICTED: uncharacterized protein LOC587493
PREDICTED: uncharacterized protein LOC577099
uncharacterized protein LOC110984997 isoform X1
endonuclease-reverse transcriptase
craniofacial development protein 2-like
RNA-directed DNA polymerase from mobile element jockey
hypothetical protein DSY43_04445
PREDICTED: uncharacterized protein LOC589347

"Treated_1 vs Control" and "Treated_1 vs Treated_2"
annexin A4
heme-binding protein 2

proteasome subunit beta type-1-B

PREDICTED: uncharacterized protein LOC105444208

"Treated_2 vs Control" and "Treated_1 vs Treated_2"
PREDICTED: uncharacterized protein LOC105443609
uncharacterized protein LOC110987973
haloacid dehalogenase-like hydrolase domain-containing protein 2
uncharacterized protein LOC111085826
biogenesis of lysosome-related organelles complex 1 subunit 1
PREDICTED: uncharacterized protein LOC105441676
PREDICTED: mucin-4
PREDICTED: uncharacterized protein LOC105437795
PREDICTED: uncharacterized protein LOC105912973
PREDICTED: uncharacterized protein LOC105447150
PREDICTED: uncharacterized protein LOC105441670
protein KRI1 homolog
homeobox protein cut-like 1 isoform X1
low-density lipoprotein receptor-related protein 1B isoform X1
uncharacterized protein LOC110984676 isoform X7
PREDICTED: uncharacterized protein LOC107350581
putative DNA transposase THAP9-like
PREDICTED: uncharacterized protein LOC105437731
PREDICTED: uncharacterized protein LOC754161
PREDICTED: uncharacterized protein LOC578465
PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC107348641
toll-like receptor 3
uncharacterized protein LOC110975147 isoform X1
hypothetical protein AC249_AIPGENE18192
hypothetical protein CIL05_21475
PREDICTED: uncharacterized protein LOC100890507
PREDICTED: uncharacterized protein LOC107334619
long-chain-fatty-acid--CoA ligase 5-like
PREDICTED: uncharacterized protein LOC100893480 isoform X5
hypothetical protein AWC38_SpisGene22463
PREDICTED: sialin

PREDICTED: plexin-B3-like

hypothetical protein BSL78_27382

PREDICTED: uncharacterized protein LOC105440726

PREDICTED: uncharacterized protein LOC105437364

PREDICTED: uncharacterized protein LOC105442164, partial

uncharacterized protein LOC110234800

pre-rRNA-processing protein TSR1 homolog

PREDICTED: uncharacterized protein LOC105444020

sodium/myo-inositol cotransporter 2 isoform X1

PREDICTED: mucin-22-like

deleted in malignant brain tumors 1 protein-like

cystine/glutamate transporter

V(D)J recombination-activating protein 1-like isoform X2

zinc-carboxypeptidase, putative

PREDICTED: uncharacterized protein LOC100890950

PREDICTED: uncharacterized protein LOC108087055

PREDICTED: latrophilin-1

PREDICTED: uncharacterized protein LOC105442991

recombination activating protein 2-like

PREDICTED: uncharacterized protein LOC105445996

PREDICTED: uncharacterized protein LOC105440296

sucrase-isomaltase, intestinal

PREDICTED: uncharacterized protein LOC105447620, partial

PREDICTED: uncharacterized protein LOC105445209

hypothetical protein BSL78_14139

hypothetical protein BSL78_24528, partial

PREDICTED: uncharacterized protein LOC105443558

uncharacterized protein LOC115369174

hyphally regulated cell wall protein 3

PREDICTED: uncharacterized protein LOC100893587

uncharacterized protein LOC110464703

Supplementary Table S14. Common down-regulated genes in the Venn diagrams among the three experimental groups: "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control" (plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants, "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2".

Down-regulated genes
"Treated_1 vs Control" and "Treated_2 vs Control"
PREDICTED: uncharacterized protein LOC105440703 isoform X1
E3 ubiquitin-protein ligase TRIM33-like
cGMP-dependent protein kinase 1 isoform X5
PREDICTED: uncharacterized protein LOC105441665
zinc finger protein 862-like
galactosylceramide sulfotransferase-like
UDP-glucuronosyltransferase 2B33-like
PREDICTED: uncharacterized protein LOC105444646
PREDICTED: uncharacterized protein LOC105444794
methylmalonyl-CoA epimerase, mitochondrial
neurogenic locus notch homolog protein 1
zinc finger and SCAN domain-containing protein 29-like
PREDICTED: uncharacterized protein LOC100888828
solute carrier family 28 member 3 isoform X1
uncharacterized protein LOC110061264
PREDICTED: uncharacterized protein LOC109618538
hypothetical protein CRYPA_1785
PREDICTED: uncharacterized protein LOC764539
uncharacterized protein LOC113684170, partial
EGF, latrophilin and seven transmembrane domain-containing protein 1-like
AAEL014742-PA, partial
serine/arginine-rich SC35-like splicing factor SCL33
PREDICTED: uncharacterized protein LOC100893955
fibrillin-1-like
structural maintenance of chromosomes protein 1A isoform X2
uncharacterized protein LOC111113885
gastrula zinc finger protein XlCGF8.2DB-like
PREDICTED: uncharacterized protein LOC105447180
PREDICTED: uncharacterized protein LOC105444769
uncharacterized protein LOC111321513
equilibrative nucleoside transporter 1-like
isocitrate dehydrogenase [NADP] cytoplasmic isoform X2

cilia- and flagella-associated protein 221

putative nuclease HARBI1

hypothetical protein AC249_AIPGENE2135

uncharacterized protein LOC110245901

beta-1,3-galactosyltransferase 1-like

zinc transporter 2

uncharacterized protein LOC110441045

cystine/glutamate transporter

uncharacterized protein LOC110977661

PREDICTED: uncharacterized protein LOC107343991

PREDICTED: uncharacterized protein LOC105446914

unnamed protein product

hypothetical protein, partial

uncharacterized protein LOC115366985, partial

protein NYNRIN-like

PREDICTED: uncharacterized protein LOC100893140

uncharacterized protein LOC110239146

PREDICTED: uncharacterized protein LOC105444695

PREDICTED: uncharacterized protein LOC581299 isoform X10

LINE-1 retrotransposable element ORF2 protein

PREDICTED: uncharacterized protein LOC589347

uncharacterized protein LOC110062710, partial

sushi domain-containing protein 2-like

GRIP and coiled-coil domain-containing protein 2-like

beta-1,4-galactosyltransferase 6-like

PREDICTED: uncharacterized protein LOC105441043

RNA-binding protein 34

kremen protein 2

PREDICTED: uncharacterized protein LOC580499

PREDICTED: uncharacterized protein LOC105440451, partial

uncharacterized protein LOC114531265

beta-1,4-galactosyltransferase 6

PREDICTED: uncharacterized protein LOC100888223

DNA (cytosine-5)-methyltransferase 3A

hypothetical protein BSL78_13033

PREDICTED: uncharacterized protein LOC105438475

hypothetical protein DSY43_04445

PREDICTED: uncharacterized protein LOC105437955

PREDICTED: uncharacterized protein LOC105439418

heparan sulfate glucosamine 3-O-sulfotransferase 1-like

DENN domain-containing protein 4C

Exodeoxyribonuclease III

PREDICTED: uncharacterized protein LOC105440670

PREDICTED: uncharacterized protein LOC588798, partial

PREDICTED: uncharacterized protein LOC105443839

PREDICTED: chondroadherin-like

PREDICTED: uncharacterized protein LOC100890838

centrosome-associated protein CEP250 isoform X4

uncharacterized protein LOC115383145

PREDICTED: uncharacterized protein LOC105444738

tripartite motif-containing protein 5-like

rho-related BTB domain-containing protein 1

prostatic spermine-binding protein-like

probable thiopurine S-methyltransferase

PREDICTED: uncharacterized protein LOC105439906

hypothetical protein LOTGIDRAFT_145246, partial

acid ceramidase

hypothetical protein DJ031_00320, partial

lysine-specific demethylase 8

Transposon TX1 uncharacterized 149 kDa protein

hydroxyacid oxidase 1

uncharacterized protein LOC114967315

deleted in malignant brain tumors 1 protein

hypothetical protein KP79_PYT01408

zinc transporter ZIP10

piggyBac transposable element-derived protein 4-like

PREDICTED: uncharacterized protein LOC105339229

phospholipase B1, membrane-associated

4-hydroxyphenylpyruvate dioxygenase-like protein

PREDICTED: uncharacterized protein LOC100368444

transient receptor potential cation channel subfamily A member 1 homolog

PREDICTED: uncharacterized protein LOC764243 isoform X1

"Treated_1 vs Control", "Treated_2 vs Control" and "Treated_1 vs Treated_2"
RNA-directed DNA polymerase from mobile element jockey-like
putative RNA-directed DNA polymerase from transposon BS
reverse transcriptase family protein
hypothetical protein
uncharacterized protein LOC110990766
putative RNA-directed DNA polymerase from transposon X-element
PREDICTED: uncharacterized protein LOC100888345
polyprotein
RNA-directed DNA polymerase from mobile element jockey
P2X purinoceptor 7-like
hypothetical protein DSY43_00385
uncharacterized protein LOC110975082

"Treated_1 vs Control" and "Treated_1 vs Treated_2"
MAM and LDL-receptor class A domain-containing protein 1
PREDICTED: uncharacterized protein LOC105446188
uncharacterized protein LOC106176709
PREDICTED: uncharacterized protein K02A2.6-like

"Treated_2 vs Control" and "Treated_1 vs Treated_2"
putative IQ motif and ankyrin repeat domain-containing protein
PREDICTED: uncharacterized protein LOC584294
PREDICTED: uncharacterized protein LOC105441319
PREDICTED: uncharacterized protein LOC100893260
Extracellular matrix protein 3
PREDICTED: uncharacterized protein LOC105439932 isoform X1
PREDICTED: hyalin
transposase
carboxypeptidase B
serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like
GD13022

proteasome subunit beta type-7

PREDICTED: uncharacterized protein LOC590790, partial

transient receptor potential cation channel subfamily A member 1

uncharacterized protein K02A2.6-like

PREDICTED: uncharacterized protein LOC105442928

Retrovirus-related Pol polyprotein from transposon 412

PREDICTED: uncharacterized protein LOC105446133

PREDICTED: uncharacterized protein LOC105437502

mitochondrial 10-formyltetrahydrofolate dehydrogenase isoform X1

PREDICTED: uncharacterized protein LOC106522096

DNA topoisomerase 2-alpha-like

PREDICTED: uncharacterized protein LOC105437506

PREDICTED: uncharacterized protein F54H12.2-like

kinesin-like protein KIF20A

Elongator complex protein 3

carbonyl reductase [NADPH] 1

PREDICTED: uncharacterized protein LOC105437736

non-specific lipid-transfer protein

sulfotransferase 1C2A

low-density lipoprotein receptor-related protein 12-like

histone chaperone ASF1A

hypothetical protein BSL78_19340

sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1

retinol dehydrogenase 8

uncharacterized protein LOC114544649

PREDICTED: uncharacterized protein LOC100891882

uncharacterized protein LOC113008516

PREDICTED: uncharacterized protein LOC100892824

poly(U)-specific endoribonuclease isoform X2

aldehyde dehydrogenase, mitochondrial

PREDICTED: uncharacterized protein LOC100888657 isoform X1

hyaluronidase-1 isoform X2

PREDICTED: uncharacterized protein LOC100890018 isoform X5

gamma-butyrobetaine dioxygenase

microsomal glutathione S-transferase 1

PREDICTED: alpha-amylase

KLF2/4

PREDICTED: uncharacterized protein LOC764164, partial

uncharacterized protein LOC110989726

protein dispatched homolog 1

PREDICTED: uncharacterized protein LOC756169

Supplementary Table S15. Data of expression levels in embryos at the pluteus stage, deriving from sea urchins exposed to PAHs and PCBs, were reported as a fold difference (in red up-expressed genes; in green down-expressed genes) from control (represented by embryos deriving from adults of sea urchins reared in tanks with sediment without contaminants) at 48 hpf. Fold differences greater than ± 1.5 were considered significant. The genes were classified according the four functional classes (A = stress, B = development/differentiation, C = skeletogenesis and D = detoxification, reported in **Figure 15**).

		PAH	PCB
Stress	*ARF1*	-2.090	-0.540
	caspase 3/7	-1.631	0.743
	CASP8	4.973	5.605
	CYP-2UI	2.808	2.977
	cytb	4.172	5.824
	ChE	-5.976	-5.870
	ERCC3	1.826	0.168
	GAPDH	-2.018	-2.028
	GRHPR	1.242	0.810
	GS	1.026	5.596
	GST	7.307	7.372
	HIF1A	-0.244	3.414
	hsp56	0.609	5.966
	hsp60	0.424	-0.126
	hsp70	0.052	-0.679
	hsp75	-4.736	-3.083
	hsp90	-2.337	-2.371
	MTase	3.649	6.887
	NF-kB	0.113	0.126
	PARP	4.552	5.698
	PKS	-8.996	-6.475
	p38 MAPK	0.702	-0.070
	p53	-5.695	-3.784
	SDH	5.715	7.020
	SULT1	-9.998	-9.287
	TNF	5.633	8.084
	14-3-3 ε	0.341	8.390
Development/Differentation	*ADMP2*	0.406	0.243

Alix	2.007	2.815
Blimp	0.959	2.707
BP10	0.019	2.436
BRA	2.836	2.787
CM-K	1.917	3.980
CREB	5.407	6.902
DELTA	0.067	0.206
δ-2-catenin	-0.690	-0.483
EGF	1.810	5.823
FOXA	4.301	4.561
FoxG	1.899	0.994
Foxo	3.792	3.750
FZ-7	-1.453	3.710
GFI1	2.472	2.174
GOOS	7.158	7.929
hat	-6.389	-5.232
HH	5.344	1.790
H3.3	-0.013	3.992
JAK	4.412	3.314
JNK	7.473	8.191
KIF19	-0.338	7.176
Lefty	-4.885	-4.797
M-Vg1	2.433	2.384
NLK	2.182	8.600
nodal	-0.125	8.743
NOTCH	-0.009	0.345
NOTCHLESS	3.543	7.827
OneCut	4.846	7.933
PLAUF3	2.399	2.125
PLC	8.740	8.440
Ptc	-5.741	-2.837
SMAD6	0.835	0.461
Smo	-6.852	-5.311

	sox9	5.187	6.054
	STAT1	0.898	5.886
	TAK1	6.965	7.495
	tcf4	7.050	7.196
	TCF7	6.020	6.525
	VEGF	-0.453	0.228
	Wnt5	0.785	0.199
	Wnt6	2.876	2.941
	Wnt8	0.150	3.331
Skeletogenesis	*BMP5-7*	0.018	1.189
	C-jun	-0.128	-0.033
	Nec	5.117	5.986
	p16	0.901	1.388
	p19	5.233	6.792
	SM30	5.838	13.627
	SM50	9.982	10.616
	uni	-3.552	0.050
Detoxification	*CAT*	0.171	0.502
	MDR1	2.587	2.710
	MT	3.573	3.589
	MT4	2.662	1.978
	MT5	2.312	0.543
	MT6	4.219	4.334
	MT7	2.348	2.563
	MT8	14.667	17.053
	NADH	-1.889	-2.757

Supplementary Table S16. Data of expression levels in adults exposed to NAP, PHE, FLT and BkF, were reported as a fold difference (in red up-expressed genes; in green down-expressed genes) from control (represented by adults of crustaceans reared in SW without contaminants) for 48 h. Fold differences greater than ± 1.5 were considered significant.

		Naphthalene	Phenanthrene	Fluoranthene	Benzo-k-fluoranthene
Stress	***hsp26***	-2.16	5.20	1.96	2.56
	hsp60	3.02	4.03	3.48	4.09
	hsp70	0.31	3.35	-1.51	-1.37
	COXI	6.92	8.87	0.17	-1.77
	COXIII	7.77	12.65	0.79	1.65

Supplementary Table S17. Data of expression levels in adults exposed to NAP, PHE, FLT and BkF, were reported as a fold difference (in red up-expressed genes; in green down-expressed genes) from control (represented by adults of crustaceans reared in SW without contaminants) for 48 h. Fold differences greater than ± 1.5 were considered significant.

		NAP	PHE	FLT	BkF
Stress	***hsp26***	-1.81	2.70	0.95	1.23
	hsp60	0.42	3.32	4.92	6.52
	hsp70	0.67	0.40	0.69	1.93
	COXI	-0.82	-3.92	0.90	2.61
	COXIII	-0.83	-3.59	-0.59	2.03
Development/differentiation	***HAD-like***	-4.4845	6.8765	3.05	7.02
	tcp	2.7405	7.4955	-2.53	2.85
	UCP2	6.8885	12.3165	2.04	2.16
	CDC48	3.582	8.558	-7.26	3.94

Supplementary Table S18. Sediment treatment (1), group of organisms (2), species (3), time of exposure (days; *h; +min) (4), concentrations (g/L) (5), endpoints (6), effects (%) (7), water quality parameters (8) and references (9) of negative impact of contaminated sediment restoring. M = Mortality, BSAF = biota-sediment accumulation factor, BR = Bioaccumulation reduction, IG = inhibition growt and R= reproduction, n.e. = not effect, T = Temperature, RT = room temperature, DOC = dissolved organic carbon, COD = chemical oxygen demand, TAN = total ammonia nitrogen, H = hardness, A = alkalinity, n.a. = not available

1	2	3	4	5	6	7	8	9
AC	Annelids			14.4	R and IG	n.e. and 60	RT	(Han et al., 2017)
				12	R and IG	4 and 97.5	RT	(Han et al., 2015)
			28	4.8	IG	70	RT	(Rakowska et al., 2012)
				72	R and IG	78 and 90	RT	(Nybom et al., 2015)
		Lumbriculus variegatus	56	12.5	BR	2.6	T = 22.4 - 23.5, pH = 7.9, H = 320-390, A = 48=100, TAN = <0.05	(Sun and Ghosh, 2007)
			4	19.2	M	60	T = 20	(Jonker et al., 2009)
			28	12	IG, M and R	81, 9 and 63	pH = 6.5 - 7.5, RT	(Nybom et al., 2015)
			28	9.6	R	75	T = 16, pH = 8.0, DOC = 9.4, COD = 26.5,	(Stalter et al., 2010)

						TAN = 5.35	
		28	36	BR	45	T = 20, pH = 6-9, TAN = <10	(Abel and Akkan, 2019)
		28	4.8	IG and BR	92 and 88	T = 20	(Kupryianchyk et al., 2013)
		28	4.8	IG	1	pH = 5.6 - 6.5, RT, DOC = 35-40	(Lewis et al., 2016)
			4.8	IG and BR	80 and 93.2	T = 20, pH = 6 - 9	(Abel et al., 2017)
	Neanthes arenaceodentata	28	24	IG	n.e.	RT	(Janssen et al., 2012)
Molluscs	*Hinia reticulata*	28	9.6	BSAF	n.e.	T = 22	(Cornelissen et al., 2006)
Echinoderms	*Amphiura filiformis*	28	8.6	M	n.e.	T = 13.8	(Samuelsson et al., 2015)
	Echinocardium cordatum	28	8.6	M	n.e.		
Crustaceans	*Americamysis bahia*	4	72	M	n.e.	RT	(Ho et al., 2004)
	Ampelisca abdita						
Insects		10	12	IG and R	5 and 16.6	RT	(Nybom et al., 2016)
	Chironomus riparius	28	36	BR	44	T = 20, pH = 6-9, TAN = <10	(Abel and Akkanen, 2019)

			28	36	M	82	T = 20, pH = 6.15 - 8.61	(Lillicrap et al., 2015)
nZVI	Fungi	*Alternaria sp.*	8	0.1	IG	n.e.	pH = 5.5 - 5.7, RT	(Josan et al., 2018)
		Penicillium viride				80		
	Algae	*Chlamydomonas sp.*	1	0.5	IG	n.e.	T = 22	(Nguyen et al., 2018)
A, Z and OC	Fungi	*Saprolegnia sp.*	3	0.5	IG	80	T = 22	(Johari et al., 2014)
	Algae	*Emiliania huxleyi*	7	0.05	IG	n.e.	T = 18, pH = 8.3 - 9.3	(Vasconcelos et al., 2004)
	Fishes	*Oncorhynchus mykiss*	0.5	5	IG and M	n.e.	pH = 7.8, T = 12	(Sheikhzadeh et al., 2017)
		Cyprinodon variegatus	7	5, 10, 15	IG and M	n.e.	T = 20	(Rosen et al., 2011)
	Annelids	*Neanthes arenaceodentata*	28	5, 10, 15	M	n.e.	T = 20	(Rosen et al., 2011)
		Lumbriculus variegatus	28	25	IG and M	n.e.	T = 25	(Paller and Knox, 2010)
	Crustaceans	*Eohaustorius estuarius*	10	5, 10, 15	M	n.e.	T = 20	(Rosen et al., 2011)
		Corbicula fluminea	28	25	M	n.e.	T = 25	(Paller and Knox 2010)

Supplementary Table S19. Tukey's test used to indicate the significance level between different groups of organisms to AC is reported as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	4.8-9.6 g/L	**7.2 g/L**	**12-16.3 g-L**	**13.4 g-L**	**19.2-36.0 g-L**	**26.4 g-L**	**4.8-36.0 g-L**	**14.8 g-L**
Bacteria vs. Annelids	ns	ns	ns	ns	ns	ns	ns	0.0257
Bacteria vs. Molluscs	ns	0.0448	ns	ns	ns	ns	ns	0.0245
Bacteria vs. Crustaceans	ns	ns	ns	ns	ns	ns	ns	0.0028
Bacteria vs. Fishes	ns	ns	ns	ns	ns	ns	ns	ns
Molluscs vs. Annelids	ns	ns	0.0257	ns	ns	ns	ns	ns
Crustaceans vs. Annelids	ns	ns	ns	ns	ns	ns	ns	ns
Annelids vs. Fishes	ns	ns	ns	ns	ns	ns	ns	ns
Molluscs vs. Crustaceans	ns	ns	0.01	ns	ns	ns	ns	ns
Molluscs vs. Fishes	ns	ns	ns	ns	ns	ns	ns	ns
Crustaceans vs. Fishes	ns	ns	ns	ns	ns	ns	ns	ns

Supplementary Table S20. Tukey's test used to indicate the significance level between different groups of organisms to nZVI is reported as follows: ** $p < 0.01$, *** $p < 0.001$, **** $p<0.0001$.

	0.0002-0.0013 g/l	**0.6 g/l**	**0.008-0.5 g/l**	**0.186 g/l**	**0.0002-0.5 g/l**	**0.093 g/l**
Bacteria vs. Algae	ns	ns	0.0002	ns	<0.0001	ns
Bacteria vs. Molluscs	ns	ns	0.0035	ns	0.0003	ns
Bacteria vs. Crustaceans	ns	ns	ns	ns	0.0004	ns
Bacteria vs. Fishes	ns	ns	ns	ns	0.0003	ns
Molluscs vs. Algae	ns	ns	ns	ns	ns	ns
Crustaceans vs. Algae	ns	ns	ns	ns	ns	ns
Algae vs. Fishes	ns	ns	0.0002	ns	ns	ns
Molluscs vs. Crustaceans	ns	ns	ns	ns	ns	ns
Molluscs vs. Fishes	ns	ns	0.0025	ns	ns	ns
Crustaceans vs. Fishes	ns	ns	ns	ns	ns	ns

Supplementary Table S21. Tukey's test used to indicate the significance level between different groups of organisms to OC, A and Z is reported as follows: * < 0.05, ** p < 0.01, *** p < 0.001, **** p<0.0001. n.s. = not significant.

	0.0001-0.1 g/l	**0.03 g/l**	**0.5-350 g/l**	**88.4 g/l**	**0.0001-350 g/l**	**44.2 g/l**
Bacteria vs. Crustaceans	ns	ns	ns	ns	ns	<0.0001
Bacteria vs. Fishes	ns	ns	ns	ns	ns	0.0013
Crustaceans vs. Fishes	ns	ns	ns	ns	ns	0.0118

Supplementary Table S22. Tukey's test used to indicate the significance level between two fixed factors (groups of organisms vs. remediation methods) is reported as follows: * $p < 0.05$, ** $p < 0.01$. n.s. = not significant.

	Raw data (RD)			Predicted data (PD)		
	AC vs. OC, A and Z	nZVI vs. OC, A and Z	AC vs. nZVI	AC vs. OC, A and Z	nZVI vs. OC, A and Z	AC vs. nZVI
Bacteria	ns	0.0256	ns	ns	ns	ns
Crustaceans	ns	ns	0.0085	ns	ns	ns
Molluscs	ns	ns	ns	ns	ns	ns
Fishes	ns	ns	ns	0.022	0.022	ns

Supplementary Table S23. Tukey's test used to indicate the significance level between nZVI different percentage and control at all considered time (T0 = after adding of amendment; T1 = 3 h; T2 = 6 h; T3 = 21 h; T4 = 24 h, T5 = 72 h and T6 = 21 days): * < 0.05, ** p < 0.01, *** p < 0.001, **** p<0.0001. n.s. = not significant.

	W + nZVI	W + PAHs	W + PAHs + nZVI	W + SED1 + nZVI	W + SED2 + nZVI
T0					
0 vs. 6.25	0.0346	0.0001	ns	ns	ns
0 vs. 12.5	<0.0001	<0.0001	<0.0001	ns	ns
0 vs. 25	<0.0001	<0.0001	<0.0001	ns	ns
0 vs. 50	<0.0001	<0.0001	<0.0001	ns	<0.0001
0 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
6.25 vs. 12.5	<0.0001	<0.0001	<0.0001	ns	ns
6.25 vs. 25	<0.0001	<0.0001	<0.0001	ns	ns
6.25 vs. 50	<0.0001	<0.0001	<0.0001	ns	<0.0001
6.25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
12.5 vs. 25	ns	0.0036	ns	ns	ns
12.5 vs. 50	ns	<0.0001	<0.0001	ns	<0.0001
12.5 vs. 100	0.0002	<0.0001	<0.0001	<0.0001	<0.0001
25 vs. 50	ns	ns	0.0033	ns	<0.0001
25 vs. 100	0.0002	<0.0001	<0.0001	<0.0001	<0.0001
50 vs. 100	0.0002	<0.0001	<0.0001	<0.0001	ns
T1					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	<0.0001	<0.0001	<0.0001	ns	ns
0 vs. 50	<0.0001	<0.0001	<0.0001	ns	<0.0001
0 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
6.25 vs. 12.5	ns	<0.0001	ns	ns	ns
6.25 vs. 25	<0.0001	<0.0001	<0.0001	ns	ns
6.25 vs. 50	<0.0001	<0.0001	<0.0001	ns	<0.0001
6.25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
12.5 vs. 25	<0.0001	ns	<0.0001	ns	ns

12.5 vs. 50	<0.0001	<0.0001	<0.0001	ns	<0.0001
12.5 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25 vs. 50	0.0346	<0.0001	<0.0001	ns	<0.0001
25 vs. 100	0.0002	<0.0001	<0.0001	<0.0001	<0.0001
50 vs. 100	ns	<0.0001	<0.0001	<0.0001	0.0205
T2					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	ns	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
0 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
6.25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
12.5 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25 vs. 50	<0.0001	ns	ns	<0.0001	<0.0001
25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
50 vs. 100	0.0346	<0.0001	0.0033	<0.0001	0.0205
T3					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	ns	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	0.0346	<0.0001	ns	ns	<0.0001
0 vs. 100	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	0.0346	<0.0001	ns	ns	<0.0001
6.25 vs. 100	<0.0001	<0.0001	0.0033	<0.0001	<0.0001

12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	0.0346	<0.0001	ns	ns	<0.0001
12.5 vs. 100	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
25 vs. 50	0.0346	ns	ns	ns	<0.0001
25 vs. 100	<0.0001	<0.0001	0.0033	<0.0001	<0.0001
50 vs. 100	0.0002	0.0036	ns	<0.0001	ns
T4					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	ns	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	0.0205
0 vs. 100	<0.0001	<0.0001	0.0033	0.0005	<0.0001
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	0.0205
6.25 vs. 100	<0.0001	<0.0001	0.0033	0.0005	<0.0001
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	0.0205
12.5 vs. 100	<0.0001	<0.0001	0.0033	0.0005	<0.0001
25 vs. 50	ns	ns	ns	ns	0.0205
25 vs. 100	<0.0001	0.0001	0.0033	0.0005	<0.0001
50 vs. 100	<0.0001	0.0036	0.0569	0.0005	ns
T5					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	ns	ns	ns	ns
0 vs. 25	ns	ns	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	<0.0001	<0.0001	ns	ns	ns
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns

6.25 vs. 100	<0.0001	<0.0001	ns	ns	ns
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	<0.0001	<0.0001	ns	ns	ns
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	<0.0001	0.0001	ns	ns	ns
50 vs. 100	<0.0001	0.0036	ns	ns	ns
T6					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	ns	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	ns	ns	ns
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	ns	<0.0001	ns	ns	ns
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	ns	ns	ns
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	ns	0.0001	ns	ns	ns
50 vs. 100	ns	0.016	ns	ns	ns

Supplementary Table S24. Tukey's test used to indicate the significance level between AC different percentage and control at all considered time (T0 = after adding of amendment; T1 = 3 h; T2 = 6 h; T3 = 21 h; T4 = 24 h, T5 = 72 h and T6 = 21 days): * < 0.05, ** p < 0.01, *** p < 0.001, **** p<0.0001. n.s. = not significant.

	W + AC	W + PAHs	W + PAHs + AC	W + SED1 + AC	W + SED2 + AC
T0					
0 vs. 6.25	ns	<0.0001	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
6.25 vs. 12.5	ns	0.0004	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25 vs. 50	ns	<0.0001	ns	ns	ns
25 vs. 100	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
50 vs. 100	<0.0001	0.0004	<0.0001	<0.0001	<0.0001
T1					
0 vs. 6.25	ns	<0.0001	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	0.0205	<0.0001	<0.0001	<0.0001	<0.0001
6.25 vs. 12.5	ns	0.2384	ns	ns	ns
6.25 vs. 25	ns	0.0004	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	0.0205	<0.0001	<0.0001	<0.0001	<0.0001
12.5 vs. 25	ns	0.2384	ns	ns	ns

12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	0.0205	<0.0001	<0.0001	<0.0001	<0.0001
25 vs. 50	ns	0.0004	ns	ns	ns
25 vs. 100	0.0205	<0.0001	<0.0001	<0.0001	<0.0001
50 vs. 100	0.0205	0.0156	<0.0001	<0.0001	<0.0001
T2					
0 vs. 6.25	ns	<0.0001	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	<0.0001	ns	<0.0001
6.25 vs. 12.5	ns	0.0004	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	ns	<0.0001	<0.0001	ns	<0.0001
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	<0.0001	ns	<0.0001
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	ns	0.0004	<0.0001	ns	<0.0001
50 vs. 100	ns	ns	<0.0001	ns	<0.0001
T3					
0 vs. 6.25	ns	<0.0001	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	ns	ns	<0.0001
6.25 vs. 12.5	ns	<0.0001	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	ns	<0.0001	ns	ns	<0.0001

12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	ns	ns	<0.0001
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	ns	0.0156	ns	ns	<0.0001
50 vs. 100	ns	ns	ns	ns	<0.0001
T4					
0 vs. 6.25	ns	0.0004	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	ns	ns	ns
6.25 vs. 12.5	ns	ns	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	ns	<0.0001	ns	ns	ns
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	ns	ns	ns
25 vs. 50	ns	0.0156	ns	ns	ns
25 vs. 100	ns	0.0004	ns	ns	ns
50 vs. 100	ns	ns	ns	ns	ns
T5					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	ns	ns	ns
6.25 vs. 12.5	ns	<0.0001	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns

6.25 vs. 100	ns	<0.0001	ns	ns	ns
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	ns	ns	ns
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	ns	0.0156	ns	ns	ns
50 vs. 100	ns	0.0156	ns	ns	ns
T6					
0 vs. 6.25	ns	ns	ns	ns	ns
0 vs. 12.5	ns	<0.0001	ns	ns	ns
0 vs. 25	ns	<0.0001	ns	ns	ns
0 vs. 50	ns	<0.0001	ns	ns	ns
0 vs. 100	ns	<0.0001	ns	ns	ns
6.25 vs. 12.5	ns	<0.0001	ns	ns	ns
6.25 vs. 25	ns	<0.0001	ns	ns	ns
6.25 vs. 50	ns	<0.0001	ns	ns	ns
6.25 vs. 100	ns	<0.0001	ns	ns	ns
12.5 vs. 25	ns	<0.0001	ns	ns	ns
12.5 vs. 50	ns	<0.0001	ns	ns	ns
12.5 vs. 100	ns	<0.0001	ns	ns	ns
25 vs. 50	ns	ns	ns	ns	ns
25 vs. 100	ns	0.0156	ns	ns	ns
50 vs. 100	ns	0.0156	ns	ns	ns

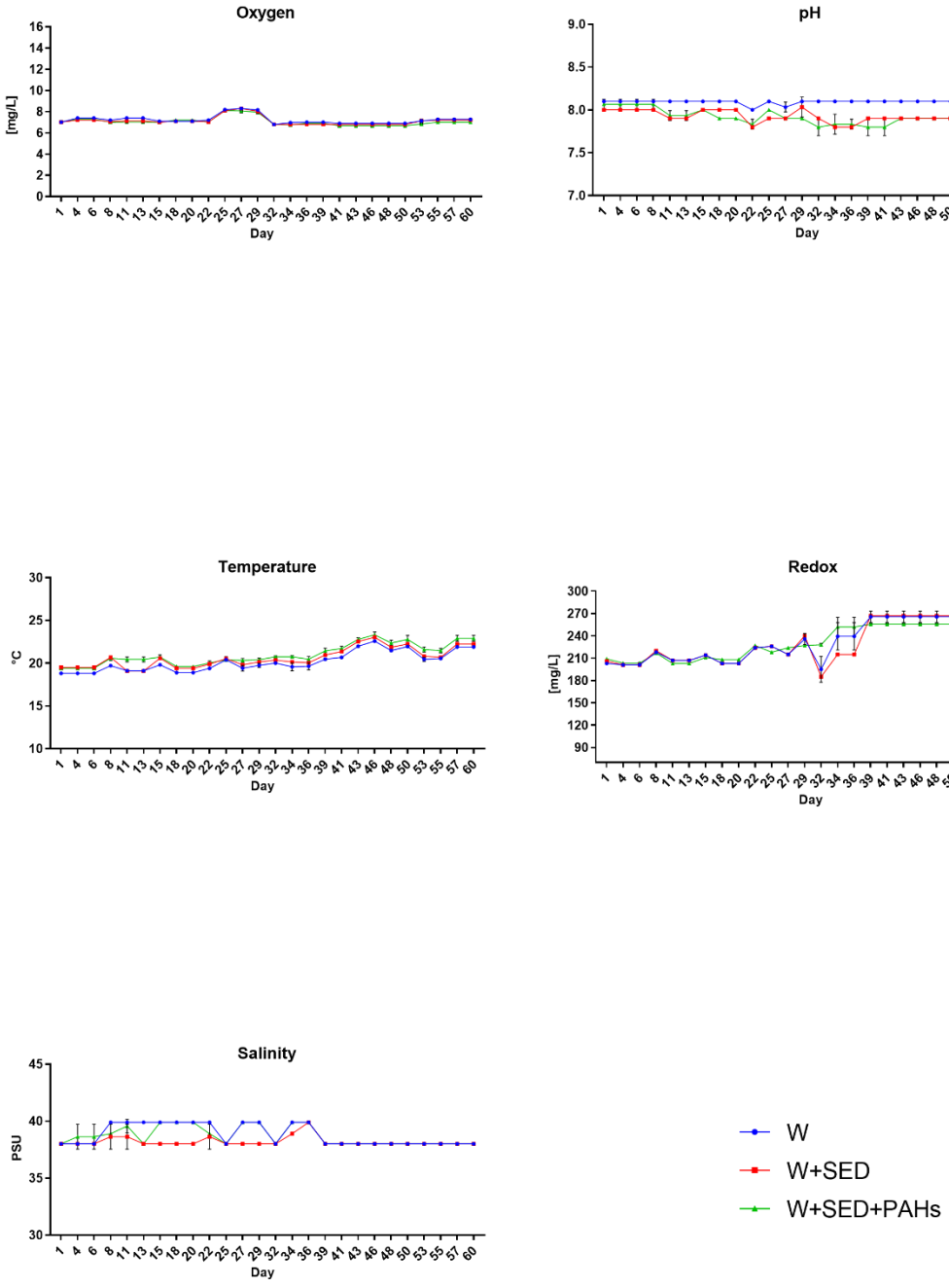
Supplementary Table S25. Data of expression levels in adults exposed to W + nZVI, W + PAHs, W + PAHs + nZVI, W + SED1 + nZVI and W + SED2 + nZVI were reported as a fold difference (in red up-expressed genes; in green down-expressed genes) from control (represented by adults of crustaceans reared in SW without contaminants) for 48 h. Fold differences greater than ± 1.5 were considered significant.

		W + nZVI	W + PAHs	W + PAHs + nZVI	W + SED1 + nZVI	W + SED2 + nZVI
Stress	*hsp26*	0.87	-8.71	-4.10	-3.32	-6.69
	hsp60	-1.64	-3.72	2.16	-8.13	-1.78
	hsp70	0.44	4.02	4.07	4.75	4.17
	COXI	-0.80	-1.26	0.51	1.80	-1.44
	COXIII	-3.14	-3.53	-3.56	2.38	-3.90
Development	*HAD*	-1.33	11.08	-10.65	-6.02	-6.80
	CDC48	-1.26	-5.78	-7.47	-6.65	-7.30
	tcp	-0.82	-1.91	-0.79	1.94	-0.38
	UCP2	1.29	0.65	2.47	2.89	0.84

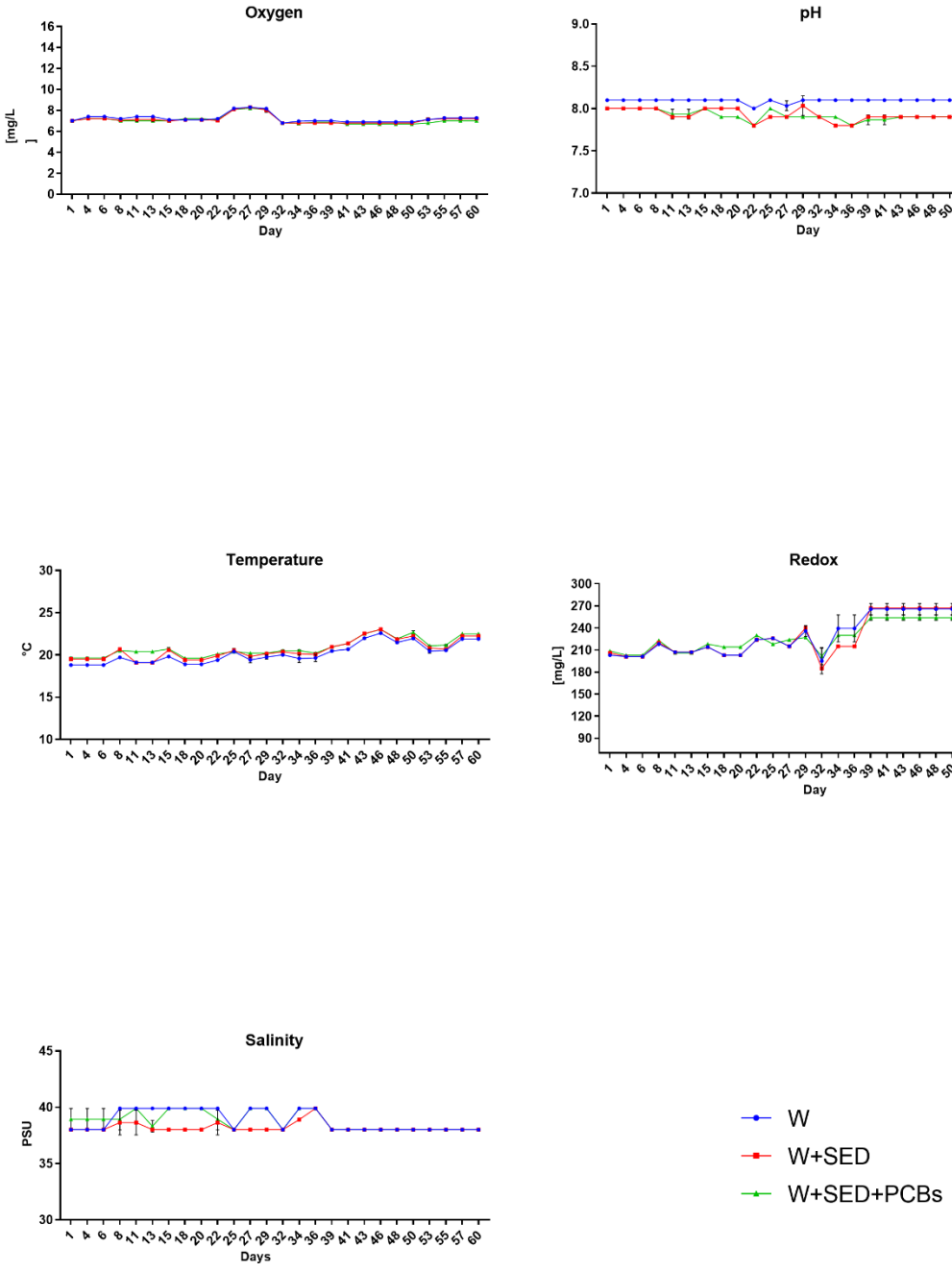
Supplementary Table S26. Data of expression levels in adults exposed to W + AC, W + PAHs, W + PAHs + AC, W + SED1 + AC and W + SED2 + AC, were reported as a fold difference (in red up-expressed genes; in green down-expressed genes) from control (represented by adults of crustaceans reared in SW without contaminants) for 48 h. Fold differences greater than ± 1.5 were considered significant.

		W + AC	W + PAHs	W + PAHs + AC	W + SED1 + AC	W + SED2 + AC
Stress	*hsp26*	-0.69	-8.71	-0.53	-0.01	-0.27
	hsp60	2.47	-3.72	1.33	2.71	2.42
	hsp70	-0.05	4.02	3.42	-1.69	4.58
	COXI	-0.07	-1.26	2.37	2.71	2.70
	COXIII	-0.39	-3.53	2.15	2.44	2.15
Development	*HAD*	0.51	11.08	0.39	1.84	3.60
	CDC48	0.19	-5.78	0.15	2.32	1.92
	tcp	0.55	-1.91	5.55	4.96	4.92
	UCP2	0.62	0.65	3.65	3.74	2.88

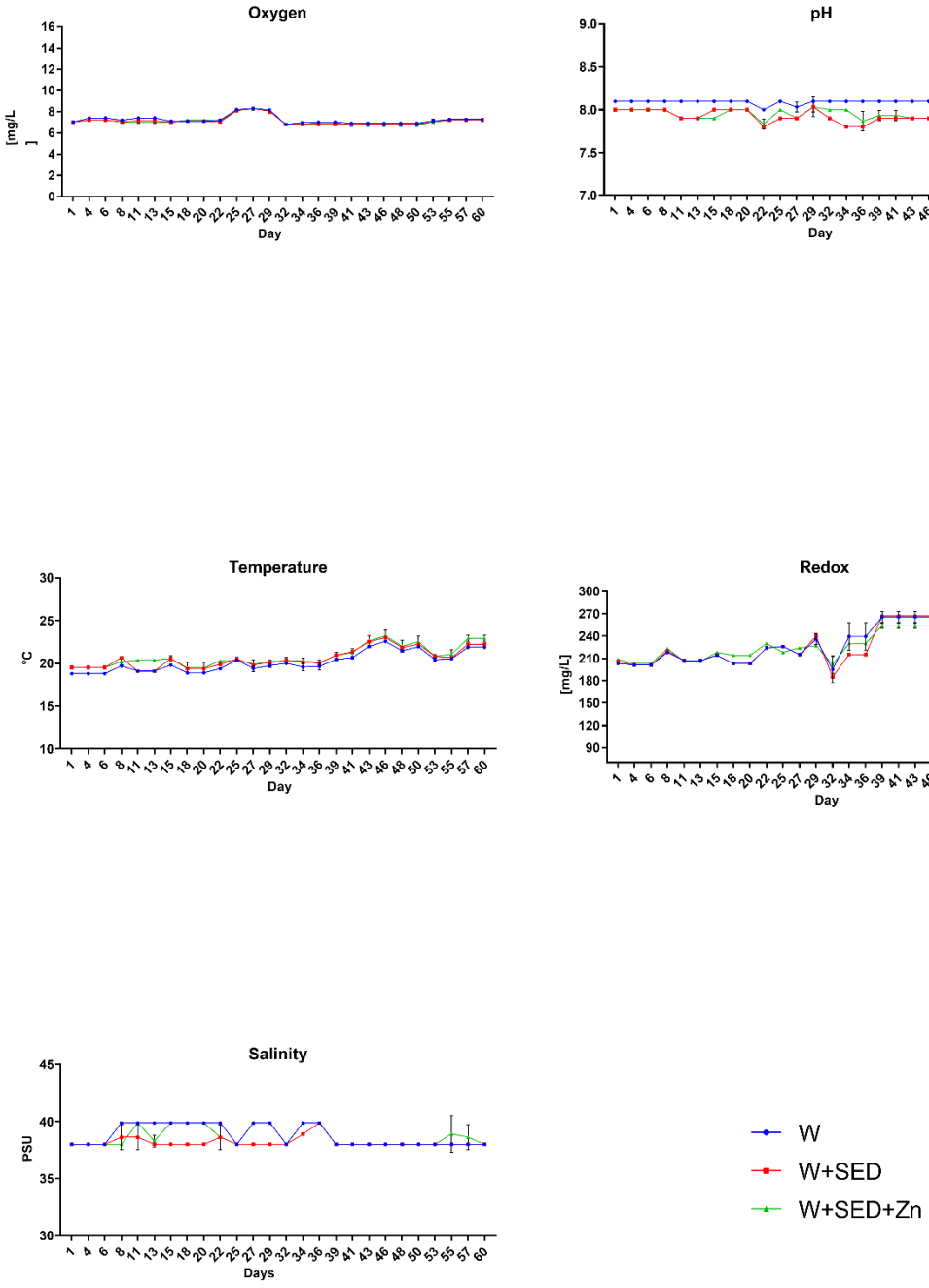
Supplementary Figure S1. Physical parameters (dissolved oxygen, pH,redox, temperature and salinity) of sea water checked three times a week in tanks at the three different experimental conditions: W, W+SED, W+SED+PAHs.



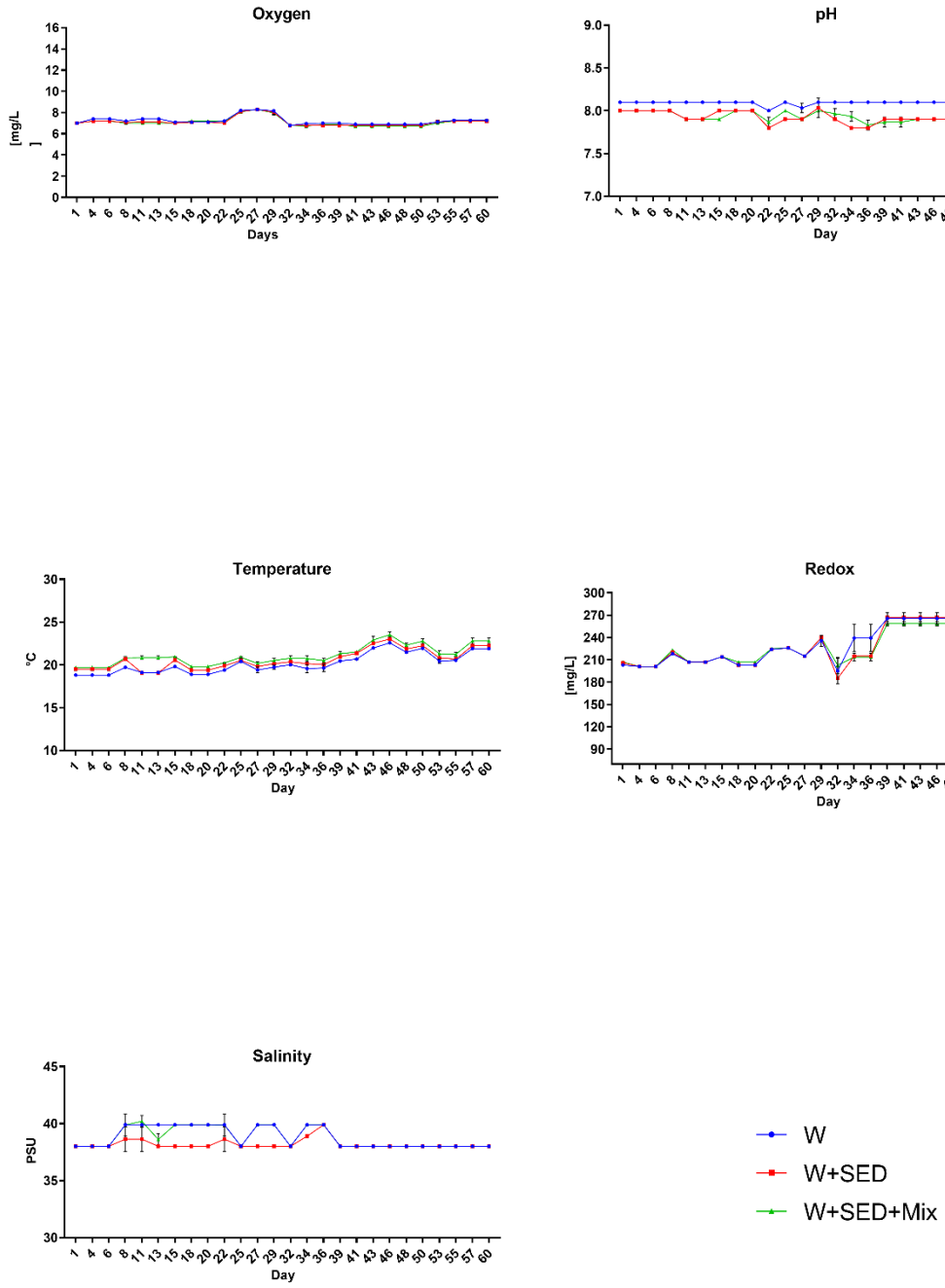
Supplementary Figure S2. Physical parameters (dissolved oxygen, pH,redox, temperature and salinity) of sea water checked three times a week in tanks at the three different experimental conditions: W, W+SED, W+SED+PCBs.



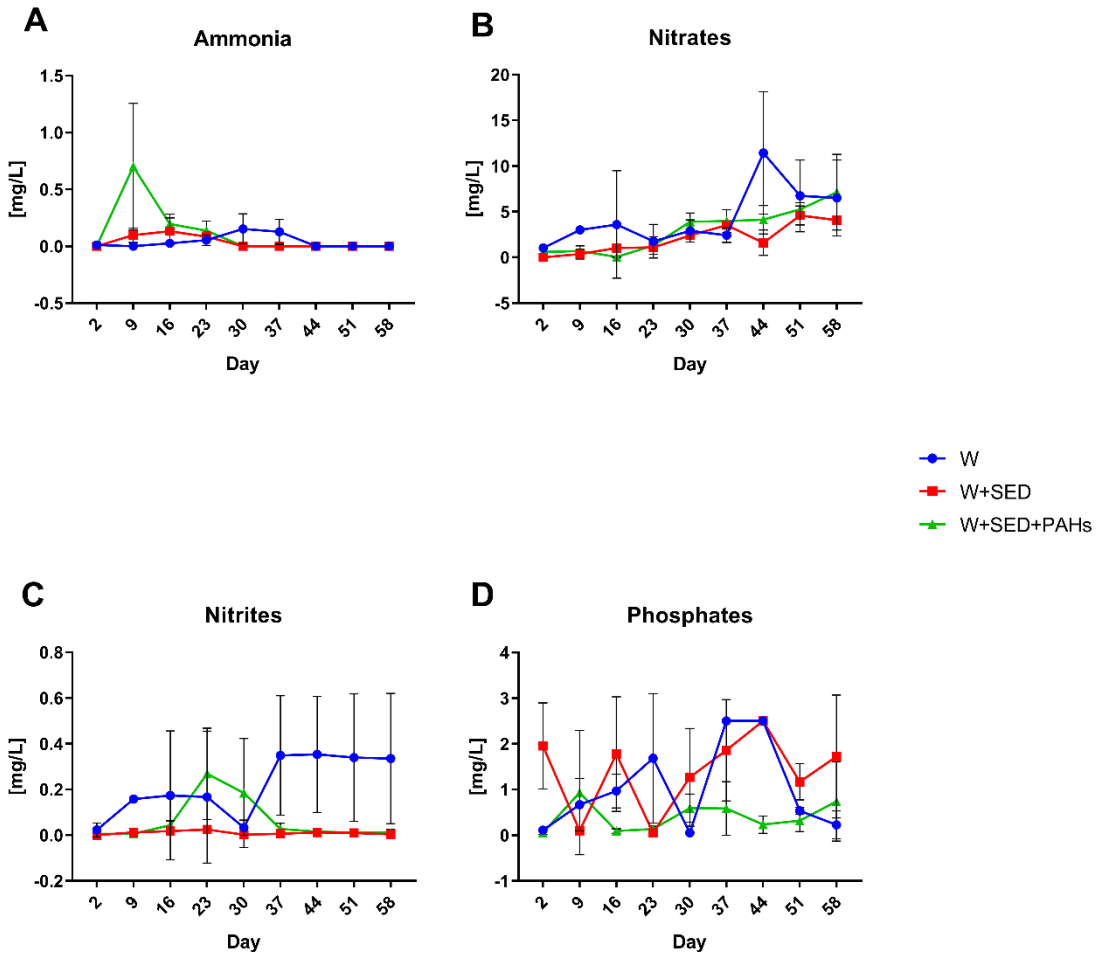
Supplementary Figure S3. Physical parameters (dissolved oxygen, pH,redox, temperature and salinity) of sea water checked three times a week in tanks at the three different experimental conditions: W, W+SED, W+SED+Zn.



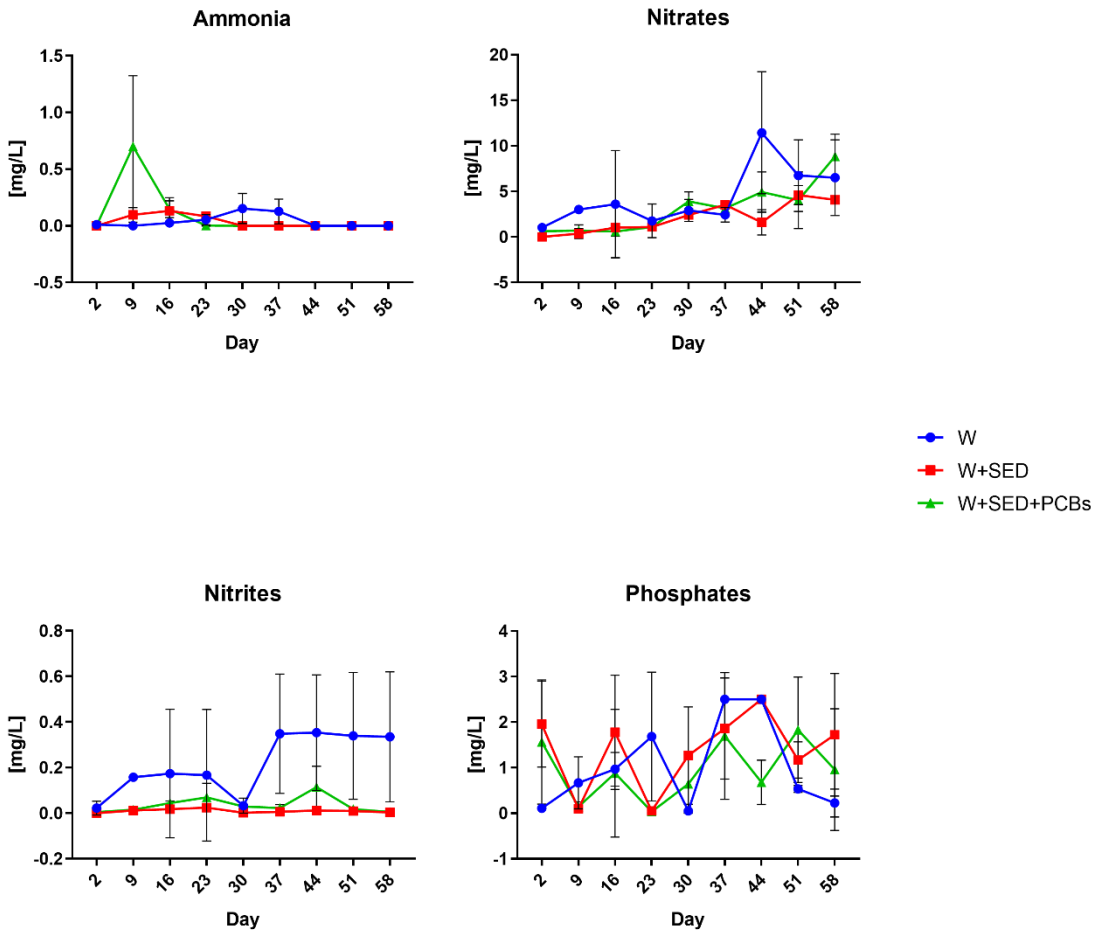
Supplementary Figure S4. Physical parameters (dissolved oxygen, pH,redox, temperature and salinity) of sea water checked three times a week in tanks at the three different experimental conditions: W, W+SED, W+SED+Mix.



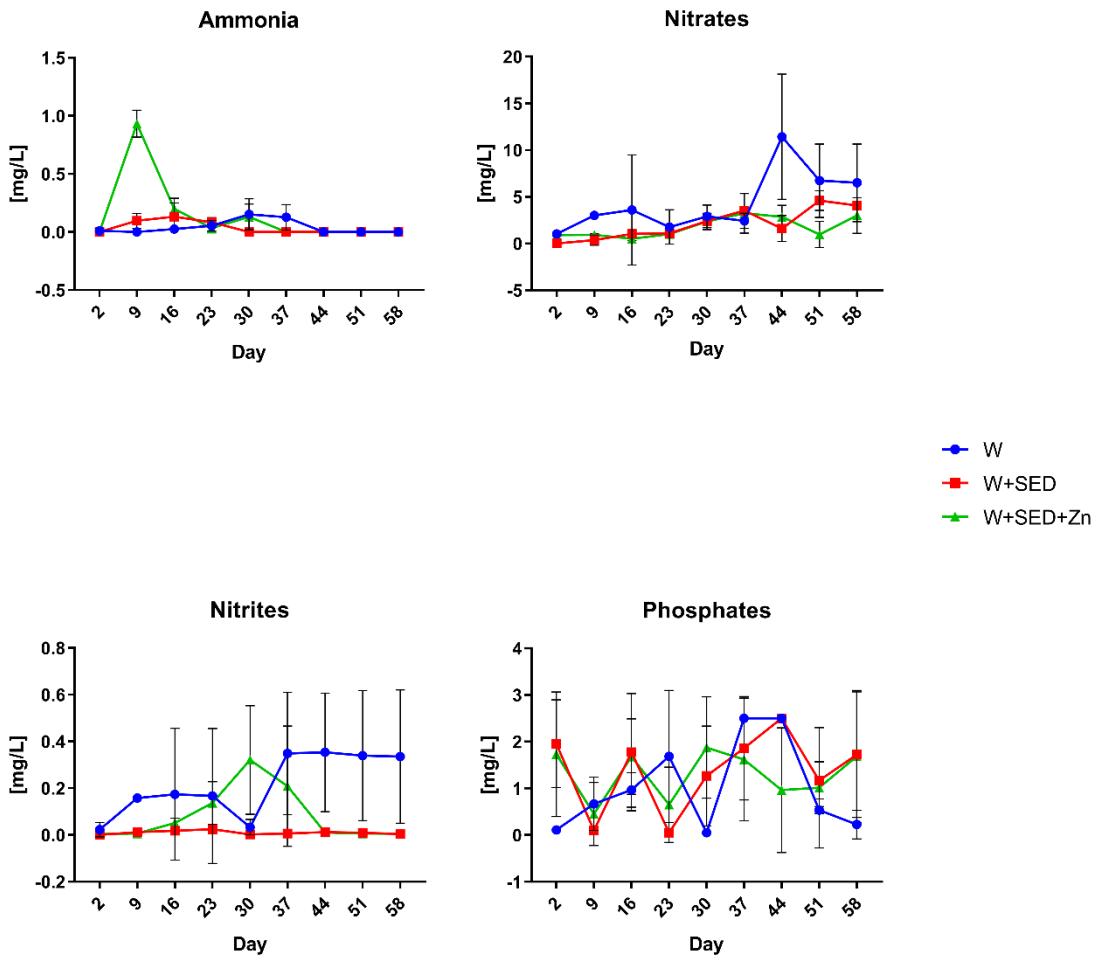
Supplementary Figure S5. Chemical parameters (ammonia, nitrates, nitrites an phosphates) of sea water checked once a week in tanks at the four different experimental conditions: W, W+SED, W+SED+PAHs. On X axis the days at which the data were collected.



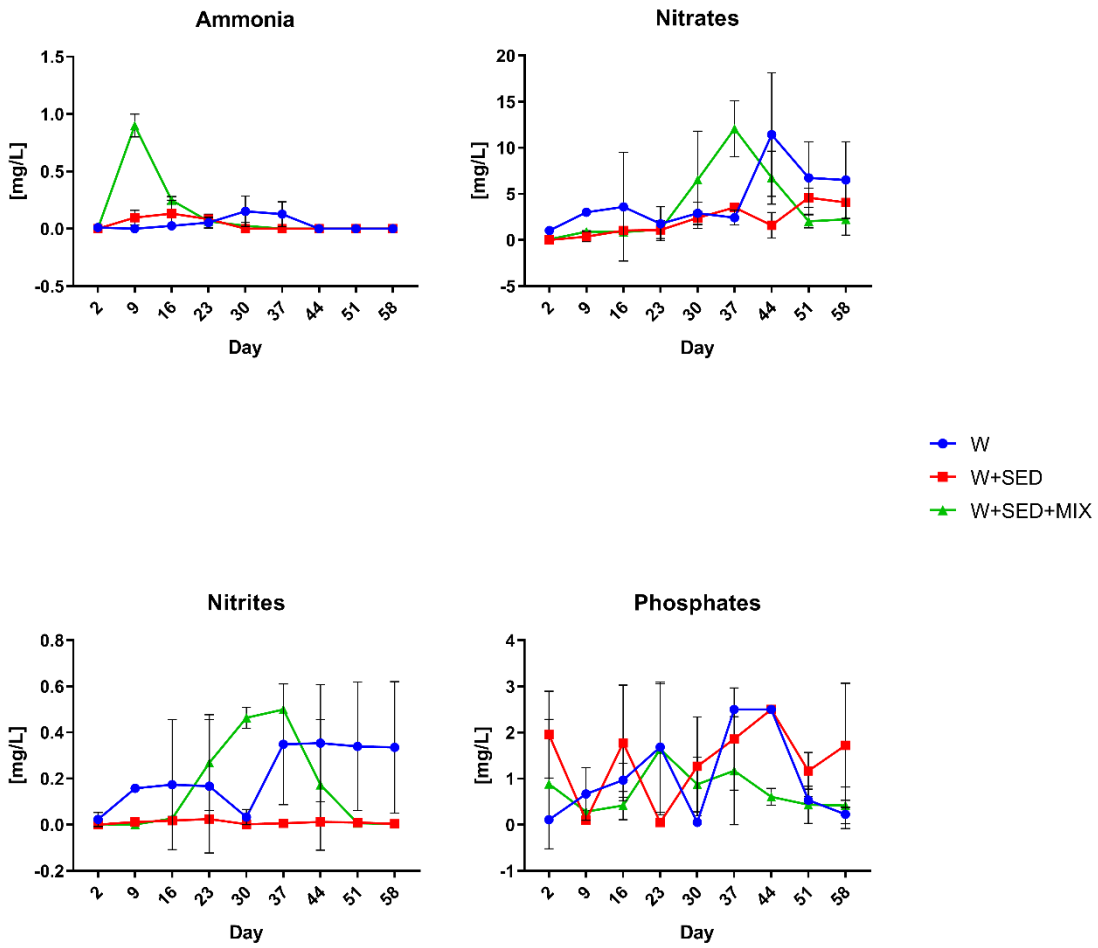
Supplementary Figure S6. Chemical parameters (ammonia, nitrates, nitrites an phosphates) of sea water checked once a week in tanks at the four different experimental conditions: W, W+SED, W+SED+PCBs. On X axis the days at which the data were collected.



Supplementary Figure S7. Chemical parameters (ammonia, nitrates, nitrites an phosphates) of sea water checked once a week in tanks at the four different experimental conditions: W, W+SED, W+SED+Zn. On X axis the days at which the data were collected.

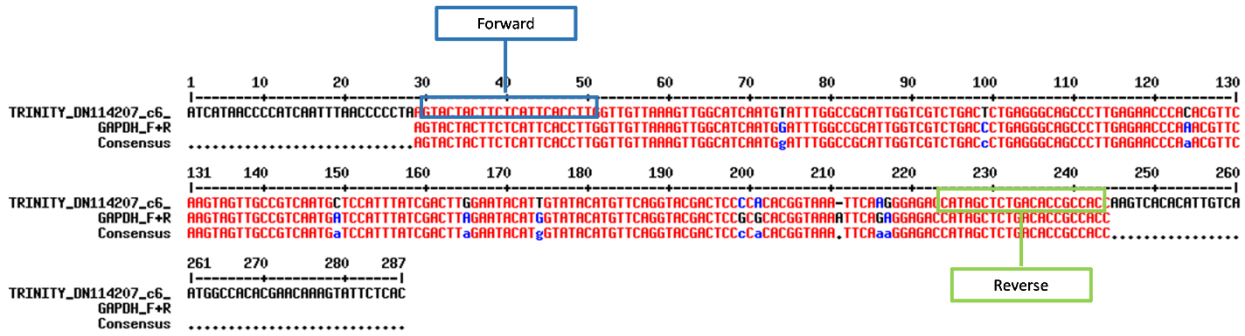


Supplementary Figure S8. Chemical parameters (ammonia, nitrates, nitrites an phosphates) of sea water checked once a week in tanks at the four different experimental conditions: W, W+SED, W+SED+Mix. On X axis the days at which the data were collected.



Supplementary Figure S9. Allignments of PCR products to the original sequence of GAPDH (A), GST (B), hsp75 (C), TNF (D), CM-K (E), CREB (F), EGF (G), FZ-7 (H), HH (I), NLK (J), NOTCHLESS (K), PLC (L), STAT1 (M), NADH (N), ChE (O), Ptc (P), Smo (Q), hsp90 (R), Lefty (S), PKS (T), CYP-2UI (U), SULT1 (V), M-Vg1 (W), JAK (X), PLAUF3 (Y) genes.

A)



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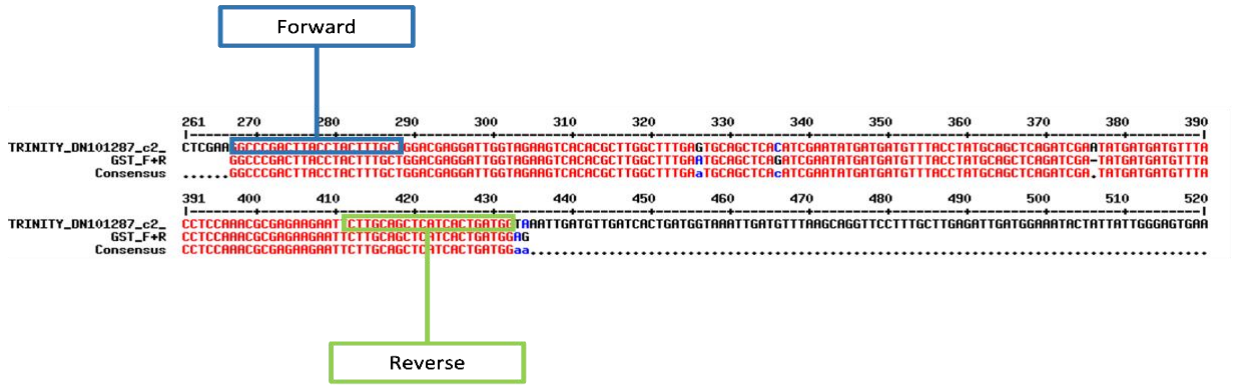

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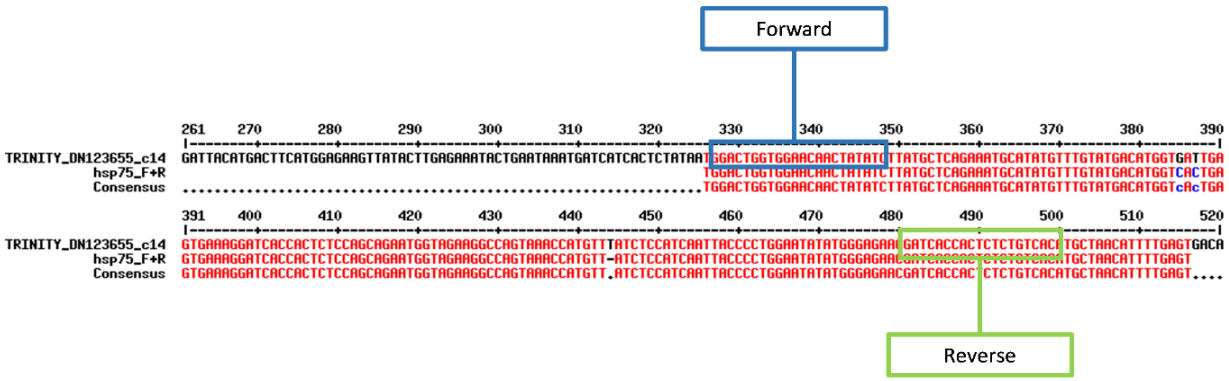
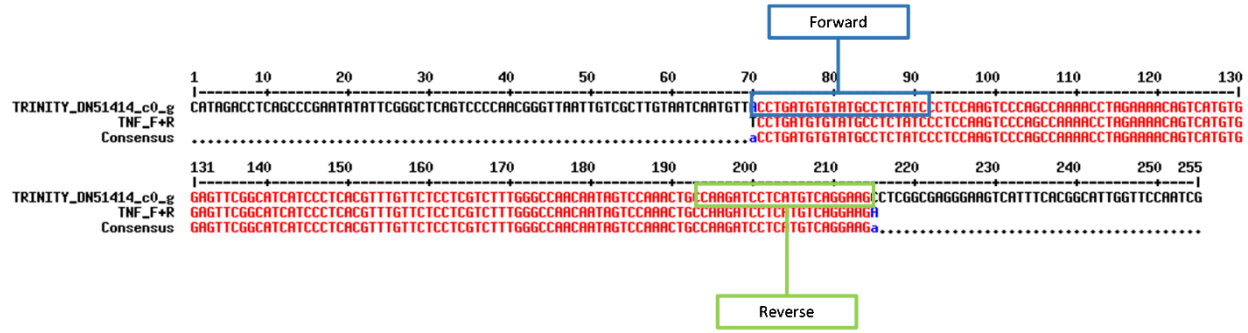


D)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Reverse

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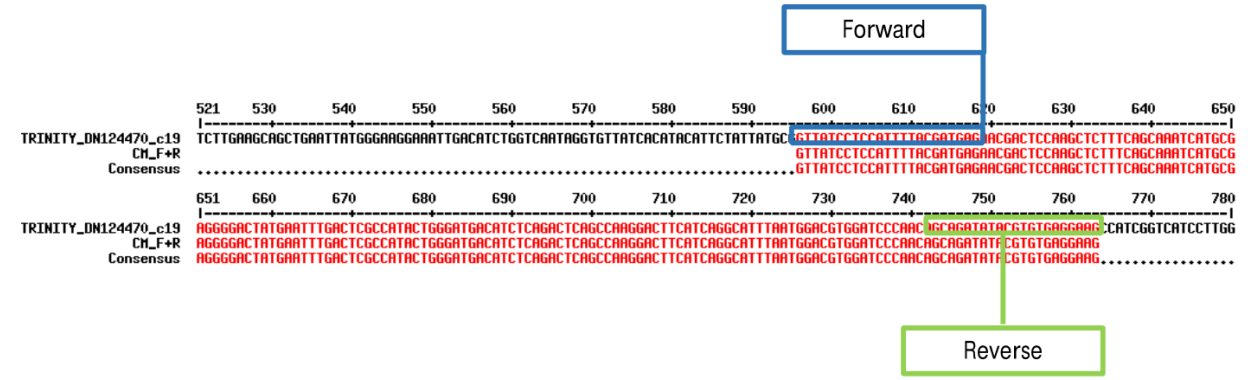
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Reverse

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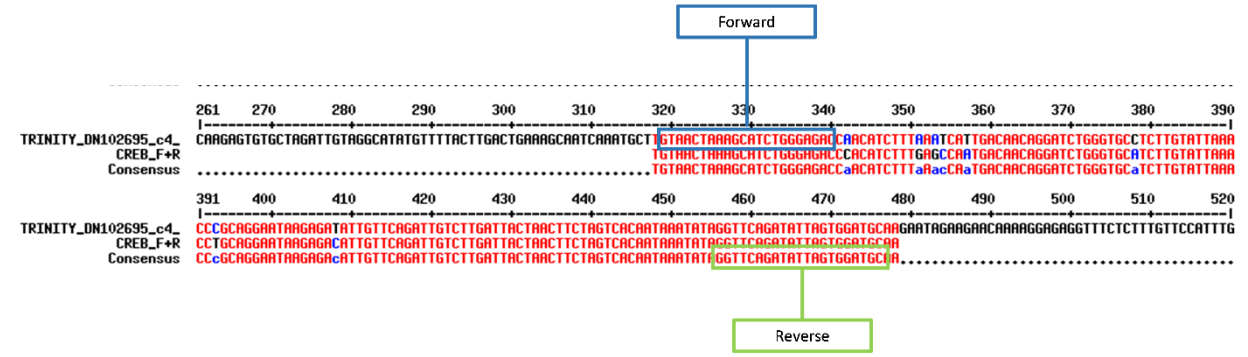
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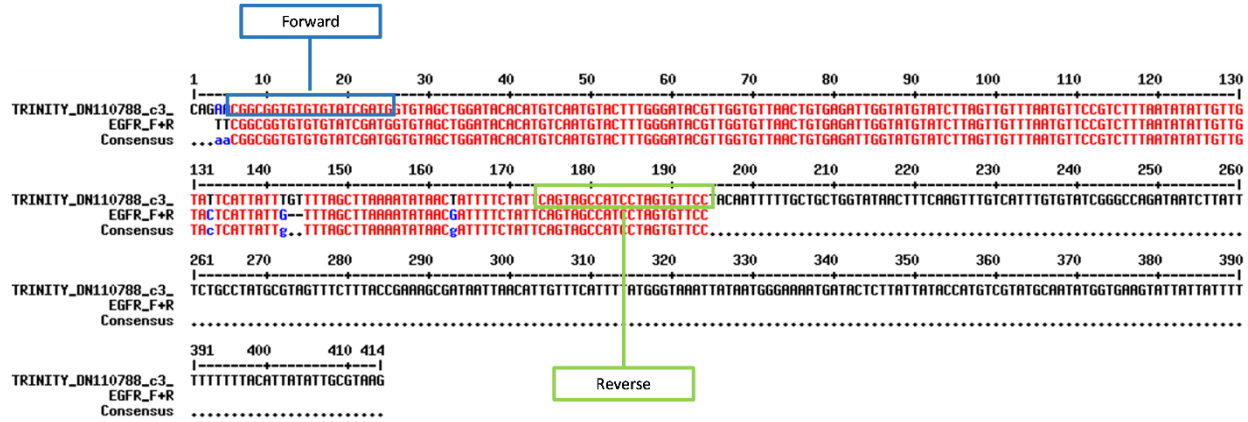
Forward
Reverse

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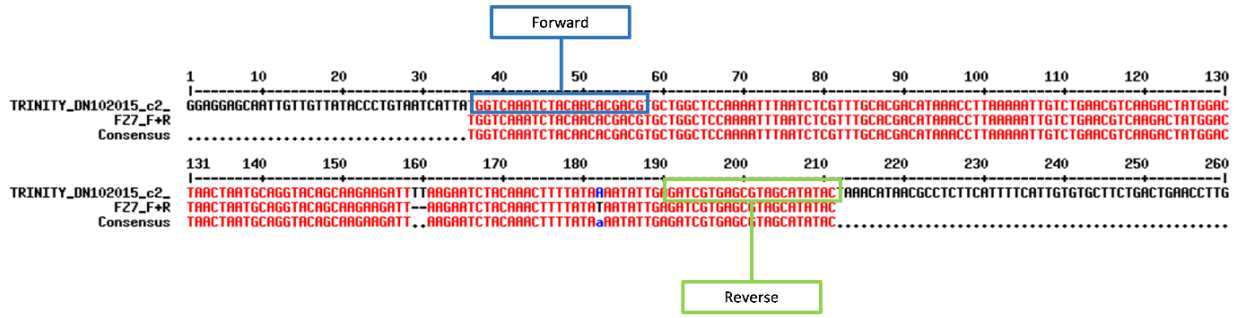
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FZ7_F+R
Consensus
Reverse

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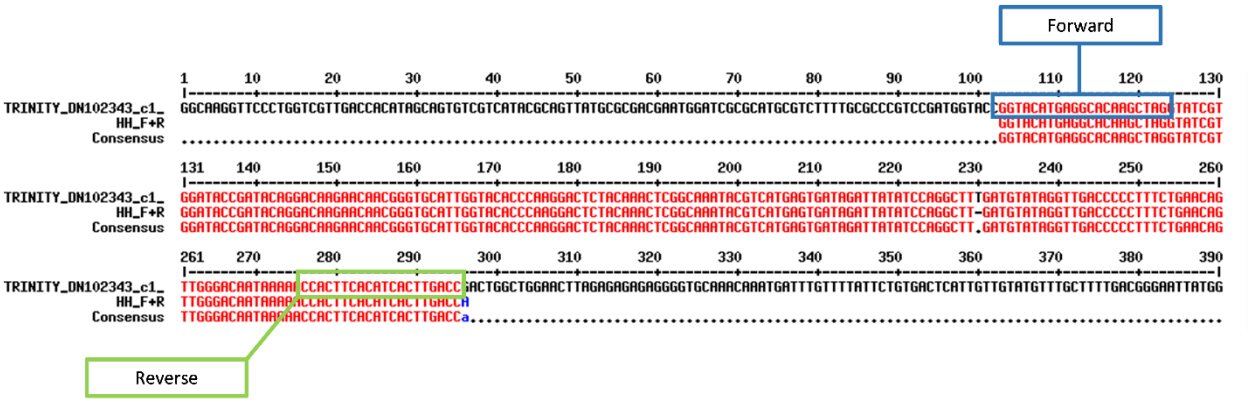
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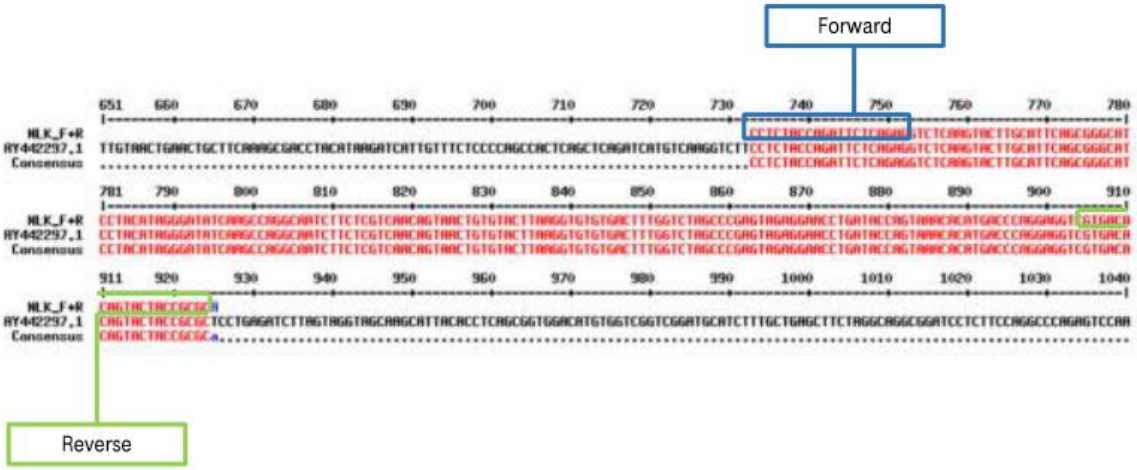
HH_F+R
Consensus
Reverse

J)



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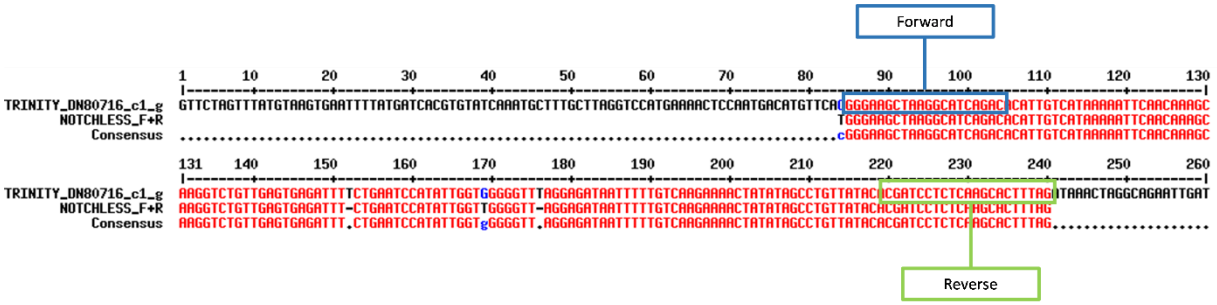

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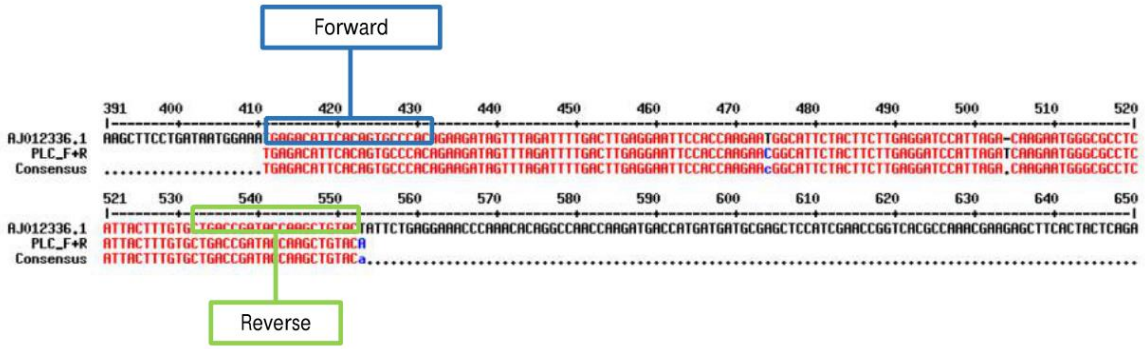
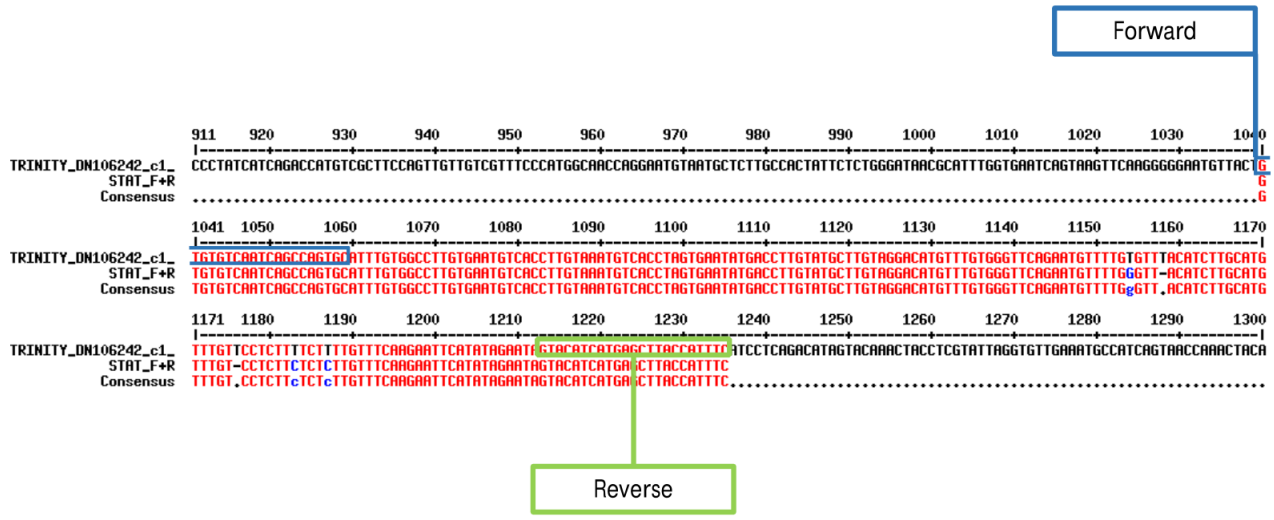


M)



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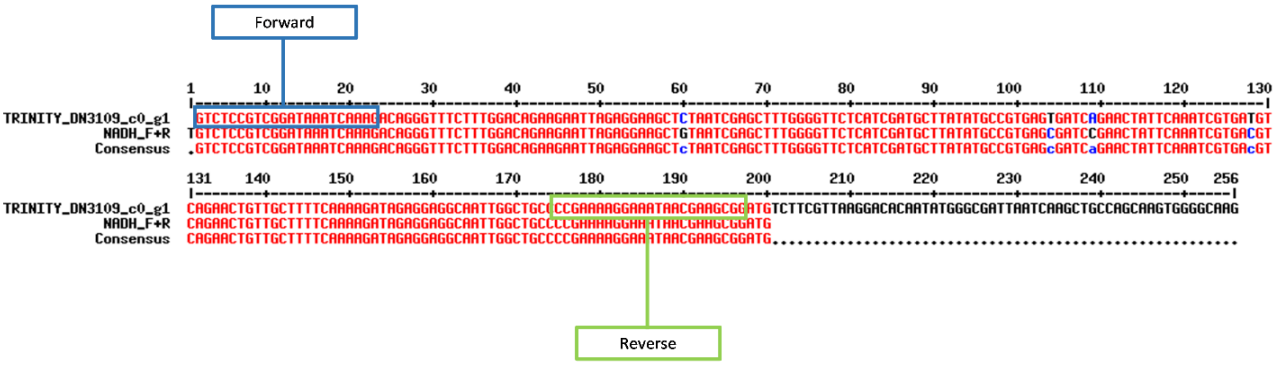
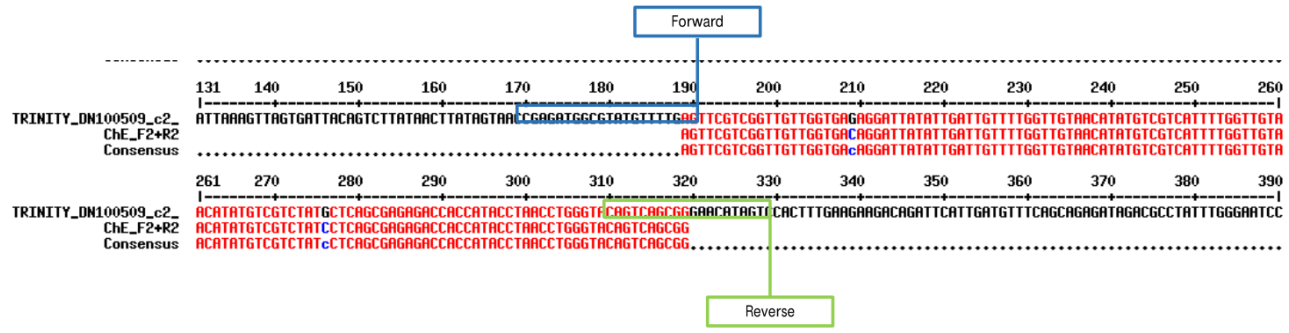


O)



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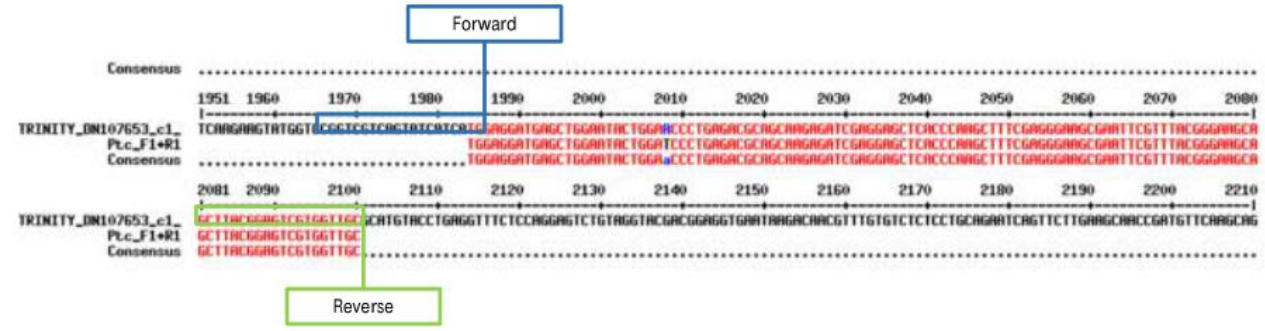
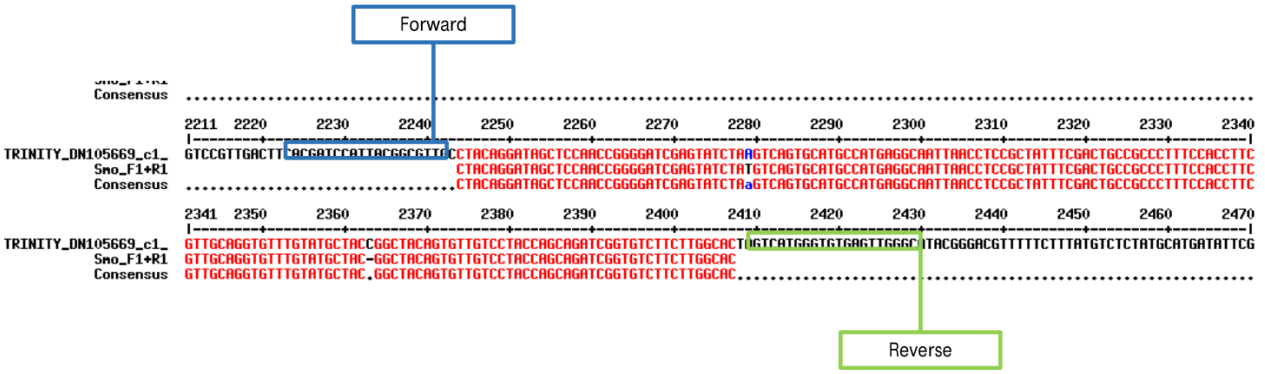


Q)



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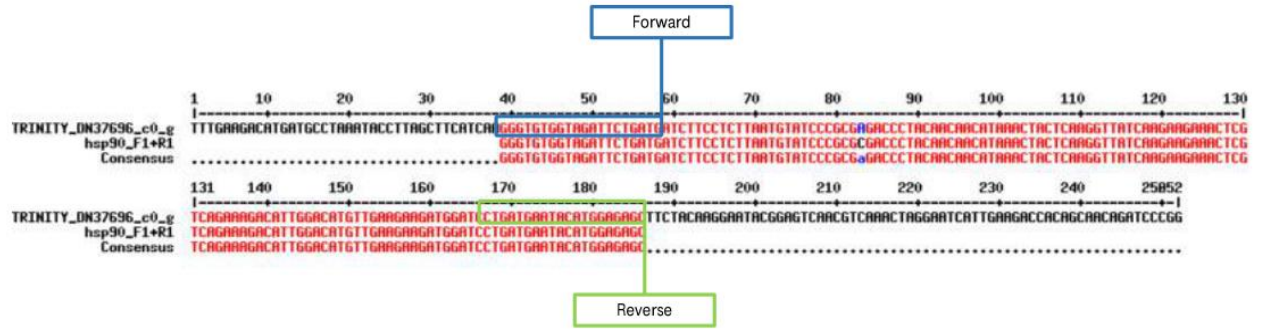

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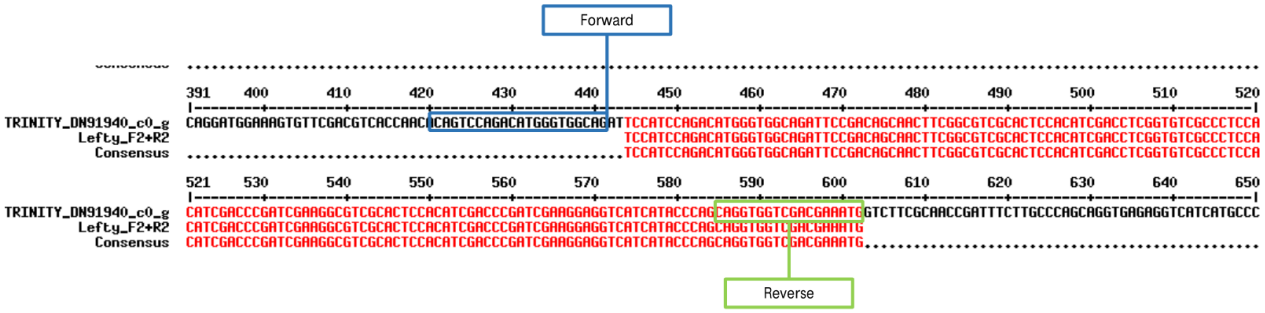
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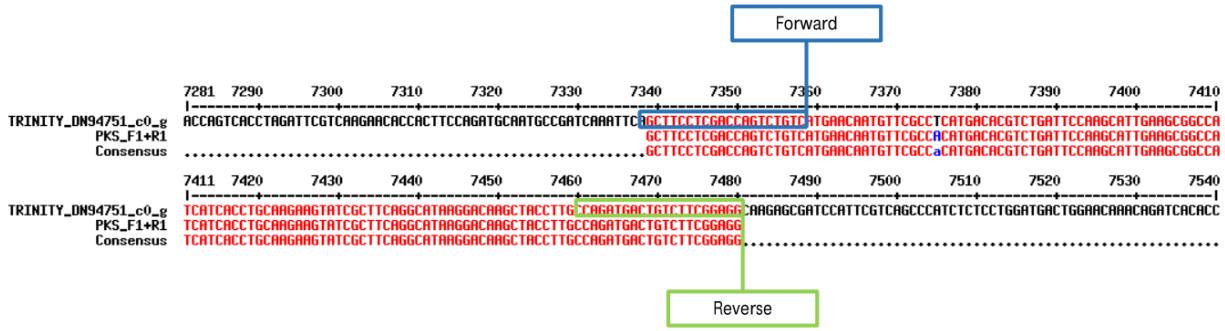
Lefty_F2+R2
Consensus
Reverse

T)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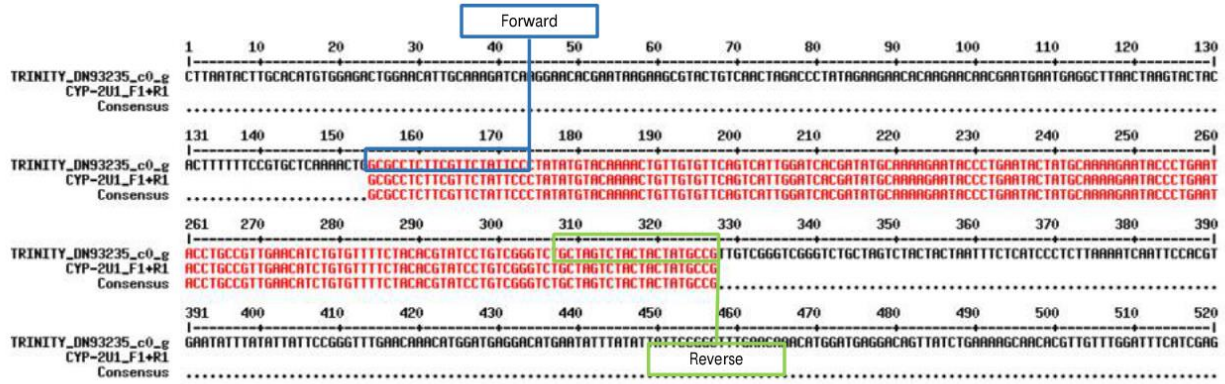
PKS_F1+R1
Consensus
Reverse

U)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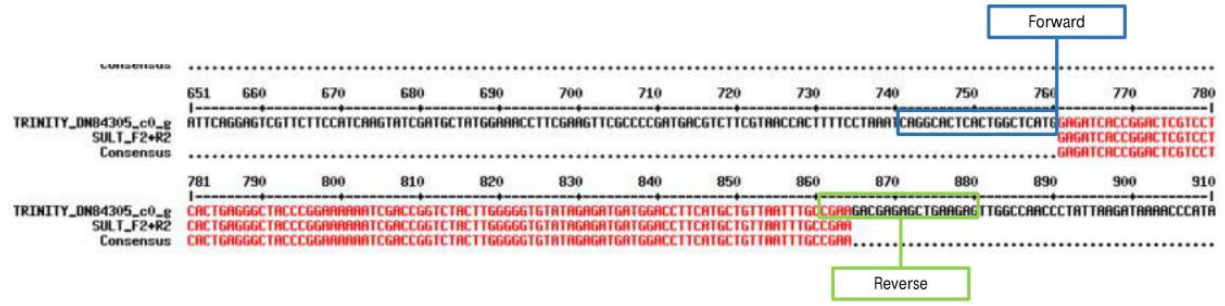
CYP-2U1_F1+R1
Consensus
Reverse

V)


Forward
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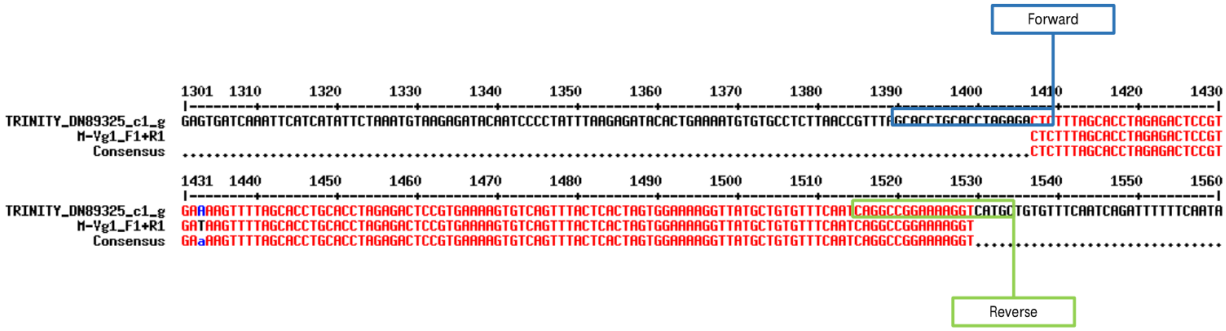
SULT_F2+R2
Consensus
Reverse

W)



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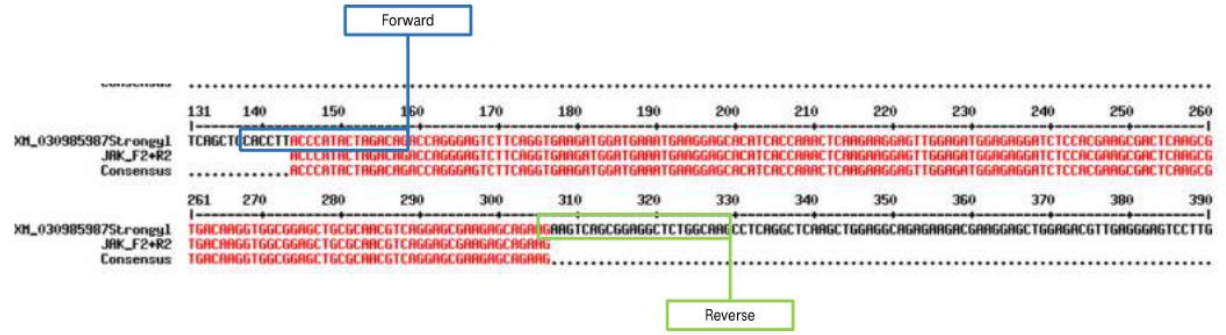

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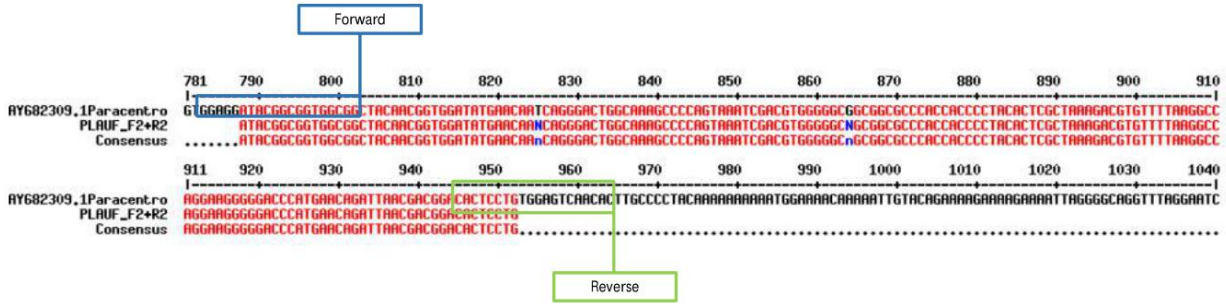
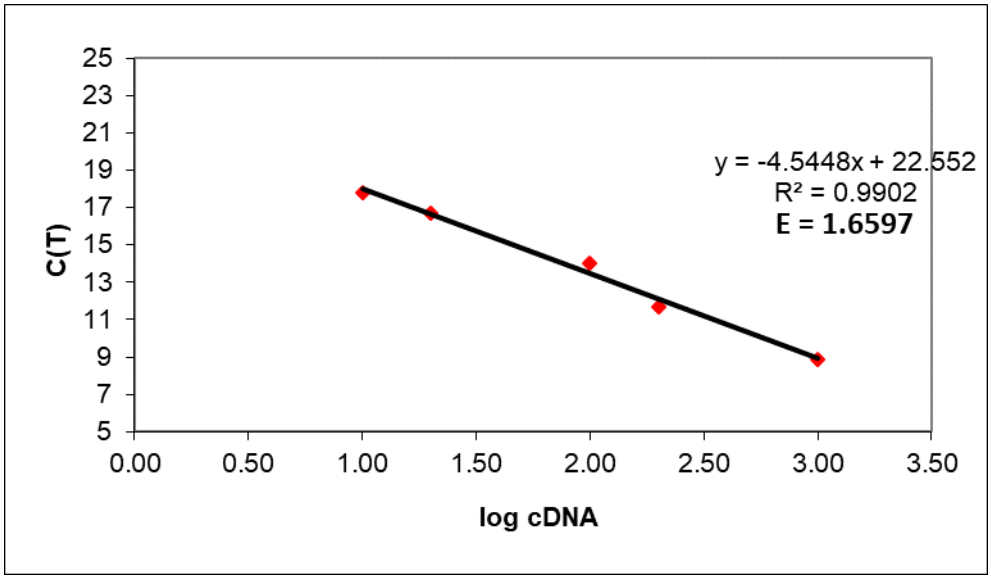
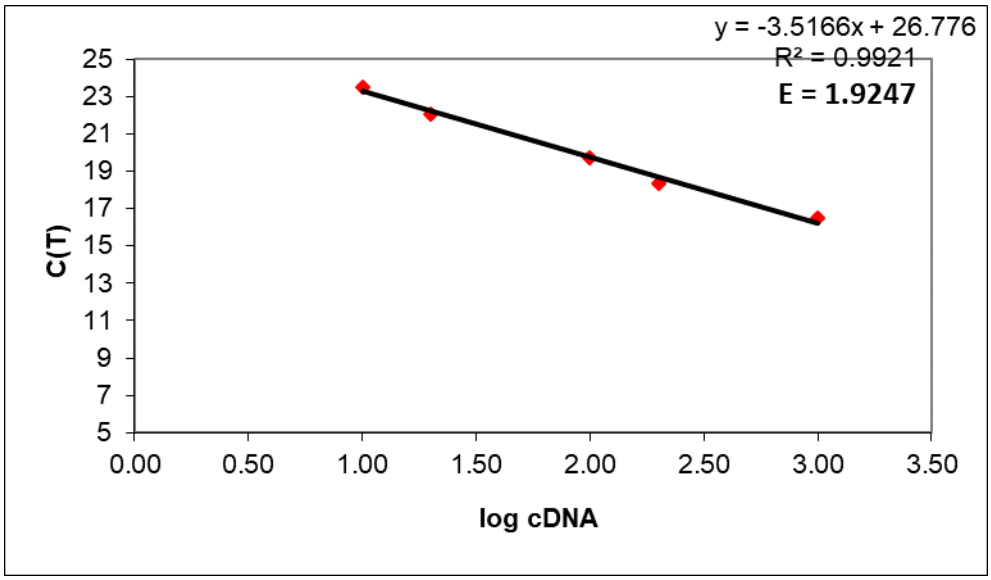


Figure S10. Efficiencies (E) of *18S rRNA* (A), *Ubiquitin* (B), *ChE* (C), *CREB* (D), *CYP-2UI* (E), *GAPDH* (F), *hsp90* (G), *Lefty* (H), *NLK* (I), *NOTCHLESS* (J), *PKS* (K), *PLAUF3* (L), *Ptc* (M), *Smo* (N), *SULT1* (O) genes. For those genes whose expression was detected significantly low, the efficiency was not measured.

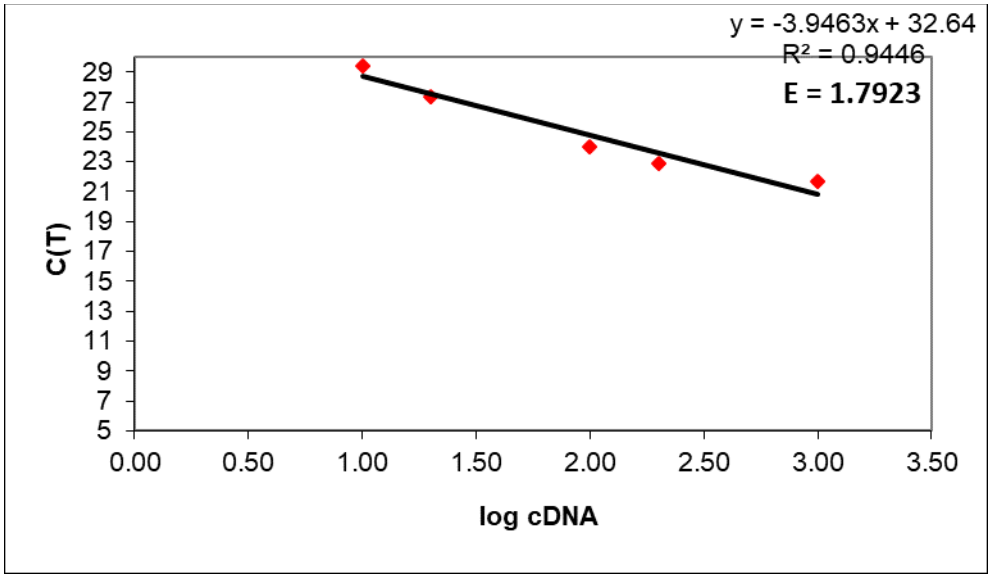
A)



B)



C)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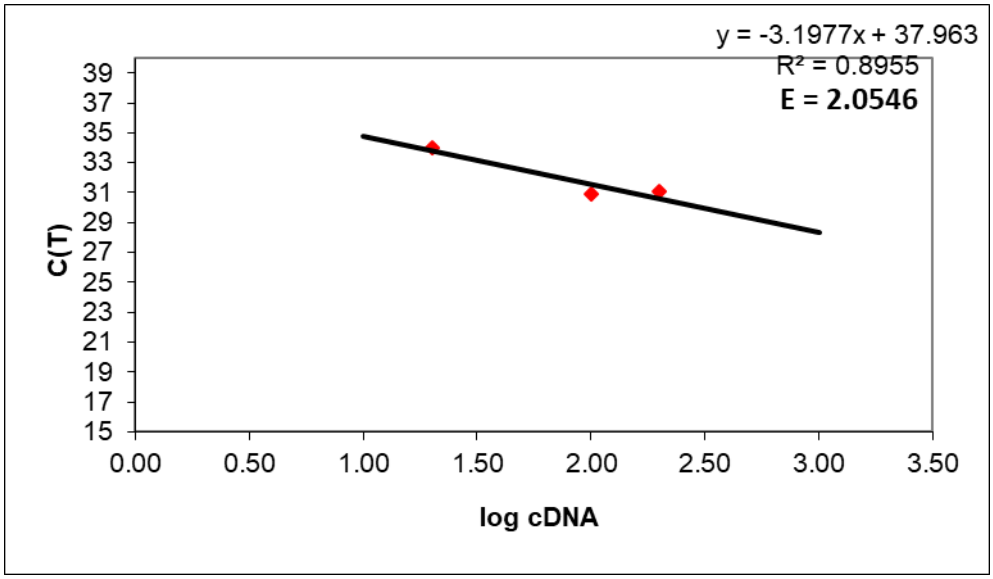
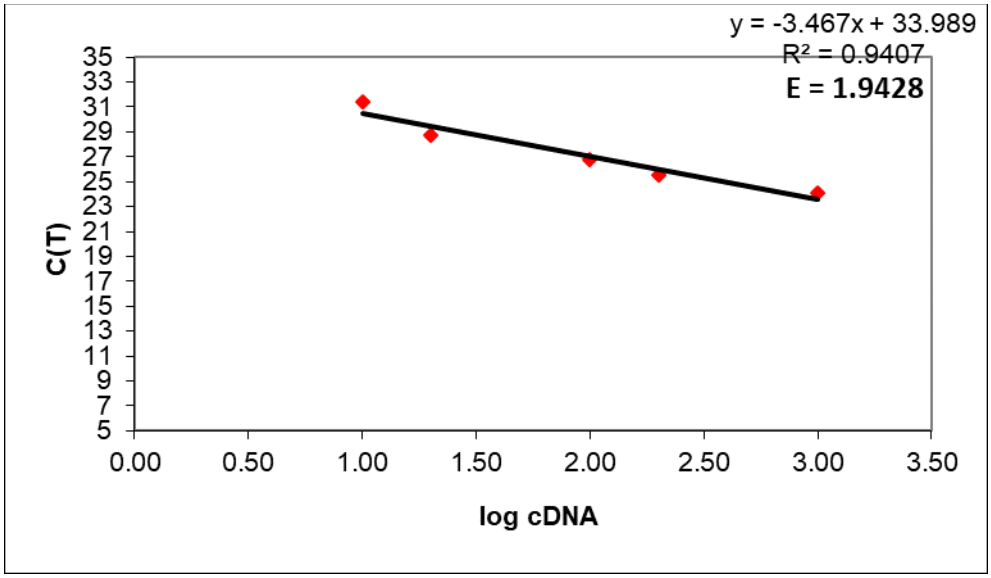
R² = 0.9446
E = 1.7923
C(T)
log cDNA

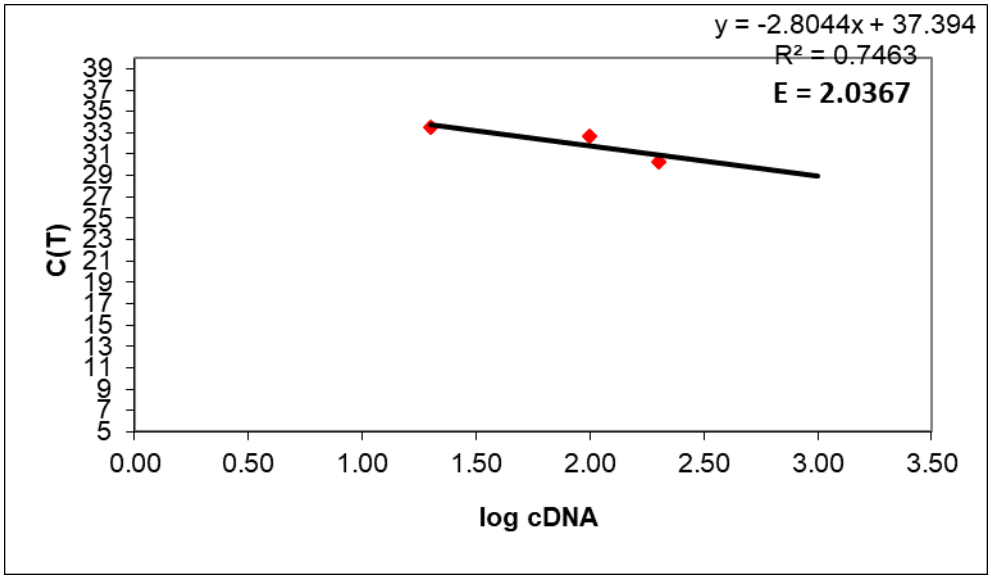
D)


y = -3.1977x + 37.963
R² = 0.8955
E = 2.0546
C(T)
log cDNA

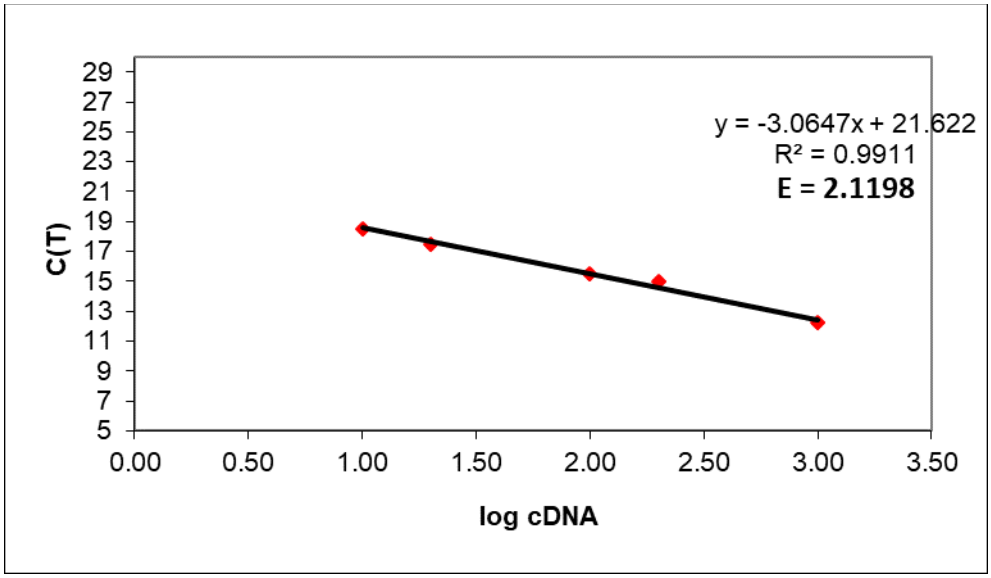
E)


y = -3.467x + 33.989
R² = 0.9407
E = 1.9428
C(T)
log cDNA

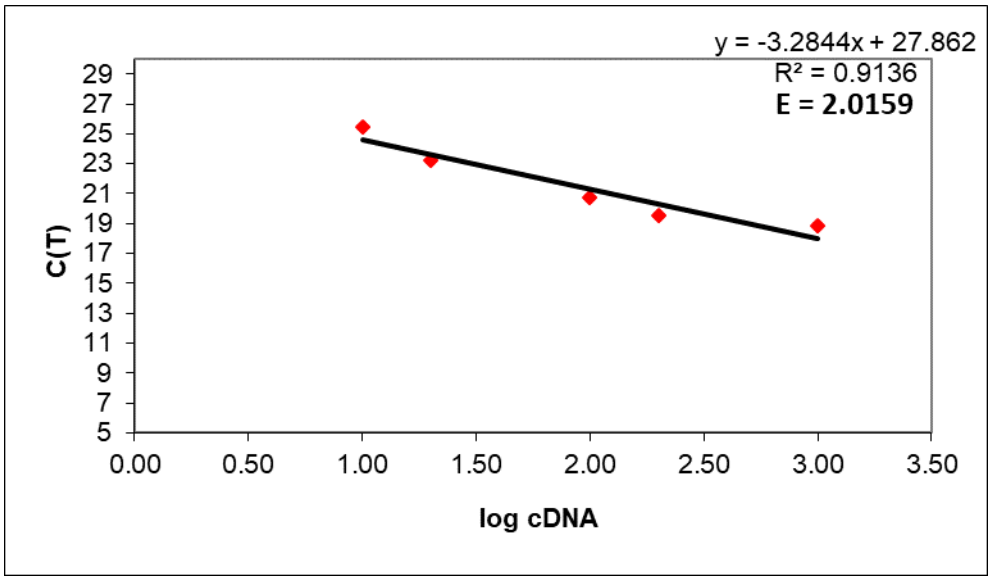
F)


y = -2.8044x + 37.394
R² = 0.7463
E = 2.0367
C(T)
log cDNA

G)


y = -3.0647x + 21.622
R² = 0.9911
E = 2.1198
C(T)
log cDNA
29
27
25
23
21
19
17
15
13
11
9
7
5
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50

H)


y = -3.2844x + 27.862
R² = 0.9136
E = 2.0159
C(T)
log cDNA
29
27
25
23
21
19
17
15
13
11
9
7
5
0.00
0.50
1.00
1.50
2.00
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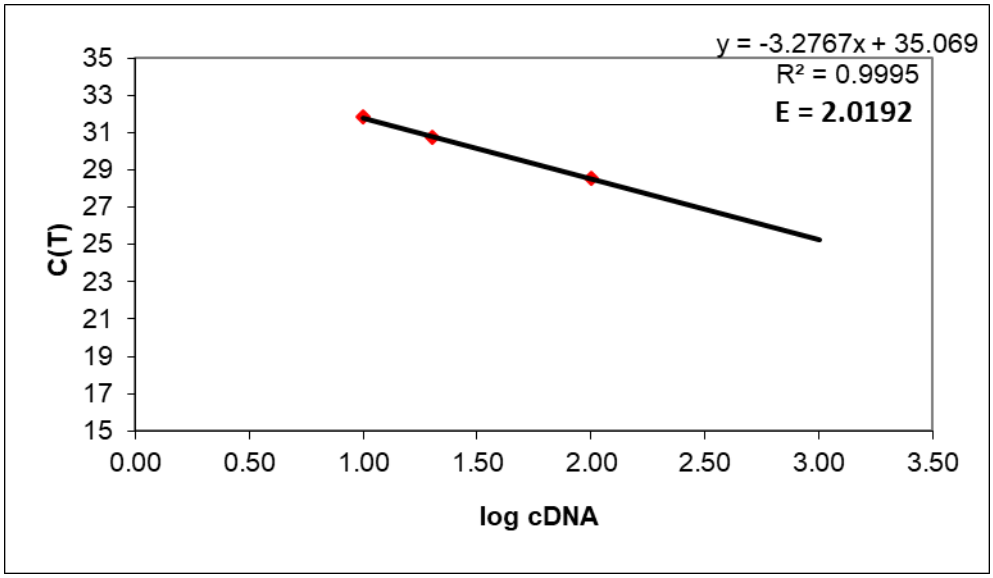
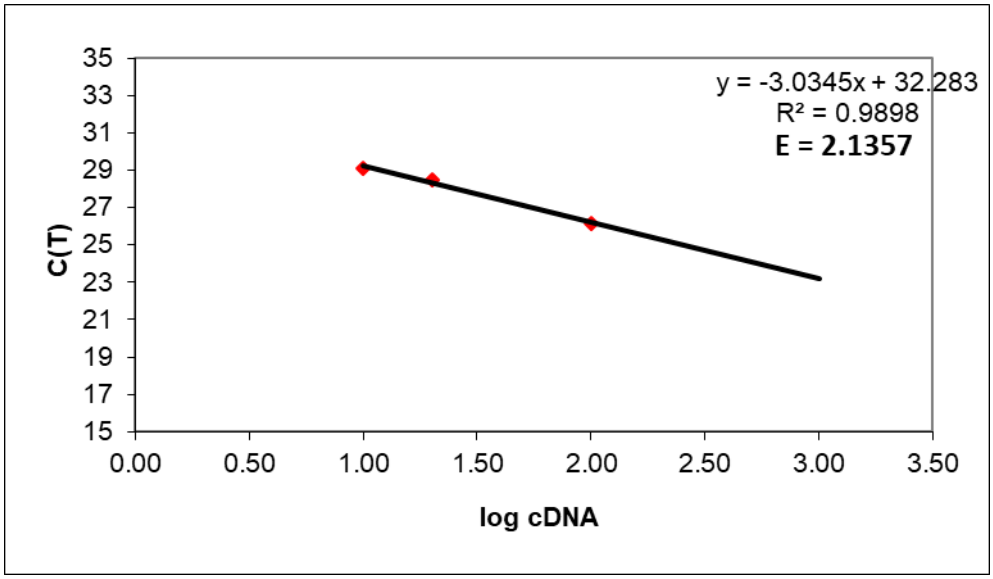
3.00
3.50

I)


y = -3.2767x + 35.069
R² = 0.9995
E = 2.0192
C(T)
log cDNA
35
33
31
29
27
25
23
21
19
17
15
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50

J)


y = -3.0345x + 32.283
R² = 0.9898
E = 2.1357
C(T)
log cDNA
35
33
31
29
27
25
23
21
19
17
15
0.00
0.50
1.00
1.50
2.00
2.50
3.00
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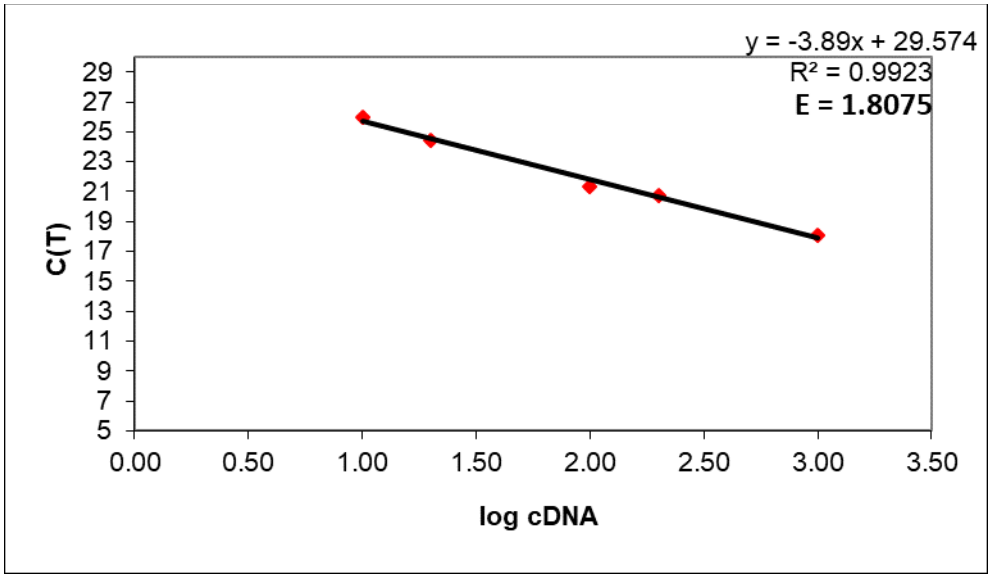
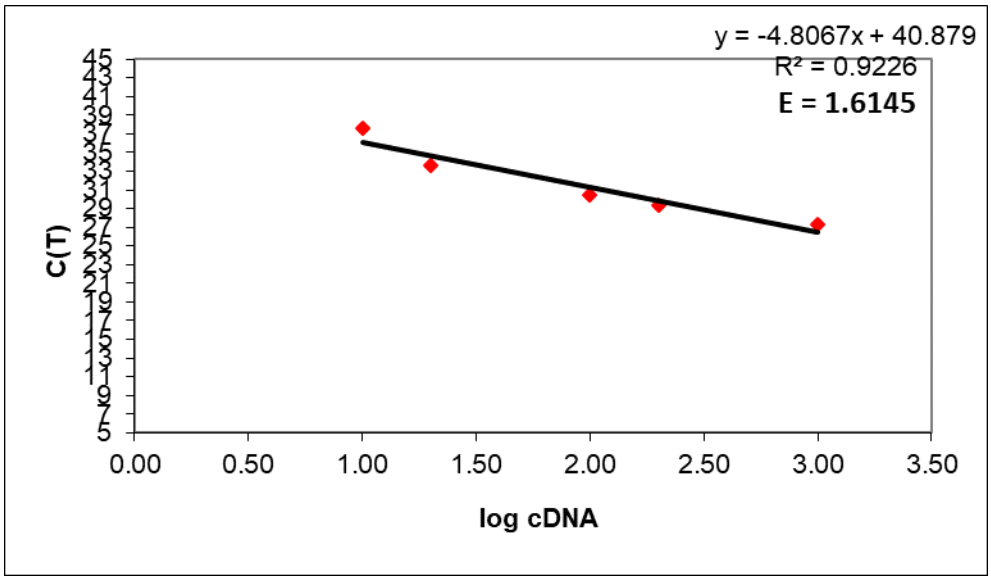
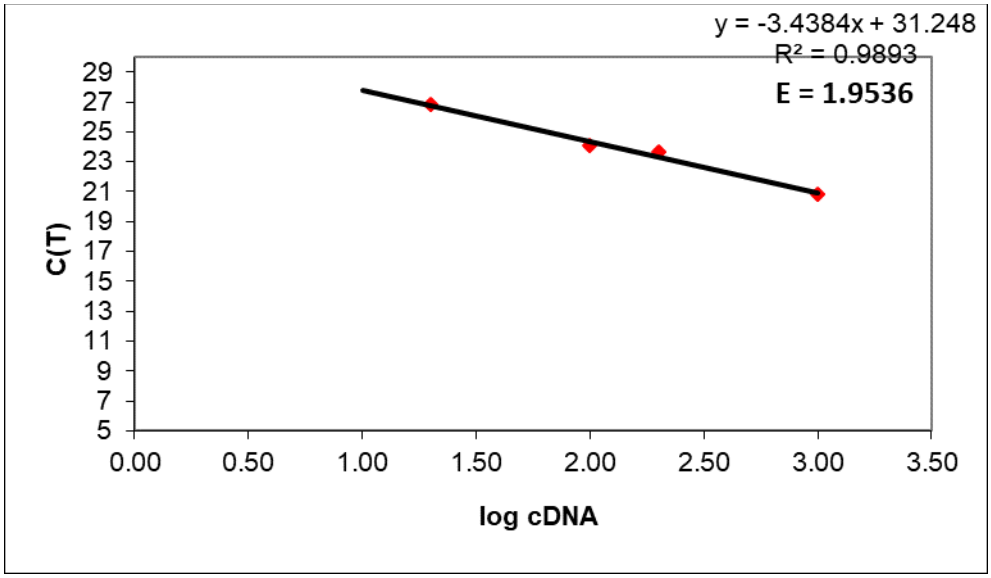
K)


y = -3.89x + 29.574
R² = 0.9923
E = 1.8075
C(T)
log cDNA

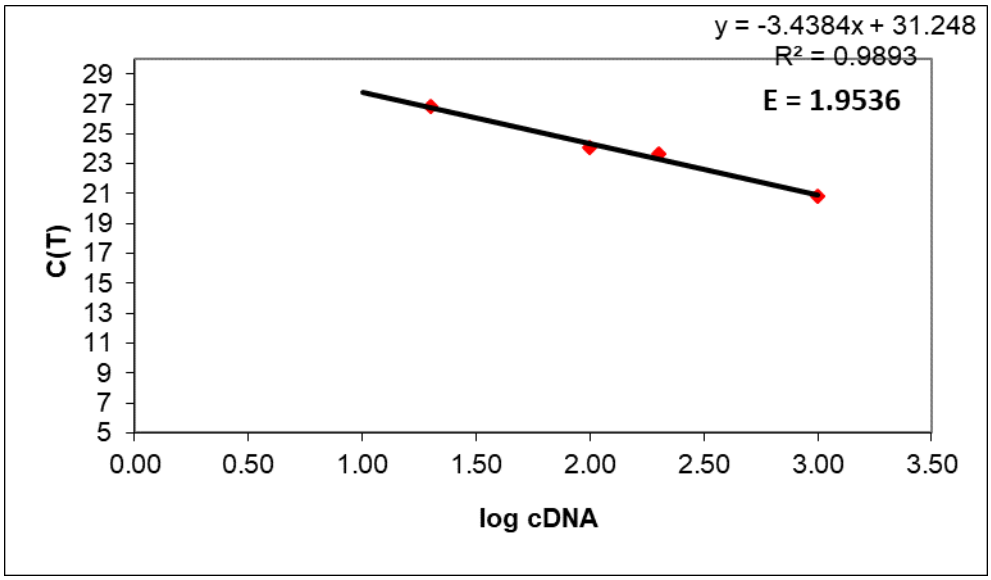
L)


y = -4.8067x + 40.879
R² = 0.9226
E = 1.6145
C(T)
log cDNA

M)


y = -3.4384x + 31.248
R² = 0.9893
E = 1.9536
C(T)
29
27
25
23
21
19
17
15
13
11
9
7
5
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
log cDNA

N)


y = -3.4384x + 31.248
R² = 0.9893
E = 1.9536
C(T)
29
27
25
23
21
19
17
15
13
11
9
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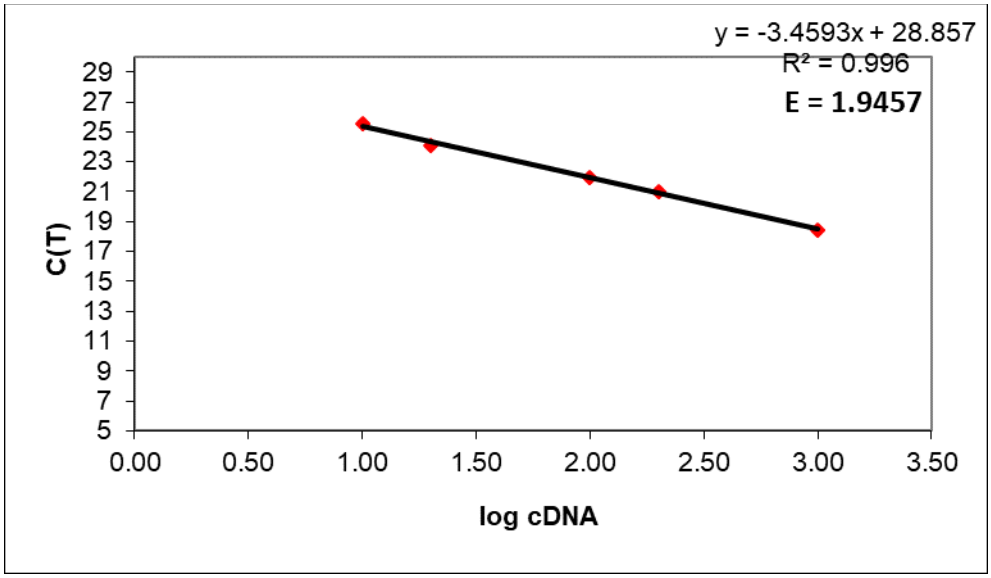
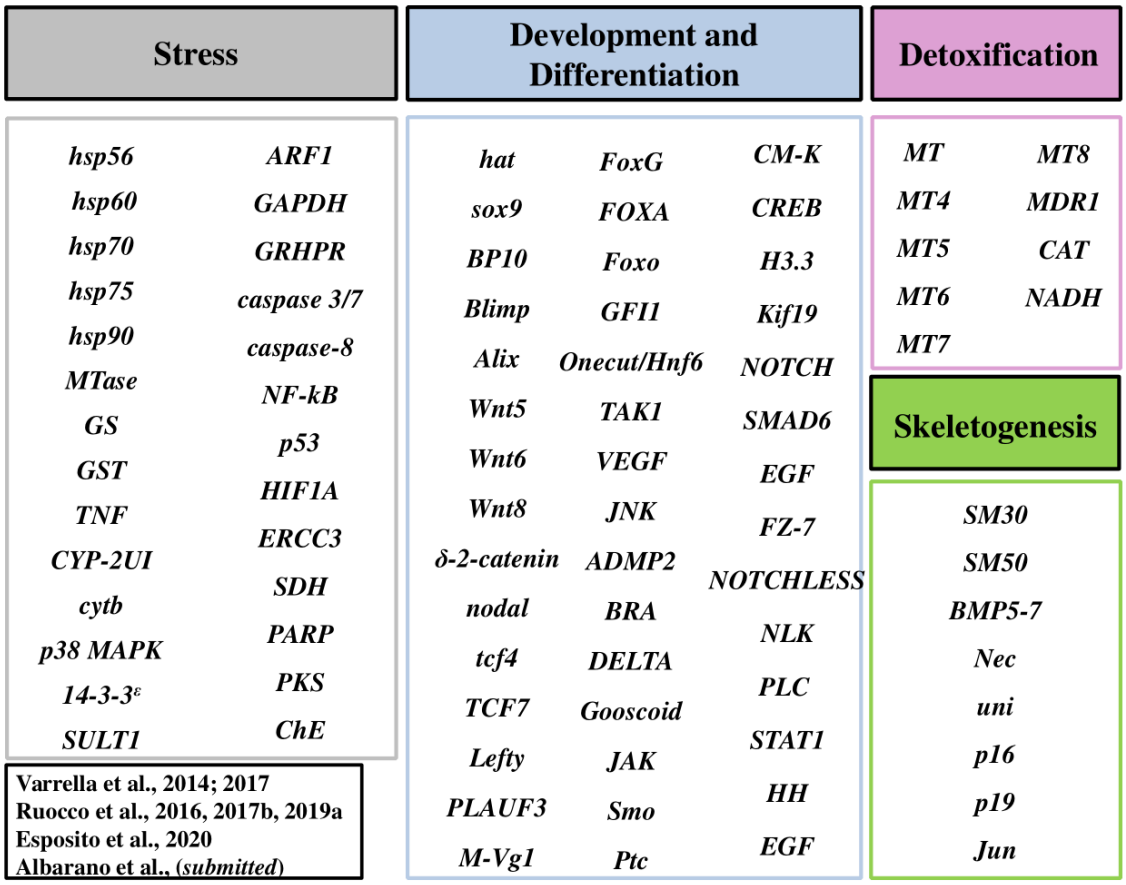
5
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
log cDNA

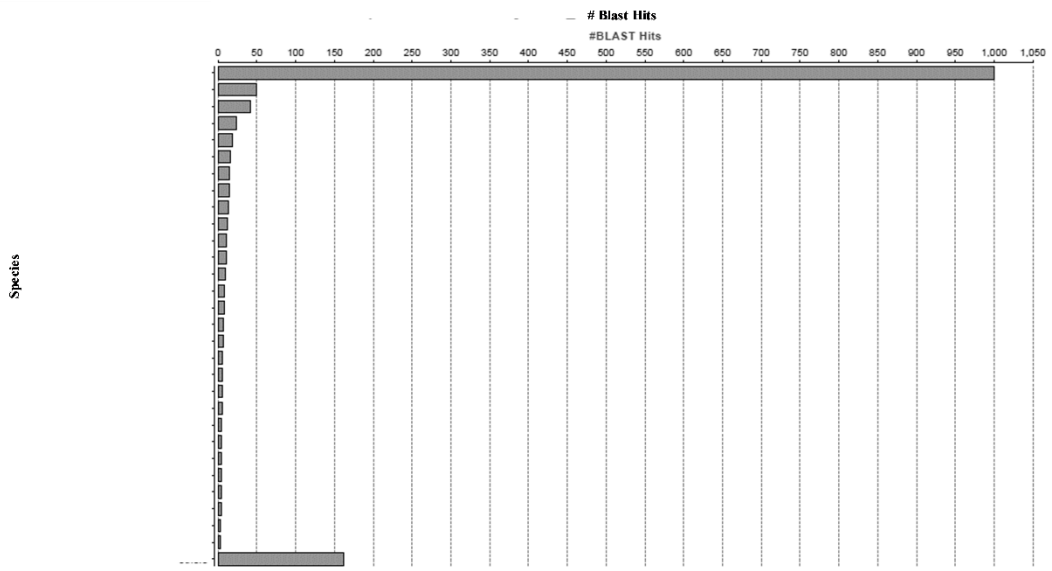
O)


y = -3.4593x + 28.857
R² = 0.996
E = 1.9457
C(T)
29
27
25
23
21
19
17
15
13
11
9
7
5
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
log cDNA

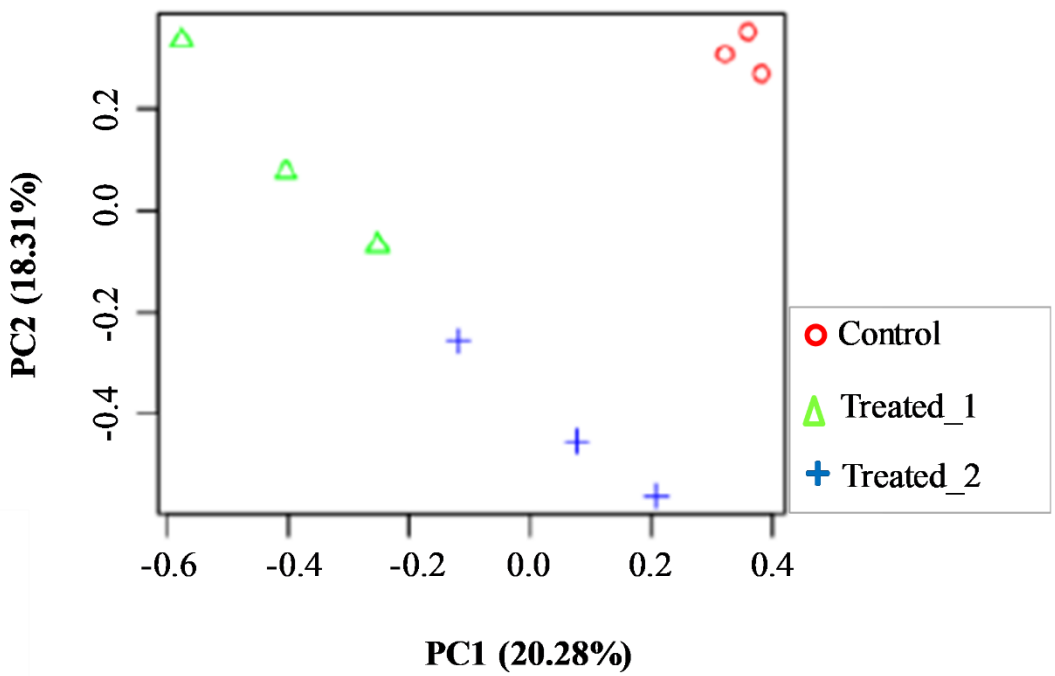
Supplementary Figure S11. Summary of the eighty-seven genes analyzed by Real Time qPCR involved in stress, skeletogenesis, development/differentiation and detoxification process. Bibliographic references for the identification of these genes see Varrella et al. (2014; 2017), Ruocco et al. (2016; 2017b; 2019a), Esposito et al. (2020), Albarano et al., (*submitted*).



Supplementary Figure S12. Blastx top hit species distribution (reported as number) of matches with known sequences.

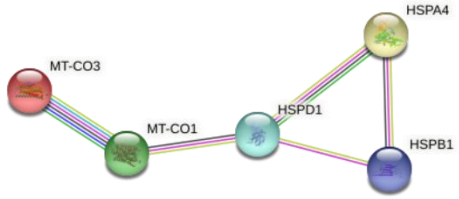


Supplementary Figure S13. Principal Component Analysis (PCA) to evaluate the correlation of samples: Control, Control1, C2 and C3, plutei from adult sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants); the triplicated for plutei deriving from adults exposed for two months to sediment contaminated with PAHs (Treated 1_1, 1_2 and 1_3); the triplicated for plutei deriving from adults exposed for two months to sediment contaminated with PCBs (Treated 2_1, 2_2 and 2_3).



Supplementary Figure S14. A) Interactomic analysis by STRING (https://string-db.org/). The network graphically displays the relationship between genes. The biological relationships between genes are indicated by different colours. Known interactions: reported by database = light blue and determined experimentally = pink. Expected interactions: gene proximity= green; gene fusion = red; genes with similar pattern = light blue. B) Human gene names and the corresponding *A. franciscana* orthologous genes.

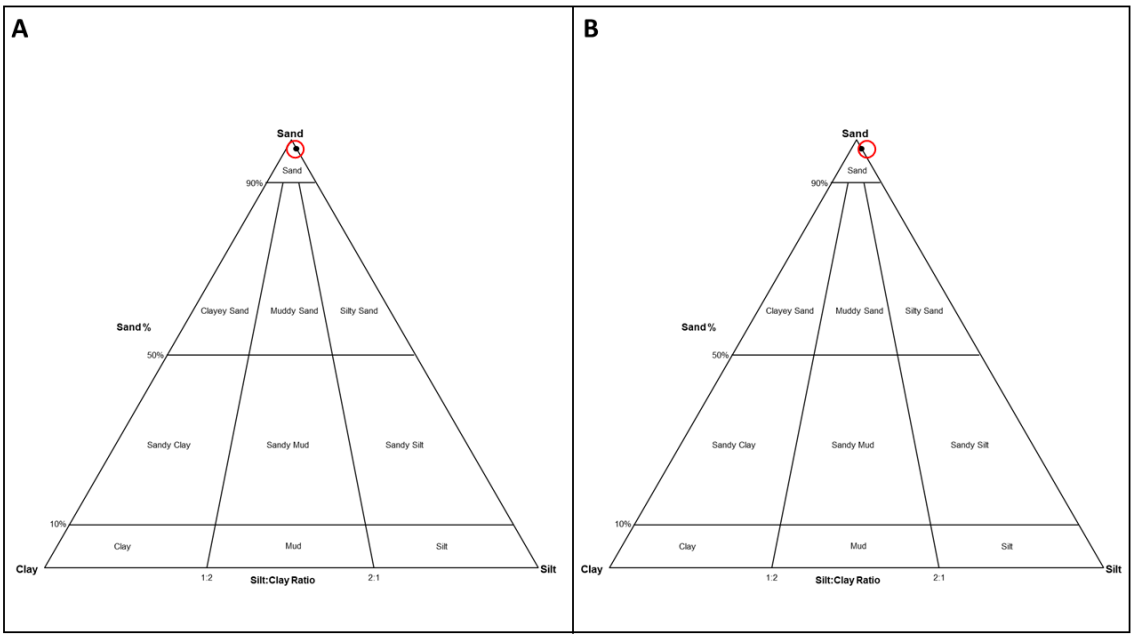
A



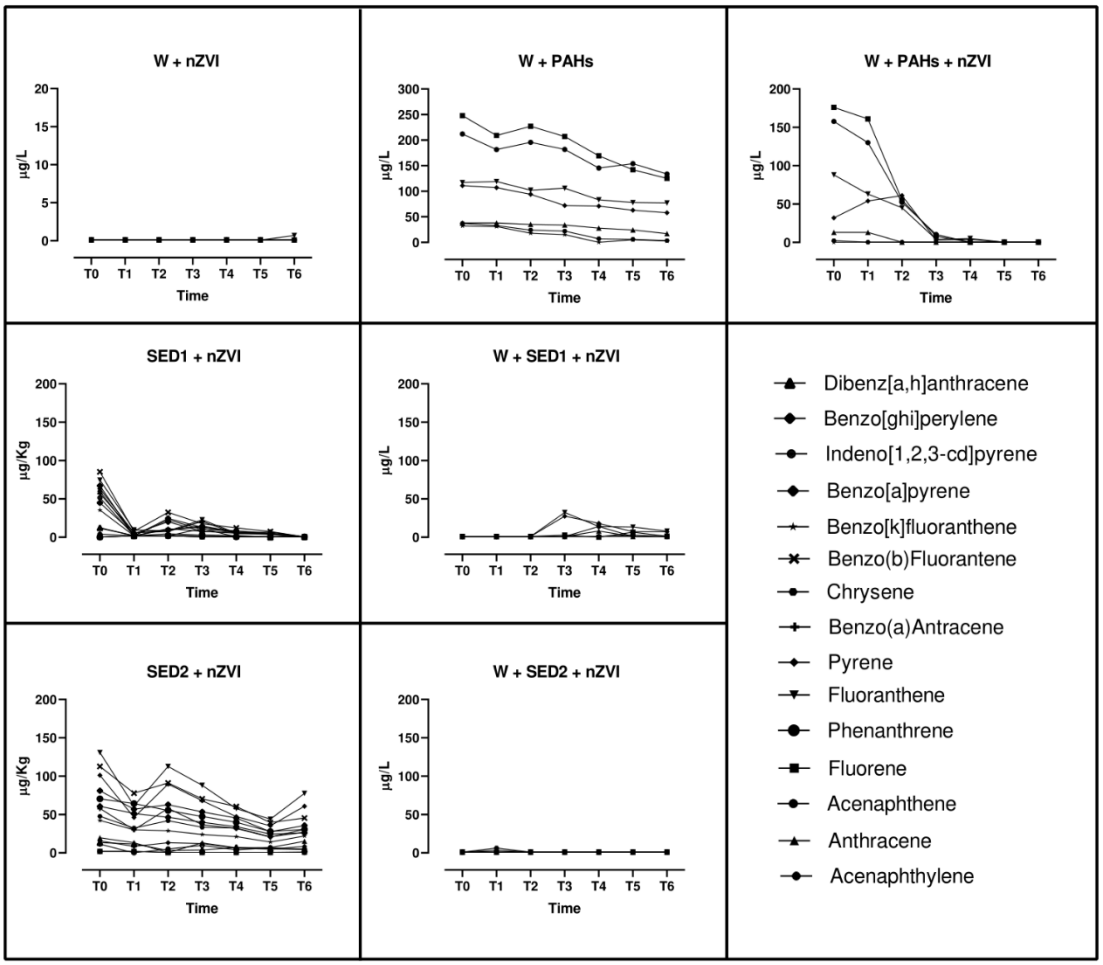
B

Gene name	*A. franciscana*	*H. sapiens*
heat shock protein 26	*hsp26*	*HSPB1*
heat shock protein 70	*hsp70*	*HSPA4*
heat shock protein 60	*hsp60*	*HSPD1*
Cytochrome c oxidase subunit I	*COXI*	*MT-CO1*
Cytochrome c oxidase subunit III	*COXIII*	*MT-CO3*

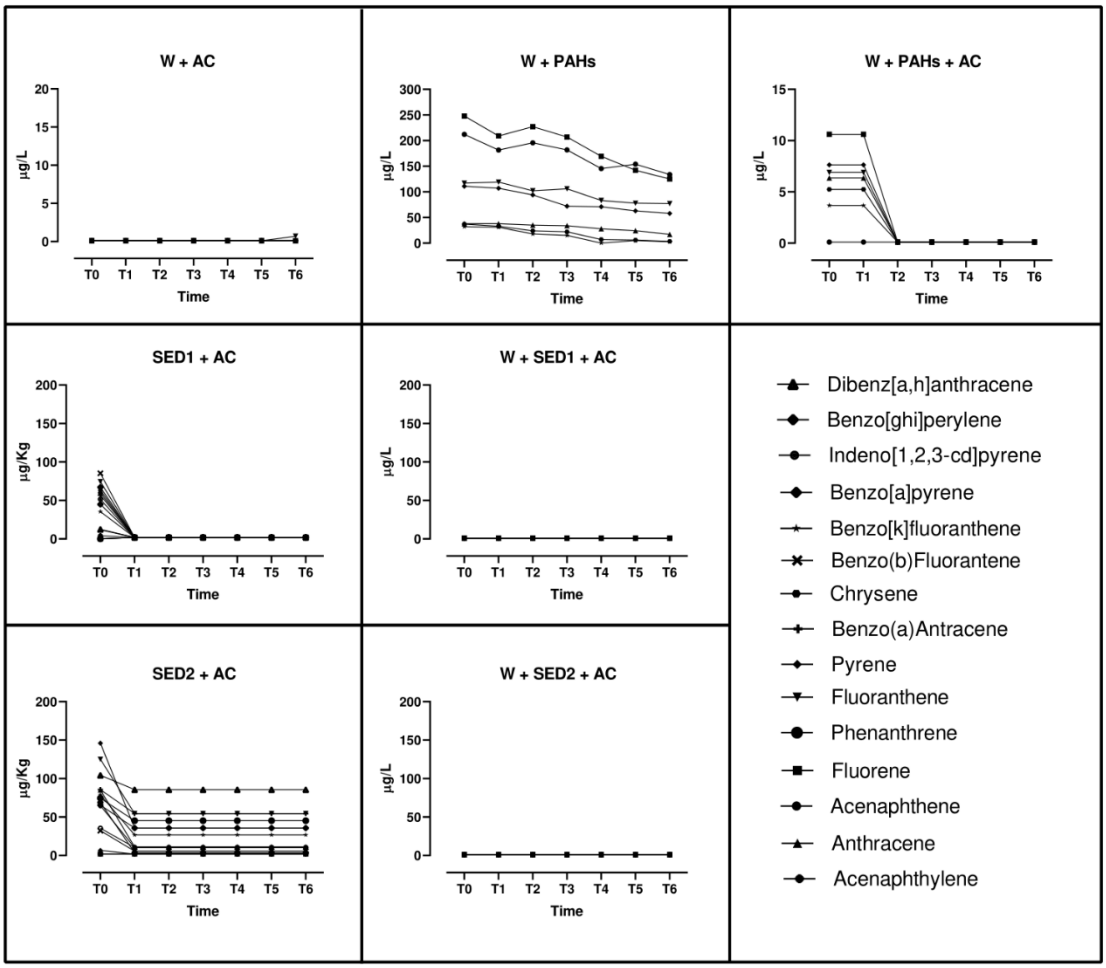
Supplementary Figure S15. Data of the sediment grain size analyzed with GradiStat software (version 8.0 based) to calculate particle size statistics for sieve or laser granulometric data. A) SED1; B) SED2.



Supplementary Figure S16. Effect of nZVI dosage on single compound removal. Experimental conditions: negative control (W + nZVI); positive control (W + PAHs); sediment spiked with PAHs + amendments (W + PAHs + nZVI); seawater + sediment1 (SED1) with amendments (W + SED1 nZVI); and seawater + sediment2 (SED2) plus amendments (W + SED2 + nZVI). Data points represent averages of three samples from triplicate reactors.



Supplementary Figure S17. Effect of AC dosage on single compound removal. Experimental conditions: negative control (W + AC); positive control (W + PAHs); sediment spiked with PAHs + amendments (W + PAHs + AC); seawater + sediment1 (SED1) with amendments (W + SED1 + AC); and seawater + sediment2 (SED2) plus amendments (W + SED2 + AC). Data points represent averages of three samples from triplicate reactors.



Appendix B

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Review

Marine sediment toxicity: A focus on micro- and mesocosms towards remediation

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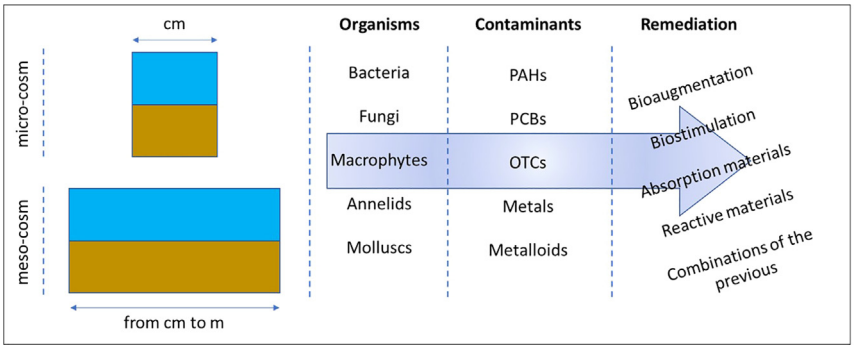
[b] Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cinthia 21, 80126, Napoli, Italy
[c] Centro Servizi Metrologici e Tecnologici Avanzati (CeSMA), Complesso Universitario di Monte Sant'Angelo, Via Cinthia 21, 80126, Napoli, Italy

HIGHLIGHTS

- M-cosms provide a link between observational field studies and natural environment.
- Remediation of contaminated marine sediments could be supported by m-cosms studies.
- Case-studies exist on m-cosms and remediation about PAHs, PCBs and heavy metals.
- Main limitations are on m-cosm's structure and components, and model species.

GRAPHICAL ABSTRACT



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Polycyclic aromatic hydrocarbons
Remediation

ABSTRACT

Micro- and/or mesocosms are experimental tools bringing ecologically relevant components of the natural environment under controlled conditions closest to the real world, without losing the advantage of reliable reference conditions and replications, providing a link between laboratory studies and filed studies in natural environments. Here, for the first time, a formal comparison of different types of mesocosm applied to the study of marine contaminants is offered, considering that pollution of coastal areas represented a major concern in the last decades because of the abundance of discharged toxic substances. In particular, the structural characteristics of micro- and mesocosms (m-cosms) used to study marine contaminated sediments were reviewed, focusing on their advantages/disadvantages. Their potentiality to investigate sediment remediation have been discussed, offering new perspective on how the use of m-cosms can be useful for the development of practical application in the development of solutions for contaminated sediment management in the contaminated marine environment.

Contents

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1. Contaminated marine sediment and EU directives

Environmental concerns arose in most European countries in the mid-1960s, and national governments started to be more active in their attempts to monitor and control environmental pollution. Major focus was devoted to developing and implementing environmental quality guidelines in policies and regulations (Van Wensem, 1989). Special attention was dedicated to the disposal of sediment, resulting from dredging activities of port channels and seaways to maintain maritime navigation. Marine sediments are fundamental and integrated parts of water bodies and are composed of soluble and insoluble matter, primarily rock and soil particles transported from land areas to the oceans by some natural processes, mainly rivers, glaciers and ice, windblown dust, coastal erosion (Brils, 2008). Recent studies showed that Mediterranean coastal sediment may be heavily contaminated, due to industrial, shipping and other anthropogenic activities (Pougnet et al., 2014; Lofrano et al., 2017). Sediment pollution is associated with potential economic, social and environmental problems (Eggleton and Thomas, 2004; Arizzi Novelli et al., 2006; Libralato et al., 2008; Lofrano et al., 2016).

Marine pollution in coastal areas is a major concern, due to the large number of toxic substances discharged and accumulated in sediment, which act as sink and source of pollution (Arizzi Novelli et al., 2006). Especially in harbours and marinas, where exchanges of water masses with the open sea is limited, the accumulation of toxic substances can pose major concerns for human and environmental health such as in presence of recreational waters and maricultural activities (Mamindy-Pajany et al., 2010). Frequently, contaminants occur as mixtures showing combined effects, which are still largely unknown. Dredging activities in industrial and commercial ports tend to remobilize sediment, as well as the associated pollution through the washing out of both short- and long-term contaminant loadings (Arizzi Novelli et al., 2006; Libralato et al., 2008). An effective assessment of sediment quality is compulsory for the management of marine environment as required by the Water Framework Directive (2000/60/EC) (WFD) and the Marine Strategy Framework Directive (2008/56/EC) (MSFD) that considered sediment as one of the key issues for the proper management of surface water bodies. Sediment toxicity proved to be essential in monitoring studies to characterize the state-of-the-art of aquatic environments and to take decisions about contaminated areas according to the TRIAD approach (Losso and Volpi Ghirardini, 2010; Libralato et al., 2008; Hurel et al., 2017). Polluted sediment represents an important issue for fresh, brackish and marine ecosystems, especially coastal ones, due to the high human pressures (i.e. commercial and industrial port activities, human settlements and tourism) and sedimentation rates caused by solid discharges from catchment basins (Nikolaou et al., 2009a; Lofrano et al., 2016). Sediment drains and temporarily segregates pollution with potential direct acute and chronic effects for benthic communities. Natural (i.e., bioturbation) or artificial (i.e., dredging) perturbative events can release the accumulated contamination causing acute concerns to water column populations and the re-allocation of contaminants within the same aquatic environment. Thus, contamination can be scattered in vaster areas or sometimes exported outside from confined aquatic ecosystem (e.g., lake or lagoon) due to flooding events or tides (Arizzi Novelli et al., 2006; Nikolaou et al., 2009b; Mamindy-Pajany et al., 2010). This activity must be carried out in a highly efficient and environmentally safe manner to reduce and keep impacts. Dredged sediment can be treated *ex situ* "on-site" or "off-site" and, finally, transported to its destination (e.g., landfill) or second life (e.g. construction materials). Sometimes the problem of polluted sediment can be tackled without dredging, keeping them in place especially when it must not be removed for assuring drafting ships and if physical dynamics of sites (i.e., currents and wave actions) are not of concern. Dredging and disposal of dredged material are constantly identified as top priority concern by European seaports since 1996 and they are a top priority independently of the port's size. As recently estimated, up to 200 million m^3 of dredged sediment are produced yearly at EU (SedNET, 2004; http://www.sednet.org/download/Sednet_booklet_final.pdf). No clear indications on how to manage them, besides landfill disposal, are available.

Anthropogenic activities can change the natural compositions, the rate, the deposition and the transport of sediment causing a radical shift of biological responses (Burton and Johnston, 2010). Since sediment represents the habitat for benthic community, they could negatively affect the marine flora and fauna (Yi et al., 2011). Several biological communities can react differently to contaminant exposure: i) tolerant species will respond differently with respect to sensitive species; ii) the overall community tolerance is often induced by the constant contaminants' exposure (Clements et al., 2009). The distribution of contaminants within sediment and their affinity with sediment particles may change following receipt to sediment disturbance that stimulates the mobilization of contaminants. Desorption, partitioning, bacterial degradation and the oxidation of organic compounds can accelerate changes of the redox potential and pH (Eggleton and Thomas, 2004). Anthropogenic activities, such as maintenance, capital dredging and the disposal of historically contaminated sediment are also able to induce the remobilization of pollutants in the water column (Eggleton and Thomas, 2004). This resuspension can negatively influence the marine environment and human health, because pollutants can accumulate in bottom feeders and magnify through marine food webs, reaching humans through fishery products (Zupo et al., 2019). For these reasons, the European Water Framework Directive 2000/60/EC (WFD) was developed and constantly updated. This directive provided a new approach for preventing the qualitative and quantitative deterioration of water, to improve the state of water and to assure a sustainable use (Borja et al., 2004). This directive does not specifically address sediment, although sediments are essential for the aquatic environmental.

Since the sediment management plays an important role in preservation of good marine water status, the European Marine Strategy Framework Directive 2008/56/EC (MSFD) includes 11 descriptors including sediment as well (Rice et al., 2012). Both Directives indicated what a "good marine environmental status" is, but the WFD classified ecosystems at different levels by comparing their features until combining them; whereas the MSFD focuses on the 11 descriptors, which together represent the function of the entire system. Thus, the WFD could be combined with the MSFD (Borja et al., 2010). Considering the potential great impact of contaminated sediment to the marine environment, the first step to deal with this problem could be the control of the contaminant source and/or its elimination of both *in situ* and *ex situ* treatments (Gomes et al., 2013).

To the best of our knowledge, scattered studies exist on the use of micro- and mesocosms (m-cosms) to investigate the effects of polluted marine sediment and remediation activities. For these reasons the main aim of this review is to assess m-cosms studies applied to contaminated sediment. In particular, the present study focuses on i) the problem of contaminated marine sediment and the EU directives, ii) structural properties of micro- and mesocoms applied to aquatic studies to detect sediment toxicity; iii) advantages and disadvantages of m-cosms for sediment quality assessment; iv) sediment remediation assessment through m-cosms, focusing on sediment contaminated with polycyclic aromatic hydrocarbons, polychlorinated biphenyls and heavy metals.

1.1. Sediment assessment: From micro- to mesocoms

M-cosms are experimental systems that enable controlling ecologically-relevant components of the natural environment. These controlled conditions are close to the real world and present the advantage of reliable referencing of conditions and replication (Watts and Bigg, 2001). M-cosms provide a link between observational field studies that take place in natural environments, evaluating how organisms or communities might react to environmental changes or exposure to pollutants. They can represent a good compromise between the ecosystem complexity and the highly artificial settings of the laboratory experiments. The debate about m-cosms' definition is still open as well as about their advantages or disadvantages compared to the one-species toxicity testing (Cairns, 1983; Tarradellas et al., 1996). M-cosms can be defined in terms of the size and complexity of the experimental system rather than the number of species they support.

A mesocosm was defined by Odum (1984) as "a bounded and partially enclosed outdoor experimental unit, which simulates the natural environment, particularly the aquatic environment". This type of system provides a bridge between the real environment and the laboratory, particularly useful to study the biological effects of chemicals in ecotoxicology (Crossland and La Point, 1992).

In such experiments the microcosms consisted of polyethylene tubes, glass bottles and beakers starting from few liters to a maximum volume of 500 L. The differences between microcosms and mesocosms were only a matter of scale in terms of volumes as well as of parameters to be analyzed. The physical size of mesocosm systems ranged from 1 m depth (Petersen et al., 1997) to 18 m or roughly 1–400 m^3 in volume (Harada et al., 1996). Mesocosms (Teuben, 1992) were mainly used in field experiments to fill the gap between experimental laboratory work and natural conditions. Some examples are reported in Fig. 1.

M-cosms are small-scale experimental systems which inform about ecological processes that are applicable at larger scales (Carbonell and Tarazona, 2014; Vidican and Sandor, 2015). More recently, the use of these systems played a role in exploring the consequences of seemingly insurmountable global issues, such as the effect of climate change on species distributions (Petchey et al., 1999), the impacts of pollution and fisheries on ecosystems (Micheli, 1999) or harvesting on population dynamics (Cameron and Benton, 2004). Micro- and mesocosms are basically small ecosystems, able to improve the understanding of natural processes by simplifying the complexities of natural environments. This review assesses some micro- and mesocosm structural properties to strengthen the knowledge about their role in environmental studies applied to marine scenarios.

Micro- and mesocosms can be closed or open systems. However, for studying soil ecosystem processes, the best results were reached using the open flow-through experimental systems.

At the beginning, the microcosms were used by French et al. (1989) to evaluate the effects of different nutrient and chemical additions on water quality. The effects of organic additions on nitrification and denitrification were examined in sediment microcosms (Caffrey et al., 1993). Laboratory-based microcosms were used to determine the persistence of the faecal indicator organism *Escherichia coli* in recreational coastal water (Craig et al., 2004). Results of this microcosm demonstrated the prolonged survival of *E. coli* in coastal sediment compared with overlying water, implying an increased risk of exposure because of the possible resuspension of pathogenic micro-organisms during natural turbulence or human recreational activity.

Anthropogenic inputs of crude and refined petroleum hydrocarbons into the sea required knowledge of the effects of these contaminants on the receiving of assemblages of organisms. A microcosm experiment was carried out to study the influence of diesel on a free-living nematode community of a Tunisian lagoon (Mahmoudi et al., 2005). Sediments were contaminated by diesel and the ecological effects were examined. Dose-dependent changes on the community structure were revealed, as well as the responses of nematodes to the diesel treatments that varied according on a species-by-species basis. Duan et al. (2009) used closed microcosms with glass beakers containing sediment and seawater to define the effects of microbial activity and carbon source on arsenic and iron mobilization. More recently, Santos et al. (2018) tested the effects of exposure to sewage-impacted pore water simultaneously at the community level using meiofauna microcosms and at the individual level of the copepod *Nitokra* sp. by using standard fecundity tests. Considering the importance of meiofauna for benthic ecosystems, the microcosm approach using natural meiobenthic communities provided highly relevant ecological information on the toxicity of contaminated sediments. More in detail, the toxicity of pore waters was tested from three sites according to a contamination gradient. Both approaches revealed differences in toxicity between sites at various levels of contamination.

1.2. Microcosm and sediment toxicity

The first applications of mesocosms in aquatic studies referred to the patterns of productivity and respiration during eutrophication in lower Narragansett Bay (Rhode Island, USA; Oviatt et al., 1986; see Fig. 1A). Another study was applied to detect the effects of chemicals in freshwater environment, varying in size, design, construction and costs according to the purpose (Beklioglu and Moss, 1996). Mesocosm experiments were used to study the interaction of sediment influence, fish predation and aquatic plants with the structure of phytoplankton and zooplankton communities (Beklioglu and Moss 1996).

The Multiscale Experimental Ecosystem Research Center (MEERC) conducted several experiments in mesocosms with the aim of quantifying the effects of scale in terms of time, depth, radius, exchange rate and ecological complexity, on biogeochemical processes and trophic dynamics in different coastal habitats

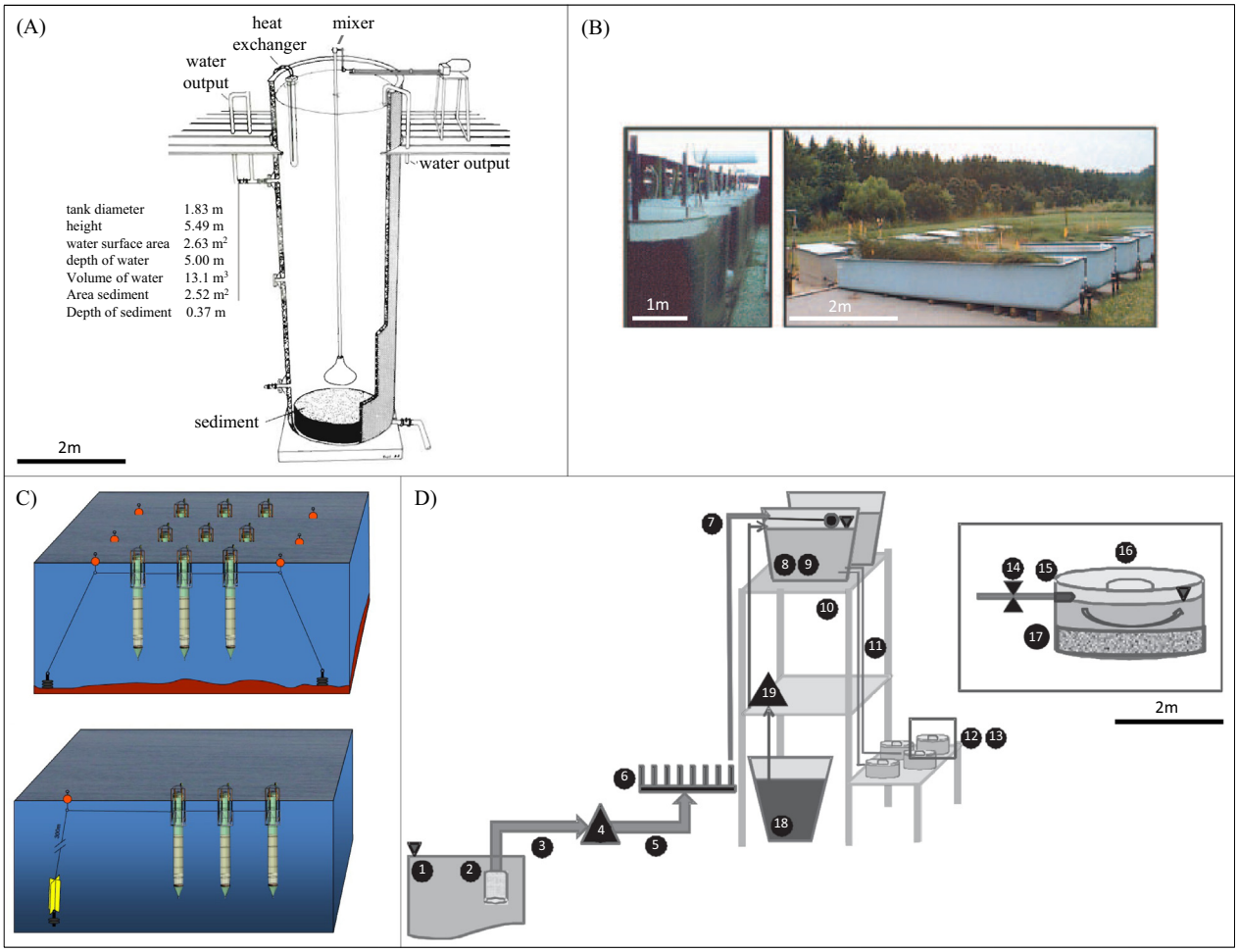

Fig. 1. Mesocosms. (A) Cross section of a mesocosm, showing input and output pipes, mixer, sediment container and heat exchanger. The tanks are constructed of fiberglass reinforced resin with interior walls to maximize the reflection of sunlight. Seawater is fed in a pulsed flow of 10 1iters per minutes for 12 min period every six hours. Temperature control is accomplished with glass heat exchangers able to heat and cool (modified from Oviatt et al., 1986). (B) Multiscale, multihabitat experimental ecosystems for planktonic–benthic systems (left panel), marsh (right panel) (modified from Peterson et al., 2003). (C) Schematic representation of the two modes of mesocosm operation: mesocosms in moored mode in packs of three with anchor weight at each end (upper panel); mesocosms in free-floating mode connected to a weighted drogue hanging from a buoy at 150 m water depth (lower panel) (modified from Biebesell et al., 2013). (D) Experimental set-up. (1) Kauru River, (2) pump inflow covered with 4.5 mm mesh, (3) 20 m of 50-mm polythene piping, (4) two centrifugal pumps, (5) 80 m of 50-mm polythene piping, (6) eightfold manifold, used to split water flow into eight pipes, (7) 8 9 10 m of 25-mm polythene piping, (8) eight header tanks (volume 135 L), each one feeding a batch of 16 mesocosms, (9) water level in header tanks regulated by ball-cock, (10) two-level scaffold, 4.1 m high and 20 m long (11), 128 9 4 m of 13-mm polythene piping supplying individual mesocosms with water, (12) 1-m-high and 1.2-m-wide wooden bench running along scaffold, (13) 128 circular flow-through stream mesocosms, (14) tap regulator, (15) inflow jet, (16) mesocosm water outflow, (17) base layer of substratum, (18) seven barrels (volume 300 L) containing highly concentrated $NaNO_3$ and KH_2PO_4 solution for nutrient treatment levels 2–8, level one received ambient nutrient concentrations, (19) seven fluid metering pumps used to drip highly concentrated nutrient solutions into seven header tanks (mod. from Wagenhoff et al., 2013).

(Petersen et al., 2003; see Fig. 1B). To this end, a series of experiments were performed, in which time, space and complexity in a variety of estuarine habitat types were systematically manipulated. Firstly, the effects of mesocosm size and shape on the dynamics of planktonic-benthic ecosystems were assessed. Fifteen cylindrical mesocosms were constructed according to five sizes, three volumes and three replicates per size and they were organized into three series: one with a constant depth (1.0 m), one with a constant shape (radius-to-depth ratio = 0.57) and one with constant volume (10 m^3). Mesocosms received artificial light on a 12:12 light–dark cycle and an exchange of filtered estuarine water at a rate of 10% per day, mixed to mimic turbulence in a tidal environment. Because tidal estuarine marshes play an important role in processing nutrients and organic matter at the land-sea interface, the effects of species diversity on this function was evaluated. Six marsh mesocosms were constructed using fiberglass tanks (6 m long, 1 m wide, 1 m deep) angled at a 20:1 slope. In this system pumps were used to simulate a 12.5-h tidal exchange with filtered water from an adjacent estuary. The experiment was run for five years to evaluate long-term dynamics of three common plant species found in Chesapeake marshes (*Spartina alterniflora*, *Spartina patens* and *Distichlis spicata*) and no macrofauna were added. While the plant species diversity over the temporal extent of the experiment was preserved, unsuccessful maintenance of the additional animals introduced in the high diversity treatment was recorded. More in general, the results indicated that the effects might be categorized in fundamental (evident in both natural and experimental conditions) and artefacts of enclosure (attributable to the artificial environment in mesocosms).

A mesocosm study was also applied to detect the impact of resuspension on the fate and bioaccumulation of mercury and methylmercury in shallow estuarine environment (Kim et al., 2006). Two experiments of four weeks were performed in spring and fall in cylindrical tanks (1 m^2 of surface area and 1 m deep),

demonstrating that the interplay between mercury and methylmercury degradation determined the overall methylmercury pool in sediments. In turn, their resuspension lead to changes in the association to mercury binding phases, influencing its methylation.

Wagenhoff et al. (2012) studied macroinvertebrate responses along broad stressor gradients of deposited fine sediment and dissolved nutrients in a stream mesocosm. Most responses were additive multiple-stressor patterns. Later these authors also studied the composition of benthic algae and cyanobacteria using the same experimental approach (Wagenhoff et al., 2013; see Fig. 1D).

To perform mesocosm experiments in different hydrographic conditions and in areas considered most sensitive to ocean change, Riebesell et al. (2013) developed a mobile sea-going mesocosm facility, the Kiel Off-Shore Mesocosms for Future Ocean Simulations (KOSMOS). The KOSMOS platform, transported and deployed by mid-sized research vessels, was designed for operation in moored and free-floating mode under low to moderate wave conditions (up to 2.5 m wave heights; see Fig. 1C). It consisted of a water column 2 m in diameter and 15–25 m deep (about 50–75 m^3) without disrupting the vertical structure of the planktonic community. This system minimized the differences in starting conditions between mesocosms, extended experimental duration, determined the mesocosm volume and air–sea gas exchange.

Open system mesocosms were mainly used to simulate the ocean acidification and it has been demonstrated that this approach is low-cost and allows for pioneering long-term experiment with continuous intake of seawater ensuring the maintenance of quasi-natural environmental conditions (Jokiel et al., 2014). This approach is based on a low-cost method applicable to small or very large experimental systems and consisted of a gravity-feed seawater supply system with a peristaltic pump to regulate CO_2 injection rates and a power head, cavitating the injected CO_2 into microscopic bubbles that are dissolved immediately. The system permitted long-term experiments under full sunlight with rapid seawater turnover rate with realistic environmental conditions in the experimental chambers.

Moreover, a modular, small size (60 L) and indoor mesocosm was used to detect the environmental risk of engineered nanomaterials (Auffan et al., 2014). Initially, they waited for the settlement of suspended particles, then the stabilization of the physical parameters (O_2, redox potential and pH) and the growth of organisms were measured. This experimental system allowed the simultaneous monitoring of several parameters (e.g. aggregation, biotransformation, oxidative stress, microbial diversity) under environmentally meaningful conditions. This experimental design can accommodate several types of ecosystems such as lotic, lentic, estuarine, or lagoon environments, without the necessity of expensive infrastructures.

Pansch et al. (2016) used open systems to study the global change of temperature, ocean acidification, rising sea level and eutrophication. The system consisted in tanks of 170 × 85 cm connected to a measurement system used to assess different parameters such as temperature, dissolved oxygen and pH. Closed micro- and mesocosm systems are self-sustaining once built-up without intake of nutrients or more water. These systems were mainly used in toxicity experiments because they avoided the environmental dispersion of contaminants. Another interesting study was recently conducted by Lehto et al. (2017) usinga closed mesocosm of 15 × 14 × 8 cm to explore possible effects of deposition of particulate organic matter and the release of metals from sediment. The examples showed the high versatility of these systems to different conditions, suggesting their challenging applications for contaminated sediment assessment.

2. Pros and cons of m-cosms for sediment quality assessment

At the beginning, the expectancy on the use of microcosms were very high (Draggan, 1976), but three years later Gillett and Witt (1979) concluded that they needed substantial improvements in the accuracy, sensibility and reliability to fulfil these expectations. Later, Giesy (1980), Hammonds (1981) and Van Vorris et al. (1983) reported about the improvements and discussed about advantages and disadvantages of the use of these systems. One of the major points concerned technical questions on the physical, chemical, biotic structure as well as the duration of experimental micro- and mesocosm (Table 1). Moreover, another critical point referred to the relationship of the results with a natural ecosystem, considering if m-cosms were analogous to natural ecosystems and/or are model ecosystems. Ausmus and O'Neill (1978) suggested some criteria for the extrapolation of m-cosms experiments to the field, considering if they reflected a natural ecosystem.

As reported, concerning the different types of m-cosm systems (Cole et al., 1978; Coleman et al., 1978; Anderson et al., 1979; Parkinson et al., 1979; Clarholm et al., 1981; Hassall et al., 1986) the best results for a valuable comparison can be expected from open systems (Huhta et al., 1988; Setälä et al., 1988; Taylor and Parkinson, 1988, 1989; Williams and Griffiths, 1989). Some authors reported the results of laboratory microcosm experiments (Teuben and Roelofsma, 1990) and field experiments with mesocosms (Teuben, 1991a, b) comparing them with measurements of process variables from the field, to test the relevance of these micro- and mesocosm studies. These results concluded that m-cosms were reliable to unravel the intrinsic soil ecosystem interactions, but mesocosms in the field were referred to laboratory microcosms. At the beginning microcosm tests are more expensive and time consuming, than other types of traditional tests for environmental issues (Gorsuch et al., 1993).

In addition, it is important to consider that the prevention of the ecological impacts of global environmental changes require scientific evidences, but the temporal and spatial scales of these environmental problems are too extensive and become difficult to gain only by using the traditional experimental manipulation (McCann, 2007). M-cosms might help, even if a 'credibility gap' can arise from within the scientific community, in understanding if insights from these experimental systems could be relevant to study larger scale processes. Benton et al. (2007) considered the key role of m-cosms in the attempt to understand large-scale processes. having two important roles: i) providing ideal systems on which the statistical approaches can be tested; ii) managing recommendations on systems with limited knowledge of the ecological processes are involved.

Despite their distinct contribution to our understanding of ecology and the environment, however, microcosm approaches have been often criticized for being irrelevant for policy-relevant issues (Carpenter, 1996; Srivastava and Vellend, 2005). An opposite view-

Table 1
Advantages and disadvantages in the use of micro- and mesocosms.

Advantages	Disadvantages
More sensitive than laboratory experiments	Low number of replicates
More reproducibile data and easier to conduct experiments	Small size and short experiment time
Use of more species for defining the interaction beetween them	Inaplicable to understanding less simple parts of nature
Fills a gap beetween laboratory and field studies	Not realistic

point was presented by Cadotte et al. (2005), claiming that laboratory models were necessary for investigating complex ecological communities. This may have been pushed by the urgency to explore the fundamental processes of ecology rather than with the aim to use model systems to address real world and global-scale problems.

Ecologists tried to build projects around microcosm experiments, because, in addition to the basic-science insights, *m*-cosms have pragmatic advantages important in the competition between environmental sciences and molecular biology (Carpenter, 1996). They can provide i) results to be rapidly published; ii) modest costs. Ecology is now considered a significant applied science with a responsibility versus the society in contributing to solve environmental problems by using appropriate scaled field studies. (Likens, 1992; Shrader-Frechette and McCoy, 1993), using all available tools to advance the analysis of ecosystems and their communities at the scales of natural processes, management and societal concern.

More recently, Drake and Kramer (2012) evidenced that m-cosms are ecologically simplistic, mainly the subset of species included, such as the exclusion of large-bodied species (Schindler, 1998), meaning that the reduction of complexity decreases the capacity of an experiment to provide reliable predictions about the ecosystem. M-cosms did not consider natural spatial and temporal variation, being small in size and short in duration and so limiting physical environmental heterogeneity and excluding processes at large spatial extents (Diamond, 1986; Ricklefs, 2004). The use of mesocosms, essentially larger than microcosms, often includes at least partially these sources of variation (Relyea, 2005), giving rise to potential artefacts.

The use of m-cosms enables to study the stress response of various species and the effects on the communities at different levels (Crossland and La Point, 1992; Beyers and Odum, 2012). Microcosm studies were more sensitive to detect the toxic impacts when compared to laboratory experiments. Santos et al. (2018) studied the toxicity of pore water of three different sites using two approaches: i) experiments in microcosms, ii) and in laboratory. In both cases, different toxicity between three sites has been established. Microcosm experiments are easy to conduct and give more reproducible data than field experiments. Chen et al. (2001) tested the effects of three different fungicides using a plastic tube as a microcosm, obtaining good results and reproducing the basic features of natural environmental at a small scale.

One of the advantages of using these systems is the great number of organisms that can be used (Steele, 2001). The use of mesocosms offered a solution to fill a gap between laboratory and field experiments, as in the case of Riebesell et al. (2013), where they made analyses about the effects of ocean challenges on pelagic community.

Hose et al. (2004) compared the species-sensitive distribution (SSD) curves of laboratory with local experiments in mesocosms and field data to evaluate the water quality in Australia. The mesocosm species were less sensitive than the species in laboratory tests, suggesting that the laboratory data can be used to determine the concentrations of mesocosms.

Several studies showed that the combination of the field, mesocosm and *in vitro* results were interesting to denife toxicant effects. Culp et al. (2000) evaluated the regional assessment of rivers by using these combined studies establishing the main effects of the discharges and the community responses to these stressors.

Grenni et al. (2012) tested the effects of microbial activity for ecological bioremediation of contaminated ecosystems in mesocosm systems. Using these systems, they assessed the degradation property of microbial activity under different natural conditions and under more stressor factors by providing realistic results. In any case, careful attention must be paid in generalizing the conclusion to natural ecosystems (Petersen 2003).

3. Sediment remediation assessment and m-cosms

Marine sediment is a fundamental and integrated part of water bodies accumulating biological, physical and chemical contaminants (Brils, 2008). Organic pollutants in sediment represent a worldwide problem, considering that sediment acts as a sink of hydrophobic, recalcitrant and hazardous compounds (Perelo, 2010). According to the biogeochemical processes, these contaminants are available to benthic organisms as well as to organisms present in the water column and toxic and carcinogenic, entering in the food chain and accumulating in the biological tissue. Among these compounds polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals represent the most abundant pollutants present in some marine sediments (Mamindy-Pajany et al., 2010), being released during perturbation events, absorbed by suspended particles and therefore settle at the bottom with a high impact on marine environment and consequently on human health (Geffard et al., 2001; Akcil et al., 2014).

Because of the potential toxic effects of these compounds on human health, researchers explored methods to detoxify/remove them from natural marine environments with both *in situ* and *ex situ* treatments, using different chemical, biological and thermal methods (Bamforth and Singleton, 2005; Gomes et al., 2013). These processes were classified as forms of "bioremediation", which refer to a process through which toxic compounds are biologically degraded and/or transformed to an innocuous state (Mueller et al., 1997). This process consists in the removing of pollutants from the natural environment converting them to a less harmful product (Bamforth and Singleton, 2005). In this review, we focused on sediment restoration contaminated with PAHs, PCBs and heavy metals, mainly using m-cosms.

3.1. PAH case studies

Polycyclic aromatic hydrocarbons (PAHs) consist of a group of over 100 different organic compounds, formed by two or more fused benzene rings and pentacyclic molecules differing in their physical and chemical properties. Their toxicity depends on the number of benzene rings (Perelo, 2010) and can be generated by: i) natural events such as volcanic eruptions, fossil fuels or incomplete combustion of organic materials; ii) anthropogenic activities such as industrial processes, oil and diesel spills and waste incineration. Anthropogenically produced substances that are considered the most impacting inputs into the natural environment (Bamforth and Singleton, 2005). These compounds tend to persist in the environment because of their low water solubility and hydrophobicity that permit them to be adsorbed and accumulated in sediment, where their degradation is very slow. The persistence of PAHs in the environment can also be controlled by such environmental factors such as soil type, pH, temperature, oxygen levels (Sutherland et al., 1995; Bamforth and Singleton, 2005). Sixteen PAH compounds were classified as priority pollutants by the United States Environmental Protection Agency (US-EPA; Perelo, 2010). Some studies have demonstrated that these organic molecules can have toxic effects for human health with many PAHs showing carcinogenic, teratogenic and mutagenic properties (Ferrarese et al., 2008). For these reasons, several remediation techniques were used for PAHs removal from contaminated sediments (Table 2). Some researchers suggested bioremediation techniques using the microbial metabolism to remove pollutants from contaminated sediments. Three fundamental methods exist: a) bacterial degrada-

Table 2
Strategy, site, operating conditions, efficiency of treatment for bioremediation by PAHs.

Strategy	Site	Operating conditions	Efficiency of treatment	References
Biological	Italian harbor in Mediterranean sea	Mixtures of inorganic nutrients and sand amendaments	40% PAHs	Beolchini et al., 2010
	Ancona port	Mixtures of inorganic nutrients and switch of temperature, in aerobic and anaerobic conditions	80% PAHs	Dell'Anno et al., 2012
	Carteau cove	Combination of marine polychaete *Nereis diversicolor* and oil contaminated sediments	Increase of bacteria communities to reduce contaminations	Cuny et al., 2007
	Dredged sediments from DeGray Reservoir	Inoculation of *Mycobacterium* sp.	25% 2-methylnaphthalene, 74% phenanthrene, 34% pyrene, 5% benzo-α-pyrene	Heiktamp et al., 1989
	Van-veen grab in Nyborg port	Combination of marine polychaete *Capitella* sp. I and oil contaminated sediments	No effects on PAHs decrease	Holmer et al., 1997
	Stanford le Hope, Essex, UK	Mixtures of inorganic nutrients and three hydrocarbon-degradation bacteria	78% PAHs with *Alcanivorax*, 70% *Thalassolituus*, 70% all bacteria	McKew et al.,2007
	Kamaishi (Japan)	Inoculation of *Cycloclasticus* sp.	38% PAHs	Miyasaka et al., 2006
	Gulf of Fos (France)	Natural develop of hydrocarbon-degradation bacteria	35% PAHs	Syakti et al., 2006
	Yiu Lian Floating Dock (France)	PAHs-addition	Increase PAHs-degradation bacteria, 50–98% PAHs	Wang and Tam 2011
Chemical/ physical	Oxford	AC-addition	80% PAHs	Bussan et al., 2016
	River Tyne	AC-addition	94% PAHs	Hale et al., 2010
	Oslo harbor	Capping	93% PAHs retained	Eek et al., 2008
	Norwey	AC-Capping	93% PAHs retained	Samuelsson et al., 2015
	Rivers Tyne and Wear (UK)	MAC-addiction	98% PAHs retained	Han et al., 2015

tion, b) lignolytic and c) non-lignolytic fungal degradation, which all together on benzene rings (Bamforth and Singleton, 2005). The bacterial degradation may take place aerobically, anoxically, or anaerobically, but PAH-restoring are usually associated with anoxic/aerobic biodegradation because most of the contaminated sediments are in these conditions (Lu et al., 2011).

The main mechanism for aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the activity of dioxygenase enzymes, which can then be further metabolised via catechol to carbon dioxide and water. The metabolic pathways and enzymatic reactions involved in the microbial degradation of naphthalene have been studied in detail (Cerniglia, 1992). Several bacteria can oxidize naphthalene through dioxygenase enzymes, including organisms from the genus *Pseudomonas* and *Rhodococcus* (Cerniglia, 1992; Mueller et al., 1997; Revelet et al., 2000).

PAH-restoring also took place thanks to the action of other microorganisms, as cyanobacteria, green algae and diatoms, which can transform naphthalene into different metabolites (Haritash et al., 2009). Several studies under controlled conditions were performed using the miniaturized ecosystems micro- and mesocosms (Grenni et al., 2012). The first study was performed by Heitkamp et al. (1989), using sediment and water collected with a Peterson dredge from DeGray Reservoir (a manmade impoundment which receives no significant exposure to chemical compounds), near Arkadelphia (Arkansas, USA). A microcosm (consisting of a flow-through system, 0.5 L glass tanks containing 20 g of sediment and 180 mL of lake water) was used to conduct biodegradation tests to monitor mineralization and recovery of chemicals. *Mycobacterium* sp. was added to sediment and water from this pristine ecosystem. Microcosms inoculated with the *Mycobacterium* sp. showed enhanced mineralization, alone and as components of a mixture of 2-methylnaphthalene, phenanthrene, pyrene, and benzo[*a*]pyrene. In (Miyasaka et al., 2006), the efficiency of bacteria in PAHs marine sediment restoring has been confirmed. In fact, they investigated three different bioremediation methods using microcosms: biostimulation with fertilized, bioaugmentation with *Cycloclasticus* sp. and mixtures of fertilized and bacteria. All microcosm experiments resulted efficiently for PAHs restoring after 90 days of exposure, having effects on bacterial abundance.

Considering the important role of the microorganisms in contaminated sediment bioremediation, several studies were performed on the effect of nutrient additions (e.g., nitrates) on the microbial communities of contaminated sediment with PAHs. The structural properties and the recycling processes of these microorganisms were highly enriched after inorganic materials addiction, suggesting that this bioremediation *in situ* can be stimulated by the contaminated sediments for bioremediation were collected from an Italian harbour in the Mediterranean Sea, using microcosms with 20 g of wet sediment samples and 100 mL of pre-filter seawater. Inorganic materials were added to all tanks (nitrogen and phosphorus) and to sand amendments and after thirty days the growth of prokaryotic organisms was detected, and the PAHs decreased of 40%. Dell'Anno et al. (2012) showed that bacterial abundance and biodiversity changed and increased at high temperature in both aerobic and anaerobic conditions. Bioremediation experiments were performed with sediment samples from two sites of the port of Ancona (Italy) characterized by two different redox potentials, using microcosms in anaerobic and aerobic conditions. The nutrients were added together with the increasing of temperature from 20 °C to 35 °C, observing major changes in the bacterial biodiversity and abundance at higher temperature in both conditions (aerobic and anaerobic), with a subsequent decrease of PAHs. No significant effects of nutrient addictions were evidenced. A part from the important role of hydrocarbon-degradation bacteria in restoring sediments, little information is available on their different strategy of bioremediation. McKew et al. (2007) analysed the degradation efficiency of three hydrocarbon-degradation bacteria (*Alcanivorax*, *Thalassolituus*, *Cycloclasticus*) adding inorganic nutrients (i.e. N and P) and using microcosm and seawater collected from a site (Essex, UK) neighbouring the Shell-Haven and Coryton BP oil refineries. Different microcosms were built under several conditions: oil-only microcosm, *Alcanivorax* + N-P microcosm, *Thalassolituus* + N-P

microcosm and *Cycloclasticus* + N-P microcosm, all bacteria + N-P + microcosm. They observed an increase of all bacteria after one month, mainly of *Alcanivorax*. However, in all microcosms with nutrients, they recorded a decrease of PAHs levels, showing a significant correlation between availability of nutrients and bacteria abundance.

Furthermore, it has been demonstrated that bacterial biodiversity and abundance were correlated to contamination. Syakti et al. (2006) recorded an increase of hydrocarbon-degradation and heterotrophic bacteria in the oil contaminated sediments. Specifically, microcosms were used with/without oil-contaminated sediments to assess PAHs-biodegradation and phospholipids fatty acid (PLFA) composition. An increase of hydrocarbon-degradation bacteria was observed after 21 days, due to a composition change in PLFA and consequently the decrease of PAHs. Wang and Tam (2011) demonstrated an increase of PAHs-degradation bacteria by adding PAHs to hydrocarbon-contaminated sediment. This experiment was performed in a microcosm (consisting of 12 conical flasks of 250 mL, each containing 150 mL sediment slurries prepared by mixing 800 g fresh marine sediments and 1600 mL sterilized) with/without added PAHs to contaminated sediment. A clear enhancement of degradation bacteria was recorded after 60 days, and a reduction in species diversity in all treatments. This change was coupled by a significant depletion of PAHs (50–98%; see also Table 2).

Moreover, Cuny et al. (2007) observed that petroleum contaminated sediments inhabited by a marine polychaete *Hediste diversicolor*, allowed the growth of specific bacteria, such as *Psychroserpens burtonensis*, known to degrade PAHs. Then, they investigated on the possible influence of these polychaetes on the development of different bacterial species using microcosms, consisting of twelve 2.5 L aquariums. They established that the presence of *H. diversicolor* in contaminated sediments promoted the degradation of oil and the development of bacterial communities able to reduce pollution after one month of exposure. However, the possible role of some polychaetes on PAHs-degradation was already evaluated in previous studies. Holmer et al. (1997) conducted a study on the polychaete *Capitella* sp. influence in the biogeochemistry of oil-contaminated sediments. Specifically, they used microcosms inoculating the PAH fluoranthene and worms, and established that the initial rate of PAHs did not decrease but these compounds were transported to deeper sediment layers where microbial degradation activity was less efficient.

Several studies were conducted to assess the potential role of chemical oxidation in PAHs degradation in contaminated sediments. There are various reactive liquids (such as modified Fenton's reagent, hydrogen peroxide, potassium permanganate, activated sodium persulfate) and he residual concentration of PAHs after each type of chemical oxidation has been followed to assess the efficiency of treatments. The best results were achieved with the use of modified Fenton's reagent, hydrogen peroxide and potassium permanganate (Ferrarese et al., 2008; Flotron et al., 2005).

Another method of chemical remediation is the addition of activated carbon (AC) to contaminated sediment, that can be defined as all substances with high carbon content and porosity enabling to absorb different compounds (Rakowska et al., 2012). PAHs-concentrations in sediment decreased of >50% after AC treatment (Hilber et al., 2010). The efficiency of this amendment depends on the quantity of AC added to the sediment per unit of volume/weight. The ratio, then, is influenced by the physical–chemical properties of sediment. Recently Bussan et al. (2016) investigated the role of AC in PAHs bioremediation of polluted sediments using marine microcosm systems before any treatment with AC. Then they set up different experimental designs: i) only sediments without amendments, ii) autoclaved sediments, iii) sediments + bio char; iv) sediments + AC. After two weeks, they observed a considerable mercury-depletion in all treatments. Hale et al. (2010) compared bioremediation and AC amendment for PAHs contaminated sediment from River Tyne. The efficiency of different amendments was followed using microcosm, including: i) an unamended system of 7 g sediment and 40 mL of River Tyne water and two amended systems with 7 g sediment, ii) 40 mL nutrient solution and iii) 40 mL nutrient solution inoculated with *Pseudomonas putida* (i.e. a strain that can degrade naphthalene and phenanthrene). These three systems showed a significant decrease in total sediment PAH concentrations over one month. Polyethylene passive (PE) samplers were embedded for 21 days in these sediment microcosms in order to measure the available portion of PAHs and accumulated from the unamended, biostimulated, and bioaugmented microcosms, respectively. Higher PAH uptake by PE samplers in biostimulated and bioaugmented microcosms coincided with slower degradation of spiked phenanthrene in sediment-free filtrates from these microcosms compared to filtrates from the unamended microcosms. Microbial community analyses revealed changes in the bacterial community directly following the addition of nutrients, but the added *P. putida* community failed to establish itself.

This amendment was usually associated to another *in situ* treatment, the capping, which is a physical cover of contaminated sediment, made of several materials, such as sand, clay or mixtures of different components. The combination between capping and AC is successful and do not lead to massive AC resuspension (Cornelissen et al., 2011). Eek et al. (2008) tested a mineral cap (crushed limestone) to limit the PAHs spread into the water column using microcosms. During 410 days of treatment, they assessed that the flux of contaminants from capped sediment was low respect the flux from uncapped sediment. Samuelsson et al. (2015) evaluated three different *in situ* AC-capping (AC-only, AC + clay and AC + sand) to assess the cap efficiency. After one year of capping, they collected samples to understand the best method using microcosms, using the worm *H. diversicolor* and the clam *Abra nitida* as test organisms. They measured the PAHs-bioaccumulation of two organisms, after one month, demonstrating that the AC-capping + clay was most efficient. Nevertheless, the AC addiction is an efficient method of bioremediation, and it has been demonstrated that it causes adverse effects on some organisms, especially on benthic organisms. In fact, one part of the AC added can diffuse in the water column causing negative impacts on various organisms. Another option can be the use of iron-based amendments into "magnetic AC" (MAC) because it has been demonstrated that it has remediation features as also AC, but it enables their magnetic retrieval (Libralato et al., 2018). Han et al. (2015) tested the MAC remediation using microcosm experiments to evaluate the remediation capacity and possible negative or positive effects on microorganisms. After six months, they showed that the MAC use efficiently reduced PAHs concentrations. They recorded the 77% of MAC and the remainder of MAC had fewer negative effects respect to AC on *Lumbriculus variegatus* while maintaining PAH availability low.

3.2. PCB case studies

Polychlorinated biphenyls (PCB) are persistent organic compounds and are considered among the most impacting contaminants because of their toxic and carcinogenic properties, vast distribution and high permanence in environmental systems (Meagher, 2000; Scragg, 2005; Perelo, 2010). These compounds mainly derive from lubricants, plasticizers and adhesives, as well as from the production of, hydraulic and dielectric fluids. The accumulation of PCB-contaminated sediment by biota represents a great problem because they can be introduced into the food webs, constituting a high risk for human health (Gomes et al., 2013). Several studies have been conducted to assess the best method of PCB-

Table 3
Strategy, site, operating conditions, efficiency of treatment for bioremediation by PCBs.

Strategy	Site	Experimental design	Operating conditions	Treatment efficiency	References
Biological	Strážsky (Slovakia)	Microcosm	Addiction of 15 different bacterial strains	10–20% PCB 153 and 118, 25% PCB 138, 40–45% PCB 101, 30–80% PCB 52, 55–80% PCB 28	Dudášová et al., 2012
	Busan coast (South Korea)	Microcosm	Addiction of 2 bacterial strains and plant terpenes	60–70% PCB	Kwon et al., 2008
	Lake Hartwell, New Bedford harbor, Roxana Marsh	Microcosm	Addiction of iron to produce hydrogen	Enhancement of PCB-degradation	Srinivasa Varadhan et al., 2011
	Brentella canal (Venice, Italy)	Microcosm	Addiction of iron to produce hydrogen	Enhancement of PCB-degradation	Zanaroli et al., 2012
	Anacostia River	Microcosm	Mixtures of electron donors, alternate halogenated electron acceptors, tetrachlorobenzene, pentachloronitrobenzene and bacteria	Enhancement of PCB-degradation	Krumins et al., 2009
	Brentella canal (Venice, Italy)	Microcosm	Addiction of PCBs	90% PCB	Zanaroli et al., 2010
	Mar Piccolo (Taranto, Italy)	Microcosm	Addiction of PCB-degradation bacteria	Important decrease of PCB	Matturro et al., 2015
	Grasse River (Massena, NY)	Microcosm	Addiction of PCB-degradation bacteria	83% PCB	Kaya et al., 2017
	Grasse River (NY), Fox River (WI), Baltimore harbor (MD)	Microcosm	Addiction of PCB-degradation bacteria	51–88% in Grasse River, 83–88% in Fox River and 70% in Baltimore harbor	Kaya et al., 2018
Chemical/ physical	Grasse River (Massena, NY)	Microcosm	AC-addition	70–90% PCB	Sun et al., 2007
	Grasse River (Massena, NY), Hunters Point Naval Shipyard (San Francisco Bay, CA)	Microcosm	AC-addition	70–90% PCB	Mcleod et al., 2008
	Lac du Bourget (Savoie, France)	Microcosm	GAC + biofilm	Efficiently PCBs adsorption	Mercier et al., 2013
	Lac du Bourget (Savoie, France)	Microcosm	Three different granular AC-addition	98.3–100% PCBs	Mercier et al., 2014
	Baltimore harbor (USA)	Mesocosm	Bioaugmentation with anaerobic halorespiring *Dehalobium chlorocoerci* and aerobic *Burkholdeira xenovorans,* and GAC addition	>80% PCBs	Payne et al., 2013
	Oslo harbor	Microcosm	Capping	93–98% PCBs	Eek et al., 2008

contaminated sediment remediation (Table 3). The use of bacteria to reduce PCBs concentration in marine contaminated sediment was reported. Kwon et al. (2008) demonstrated that the best rate of bacterial degradation can be obtained by adding plant terpenes. To date, data on the application of plant terpenes in marine sediments contaminated with PCBs remained limited, being mainly used in soil systems. Various microcosms were built only sediment, sediment + plant terpenes and sediment + plant terpenes + bacteria. In this case, the experimental setup was prepared into a 250 mL Erlenmeyer flask. A significant PCBs decrease has been observed after one month in microcosms that contained both plant terpenes and bacteria with respect to other two conditions. Krumins et al. (2009) used microcosm experiments and demonstrated that the enhancement of PCBs-degradation can be obtained with biostimulation and bioaugumention. Microcosms were constructed, using 200 mL site sediment in 250 mL stock bottles with homogenized sediment recovered from the Anacostia River (USA) capping site control plot. Various microcosm conditions were used as the following: only sediment (control), sediment + bacteria, sediment + mixture of electron donors, alternate halogenate acceptors, tetrachlorobenzene, pentachloronitrobenzene and sediment + bacteria + bioaugumentation. After 400 days, they assessed a decrease of PCB-concentrations in microcosms with bioaugumentation and biostimolation. Moreover, the PCB-degradation bacteria, as PAHs-degradation microorganisms, can be isolated from contaminated sediments and can be stimulated by adding pollutants (PCBs). In fact, Zanaroli et al. (2010) demonstrated that the degradation of PCBs was enhanced by the addition of contaminants, because it promoted the development of PCBs-dichlorination bacteria. They showed an important decrease of PCBs after 30 months, caused by the growth of bacteria after the addition of the contaminants. Kaya et al. (2017) conducted similar experiments using microcosms with addition of PCBs. After 30 days of incubation, they assessed a crucial reduction of PCBs in marine sediments with a corresponding increase of dichlorination bacteria. One year later, Kaya et al. (2018) evaluated the depletion of PCB after the addition of pollutants, using three different sediment microcosm samples. After 30 days, they measured a decrease of PCB concentrations, which were recorded at different percentages, suggesting that there were different dichlorination activities. Certainly, it is not related to different contamination history of three sediments because the sites with the highest percentages of dichlorination had also the lowest initial bacterial concentrations. Probably, individual congeners of PCBs may affect the activity of individual PCBs-degradation bacteria. Dudášová et al. (2012) performed microcosm experiments to assess the bacteria role in PCB-restoring using 15 different bacterial strains, isolated from PCBs-contaminated sediments. After 21 days, five of the 15 bacterial strains were suitable for restoring PCBs-contaminated sediments, because these microorganisms demonstrated high biodegradation ability compared to others.

Srinivasa Varadhan et al. (2011), enhanced the PCBs-degradation by dehalorespiring microorganisms in contaminated sediments by providing hydrogen produced in anaerobic corrosion of iron added to sediments. In that case, three different microcosm sediments were used, consisting of 125-mL borosilicate serum bottles, in which they added different iron concentrations. After 18 months, they observed an improved performance of bacterial

degradation, at low levels of iron, suggesting that the bacteria preferred low concentrations of hydrogen. Zanaroli et al. (2012) conducted a similar study using microcosm experiments in which they added iron to stimulate PCBs microbial dichlorination. After 36 weeks, an inhibitor effect of iron on sediment indigenous microbial communities was observed, favoring the growth of PCB microbial dichlorination and their biodegradation activity. The iron, probably, reduced the lag phase of PCB-dichlorination by blocking the sulfate reducing bacteria and by promoting hydrogen release. Matturro et al. (2015) studied the PCBs-degradation in marine sediments (consisting in sterile 250-mL serum), using different microcosm experiments with sediments and sediment with/without addition of *Dehalococcoides mccartyi*, microorganism known able to degrade PCBs. After 320 days, they observed an increase of the PCBs-degradation community and a decrease of PCBs in microcosms with *D. mccartyi*.

Furthermore, different studies were conducted on possible roles of AC in PCBs-degradation. In fact, as reported in Sun et al. (2007) the use of little AC addition in PCBs-contaminated sediments reduced the bioavailability of these compounds for the oligochaete *Lumbriculus variegatus*. Setting-up different microcosms (only sediment, sediment + AC and sediment + AC + worms), after one month of exposure a decrease of PCB-concentrations was registered together with a reduction of PCB bioavailability in worm's chemical activity. McLeod et al. (2004) performed microcosm experiments with AC additions to reduce the PCBs bioavailability in contaminated sediments from two different sites using the clams *Corbicula fluminea* and *Macoma balthica* as bioindicators. A considerable reduction of PCBs in microcosms was assessed after 28 days of exposure, and after the addition of AC. In addition, several studies have been conducted on the PCBs absorption of granular activated carbon (GAC) and the biofilms that grow on these particles. Mercier at al. (2013) evaluated the efficiency absorption of three different GAC conditions using different microcosm experiments: raw GAC, GAC particles with and without biofilms. The adsorption capacity was excellent for all conditions after one month, even in the case of GAC particles plus biofilms where the PCBs removal was not hampered by biofilm development. Afterwards Mercier et al. (2014) evaluated the PCBs removal efficiency of three different GACs in contaminated sediments. After 8 months of treatment in microcosms, all granular particles were able to efficiently absorb the PCBs in marine sediments and the bacterial adhesion depended on physical and chemical properties of particles. Later, Payne et al. (2013) demonstrated that the use of GAC with simultaneous application of aerobic and anaerobic microorganisms was able to better reduce the PCB concentrations by about 80%. Different mesocosm conditions were used: only GAC, GAC + sodium lactate, GAC + zerovalent iron, GAC + anaerobic *Dehalobium chlorocoercia* (DF1), GAC + DF1 + sodium lactate, GAC + DF1 + zerovalent iron, GAC + DF1 + aerobic *Burkholderia xenovorans* (LB400), GAC + DF1 + LB400 + sodium lactate and GAC + DF1 + LB400 + zerovalent iron. The results demonstrated that i) the best effects were obtained with bioaugmentation using both organisms after one year, and ii) a treatment employing both aerobic and anaerobic microorganisms could be considered a suitable strategy to remove PCBs in contaminated aquatic sediments.

Another strategy used to remove PCBs in contaminated marine environment is the capping, also used for PAHs (see above). Eek et al. (2008) tested a mineral cap (crushed limestone) to prevent PCBs diffusion and to limit bioavailability of these compounds in the marine environment. After 13 months of treatment in microcosm, they showed that the use of capping significantly limited PCBs concentrations respect to uncapped sediment microcosm.

3.3. Heavy metals case studies

Heavy metals (zinc, copper, manganese, silver, iron, cadmium, and so on) often dispersed in marine environments derive from anthropogenic activities and represent an issue for marine environments and consequently for human health. These elements can diffuse and accumulate in sediment where they influence physical and chemical factors (Peng et al., 2009). Several studies have been conducted to investigate their removal strategies (Table 4). As for PAHs and PCBs, the most common applied strategies concerned biological and chemical activities. The potential role of bacteria versus heavy metals degradation was tested in several studies. Beolchini et al. (2009) tested the potential role of different bacteria strains in bioremediation of dredged sediments contaminated by heavy metals using microcosms. Three conditions were set up: i) microcosms with autotrophic bacteria, ii) microcosms with heterotrophic bacteria, iii) and microcosms with mixed culture. In presence of both bacterial types, the efficiency of heavy metal extractions increased after 15 days, suggesting a potential synergistic action of these microorganisms. Fonti et al. (2013) studied three contaminated sediment microcosms in which they added autotrophic Fe/S oxidizing and heterotrophic Fe-reducing bacteria. The presence of autotrophic Fe/S oxidizing bacteria enhanced after 14 days of treatment, to maintain the acid and oxidative condi-

Table 4
Strategy, site, operating conditions, efficiency of treatment for bioremediation by heavy metals.

Strategy	Site	Operating conditions	Treatment efficiency	References
Biological/ Chemical	Site of US	Injection of ethanol	Increase of bacteria communities able to reduce contaminations	Cardenas et al., 2008
	Ancona harbour (Mediterranean Sea)	Mixture of autotrophic and heterotrophic bacteria	>90% Cu, Cd, Hg and Zn	Beolchini et al., 2009
	Oak Ridge (Tennessee, USA)	Addition of the electron donors	5–7% with ethanol, 49% with glucose, 93% with metthanol	Madden et al., 2009
	Three sites (New Caledonia)	Addition of glucose in oxic and anoxic conditions	Enhancement of nickel removal	Pringault et al., 2010
	Ancona harbour (Mediterranean Sea)	Addition of the electron donors	Change of metal partitioning	Rocchetti et al., 2012
	Three sites in New Caledonia	Addition of glucose in oxic and anoxic conditions	Enhancement of zinc removal	Pringault et al., 2012
	Ports of Livorno, Piombino and Ancona (Mediterranean Sea)	Mixture of autotrophic and heterotrophic bacteria	40–76% Zn, 0–7% Pb, 13–39% Cd, 1–8% Cr, 20–23% As, 35% Ni	Fonti et al., 2013
	Port of Piombino (Mediterranean Sea)	Addition of the electron donors	32–40% Zn, 14–40% Pb, 26% Cd, 15% Cr, 2–20% As	Fonti et al., 2015
	Contaminated sediment	Addition of microalgae	Enhancement of copper and zinc removal with RED LEDs	Kwon et al., 2017
	Port of Ancona (Mediterranean Sea)	Mixture of different chemical leaching agents and autotrophic and heterotrophic bacteria	30% Zn, 30% Cr, 40% Ni, 35–58% As	Beolchini et al., 2013

tions, so influencing metal solubilization in the marine environment. Pringault et al. (2010) evaluated the bacteria role in Ni removal. Different experimental designs were built up: microcosm without metals (control), microcosm with Ni and formaldehyde (to sterilize sediment and seawater), and microcosm with nickel and glucose (to stimulate the bacterial growth). Nickel removal was always significantly higher in non-sterilized microcosms after 8 days, indicating the important role of bacteria. Pringault et al. (2012) evaluated the possible effects of Zn on bacterial structures and if these microorganisms could play a role in adsorption of this metal. They set up oxic and anoxic microcosm experiments using different conditions: one microcosm without Zn (control), one microcosm with Zn, one microcosm with metal and formaldehyde or glucose. The microbial structure changed after 8 days, more in microcosms with glucose than in microcosms with metal. Probably, these different effects were due to the strong adsorption of Zn in presence of bacteria (uptake).

Cardenas et al. (2008) stimulated the bacterial growth in uranium-contaminated sediments injecting ethanol *in situ* microcosm experiments. After two years of treatments with ethanol addition, they showed that the denitrifying bacteria (especially *Desulfovibrio* spp.) increased with the reduction of U (VI) to U (IV). Madden et al. (2009) added different electron donors (ethanol, methanol, glucose and methanol with humic acids) to improve the bacterial performance in uranium reduction. Microcosm experiments were performed to different exposure time, depending on the type of electron donors: 77–153 days for ethanol, 53 days for glucose and 90 days for methanol. All electron donors stimulated the U-reduction with no difference with humic acids addition. The enhancement of U-reduction was probably due to encouragement of bacterial activities with electron donors' additions. In all microcosms they found different bacteria, known to reducing U. Rocchetti et al. (2012) added different electron donors, lactose and sodium acetate, to stimulate the prokaryotic development. They performed anaerobic microcosm experiments at either 20 °C or 35 °C, to investigate the effects of biostimulation activities on sediments contaminated with hydrocarbons and metals. After 60 days of incubation, they determined a significant change of metal partitioning and hydrocarbon concentrations in microcosms with lactose and sodium acetate at 20 °C. These results showed that additions of electron donors and the temperature influenced the prokaryotic metabolism, determining a decrease of PAHs concentrations and a change also on metal partitioning. In fact, the bioremediation strategies on contaminated sediments can influence the mobility and bioavailability of metals found in sediments. As reported in Fonti et al. (2015) during the restoring activities, the use of bacteria and the addition of electron donors to enhance the microbial growth were associated to metal decreases, suggesting a potential role of prokaryotes in metal assembly processes. Specifically, they used microcosms in which lactose and sodium acetate were added, and after 60 days the metal partitioning and the abundance and diversity of bacteria were measured. A considerable increase of abundance and diversity of bacteria was recorded in all microcosms and a decrease of metal concentration, confirming that the bacterial activities play an important role in metal mobility in the sediments. The heavy metal restoration involving microorganisms and algae was not an exception. Kwon et al. (2017) used microcosm experiments with addition of microalgae demonstrating that the use of these microorganisms could be efficient in metals removal. They studied the effects of monochrome and mixed wavelength on absorption of copper and zinc by microalgae *Phaeodactylum tricornutum*, *Nitzschia* sp., *Skeletonema* sp. and *Chlorella vulgaris*, showing in the last one the highest copper and zinc removal.

Another strategy used to remove heavy metals in contaminated marine environment is the chemical treatments. Beolchini et al. (2013) performed microcosm experiments with addition of different chemical leaching agents, oxalic, citric and sulfuric acids, and autotrophic and heterotrophic bacteria to evaluate extraction of toxic metals from contaminated sediments. After 14 days, they assessed that citric and sulfuric acids were the most appropriate to remove the toxic metals. They also measured similar effects in presence of ferrous iron showing that the role of autotrophic bacteria was the most important in the metal extraction and the heterotrophic bacteria were used as support for the autotrophic bacterial activities.

4. Conclusions and future perspectives

This review highlighted that how small-scale experiments in microcosms and/or mesocosms could represent an potential useful method to assess contaminated sediment along with their remediation process. Such kind of approach could strengthen the knowledge about potential side effects of remediation activities (e.g. adverse effects on benthic communities), suggesting the most viable treatment to be administered as well as support technology advancements to reduce the impact of old and new treatment technologies especially those related to the addition of adsorbents (e.g. AC), reactive compounds or capping. Currently, the use of m-cosms present some limitations that must be overcome in the near future about: i) exact definition of m-cosm's structure and components in time and space; ii) identification of common standards to be shared at international level (i.e. standardization process); iii) model species batteries. The matter of costs is of course important and are generally country based, but standardization and economy of scale may increase savings.

Author contribution

L.A., M.C., V.Z., G.L., the general content of the review; L.A. performed most bibliographic research; L.A., M.C., G.L. wrote the original draft; all authors contributed to paper writing.

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.

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Sub-Chronic Effects of Slight PAH- and PCB-Contaminated Mesocosms in *Paracentrotus lividus* Lmk: A Multi-Endpoint Approach and De Novo Transcriptomic

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Abstract: Sediment pollution is a major issue in coastal areas, potentially endangering human health and the marine environments. We investigated the short-term sublethal effects of sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) on the sea urchin *Paracentrotus lividus* for two months. Spiking occurred at concentrations below threshold limit values permitted by the law (TLVPAHs = 900 µg/L, TLVPCBs = 8 µg/L, Legislative Italian Decree 173/2016). A multi-endpoint approach was adopted, considering both adults (mortality, bioaccumulation and gonadal index) and embryos (embryotoxicity, genotoxicity and de novo transcriptome assembly). The slight concentrations of PAHs and PCBs added to the mesocosms were observed to readily compartmentalize in adults, resulting below the detection limits just one week after their addition. Reconstructed sediment and seawater, as negative controls, did not affect sea urchins. PAH- and PCB-spiked mesocosms were observed to impair *P. lividus* at various endpoints, including bioaccumulation and embryo development (mainly PAHs) and genotoxicity (PAHs and PCBs). In particular, genotoxicity tests revealed that PAHs and PCBs affected the development of *P. lividus* embryos deriving from exposed adults. Negative effects were also detected by generating a de novo transcriptome assembly and its annotation, as well as by real-time qPCR performed to identify genes differentially expressed in adults exposed to the two contaminants. The effects on sea urchins (both adults and embryos) at background concentrations of PAHs and PCBs below TLV suggest a need for further investigations on the impact of slight concentrations of such contaminants on marine biota.

Keywords: de novo transcriptomic; marine sediment; polycyclic aromatic hydrocarbons; polychlorinated biphenyls; sea urchin; short-term effects

1. Introduction

Sediments are composed of soluble, insoluble (rock and soil particles) and biogenic matter, which can be naturally transported from lands to oceans due to coastal erosion and windblown dust [1]. Sediment represents an essential and dynamic part of marine environments and may accumulate organic and/or inorganic compounds deriving from natural and anthropogenic sources, such as industrial, commercial, agricultural and urban

activities [2,3]. Contamination associated with (re-)suspended sediment is a concern for human health, mainly due to its tendency to accumulate in bottom-feeder organisms and biomagnify through marine food webs [4,5]. Worldwide governments are promoting sediment assessment, restoration and valorization as a key compartment of water bodies [6] (i.e., the European Union via the Water Framework (2000/60/EC) (WFD) and the Marine Strategy Framework Directives (2008/56/EC)). Natural (i.e., tides, bioturbation) and artificial (i.e., dredging) perturbative events can remobilize sediment and dissolve the associated contaminants into the water column, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), causing short- and long-term effects on marine organisms [7]. PAHs consist of a large group of widespread organic compounds of high environmental concern, occurring mainly in relation to human activities, such as combustion by-products (i.e., atmospheric deposition) [8] or oil spillage (approximately 15% *w/w* of PAHs); nevertheless, crude oil water-soluble fraction effects (i.e., mixture) are still largely unexplored [9,10].

It has been estimated that direct discharges of PAHs in marine environments can range from <1 µg/L to over 625 µg/L, with concentrations in industrial effluents up to 4.4 mg/L and 170.000 ng/g in sediment (dry weight; [11]). PCBs are a group of anthropogenic compounds, classified as persistent organic pollutants (POPs) by the Stockholm Convention (2001). Less than 1% of PCBs released in the environment volatilize from soil/sediment to the atmosphere, while most of them accumulate into the water column and in aquatic organisms, and they reach up to 4601 ng/g dry weight in the sediment [12].

Besides pollution hotspots (i.e., industrial and commercial sites), marine sediments are generally only slightly contaminated by PAHs and PCBs, with concentrations below nationally and internationally set threshold limit values (TLVs) (TLV_{PAHs} = 900 µg/L, TLVPCBs = 8 µg/L; Legislative Italian Decree 173/2016), but our knowledge about the potential side effects of this background contamination on human health and the environment is still insufficient.

This research investigated the potential negative effects of PAH (to simulate post-combustion products) and PCB slightly contaminated sediment on the sea urchin *Paracentrotus lividus* Lamark. Ad hoc experimental mesocosms [13] were set up to expose adult sea urchins to a reconstructed marine habitat (i.e., sediment and water) purposely spiked with PAHs and PCBs [14,15]. We tested two hypotheses proposing that the effects of slightly polluted sediment could result in the following: (i) morphological changes in the development of sea urchin embryos, deriving from adults exposed to these contaminants and (ii) variation in the expression level of genes involved in stress response, skeletogenesis, detoxification and development/differentiation. Specifically, sea urchin endpoints included adult's mortality; gonadal index; sensitivity of embryos (up to pluteus stage) generated from the exposed organisms; contaminant accumulation in adult thecae and spines, gonads and intestine; genotoxicity; and de novo transcriptome assembly.

2. Results and Discussion

2.1. Sediment Grain Size and Water Features and Spiking Levels

The sediment showed a typical sandy profile. Sandy fraction represented 99.9%, where the coarse sand (0.5 mm–1 mm, representing 41.1%) was the dominant component (Supplementary Figure S1). The fine and medium sands (from 0.25 mm to 0.5 mm) were 2.1% and 15.7%, respectively, whereas the mud fraction represented a small percentage (about 0.1%). During two months of exposure to PAH- and PCB-contaminated sediments, the physical and chemical values of the seawater in the mesocosm were almost constant. PAHs and PCBs detected in all sediment and water samples from all mesocosms were below the relative detection limit values (see Supplementary Table S1–S8 for more details) considering all investigation times (t0, t1 and tf).

2.2. Effects of Contaminated Sediment on Adult Growth, Gonadal Index and Sea Urchin Development

None of the conditions imposed in the negative controls (W and W + SED) negatively affected sea urchins, suggesting that all the subsequently observed effects could be attributed to the treatments. After the exposure period (two months), a mortality rate of 1% was detected in all experimental conditions (W, W + SED, W + SED + PAHs and W + SED + PCBs), revealing good health conditions of the sea urchins after two months of exposure (Supplementary Figure S2). After two months of exposure, no significant differences in growth rates were found between adults exposed to PAHs and PCBs as compared to organisms collected in the field at the beginning of the experiment ($p > 0.05$), similar to GI values (p value > 0.05; Supplementary Table S9).

After gamete collections, three important endpoints of sea urchin embryonic development were detected: (i) fertilization success; (ii) first mitotic division; and (iii) the pluteus stage, occurring at 48 hpf. Exposure to both contaminants, PAHs and PCBs, did not show significant effects on the percentages of fertilization success and first mitotic cleavage with respect to the controls (in tanks with seawater (W) and in tanks with seawater + sediment (W + SED) without contaminants; $p > 0.05$). Observation of the embryos at the pluteus stage revealed that PAH and PCB treatments induced malformations, mainly affecting arms, spicules, apices and the entire body shape as compared to control embryos (Figure 1).

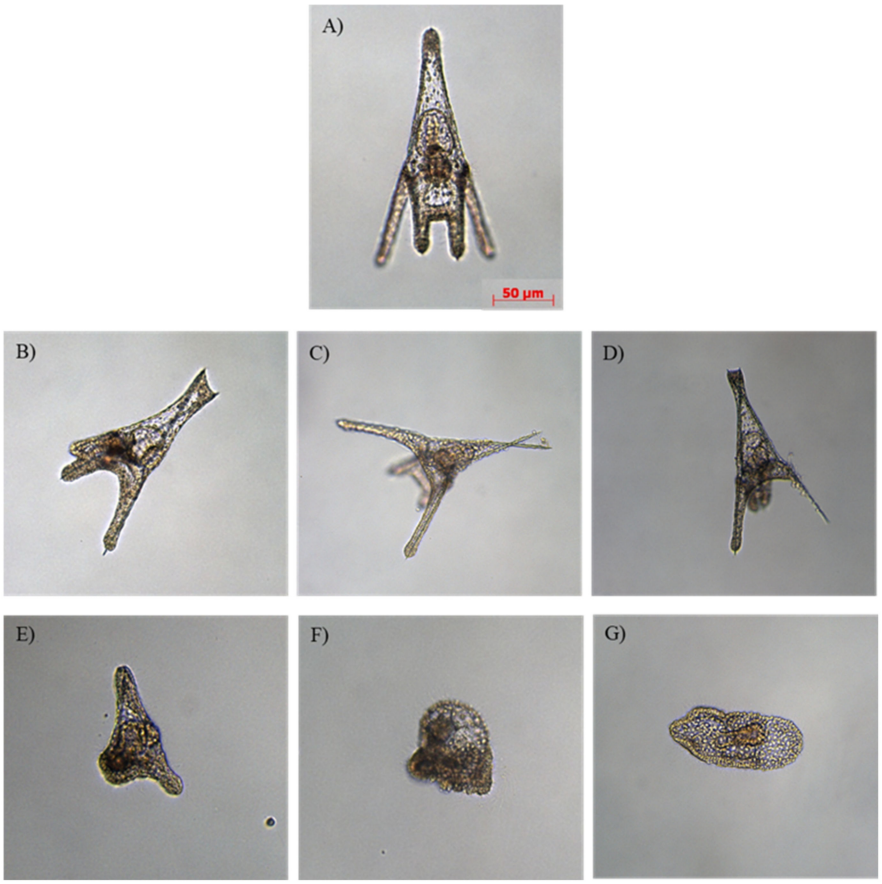

Figure 1. Examples of malformations observed in (**B**–**E**) *p. lividus* plutei deriving from adults exposed to PAHs and PCBs and in (**F**,**G**) embryos still at the gastrula stage deriving from adults exposed to PCBs in comparison with (**A**) control embryos deriving from adults reared in a tank with sediment without contaminants. (**B**) poorly-formed apex; (**C**) crossed at the apex with wider aperture of the arms; (**D**) degraded arms; (**E**) delayed and abnormal body; (**F**,**G**) malformed gastrulae.

In particular, at the pluteus stage, an increase in malformed embryos was observed in larvae deriving from sea urchins exposed to contaminated sediments with respect to the controls, represented by water and water + sediment without contaminants (Figure 2).

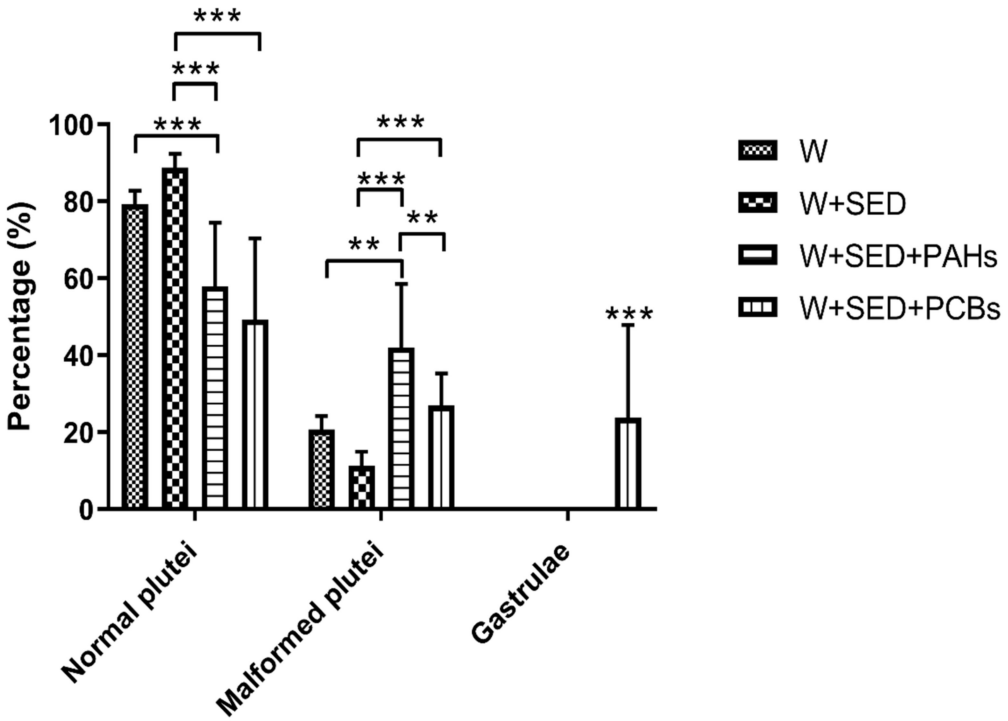

Figure 2. Percentage of normal plutei and malformed embryos at the pluteus and gastrula stages from sea urchins, deriving from adult sea urchins exposed to sediment contaminated with PAHs (water + sediment + PAHs) and PCBs (water + sediment + PCBs) and in control conditions represented by adults reared in control tanks (water and water + sediment). Data are reported as mean ± standard deviation one-way ANOVA by Holm–Sidak test (** $p < 0.01$, *** $p < 0.001$).

PAHs induced an increased percentage of malformed embryos (about 42%) with respect to control water + sediment (about 10%, $p < 0.001$). The exposure to PCBs generated approximately 27% ($p < 0.001$) of malformed plutei and developmental delays, with some embryos still at the gastrula stage (about 24%; $p < 0.001$), which were also malformed.

These results, valid for the above-mentioned doses applied to *P. lividus*, demonstrated that PAHs are more harmful than PCBs, being supported also by chemical analyses of the contaminant's bioaccumulation.

After two months of exposure, the bioaccumulation of PAHs and PCBs was also detected in three sea urchin tissues: thecae (including spines), gonads and guts. Chemical results showed that (i) 12.4 µg/kg of PAHs (including acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, 9-methylanthracene and benzo[a]anthracene) were accumulated in the theca, including the spine (Table 1); (ii) 16.3 µg/kg of total PAHs (including acenaphthylene, acenaphthene, fluorine, anthracene, phenanthrene, fluoranthene, pyrene and benzo[a]anthracene) were accumulated in the gonads; and (iii) no PAH accumulation was found in the guts. The target body compartments in sea urchins were the body wall and the spines when individuals were exposed to contaminated water and the guts when they were exposed to contaminated foods [16].

Table 1. Quantity (µg/kg) of PAHs detected in thecae (including spines), gonads and gut from adult sea urchin *P. lividus* in two experimental conditions after two months: W/W + SED and W + SED + PAHs. The values higher than the threshold values are reported in red. PAH total values are also reported.

	Thecae + Spines (µg/kg)			Gonads (µg/kg)			Intestine (µg/kg)	
	W + SED + PAHs	W	W + SED	W + SED + PAHs	W	W + SED	W + SED + PAHs	W + SED
Naphthalene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Acenaphthylene	**1.0**	<0.4	<0.4	**2.2**	<0.4	<0.4	<2	<2
Acenaphthene	0.5	<0.4	<0.4	**0.7**	<0.4	<0.4	<2	<2
Fluorene	**5.8**	<0.4	<0.4	**1.1**	<0.4	<0.4	<2	<2
Anthracene	**1.1**	<0.4	<0.4	**3.2**	<0.4	<0.4	<2	<2
Phenanthrene	**1.2**	<0.4	<0.4	**0.8**	<0.4	<0.4	<2	<2
9 metilAntracene	**2.2**	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Fluoranthene	<0.4	<0.4	<0.4	**3.0**	<0.4	<0.4	<2	<2
Pyrene	<0.4	<0.4	<0.4	**4.4**	<0.4	<0.4	<2	<2
Benzo(a)Antracene	**0.7**	<0.4	<0.4	**0.9**	<0.4	<0.4	<2	<2
Benzo(b)Fluorantene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Benzo[k]fluoranthene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Benzo(e)Pirene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Benzo[a]pyrene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Indeno[1,2,3-cd]pyrene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Dibenz[a,h]anthracene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Benzo[ghi]perylene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Coronene	<0.5	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Retene	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<2	<2
Total PAHs	**12.4**	<0.4	<0.4	**16.3**	<0.4	<0.4	<2	<2

However, the accumulation in these marine organisms was more efficient when exposed via water than via the food. No detectable events of PCB bioaccumulation were observed in the analyzed tissues (Supplementary Table S10). PCB bioaccumulation data on marine organisms are scarce, impeding an effective assessment of their toxicity. Zeng et al. [17,18] studied the uptake patterns of PCB congeners in the sea urchin *Lytechinus pictus*. More than 66 days are necessary for some congeners to attain steady state concentration in *L. pictus* gonad, whereas 28–42 days are required [19] in such marine organisms as bivalves, polychaetes and amphipods. Evidence of toxicity with changes in total or gonad weight was only detected at 647 mg/g. Studies on fish indicated that embryos and developing larvae were negatively affected by PCBs at 0.12 mg/g–12 mg/g [20,21]. Monosson et al. [20] observed that PCB effects were due to the congener 3,3′,4,4′ tetrachlorobiphenyl, which has a greater toxicity than that of the congeners' mixture [16], and exposed adult *P. lividus* to 14C-labelled PCB#153 via seawater and food, observing that the bioaccumulation efficiency was similar in the body wall, spines, gut and gonads.

2.3. Transcriptomic Assembly and Differentially Expressed Genes in Plutei from Adults Exposed to PAHs and PCBs (RNA-seq)

Another interesting result was the large-scale genomic information herein reported, which greatly improved the few molecular tools available for the sea urchin *P. lividus*, despite its importance as a marine model organism. For this reason, the de novo transcriptome obtained in this work represents a promising tool to identify new *P. lividus* genes,

which can be considered general biomarkers placed in motion from the sea urchin to deal with environmental pollution.

All the results obtained by RNA sequencing are summarized below.

BLASTx top-hit species distribution of matches for all the transcriptomes with known sequences indicated (Supplementary Figure S3) that the majority of *P. lividus* contigs (reads) showed the highest similarity with *Strongylocentrotus purpuratus* (BLAST hits = 1000). The other most represented species included *Apostichopus japonicas* (BLAST hits 50) and *Acanthaster planci* (BLAST hits 45). All alignments were carried out setting the E-value thresholds at a value of $\leq 1\ e^{-5}$.

To perform the RNA-seq assembly de novo, Trinity was used [22]. We obtained the trinity assembly with the statistics reported: Counts of transcripts: Total "trinity genes": 216864, Total "trinity transcripts": 611356, Percent GC: 38.26%. Then, we performed a differentially expression analysis in Trinity, selecting Deseq2 R package [23], and we obtained the genes differentially expressed with respect to the several conditions (less than 3000 genes and 8000 isoforms). Of the isoforms differentially expressed, we performed a BLASTx alignment with respect to the nucleotide non-redundant database in NCBIi, using OmicsBox (version1.2.4) [24]. Differentially expressed genes were identified between the three conditions: embryos at the pluteus stage spawned by adults exposed for two months to sediment contaminated with (i) PAHs or (ii) PCBs, with comparisons made with (iii) those exposed in tanks with sediment without contaminants as the control, including three biological replicates for each treatment.

The score plot showed that the replicates for the controls were very similar, with a clear separation from the treated samples, suggesting a greater number of down- and up-regulated genes in the treated samples compared to that of the controls (Supplementary Figure S4).

As reported in Supplementary Table S11, (i) 1898 genes were differentially expressed (DE) genes with a false discovery rate (FDR) of ≤ 0.05, of which 993 genes were up-regulated (FC ≥ 1.5) and 965 were down-regulated (FC ≤ 1.5) in plutei deriving from adult *P. lividus* exposed to sediment contaminated with PAHs (indicated as Treated_1); (ii) 2396 genes were DE with a false discovery rate (FDR) of ≤ 0.05, of which 1079 genes were up-regulated (FC ≥ 1.5) and 1317 were down-regulated (FC ≤ 1.5) in plutei deriving from adult *P. lividus* exposed to sediment contaminated with PCBs (indicated as Treated_2) compared to the control; (iii) 1356 genes were DE with a false discovery rate (FDR) of ≤ 0.05, of which 755 genes were up-regulated (FC ≥ 1.5) and 601 were down-regulated (FC ≤ 1.5), considering Treated_1 compared to Treated_2. After the annotation, (i) 488 genes were found up-regulated (with a FC range between 1.6 and 99) and 271 genes down-regulated (with a FC range between 1.7 and 95) for Treated_1 vs. Control, and, of these, some genes showed very high values of fold changes, such as the four up-regulated genes (RNA-directed DNA polymerase from mobile element jockey-like, calmodulin-like protein 4, arylsulfatase A and fibropellin-1-like isoform X6) and the three down-regulated genes (beta-1,3-galactosyltransferase 1-like, isocitrate dehydrogenase (NADP) cytoplasmic isoform X2 and fibrillin-1-like); (ii) 311 genes were found up-regulated (with a FC range between 1.7 and 95) and 420 genes down-regulated (with a FC range between 1.6 and 95) for Treated_2 vs. Control, and, of these, some genes showed very high values of fold changes, such as the three up-regulated genes (dnaJ homolog subfamily B member 13, rho guanine nucleotide exchange and factor 39ATP-dependent RNA helicase DHX8-like) and the two down-regulated genes (betaine-aldehyde dehydrogenase and serine/threonine-protein kinase TNNI3K); (iii) 177 genes were found up-regulated (with a FC range between 1.9 and 90) and 239 genes down-regulated (with a FC range between 1.5 and 98) for Treated_1 vs. Treated_2, and, of these, some genes showed very high values of fold changes, such as the three up-regulated genes (actin-related protein 2/3 complex subunit 3, acyl-CoA dehydrogenase and arylsulfatase I) and the two down-regulated genes (cyclin-dependent kinase 11B and glucose-6-phosphate 1-dehydrogenase isoform X1).

This large-scale genomic information represents a significant finding, being the first molecular attempt to define PAH and PCB effects on sea urchin *P. lividus* by molecular approaches. PAHs and PCBs targeted different genes and had several common targets, as shown in the Venn diagrams considering up-regulated genes and down-regulated genes, comparing the groups "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control (plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants)", "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2"(Figure 3 and Supplementary Tables S12 and S13).

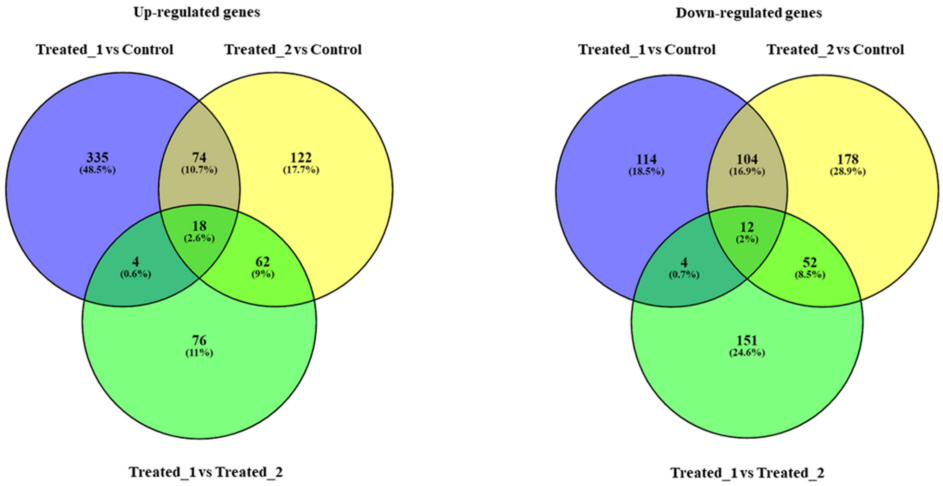

Figure 3. Venn diagrams considering up-regulated genes and down-regulated genes, comparing the groups "Treated_1 (plutei deriving from adults exposed for two months to sediment contaminated with PAHs) versus Control (plutei from adults sea urchin *P. lividus* reared for two months in tanks with sediment without contaminants)", "Treated_2 (plutei deriving from adults exposed for two months to sediment contaminated with PCBSs) versus Control" and "Treated_1 versus Treated_2". PAHs (Treated_1) and PCBs (Treated_2) induced an increase in the expression of 335 (48.5%) and 122 (17.7%) genes, respectively, compared to the Control; they also induced the down-regulation of 114 (18.5%) and 178 (28.9%) genes, respectively. The two contaminants had several common targets (see also Supplementary Tables S12 and S13 for the names of the common genes): (i) for up-regulated genes, 74 common genes (10.7%) comparing the groups "Treated_1 versus Control" and "Treated_2 versus Control"; 18 common genes (2.6%) comparing the groups "Treated_1 versus Control", "Treated_2 versus Control" and "Treated_1 versus Treated_2"; 4 common genes (0.6%) comparing "Treated_1 versus Control" and "Treated_1 versus Treated_2"; 62 common genes (9.0%) comparing "Treated_2 versus Control" and "Treated_1 versus Treated_2". (ii) for down-regulated genes, 104 common genes (16.9%) comparing the groups "Treated_1 versus Control" and "Treated_2 versus Control"; 12 common genes (2.0%) comparing the groups "Treated_1 versus Control", "Treated_2 versus Control" and "Treated_1 versus Treated_2"; 4 common genes (0.7%) comparing "Treated_1 versus Control" and "Treated_1 versus Treated_2"; 52 common genes (8.5%) comparing "Treated_2 versus Control" and "Treated_1 versus Treated_2".

Transcriptomic results indicate that PAHs and PCBs affected genes differently, mainly increasing their gene expressions, supporting those differences observed at the morphological level. In fact, the highest percentage of malformed plutei caused by exposure to PAHs can be linked to the up-regulation of the majority of the studied genes. An example was represented by nodal and nectin genes (data also confirmed by real-time qPCR experiments, see below). The nectin gene is involved in cellular adhesion [25,26], whereas nodal gene controls the left–right asymmetry in the sea urchins, regulating the expression level of the *BMP2* gene [27–30]. The exposure to PCBs caused not only the up-regulation of the nodal

gene, but also the up-regulation of the frizzled gene and the down-regulation of the *PLC* gene. The function of the frizzled gene is similar to that of the nodal gene. Binding to the Wnt6, this receptor is responsible for endoderm specification [31,32]. Instead, the *PLC* gene is involved in egg activation in the events immediately following fertilization and during embryo development in sea urchins [33–35]. Its down-regulation can be one of the causes of the delay effect shown after PCB treatment. Transcriptome is generally dynamic, and it is a good indicator of the cell's state. In addition, in this case, the ease of genome-wide profiling made the transcriptome analysis an integral part of understanding the biological processes affected by PAHs and PCBs. In fact, to identify the pathways in which the genes affected by these two contaminants were involved, a Gene Ontology (GO) term enrichment analysis was performed using DE genes (Figure 4).

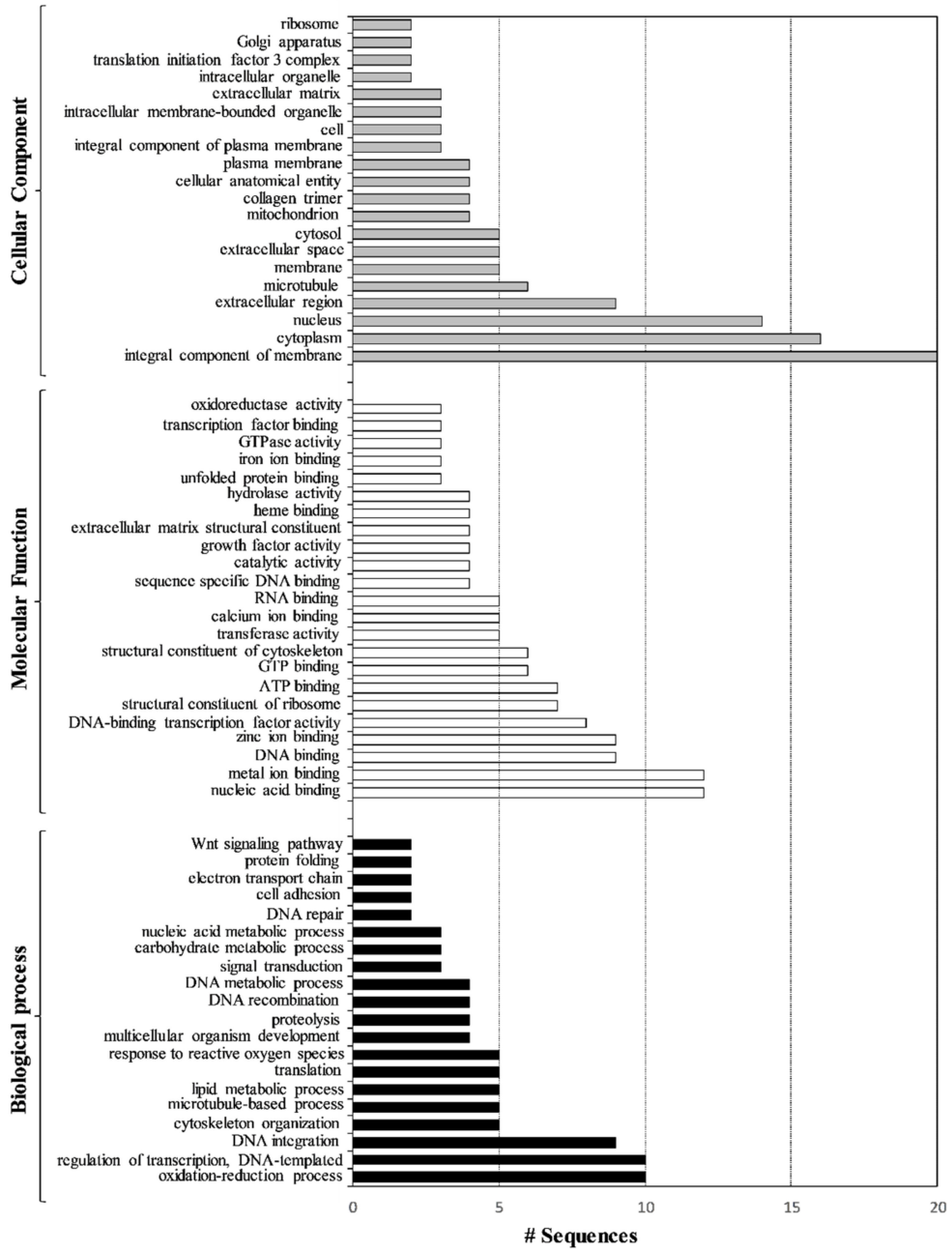

Figure 4. Overrepresented GO terms of sea urchin plutei after artificial contaminated experiments with PAHs and PCBs in the three major functional categories: Biological Process (black bars), Molecular Function (white bars) and Cellular Component (grey bars), which include all the differentially expressed genes (both up- and down-regulated).

Seventy-seven GO terms were enriched, including 20 in "Biological Process" followed by 23 in "Molecular Function" and 24 in "Cellular Component" ($p < 0.05$). Over-represented GO categories included the oxidation–reduction process, regulation of transcription, DNA integration, cytoskeleton organization, nucleic acid binding, metal ion binding, DNA binding, zinc ion binding and DNA-binding transcription factor activity. Moreover, these genes are integral components of the membrane and were mainly localized in the cytoplasm, nucleus, extracellular region and microtubule.

2.4. Effects of PAHs and PCBs on Gene Expression by Real-Time qPCR

The expression levels of 62 genes [36–40] involved in different physiological processes were followed by a real-time qPCR (reported in Supplementary Figure S5). These genes were previously selected in [36,38,39,41], and their expression levels were studied in response to natural toxins produced by marine diatoms. We proposed these genes as possible biomarkers to detect the consequences of the exposure of marine invertebrates to different environmental pollutants [38]. In particular, these genes were defined as a part of the defensome, which was placed in motion by the sea urchin to protect themselves from environmental toxicants [42].

At the pluteus stage at 48 hpf (Figure 5, for the numerical values see also Supplementary Table S14), PAHs and PCBs had several common targets.

		PAHs	PCBs			PAHs	PCBs
Stress	*ARF1*	-			*DELTA*		
	caspase 3/7	-			*δ-2-catenin*		
	CASP8	+	+		*FOXA*	+	+
	cytb	+	+		*FoxG*	+	
	ERCC3	+			*Foxo*	+	+
	GRHPR				*GFI1*	+	+
	GS		+		*GOOS*	+	+
	HIF1A		+		*hat*	-	-
	hsp56		+		*H3.3*		+
	hsp60				*JNK*	+	+
	hsp70				*KIF19*		+
	MTase	+	+		*nodal*		+
	NF-kB				*NOTCH*		
	PARP	+	+		*OneCut*	+	+
	p38 MAPK				*SMAD6*		
	p53	-	-		*sox9*	+	+
	SDH	+	+		*TAK1*	+	+
	14-3-3 ε		+		*tcf4*	+	+
Skeletogenesis	*BMP5-7*				*TCF7*	+	+
	C-jun				*VEGF*		
	Nec	+	+		*Wnt5*		
	p16				*Wnt6*	+	+
	p19	+	+		*Wnt8*		+
	SM30	+	+	**Detoxification**	*CAT*		
	SM50	+	+		*MDR1*	+	+
	uni	-			*MT*	+	+
Development/Differentation	*ADMP2*				*MT4*	+	+
	Alix	+	+		*MT5*	+	
	Blimp		+		*MT6*	+	+
	BP10		+		*MT7*	+	+
	BRA	+	+		*MT8*	+	+

Figure 5. Schematic overview of *P. lividus* genes affected by artificial contaminated sediment with PAHs and PCBs under analysis. + = up-regulated gene; - = down-regulated gene.

2.4.1. Stress Genes

Eighteen genes were analyzed, and all were targeted by PAHs and PCBs with the exception of *GRHPR*, *hsp60*, *hsp70*, *NF-kB* and *p38MAPK*. Both contaminants, PAHs and PCBs, increased the expression levels of five genes (*CASP8*, *cytb*, *MTase*, *PARP1* and *SDH*) and decreased that of p53. Moreover, treatment with PAHs also down-regulated *ARF1* and *caspase 3/7* and up-regulated *ERCC3*, whereas the exposure to PCBs up-regulated *GS*, *HIF1A*, *hsp56* and *14-3-3 ε*.

2.4.2. Genes Involved in Development/Differentiation

Among the 28 genes analyzed, only 7 genes (*ADMP2*, *Delta*, *δ-2-catenin*, *Notch*, *Smad6*, *VEGF* and *Wnt5*) were not targeted by PAHs and PCBs. Common molecular targets for the two contaminants were *Alix*, *Bra*, *FOXA*, *FOXO, GFI1*, *Goosecoid*, *JNK*, *OneCut*, *sox9*, *TAK1*, *tcf4*, and *tcf7*, which were up-regulated, and HAT was the only down-regulated gene. Moreover, *FOXG* was up-regulated only after PAH treatment, whereas PCBs also up-regulated *Blimp*, *BP10*, *H3.3*, *KIF19*, *nodal* and *Wnt8*. PAHs affect axial development and patterning in sea urchin *Lytechinus anemesis* embryos by disrupting the regulation of beta-catenin, a crucial transcriptional co-activator of specific target genes in the Wnt/wg signaling pathway [43].

2.4.3. Skeletogenic Genes

Among the eight genes analyzed, only three genes (BMP5-7, C-jun and p16) were not targeted by the two contaminants. Both PAHs and PCBs increased the expression levels of Nec, p19, SM30 and SM50. Furthermore, PAHs decreased the expression level of the uni gene. The effects of the variations of expression of these genes directly affect the formation of the skeleton of sea urchin embryos. These data were supported by the genes identified in the transcriptomic analysis that belong to biological processes, such as cytoskeleton organization and its structural constituent and the microtubule-based process (see GO terms in Figure 5).

2.4.4. Genes Involved in Detoxification

All eight genes analyzed were targeted by the contaminants with the only exception being the *CAT* gene. *MDR1*, *MT*, *MT4*, *MT6*, *MT7* and *MT8* represented common targets for PAHs and PCBs and were able to increase their expression levels. In addition, the *MT5* gene was only up-regulated by PAHs. PAHs targeted 36 genes and PCBs 40 genes, 31 of which were common molecular targets between them. Genes involved in the detoxification process were also detected in the GO term analysis (see Figure 5).

PAHs and PCBs mainly up-regulated the targeted genes (as in the case of transcriptomic results; Supplementary Figure S5 and Supplementary Table S14) involved in skeletogenesis, developmental/differentiation and detoxification processes, supporting the morphological findings, which revealed that the majority of embryonic malformations affected the skeleton and the developmental plan (Supplementary Figure S6).

To the best of our knowledge, no studies to date have been performed to investigate the effects of PAHs and PCBs on sea urchin *P. lividus* by molecular approaches, with the only exception being Ruocco et al. [44], where the effects of highly contaminated sediments from the site of national interest Bagnoli-Coroglio (Tyrrhenian Sea, western Mediterranean) were detected. Suzuki et al. [45] reported on the effects of benz[a]anthracene and 4-OHBaA on the sea urchin *H. pulcherrimus* plutei, showing that the expression of mRNAs (spicule matrix protein and transcription factors) in the 4-OHBaA-treated embryos was also more strongly inhibited. These results were very similar to those found in our experiments, because *P. lividus* embryos after PAH treatment showed spicule malformation, and the expression levels of the SM30 and SM50 genes were also affected.

These molecular results, completed and deepened by de novo transcriptome, well supported our morphological findings, revealing that the majority of affected genes by both PAHs and PCBs were involved in skeleton formation, in the developmental plan and differentiation of sea urchin, as well as the observed malformations of the embryos, as reported in the GO term analysis (see Figure 4). The up-regulation of these genes identified by real-time qPCR experiments, as well as the up-regulation of genes identified in the de novo transcriptome, lead to the morphological effects detected in embryos deriving from adults exposed to these two contaminants.

3. Materials and Methods

3.1. Experimental Design and Mesocosms

Our experimental design included four scenarios: (i) negative control—seawater (W) (check filtered seawater background quality); (ii) negative control—not spiked sediment in mesocosm filled with W (W + SED) (check reconstructed sediment background quality); (iii) sediment spiked with PAHs (192 µg/L, nominal) (W + SED + PAHs); and (iv) sediment spiked with PCBs (0.15 µg/L, nominal) (W + SED + PCBs). All experiments were carried out in triplicates.

Each of the 12 testing mesocosms, located at the Stazione Zoologica Anton Dohrn, was characterized by an independent and closed seawater recirculation system (Supplementary Figure S6).

A pump (Micra 400 L/h, SICCE, Italy) promoted seawater circulation from the filtration compartment (containing porous ceramic filters, synthetic sponge and Perlon wool) to the other compartment containing the sediment. Each tank (50 × 36 × 48 cm) was filled with 55 L of natural seawater pre-filtered through a 200-micron mesh sock filter, collected from the Gulf of Naples and treated with zeolite and activated carbon for one week to remove most pollutants prior to chemical analyses (see below; Supplementary Tables S15 and S16). The seawater volume was kept constant during the experiment. It was checked daily and, if necessary, topped up with distilled water. The artificial sediment (10 L) present in each mesocosm was produced by mixing 36.5% of quartz sand (0 mm–3 mm, G. Build, s.r.l.) and 62.5% of coarse sand (grain size 0.4–0.8, Arena Silex, Manufacturas Gre, S.A.) and 1% calcium carbonate) [46]. Before spiking and prior to adding sea urchins, mesocosms were aged for one week. Spiking occurred by adding contaminants directly to the mesocosm water column (i.e., simulating a discharge event). One week after spiking, organisms were added to the mesocosms: seven females and three males for each tank. To evaluate the compartmentalization of PAHs and PCBs in sediment and seawater, their concentrations were evaluated: (i) before the addition of contaminants (t0), (ii) after the addition of contaminants (t1) and (iii) at the end (tf) of the experiment. PAH spiking (acenaphthene (ACE), acenaphthylene (ACY), anthracene (ANT), benzo(a)anthracene (BaA), chrysene (CHR), fluoranthene (FLT), fluorine (FLR), phenanthrene (PHE), pyrene (PYR)) required the addition (in each 55 L mesocosm) of 330 µL of a solution prepared by weighing 30 mg of each PAH in 10 mL of acetone/n-hexane (1:1 *v/v*; the nominal concentration of water stock solution is 35.3 g/L). PCB spiking required the addition (in each mesocosm) of 82 µL of a certified standard solution of 100 µg PCB/mL (Ultra Scientifc, Italy). We did not carry out control experiments with PAH diluents (acetone/n-hexane) due to the insignificant volume added (as compared to mesocosm's volume) and their high volatility.

3.2. Grain Size of Sediment

After one week of aging, 50 mL samples were collected from each tank and treated with 10% H_2O_2 and distilled water (2:8) for 48 h at room temperature in order to remove salts and organic matter. After drying (24 h at 105 °C), sediment fractions were mechanically separated with multiple vibrating sieves (Ro-Tap Particle Separator, Giuliani, HAVER & BOECKER Oelde Germany) with a 63 µm mesh to distinguish between sandy and silt–clay fractions [47,48]. Each fraction was weighted separately. Gain size data were analyzed with GradiStat software (version 8.0, [49]) and expressed as a percentage of the total dry weight.

3.3. Physico-Chemical Analyses

Temperature, dissolved oxygen, redox potential, salinity and pH were checked three times a week (Supplementary Figures S7 and S8). Temperature and dissolved oxygen were detected by a multiparameter probe (YSI 85, Ohio, US); redox (REDuction-Oxidation) state (270 mV) and pH were evaluated using WTW 197-S (SenTix® 41, Göteborg-Sweden) electrodes (7.8–8.1); salinity was measured by a refractometer (Sper Scientific, Scottsdale, Arizona) and fixed with distilled water when its value exceeded 38 ± 1 PSU. The analysis of nutrients included the detection of nitrites (NO_2^-), nitrates (NO_3^-), phosphates (PO_4^{3-}) and

ammonia (NH_3^-), using a colorimetric test (HACH Odyssey DR/2500 spectrophotometer, HACH Company Loveland, Colorado, United States) (Supplementary Figures S9 and S10).

For PAHs and PCBs analyses, seawater samples were extracted by a solid-phase extraction (SPE): 1.0 l of water was filtered and preconcentrated on a C18 disk (ENVI, -18 DSK SPE Disk, diam. 47 mm). The analytes were eluted with a solution of 1:1 dichloromethane and n-hexane. The determination of PAHs and PCBs in the sediment was performed by considering 5 g of dry sediment extracted with acetone/n-hexane 1:1 *v/v* (10 mL), using an ultrasonic disruptor (Brason, US). The extract was concentrated to 1 mL in Multivap under nitrogen flow (Multivap, LabTech, Italy). A total of 10 µL of a 1 mg/L solution of internal standard (mixture of deuterated PAHs) was added to the extract and injected to a gas chromatography–mass spectrometry (GC-MS) (MS-TQ8030-Shimadzu, Japan). The limits of detection (LOD) and quantification (LOQ) were calculated, and the average values for the seawater samples were 0.02 µg/L and 0.05 µg/L for PCBs and 0.004 µg/L and 0.01 µg/L for PAHs, respectively. For sediment samples, LOD and LOQ values were 0.03 µg/kg and 0.1 µg/kg for PCB and 0.16 µg/kg and 0.1 µg/kg for PAHs, respectively. Data quality was ensured by certified reference materials (ERM-CA100 (European Commission) for PAHs and QC1033 (Supelco) for PCBs). The recovery percentage was 70%–110% for PAHs and 65%–120% for PCBs [50,51].

For the determination of PAHs and PCBs in sea urchin tissues (thecae, spines, gonads and guts), approximately 3 g of tissues were homogenated and placed in an automatic extractor, under reflux, at 80 °C for 2 h with a 2 M KOH solution in methanol. After extracting with 20 mL of cyclohexane three times, the extract was purified on sodium sulphate, dried in a rotary evaporator and recovered with a 1 mL mixture of hexane/acetone (1:1 *v/v*). The extract was analyzed by GC–MS. The limit of quantification (LOQ) was of 0.4 µg/kg w.w. and 2 µg/kg w.w. for PAHs and PCBs, respectively. The average recoveries of PAHs and PCBs were >70% [52].

3.4. Sea Urchin Collection and Exposure, Gamete Collection for Morphological and Molecular Analysis by Real-Time qPCR

Methods for sea urchin collection (according to Italian laws (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000) and the conditions of their exposure in the mesocosms are reported in Ruocco et al. [44]. Animals were fed *Ulva rigida* according to Ruocco et al. [53].

After two months of exposure, sea urchins were collected and their gametes were obtained. Fertilization, embryonic growth until the pluteus stage (48 hpf) and morphological observations were carried out according to Romano et al. [37]. In particular, the percentage of embryos still at the gastrula stage, as well as normal and malformed plutei, were determined 48 h post-fertilization by counting at least 200 embryos for each sample under light microscopy (Zeiss Axiovert 135TV). Pictures were taken using a Zeiss Axiocam connected to the microscope.

Gonadal indices (GIs) were initially evaluated on gonads from five adult sea urchins (t0) (representing the starting point), and evaluations were repeated on five specimens after two months of exposure to PAHs and PCBs as well as in control sediments (W + SED). Animals were weighed, sacrificed and dissected; gonads were weighted for the GI determined (where GI indicates gonadal wet weight (g)/sea urchin wet weight (g) x 100 according to [54]).

Collection of embryos at the pluteus stage (about 5000 sea urchin plutei) for real-time qPCR was performed according to Ruocco et al. [44]. After total RNA extraction was performed using Aurum™ Total RNA Mini kit (BioRad), its amount and integrity were estimated according to Ruocco et al. [55]. Real-time qPCR experimental protocols as reported in Ruocco et al. [56] and Ruocco et al. [57] were followed (Supplementary Figure S4 reported all the analyzed genes). In particular, about 1 µg of RNA was used for cDNAs synthesis by iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. The expression of each gene was analyzed and normalized against the housekeeping genes *Ubiquitin* and *18S* rRNA, using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method. Relative

expression ratios greater than ±1.5 were considered significant. Each real-time qPCR plate was repeated at least twice.

3.5. De novo Transcriptome Assembly and Data Analysis

The sequencing was carried out in Genomix4Life S.r.l. (Baronissi, Salerno, Italy) using Illumina Truseq mRNA stranded 2 × 150—NextSeq500. De novo transcriptome assembly and annotation of 9 samples (1–3 triplicates for the control condition, embryos at the pluteus stage deriving from adult sea urchin reared in the mesocosm in tanks with seawater plus sediment (W + SED) without contaminants, indicated with "Control"; 4–6 triplicates for embryos at the pluteus stage deriving from adult sea urchins exposed to PAHs, indicated as "Treated_1"; and 7–9 triplicates for embryos at the pluteus stage deriving from adult sea urchins exposed to PCBs, indicated as "Treated_2") were carried out to discover differentially expressed genes between the two treatments and to perform functional analysis (Supplementary Table S17).

RNA sequencing was performed in paired-end mode. Fastq underwent quality control using the FastQC tool [1]. The tool Trinity (Trinity Release v2.10.0; [22]) was used to perform transcriptome assembly. Expression analysis was performed by RSEM (version 1.1.21) using default parameters, and expression values were converted to FPKM (fragments per kilobase of exon per million fragments mapped; Roberts et al. 2011). DESeq2 [23] was used to perform the normalization matrix and differentially expressed genes of all samples were considered. OmicsBox (version1.2.4) uses the Basic Local Alignment Search Tool (BLAST) to find sequences similar to the query set in FASTA format. The Gene Ontology (GO) terms were assigned based on annotation with an E-value of 10-5. The full dataset of raw data is deposited in the Sequence Read Archive (SRA database; available at https://www.ncbi.nlm.nih.gov/sra; accession number: SUB6701449; accessed on 15 February 2021).

3.6. Statistical Analyses

Morphological data were reported as means ± standard deviations (SD). These data were analyzed by the Shapiro–Wilk normality test and F-test. The statistical significance between groups was performed by one-way ANOVA followed by the Holm–Sidak test (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com, accessed on 15 February 2021) for multiple comparisons, indicating ** $p < 0.01$, *** $p < 0.001$. Statistical differences of GI values between t0 and after two months were evaluated by the Mann–Whitney U test (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com, accessed on 15 February 2021). P values > 0.05 were considered not significant.

4. Conclusions

We investigated for the first time the subchronic effects on *P. lividus* of slight PAH and PCB contamination in mesocosms (sediment and water) considering a multi-endpoint approach. Generally, the attention is focused on sediment hot spots (i.e., highly spiked sediment from industrial and commercial areas) with long-term historical pollution (i.e., black samples). We decided to refocus on the so-called "blank" samples with very low concentrations of PAHs and PCBs below national and international threshold limit values (TLVPAHs = 900 µg/L, TLVPCBs = 8 µg/L, Legislative Italian Decree 173/2016). Our reconstructed spiked mesocosms always presented PAH and PCB levels below the respective detection limit values (LODs) for both sediment and water samples, meaning that they soon compartmentalized (in less than one week) between sediment, water, biota, air and mesocosm surfaces. Nevertheless, significant biological effects were detected ranging from bioaccumulation and embryotoxicity (PAHs) to the up- and down-regulations of genes (PAHs and PCBs). Variation of gene expression is directly translated at the morphological level in the malformations observed in the embryos, leading to the identification of genes responsible for those defects. However, de novo assembly is a necessary step to assess

differential gene expression and also provides an important resource for researchers working with this sea urchin species. In fact, the transcriptional changes detected in this study are corollary, and, in the future, functional studies will need to clearly establish that these genes can be considered as universal biomarkers involved in the response to contaminants in the marine environment.

Finally, the results evidenced that the combination of morphological and molecular approaches can efficiently support a deeper understanding of how marine species can react to the widespread background sediment contamination levels.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22136674/s1, Table S1: Chemical analyses of total PAHs and total PCBs in sediment, Table S2: Chemical analyses of total PAHs and total PCBs in seawater, Table S3: Chemical analyses of total PAHs, total PCBs and Zn in sediment, Table S4: Chemical analyses of total PAHs, total PCBs and Zn in seawater, Supplementary Table S5: Chemical analyses of total PAHs, total PCBs and Zn in sediment, Table S6: Chemical analyses of total PAHs, total PCBs and Zn in seawater, Table S7: Chemical analyses of total PAHs, total PCBs and Zn in sediment collected at tf, Table S8: Chemical analyses of total PAHs, total PCBs and Zn in seawater collected at time zero (t1), Table S9: Adult sizes and gonadal index of adults of sea urchin *P. lividus*, Table S10: Quantity (µg/kg) of PCBs detected in thecae (including spines), gonads and gut from adult sea urchin *P. lividus*, Table S11: Number of differentially expressed (DE) genes and isoforms, Table S12: Common up-regulated genes in the Venn diagrams, Table S13: Common down-regulated genes in the Venn diagrams, Table S14: Data of expression levels in embryos at the pluteus stage, Table S15: Chemical analyses of heavy metals in sea water collected at time zero, Table S16: Chemical analyses of ammonia, nitrates, nitrites and phosphates on sea water, Table S17: Sample names, conditions and paired reads for each sample, Table S18: The number of reads obtained for the samples, Figure S1: Data of the sediment grain size analyzed with GradiStat software, Figure S2: Mortality index, Figure S3: BLASTx top-hit species distribution, Figure S4: Principal component analysis (PCA), Figure S5: Summary of the 62 genes analyzed by real-time qPCR, Figure S6: Schematic overview of *P. lividus* genes affected by artificial contaminated sediment with PAHs and PCBs under analysis, Figure S7: Schematic representation (frontal view) of 1 of the 12 experimental tanks of the mesocosms, Figure S8: Physical parameters (dissolved oxygen, pH, redox, temperature and salinity) of sea water, Figure S9: Physical parameters (dissolved oxygen, pH, redox, temperature and salinity) of sea water, Figure S10: Chemical parameters (ammonia, nitrates, nitrites and phosphates) of sea water Figure S11: Chemical parameters (ammonia, nitrates, nitrites and phosphates) of sea water.

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PAHs and PCBs Affect Functionally Intercorrelated Genes in the Sea Urchin *Paracentrotus lividus* Embryos

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) represent the most common pollutants in the marine sediments. Previous investigations demonstrated short-term sublethal effects of sediments polluted with both contaminants on the sea urchin *Paracentrotus lividus* after 2 months of exposure in mesocosms. In particular, morphological malformations observed in *P. lividus* embryos deriving from adults exposed to PAHs and PCBs were explained at molecular levels by *de novo* transcriptome assembly and real-time qPCR, leading to the identification of several differentially expressed genes involved in key physiological processes. Here, we extensively explored the genes involved in the response of the sea urchin *P. lividus* to PAHs and PCBs. Firstly, 25 new genes were identified and interactomic analysis revealed that they were functionally connected among them and to several genes previously defined as molecular targets of response to the two pollutants under analysis. The expression levels of these 25 genes were followed by Real Time qPCR, showing that almost all genes analyzed were affected by PAHs and PCBs. These findings represent an important further step in defining the impacts of slight concentrations of such contaminants on sea urchins and, more in general, on marine biota, increasing our knowledge of molecular targets involved in responses to environmental stressors.

Keywords: aromatic hydrocarbons; polychlorinated biphenyls; sea urchin

1. Introduction

Marine organisms are permanently exposed to multiple stressors, such as climate changes [1–3] and the consequential ocean acidification, deoxygenation and sea-level rise [4–6], natural toxic metabolites [7–11] and compounds deriving from human activities [12–15]. The exposure to these stressors induces marine organisms to adopt strategies against external or internal environmental factors through metabolic and/or molecular changes to maintain cellular homeostasis [16,17].

Human activities represent a major source of stress for marine organisms. Anthropic activities can change, for example, the natural compositions, rate, deposition and transport of sediment, causing a radical shift of biological responses [18]. Since sediments produce the habitat for several benthic communities, their pollution could negatively influence the

fitness and survival of marine flora and fauna [19]. Sediment drains and temporarily accumulates organic and/or inorganic compounds, including polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCB) and organotin compounds (OTCs) derived from natural and anthropogenic sources, such as industrial, commercial, agricultural and urban activities [20–23]. Following several disruptive events (bioturbation and/or dredging), marine sediments can release these accumulated pollutants facilitating the re-allocation of contaminants within the water column [20,24,25]. This resuspension affects the marine environment and consequently human health, because pollutants can accumulate in bottom feeders and magnify through marine food webs, reaching humans through fishery products [26,27].

To our knowledge, there are several studies evaluating adaptability tolerance of sea urchins to several stress conditions, such as warming and ocean acidification [28–30]. However, concerning anthropogenic pollutants, the long-term exposure of sea urchins to heavy metals, PAHs and PCBs did not display any tolerance or adaptation [31–34].

PAHs and PCBs are among the most toxic pollutants commonly present in the sea. PAHs consist of a group of over 100 different organic compounds, constituted of two or more fused benzene rings and pentacyclic molecules, differing in their physical and chemical properties. PAHs can be released in the marine environment both through natural events and anthropogenic activities [35,36], with a noteworthy toxicity depending on the number of benzene rings [37]. PCBs are organic compounds, mainly derived from human activities, and considered among the most dangerous pollutants because of their toxic and carcinogenic properties, vast distribution and high persistence in the environment [37,38]. Several studies reported that PCBs were able to induce harmful effects on the embryo development of sea urchins [39–41]. Their direct involvement in the negative impact on both marine invertebrates' and vertebrates' embryo development was demonstrated by toxicity assays with pure compounds [42–44]. For example, the toxicity model proposed by Di Toro et al. [45], relied on assays using pure hydrocarbon compounds in dissolved phase, showing that dissolved PAHs were toxic in the absence of others compounds.

However, few studies demonstrated possible negative influences of these compounds on the survival of adults and their progenies. Our recent study proved that PAHs and PCBs induced toxigenic effects on the sea urchin *Paracentrotus lividus* embryos derived from adults exposed to sub-chronic concentrations in contaminated mesocosm, using a multi-endpoint approach and de novo transcriptomic [33].

This investigation was aimed at further exploring the target genes involved in the response of the sea urchin *P. lividus* to PAHs and PCBs, and other environmental stressors. To perform this study, sea urchin plutei (48 h post-fertilization, hpf) were exposed to slight PAH- and PCB-contaminated mesocosms, also adopted in our previous experiments [33]. Firstly, an interactomic analysis was conducted on 62 genes, previously described in *P. lividus* [46–49] in order to understand the biological and functional relationships and the gene networks in which they were involved. Twenty-five new genes were identified, being functionally interconnected to 10 genes already described in previous studies [46–49]. Two networks were obtained, including most of the correlated genes, involved in stress responses, detoxification and developmental processes. Finally, the expression levels of these twenty-five genes were followed by Real Time qPCR to identify the possible gene targets affected by PAH and PCB.

2. Results

2.1. Effects of PAHs and PCBs on Gene Expression by Real Time qPCR

The pollutants concentrations (i.e., as mean value, $n = 3$) in the spiked mesocosms at the end of the experiments were 5 μg/kg, 5 μg/L and 29.1 μg/L for PAHs in sediment, water and sea urchins (i.e., gonads), respectively; while PCBs concentrations were 5 μg/kg, 5 μg/L and < 2 μg/L in sediment, water and sea urchins (i.e., gonads), respectively. PAHs and PCBs were below the relative LOD in mesocosms.

PAHs and PCBs affected almost all the genes under analysis (Figure 1; fold changes are reported in Supplementary Table S1). At the pluteus stage, PAHs and PCBs had several common targets.

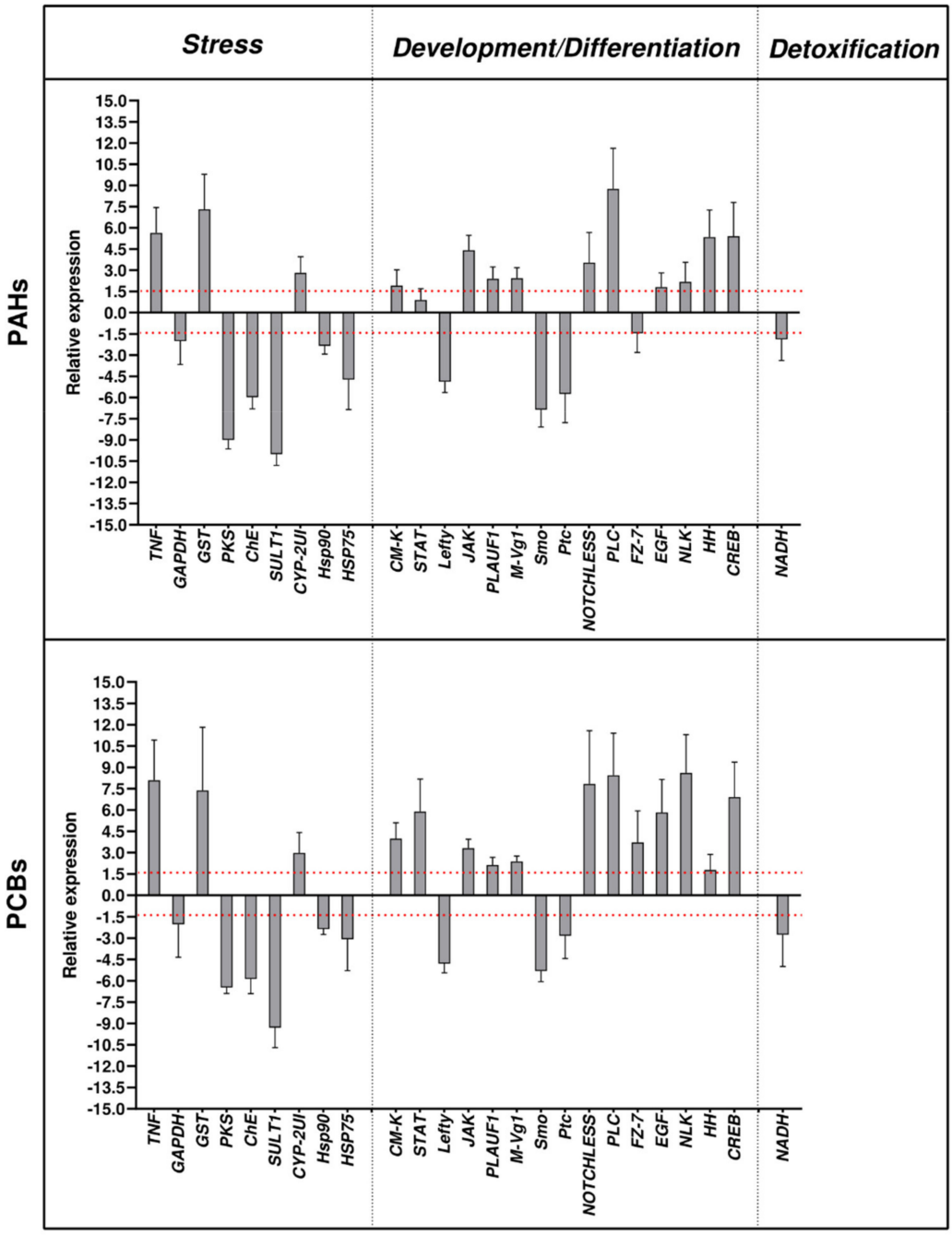

Figure 1. Real-time qPCR at the pluteus (48 hpf) stages of the sea urchin *P. lividus* embryos, deriving from adults exposed for 2 months to sediments contaminated with PAHs and PCBs. Data are reported as a fold difference compared with control embryos, deriving from adults reared in tanks with non-contaminated sediments (mean ± SD). Histograms show the fold-changes of 25 genes involved in three functional processes: stress response, development/differentiation and detoxification. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and −1.5) were considered significant. Real-time qPCR reactions were carried out in triplicate. Statistical differences were evaluated by nonparametric Mann–Whitney test. p-values < 0.05 were considered significant.

- Stress genes:

All nine genes were targeted by PAHs and PCBs. Both contaminants increased the expression levels of three genes (*TNF*, *GST* and *CYP-2UI*) and decreased *GAPDH*, *PKS*, *ChE*, *SULT1*, *hsp90* and *hsp75*.

- Genes involved in development/differentiation:

Out of 15 genes analyzed, only *STAT1* and *FZ-7* were not targeted by PAHs, whereas these two genes were up-regulated by PCBs. Common molecular targets switched on by PAHs and PCBs were *CM-K*, *JAK*, *PLAUF3*, *M-Vg1*, *NOTCHLESS*, *PLC*, *EGF*, *NLK*, *HH* and *CREB* which were up-regulated; whereas *Lefty*, *Ptc* and *Smo* were down-regulated.

- Genes involved in detoxification:

The only gene analyzed, *NADH*, was targeted by both PAHs and PCBs, which were able to decrease its expression levels.

2.2. Network Analysis

Twenty-five genes analyzed by Real Time-qPCR were functionally intercorrelated among them and also correlated with several genes previously isolated from *Paracentrotus lividus*. Depending on gene functions, they were ascribed to two functional networks, reporting the highest correlations among stress/detoxification and developmental genes.

Among the 10 genes involved in stress responses, *hsp90*, *hsp75* and *GAPDH* shared the largest number of connections (Figure 2).

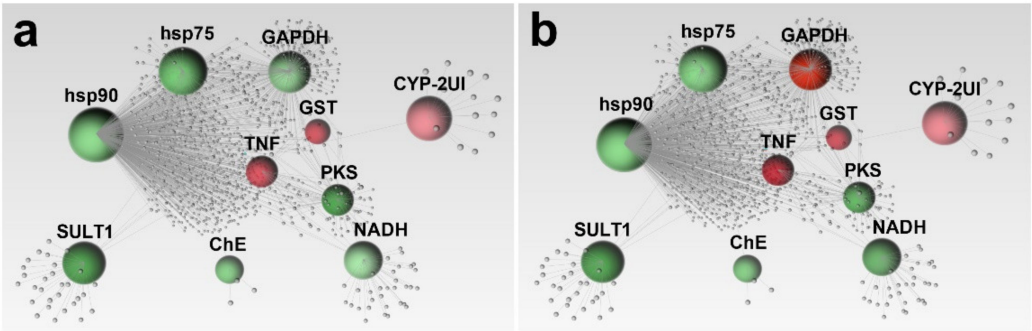

Figure 2. Gene networks obtained by STRING interactome analysis of ten genes involved in stress response and detoxification. Correlation confidence score cut-off of 400 was reported. Among functionally correlated genes, those with up (red) and down (green) expression affected by PAHs (**a**) and PCBs (**b**) were reported. Color shading depends on fold-change values. Gray spheres represent additional connections. The list of human orthologous genes used for Network analysis is reported in Supplementary Table S2.

The heat shock protein *hsp90* clearly showed a key role in several biological processes since a huge number of additional connections (grey spheres) were observed (Figure 2). *TNF* was strongly correlated to *hsp90*, *hsp75*, *GAPDH*, *GST*, *PKS* and *NADH*, while less connections have been observed for *SULT1* and *NADH*, particularly with *hasp90*, *hsp75*, *GAPDH* and *PKS* (Figure 2). *ChE* and *CYP2-UI* showed only one connection to *hsp90* through the involvement other associated genes (Figure 2).

Among development and differentiation genes, a huge functional correlation was appreciable (Figure 3).

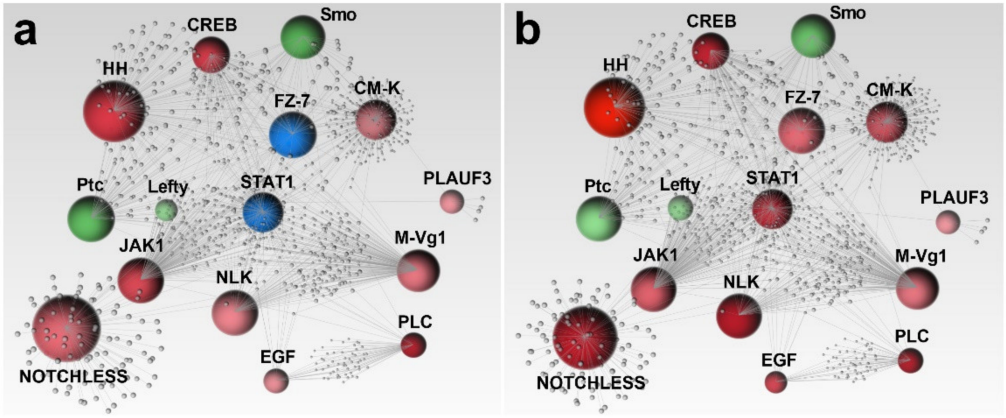

Figure 3. Gene network obtained by STRING interactome analysis of 15 genes involved in developmental processes. Correlation confidence score cut-off of 400 was reported. Among functionally correlated genes, those with up (red), down (green) and unchanged (blue) expression affected by PAHs (**a**) and PCBs (**b**) were reported. Color shading depends on fold-change values. Gray spheres represent additional connections. The whole list of human orthologous genes used for Network analysis is reported in Supplementary Table S2.

Several genes resulted extremely important within this biological cascade, including *HH*, *CREB*, *JAK*, *STAT1*, *M-Vg1*, *Ptc*, *Smo*, *FZ-7*, *NLK*, *NOTCHLESS* and *CM-K*, since a large number of connections was observed among them. In addition, these latter genes reported an independent association to other effectors, with *NOTCHLESS* and *CM-K* genes displaying the majority of them (Figure 3). Concerning the less correlated genes, *PLC* and *EGF* were found functionally interconnected between each other and with other genes, such as *JAK*, *STAT1* and *CAMP*, and also *Ptc* gene, in the case of *EGF*. Moreover, *PLAUF3* revealed only three functional relationships with *Smo*, *Ptc* and *STAT1* (Figure 3).

3. Discussion

Our results greatly expand the reach of previous investigations on genes involved in the response of the sea urchin *Paracentrotus lividus* to PAH- and PCB-contaminated sediments [33]. Previous results revealed morphological malformations in the sea urchin plutei, which were correlated to a significant alteration of the expression of several genes involved in different functional processes, such as stress response, development/differentiation, detoxification and skeletogenesis.

Here, 25 new genes involved in stress response, development/differentiation and detoxification processes were isolated in *P. lividus*. Our findings showed that PAHs and PCBs were able to switch on almost all genes under analysis (Figure 4; Supplementary Table S3).

Thus, as already reported by Albarano et al. [33], a possible correlation could be hypothesized between morphological malformations of plutei and the molecular responses exhibited. In fact, among the genes analyzed, twenty-three were altered by both pollutants, revealing comparable values of gene expression in a huge number of shared targets (Figure 1). These results could indicate that organic compounds affected some common molecular pathways, so changing physiological mechanisms in sea urchin adults, which in turn may induce abnormal offspring.

Concerning gene expression results, no significant differences were observed between PAHs and PCBs, suggesting that similar biological pathways were activated in response to PAHs and PCBs exposure. The only exception was *GAPDH*, whose expression increased in the PAHs treatment.

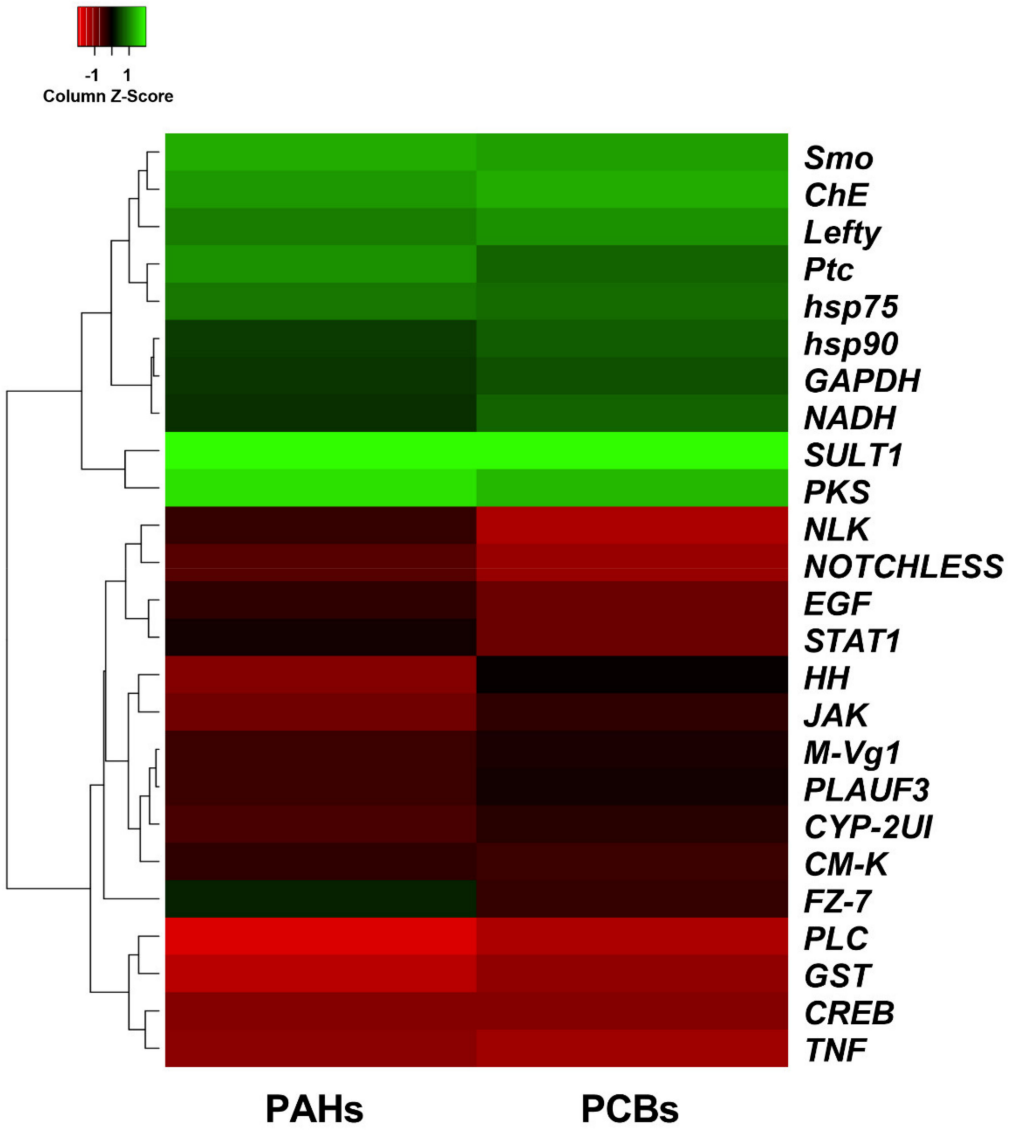

Figure 4. Heatmap showing the expression profiles and hierarchical clustering of 25 genes analyzed by Real Time qPCR in embryos deriving from adult *P. lividus* sea urchins exposed for 2 months to sediment contaminated with PAHs and PCBs. Color code: green, up-regulated genes with respect to the control; red, down-regulated genes with respect to the control; black, genes for which there is no variation in gene expression with respect to the control.

As for the stress response (Figure 2), both PAHs and PCBs targeted all genes analyzed in the present study. Over the last 10 years it has been shown that alteration of *ChE*, *PKS* and *GST* activity may cause morphological alterations of the spicules, similar to those shown in Figure 4 [50–52]. In fact, skeletal malformations and delayed larvae correlated to significant down-regulation of *PKS* gene was recently found after a 24- and 48-h exposure of *P. lividus* eggs to nickel [53]. Similarly, *CYP-2AI*, *hsp70* and *hsp90* are involved in defense against chemical stressors, and their alteration is able to impact the gastrulation process [54–56]. For instance, malformed embryos and up-regulation of hsp90 was found in sea urchins exposed to thermal stress and bacterial LPS, corroborating a fundamental role of this gene in the stress response [57–60]. Concerning *GAPDH*, this gene possesses key functions in the glycolytic pathway, and also roles in nuclear RNA export, membrane fusion, and DNA repair [61]. Although *GAPDH* was considered as a housekeeping gene in several organisms, including sea urchins [62–64], in the present study this gene was found differentially expressed after PAH and PCB exposure. The alteration of the expression level of *GAPDH*

gene in sea urchin could be considered a suitable bioindicator for human health since its up-regulation was detected in cervical and ovarian tissue during cancer development [65].

As reported for the stress response network, the affected developmental and differentiation genes were almost exclusively the same for both organic compounds. A strong up-regulation of these genes was induced by sea urchin exposure to PAHs and PCBs (Figure 3). Ten functionally connected genes, *HH*, *CREB*, *NOTCHLESS*, *JAK*, *NLK*, *EGF*, *PLC*, *M-Vg1*, *PLAUF3* and *CM-K*, were up-regulated in the same biological pathway (Figure 3), thus corroborating previous results reporting several aberrations in sea urchin progenies [33]. *FZ-7* and *STAT1* were not affected by PAHs treatment (Figure 3a), indicating that the biological cascade altered by PAHs and PCBs might be slightly different. Specifically, these genes were up-regulated only by PCBs treatment. As previously mentioned for *GAPDH* gene the variation of mRNA levels might also be correlated to human diseases. In fact, the overexpression of *FZD7* was detected in 90% of tumors (most in the human hepatocellular carcinoma cells and in breast cancer cells) where causes the accumulation of β-catenin protein affecting the Wnt/β-catenin signal transduction pathway [66–68]. Similarly, the up-regulation of *STAT1* represents a serious risk for human health since its activation is able to down-regulate programmed cell death genes in several cancer cell lines (i.e., breast cancer and adenocarcinoma cells) [69–71]. As reported in Figure 3, *CREB*, *HH*, *PLAUF3*, *NLK*, *EGF*, *JAK*, *M-Vg*, *PLC* and *CM-K* were up-regulated in both treatments. Similar to our study, the expression of the transcription factor *CREB* was targeted in response to heat stress and starvation in sea urchin of the genus *Strongylocentrotus* [72,73]. The *PLC* gene is typically regulated by *CM-K*, and together they are involved in the events following fertilization and embryo development in sea urchins [74–77]. The *M-Vg* gene together with nodal is involved into left–right axis formation, regulating some down-stream effectors, including the *BMP2* gene [78]. The up-regulation of these genes could be a possible consequence of PAHs and PCBs exposures by inducing malformations in the embryo structure. The *NOTCHLESS* gene is involved in the *NOTCH-DELTA* pathway which, in turn, regulates the expression of *NLK* and *Lefty* genes [46,79,80]. This pathway has a key role during the induction and differentiation of secondary mesenchyme cells [80–82] and could be involved in the formation of malformed gastrulae. *Ptc* and *Smo* genes showed a down-regulation after both PAHs and PCBs treatments (Figure 3). The *Smo* and *Ptc* proteins are co-receptors of *HH* ligand and are expressed by the skeletogenic and non-skeletogenic mesoderm [83–85]. Probably, the defects in embryo development were caused by the perturbation of this pathway.

As described above, almost all genes were targeted by both pollutants. The sole exceptions were represented by *FZ-7* and *STAT1,* which were up-regulated by PCBs (Figure 3b). *FZ-7* receptor binding to *Wtn6* controls the β-catenin signal during the specification of the endomesoderm [86]. The *STAT1* gene together with *JAK* gene constitute the *JAK/STAT* pathway, which plays a fundamental role in the regulation of the cellular complex [87–89].

On the whole, molecular analyses of the aforementioned 25 genes revealed interesting results, which might justify the clear malformations affecting the apex and arms of sea urchin plutei obtained from adults exposed to PAHs and PCBs. These pollutants are released into the sea by anthropogenic activities and accumulated in marine sediments where sea urchins, typical hosts of benthic environments, could suffer for their long-lasting impacts.

In addition, an increase of knowledge on *P. lividus* gene functions has a great significance for molecular analysis on this well-established model organism. In fact, despite of its importance for the scientific community, the complete genome of the sea urchin *P. lividus* is still not fully annotated and this represents a limit in its use for molecular studies.

In the sea, when evaporation exceeds the contribution of rivers, the concentration of environmental pollutants increases with a consequent effect on organisms, biodiversity and as long-term effect on human health [90]. In fact, embryos and larvae of marine invertebrates seem to be a suitable indicator in understanding the toxicological response induced by chemical pollutants marine invertebrates, accumulating high levels of them

in their tissues. Furthermore, these invertebrates have a key role in the pelagic as well in benthic food webs, representing intermediate consumers. Then, the toxicological risk faced by marine organisms and even by humans through the ingestion of contaminated edible species is concrete. Consequently, studies on the status of chemical pollutants in marine ecosystems represent an important step in evaluating possible risks on human health. On this line is also our approach with a 2-month exposure of the sea urchins to PAHs and PCBs, aiming at detecting the long-term morphological and molecular effects of these contaminants on marine invertebrates. More in general, our findings are in agreement with the idea that variations in gene expression represent an early indicator of the presence of stressful conditions in various marine environments. In fact, the identification of molecular pathways in which the targeted genes were involved represents a key step in understanding how marine organisms attempt protection against toxicants. In the case of PCBs and PAHs we found the alteration of almost the same target genes, revealing that both pollutants could activate similar biological pathways in sea urchin. This result might suggest the hypothesis that common responses of PCBs and PAHs could be also triggered in other marine invertebrates. Furthermore, this work increased the pool of genes named "defensome", as described in Marrone et al. [91], used by marine organisms to avoid deleterious consequences and irreversible damages. In conclusion, target genes for PAHs and PCBs may be considered as possible universal biomarkers to detect the presence and the effects of key environmental pollutants impacting the physiology of marine invertebrates.

4. Materials and Methods

4.1. Experimental Conditions

Adult *Paracentrotus lividus* (seven females and three males) were reared in each of experimental tanks of a mesocosm, previously spiked with PAHs (acenaphthene (ACE), acenaphthylene (ACY), anthracene (ANT), benzo(a)anthracene (BaA), chrysene (CHR), fluoranthene (FLT), fluorine (FLR), phenanthrene (PHE), pyrene (PYR)), and PCBs (standard solution) [33]. Each mesocosm was spiked with 192 µg/L and 0.15 µg/L of PAHs and PCBs (i.e., nominal concentrations), respectively, to investigate any sub-chronic effect at concentrations below sediment threshold limit values (TLVs) (TLV_{PAHs} = 900 µg/L and TLV_{PCBs} = 8 µg/L) according to the Italian regulation D.M. 173/2016. To evaluate the compartmentalization of PAHs and PCBs in sediment and seawater, their concentrations were evaluated at three times (before (t0) and at the end (tf) of the experiment) and were measured according to Trifuoggi et al. [92]. The limit of detection and limit of quantification for PAHs and PCBs were: LOD_{PAHs} = 0.004 µg/L and LOQ_{PAHs} = 0.01 µg/L, LOD_{PCBs} = 0.002 µg/L and LOQ_{PCB} = 0.05 µg/L for the seawater samples; LOD_{PAHs} = 0.016 µg/Kg and LOQ_{PAHs} = 0.01 µg/Kg, LOD_{PCBs} = 0.03 µg/Kg and LOQ_{PCBs} = 0.01 s µg/Kg for the sediment; LOD_{PCBs} = 0.4 µg/kg w.w. and LOQ_{PCBs} = 2 µg/kg w.w. for sea urchin tissues. All details about methods for PAHs and PCBs chemical assessment were summarized in Trifuoggi et al. [92].

4.2. RNA Extraction and cDNA Synthesis

Adult sea urchins were collected after 2 months of exposure in PAH- and PCB-contaminated mesocosms (192 µg/L and 0.15 µg/L, respectively), and their gametes were used for in vitro fertilization [33]. Collection of embryos at the pluteus stage (about 5000 sea urchin plutei) for RNA extraction was performed according to Ruocco et al. [93]. For each sample, 1000 ng of total RNA was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions.

4.3. Network Analysis

Sixty-two genes [46–49] (see also Supplementary Table S4) were analyzed by NetworkAnalyst 3.0 software (https://www.networkanalyst.ca/; accessed on 8 February 2021; [94]), using STRING interactome of protein–protein interactions [95]. Human orthologous genes were used to compute the network analysis (Supplementary Table S2). The relations

among genes (confidence score cut-off of 400) displaying experimental evidence were highlighted. Twenty-five mostly connected genes were then chosen and analyzed. The sequences were retrieved from the transcriptome of the sea urchin *P. lividus* deposited in the SRA database (Sequence Read Archive, available at https://www.ncbi.nlm.nih.gov/sra (accessed on 5 February 2021), accession number PRJNA495004, [96]; accession number SUB2817153, [97]; accession number SUB6701449, [33] and from NCBI (available at https://www.ncbi.nlm.nih.gov; accessed on 8 February 2021). For each gene, specific primers were designed on the nucleotide sequences (see Table 1).

Table 1. Gene Name, Acronym, Accession Numbers, Primer Names, Primer Sequences and Amplicon Lengths of PCR Fragments.

Gene Names	Acronym	Accession Number	Primer	Sequence (5′→3′)	Amplicon Lenght (bp)
Calcium/calmodulin-dependent protein kinase type 1D	*CM-K*	PRJNA495004	Pl_CM_F1 Pl_CM_R1	GTTATCCTCCATTTTACGATGAG GCAGATATACGTGTGAGGAAG	168
Camp-responsive element	*CREB*	PRJNA495004	Pl_CREB_F2 Pl_CREB_R2	GTAACTAAAGCATCTGGGAGAC GGTTCAGATATTAGTGGATGC	158
Cholinesterase	*ChE*	SUB6701449	Pl_ChE_F2 Pl_ChE_R2	CGAGATGGCGTATGTTTTGAG GACTATGTTCCCGCTGACTG	160
Citochrome P450 2UI isoform X2	*CYP-2UI*	SUB6701449	Pl_CYP-2UI_F1 Pl_CUP-2UI_R1	GCGCCTCTTCGTTCTATTCC CGGCATAGTAGTAGACTAGC	174
Epidermal growth factor	*EGF*	PRJNA495004	Pl_EGF_F1 Pl_EGF_R1	CGGCGGTGTGTGTATCGATG CAGTAGCCATCCTAGTGTTCC	189
Frizzled7	*FZ-7*	PRJNA495004	Pl_FZ_F1 Pl_FZ_R1	GATCGTGAGCGTAGCATATAC CATGGTCTTTTTGGGCACTA	175
Glutathione-S-transferase	*GST*	SUB6701449	Pl_GST_F4 Pl_GST_R4	GCCCGACTTACCTACTTTGC CTTGCAGCTCATCACTGATGG	165
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	PRJNA495004	Pl_GAPDH_F1 Pl_GAPDH_R1	GTACTACTTCTCATTCACCTTG CATAGCTCTGACACCGCCAC	213
heat shock protein 75	*hsp75*	PRJNA495004	Pl_hsp75_F2 Pl_hsp75_R2	GGACTGGTGGAACAACTATATC CGATCACCACTCTCTGTCAC	173
heat shock protein 90	*hsp90*	SUB6701449	Pl_hsp90_F1 Pl_hsp90_R1	GGGTGTGGTAGATTCTGATG GCTCTCCATGTATTCATCAG	148
Hedgehog	*HH*	PRJNA495004	Pl_HH_F1 Pl_HH_R1	GGTACATGAGGCACAAGCTAG CCACTTCACATCACTTGACC	193
Janus kinase	*JAK*	XM_030985987	Pl_JAK_F2 Pl_JAK_R2	CACCTTACCCATACTAGACAG CTTGCCAGAGCCTCCGCTGAC	192
Lefty	*Lefty*	SUB6701449	Pl_Lefty_F2 Pl_Lefty_R2	CAGTCCAGACATGGGTGGCAG CATTTCGTCGACCACCTGCTG	182
maternal Vg1	*M-Vg1*	SUB6701449	Pl_M-Vg1_F1 Pl_M-Vg1_R1	GCACCTGCACCTAGAGACTC GCATGACCTTTTCCGGCCTG	145
NADH dehydrogenase	*NADH*	SUB2817153	Pl_NADH_F1 Pl_NADH_R1	GTCTCCGTCGGATAAATCAAAG CCGAAAAGGAAATAACGAAGC	194
Nemo-like kinase protein	*NLK*	AY442297	Pl_NLK_F1 Pl_NLK_R1	CCTCTACCAGATTCTCAGAG GTGACACAGTACTACCGCGC	192
Notchless protein	*NOTCHLESS*	PRJNA495004	Pl_Notchless_F1 Pl_Notchless_R1	GGGAAGCTAAGGCATCAGAC CGATCCTCTCAAGCACTTTAG	145
Patched	*Ptc*	SUB6701449	Pl_Ptc_F1 Pl_Ptc_R1	CGGTCGTCAGTATCATCATG GCAACCACGACTCCGTAAGC	135
Phospholipase C	*PLC*	AJ012336	Pl_PLC_F1 Pl_PLC_R1	GAGACATTCACAGTGCCCAC CTGACCGATACCAAGCTGTAC	139
PLAUF 3 RNA-binding protein AUF1 mRNA	*PLAUF 3*	AY682309.1	Pl_PLAUF3_F2 Pl_PLAUF3_R2	GGAGGATACGGCGGTGGCGG GTGTTGACTCCACAGGAGTG	182
Polyketide synthase	*PKS*	SUB6701449	Pl_PKS_F1 Pl_PKS_R1	GCTTCCTCGACCAGTCTGTC CCTCCGAAGACAGTCATCTG	142
signal transducer and activator of transcription	*STAT1*	PRJNA495004	Pl_STAT_F1 Pl_STAT_R1	GTGTGTCAATCAGCCAGTGC GTACATCATGAGCTTACCATTTC	196
Smoothened	*Smo*	SUB6701449	Pl_Smo_F1 Pl_Smo_R1	CACGATCCATTACGGCGTTG GCCCAACTCACACCCATGAC	217
sulfotransferase 1C2-like	*SULT1*	SUB6701449	Pl_SULT1_F2 Pl_SULT1_R2	CAGGCACTCACTGGCTCATG CTCTTCAGCTCTCGTCTTCG	140
Tumor necrosis factor alpha	*TNF*	SUB2817153	Pl_TNF_F1 Pl_TNF_R1	CCTGATGTGTATGCCTCTATC CAAGATCCTCATGTCAGGAAG	144

PCR fragments were purified from agarose gel using the QIAquick Gel Extraction kit (Qiagen, Milan, Italy), and the specificity of the PCR product was checked by DNA sequencing. PCR products were aligned to the original sequences by MultAlin Software [98] (see Supplementary Figure S1). Five serial dilutions were set up to determine Ct values and PCR efficiencies for all primer pairs (for RT-qPCR conditions see below). Standard curves were generated for each oligonucleotide pair using Ct values versus the logarithm of each dilution factor (Supplementary Figure S2). The biological functions for 25 genes are reported in Supplementary Table S3.

4.4. RT-qPCR Analysis

Undiluted cDNA was used as a template in a reaction containing a 0.3 mM final concentration for each primer and 1× FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed in a *ViiATM7 Real Time PCR System* (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 72 °C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all *RT-qPCR* reactions were carried out in triplicate. Fluorescence was measured using the ViiATM7 software (Applied Biosystems, Monza, Italy). The relative expression ratios were calculated from quantification cycles (Cq) through an efficiency (E) corrected calculation method (Etarget $^{\Delta Cq \text{ target (Mean Control–Mean Sample)}}$ /Ereference $^{\Delta Cq \text{ reference (Mean Control–Mean Sample)}}$) [99,100] using REST software (Version updated 2009, Relative Expression Software Tool, Weihenstephan, Germany). The expression of each gene was analyzed and internally normalized against *ubiquitin* [101] and *18S rRNA* [102,103]. Relative expression ratios above 1.5 were considered significant. Nonparametric Mann–Whitney test was applied to ΔCq (Cq gene of interest—Cq reference) values between treated and control samples (n = 3). p-Values < 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism Software (version 9.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 1 February 2021).

Fold change values were represented through a Heatmap generated by Heatmapper Software (http://www.heatmapper.ca/ (accessed on 5 February 2021); [104]). Values were scaled up by column and hierarchical clustering was performed on rows by a routine adopting the Average Linkage method considering the Euclidean Distances.

4.5. Gene Networks

An interactomic analysis was performed by NetworkAnalyst 3.0 software [93] available at https://www.networkanalyst.ca/ (accessed on 8 March 2021), using STRING interactome of protein–protein interactions [94]. Human orthologs of selected genes were used to compute the network analysis. The most significant relations among genes (confidence score cut-off = 900) displaying experimental evidence were highlighted.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222212498/s1, Figure S1: Alignments of PCR products to the original sequence of *GAPDH* (A), *GST* (B), *hsp75* (C), *TNF* (D), *CM-K* (E), *CREB* (F), *EGF* (G), *FZ-7* (H), *HH* (I), *NLK* (J), *NOTCHLESS* (K), *PLC* (L), *STAT1* (M), *NADH* (N), *ChE* (O), *Ptc* (P), *Smo* (Q), *hsp90* (R), *Lefty* (S), *PKS* (T), *CYP-2UI* (U), *SULT1* (V), *M-Vg1* (W), *JAK* (X), *PLAUF3* (Y) genes., Figure S2: Efficiencies (E) of *18S rRNA* (A), *Ubiquitin* (B), *ChE* (C), *CREB* (D), *CYP-2UI* (E), *GAPDH* (F), *hsp90* (G), *Lefty* (H), *NLK* (I), *NOTCHLESS* (J), *PKS* (K), *PLAUF3* (L), *Ptc* (M), *Smo* (N), *SULT1* (O) genes., Table S1: Fold changes in embryos at the pluteus stage, deriving from sea urchins exposed to PAHs and PCBs, (in red down-expressed genes; in green up-expressed genes) in comparison with the control (represented by embryos deriving from adults of sea urchins reared non-contaminated mesoscosm) at 48 hpf., Table S2: Gene name and acronym of genes from *Paracentrotus lividus* and their human orthologs., Table S3: Gene name, acronym, function and reference of 25 new genes., Table S4: Gene name, acronym and reference of 62 genes.

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Review

Comparison of *in situ* sediment remediation amendments: Risk perspectives from species sensitivity distribution☆

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ABSTRACT

Contaminated sediment is a major issue for aquatic environments, but attention must be kept even during remediation activities that can negatively affect resident biota especially when applied *in situ*. For the first time, the species sensitivity distribution (SSD) approach was applied to amendments used for *in situ* sediment remediation considering 39 papers including both freshwater (F) and saltwater (S) effect data (i.e. n = 17 only F, n = 19 only S, and n = 3 both F and S). Toxicity data related to the application of activated carbon (AC), nano-Zero-Valent-Iron (nZVI), apatite (A), organoclay (OC) and zeolite (Z) were collected and analyzed. SSD curves were constructed by lognormal model providing comprehensive comparisons of the sensitivities of different species to the relative testing methods. Results indicated that Bacteria were the most sensitive group of testing organisms, while Crustaceans were the less sensitive. The hazardous concentration for 5% of the affected species (HC5) were derived to determine the concentration protecting 95% of the species. OC, A and Z presented both acute and chronic toxicity. The HC5 values in descending order are: AC (4.79 g/L) > nZVI (0.02 g/L) > OC, A and Z (1.77E-04 g/L). AC and nZVI can be considered safer than OC, A and Z in sediment remediation activities, even if *in situ* long-term effects remained still underexplored.

1. Introduction

Sediment is a fundamental and integrated part of water bodies. It is composed of soluble and insoluble matter, which can be naturally transported from land to ocean, due to inland soil and coastal erosion and windblown dust (Brils, 2008). Pollution is the greater ecological issue due to various discharged toxic substances that can be accumulated in sediment acting as a source of contamination (Arizzi Novelli et al., 2006; Lofrano et al., 2016; Pougnet et al., 2014). Contaminated sediment can strongly impact on aquatic ecosystems, especially in presence of harbors and marinas, embayment, and off coastal areas where commercial and industrial port activities, human settlements and tourism are increasingly widespread (Nikolaou et al., 2009; Lofrano et al., 2016). During dredging activities or natural resuspension phenomena (i.e. adverse weather conditions), accumulated contaminants could be released from sediment to the water column influencing the survival and fitness of aquatic biota and potentially human health (Arizzi Novelli et al., 2006; Mamindy-Pajany et al., 2010). Thus, the first step to deal with this issue must be the reduction/removal of toxic compounds considering both *in situ* and *ex situ* treatments using different chemical, thermal and biological methods (Gomes et al., 2013; Lofrano et al., 2016; Mueller et al., 1996). Nevertheless, these techniques for the remediation of polluted environments could negatively affect the resident biota, especially when applied *in situ* (Albarano et al., 2020; Libralato et al., 2018; Lofrano et al., 2018). Information about their (eco-)toxicity role impacting on aquatic environment are scarce, making difficult to choose the best potential technology for *in situ* remediation (Lofrano et al., 2016). The consequences of treatment activities on aquatic environment are generally considered as secondary effects (Libralato et al., 2008; Rakowska et al., 2012), while current literature still does not describe any potential undesired long-term effect, and to the best of our knowledge an overview about the different sensitivity of

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aquatic species after their administration including laboratory and field scale applications is not currently available.

This review investigated the potential effects related to the use of amendments in sediment remediation considering the sensitivity of model species from both freshwater and saltwater environments. The species sensitivity distribution (SSD) approach was used to better understand the taxonomic differences in species sensitivity for each remediation method. For the first time, the SSD analysis was evaluated from an updated toxicity database and shown as a cumulative probability distribution for multiple species. SSD curve describes the variation in sensitivity among a set of species toward a contaminant or mixture of contaminants by a statistical or empirical distribution function (Posthuma et al., 2002). The use of this method was proposed for the first time by Kooijman (1987) and later enhanced by further studies (Aldenberg and Jaworska, 2000; Posthuma et al., 2002; Newman et al., 2000; Aldenberg and Slob, 1993; Wagner and Løkke, 1991). Generally, SSDs are generated from laboratory-derived toxicity data offering protection for a wider range of organisms in the field (Hose and Van Den Brink, 2004). The aim of SSDs is to calculate the toxicant concentration affecting a specific number of species usually identified as the hazardous concentration (HC) impairing the 5% (HC5) of organisms, thus the protective concentration (PC) for the 95% of species (PC95) can be calculated as well (Posthuma et al., 2002; Newman et al., 2000). Amendments were selected from previous review papers (Libralato et al., 2018; Lofrano et al., 2018) and were namely activated carbon (AC), nano Zero Valent Iron (nZVI), organoclay (OC), apatite (A) and zeolite (Z).

2. Toxicity data identification, collection and management

Toxicity data about AC, nZVI, OC, A and Z were collected from various sources: Google scholar, National Center for Biotechnology Information (NCBI), and Scopus (last update July 30, 2020). We identified and reviewed 56 papers, but only 39 were selected concerning freshwater and saltwater sediment up to the end of July 2020. The other 17 were eliminated for two main reasons: either i) the tested species were of non-aquatic origin; or ii) the amendments didn't show toxicity on aquatic species concerned (see Table S1). Among the investigated papers 17 (43%) focused on freshwater species, 3 (9%) both freshwater and saltwater environment and approximately 48% only seawater species. Literature was reviewed in order to extract several information as summarized in Table 1 including: taxonomy, endpoints (i.e. mortality (M), reproduction (R), growth inhibition (IG), biota-sediment accumulation factor (BSAF) and bioaccumulation reduction (BR)), exposure time, concentrations, effects of amendments (direct contact) or elutriates (%), and water quality parameters. Units of measures for all amendments (i.e. AC -% sediment dry weight; nZVI -mg/L; OC, A, and Z − g/L) were changed in g/L. For AC, a density of 480 g/L activated carbon was considered (ASTM D2854-89). Since toxicity data related to A, OC and Z amendments were scarce (OC, n = 2; Z, n = 9; A, n = 1), they have been considered as one group (ASTM, 1989).

According to Table 1, we detected nine concentrations (4.8, 7.2, 8.2, 9.6, 12.0, 16.3, 19.2, 24.0 and 36.0 g/L) for AC, six (0.0002, 0.0004, 0.0013, 0.008, 0.050 and 0.5 g/L) for nZVI and eight (0.0001, 0.004, 0.005, 0.1, 0.5, 1, 2 and 350 g/L) for OC, A and Z. Due to the limited number of data, both freshwater and saltwater effect data were considered as a whole targeting the potential risk to aquatic organisms. All the difference existing in the considered materials (such as diameter and morphology) were not explicitly discussed being already included in the reviewed database. Focusing on toxic effects of AC, five taxonomic groups have been tested, for a total of 19 species. Specifically, Bacteria, Annelids, Crustaceans, Molluscs and Fishes were represented with 2, 4, 5, 5 and 3 species, respectively. About nZVI, tested species were 15 including five taxonomic groups (Bacteria, n = 6); Algae, n = 4), Molluscs (n = 3), Crustaceans (n = 1) and Fishes (n = 1)). About OC, A and Z, we identified twelve testing species belonging to three taxonomic groups (Bacteria (n = 1), Crustaceans (n = 5) and Fishes (n = 6)). Toxicity data for AC, nZVI, OC, A and Z were comparable in size and more than 50% of organisms were from Molluscs and Crustaceans for AC, Bacteria and Algae for nZVI, Crustaceans and Fishes for OC, A and Z.

Besides the values to generate SSD curves, data about temperature (T), pH, salinity (S), total organic carbon (TOC), dissolved organic carbon (DOC), chemical oxygen demand (COD), total ammonia nitrogen (TAN) and hardness (H) were collected. Their amount were highly insufficient to go further with data analysis like quantitative structure activity relationship or character-activity relationships (Mu et al., 2014; He et al., 2017; Zhao et al., 2018). AC tests were mainly carried out at room temperature (62.5%) with pH value ranging from 5.6 to 8.6. nZVI experiments were performed between 16 °C−25 °C and pH 5−9. OC, A and Z toxicity were analyzed at increasing temperatures (15 °C−37 °C) and with pH of 7−8.

2.1. Data organization

When more than one toxicity data was registered for the same species (*N. arenaceodentata*, *I. galbana, D. magna*, *E. coli* and *D. rerio*), the geometric mean was calculated and used as the estimate for this species as suggested by (Kooijman, 1987). Compiled data were elaborated considering two approaches (Fig. 1): i) raw data (RD) (method 1); and ii) predicted data (PD) (method 2).

About method 1, toxicity data have been shown as raw data without any further processing as they were collected from the 39 reviewed papers. About method 2, average concentrations have been calculated for each amendment: 1) 14.9 g/L for AC; 2) 0.0933 g/L for nZVI, and 3) 44.2 g/L for OC, A and Z. These PD values have been determined for each specific concentration using a proportional calculation (for more details see Supplementary Materials, Table S3). The RD and PD were used as the effect metrics adapted to SSD according to (Van Vlaardingen and Verbruggen, 2007) and the species sensitivity was measured accordingly.

2.2. Data treatment and statistical analysis

The toxicity values were log-transformed according to (Burmaster and Hull, 1997; Leo Posthuma, Glenn W. Suter II, 2002; Newman et al., 2000) using Equation (1):

$$\chi = log_{10}(RD\ or\ PD) \quad (1)$$

The associated risk was visualized as cumulative distribution function as defined in Equation 2

$$y = \sum_{i=1}^{\kappa} n_i \quad (2)$$

where y is the cumulative probability of species and n_i is the absolute frequency of single RD or PD value.

The distribution model was fitted to toxicity data points and evaluated using the χ^2 goodness of fit with the adjusted coefficient of determination R^2 (Adj-R^2). The median hazard concentration (HC50) and the HC affecting the 5% of species were calculated according to (Aldenberg and Slob 1993), using Equation (3):

Table 1
Sediment treatment, group of organisms, species, time of exposure, concentrations, endpoints, effects and references of negative impact of contaminated sediment restoring. Abbreviations: M = Mortality, BSAF = biota-sediment accumulation factor, BR = Bioaccumulation reduction, IG = inhibition growt and R = reproduction, n.e. = not effect, T = Temperature, S = Salinity, DOC = dissolved organic carbon, COD = chemical oxygen demand, TOC = total organic carbon, TAN = total ammonia nitrogen, H = hardness, n.a. = not available.

Sediment treatment	Group of organisms	Species	Expusure Time (days; *h; +min)	Concentrations (g/L)	Endpoints	Effects (%) of amendments/ elutriates	Water quality parameters (T = °C, S = ppt, TOC-DOC-COD-TAN-H = mg/ L)	References
AC	Bacteria	*Escherichia coli*	0.5*	4.8	M	79.5	pH = 6.8	Van Der Mei et al. (2008)
		Raoultella terrigena	0.5*	4.8	M	65.5	pH = 6.9	Van Der Mei et al. (2008)
	Annelids	*Nereis diversicolor*	28	9.6	BSAF	2	T = 22	Cornelissen et al. (2006)
		Limnodrilus spp	28	7.2	BR	94	T = 20	Jonker et al. (2004)
		Neanthes arenaceodentata	28	16.3	BR and IG	50 and 73	T = 20	Millward et al. (2005)
		Arenicola marina	10	36	M	70	T = 20, pH = 6.15–8.61	Lillicrap et al. (2015)
	Molluscs	*Macoma balthica*	28	16.3	BR	76	T = 13	McLeod et al. (2007)
		Lymnaea stagnalis	41	8.2	BR	37.3	pH = 5.6–6.5, RT, DOC = 35-40	Lewis et al. (2016)
		Corbicula fluminea	28	12	BR	95	T = 13	Mcleod et al. (2008)
		Potamopyrgus antipodarum	28	9.6	R	9.7	T = 16, pH = 8.0, DOC = 9.4, COD = 26.5, TAN = 5.35	Stalter et al. (2010)
		Meretrix meretrix	28	24	IG	36.4	T = 20, pH = 8.05	Zheng et al. (2018)
	Crustaceans	*Daphnia Magna*	4	19.2	BR	4	T = 20	Jonker et al. (2009)
		Asellus aquaticus	28	19.2	IG	36	T = 20	Kupryianchyk et al., 2011
		Corophium volutator	28	19.2	IG	50	T = 20	Jonker et al. (2009)
		Leptocheirus plumulosus	10*	16.3	BR	70	T = 20	Millward et al. (2005)
		Tisbe battagliai	72*	36	M	60	T = 20, pH = 6.15–8.61	Lillicrap et al. (2015)
	Fishes	*Pimephales promelas*	10	24	M	43.8	T = 25, pH = 8.2	He et al. (2012)
		Dicentrarchus labrax	28	4.8	M	76.4	T = 21.8, pH = 8.0 S = 37, TAN = 0.18	Aly et al. (2016)
		Gambusia affinis	10	36	IG	62.2	T = 20	Casini et al. (2006)
nZVI	Bacteria	*Escherichia coli*	0.5*	0.008	M	70	T = 32, pH = 7.2	Lee et al. (2008)
			1*			80	T = 30, pH = 5–7.4	Auffan et al. (2008)
		Bacillus subtilis var. niger	5+	0.0002	M	20	T = 20, pH = 9	Diao and Yao (2009)
		Pseudomonas fluorescens	5+	0.0002	M	100		
		Vibrio fischeri	2	0.0002	IG	87.2	T = 20, pH = 5–9, DOC = 0.01	Qiu et al. (2013)
		Dehalococcoides	2	0.5	IG	98.2	T = 22, pH = 8.1	Xiu et al. (2010)
		Microcystis aeruginosa	30	0.5	IG	92	T = 30, TOC = 18.75, TAN = 1.73	Su et al. (2018)
	Algae	*Isochrysis galbana*	4	0.0002	IG	50 and 60	pH = 7.5, RT	Keller et al. (2012)
		Dunaliella tertiolecta	4	0.0013	IG	53		Othman (2018)
		Thalassiosira pseudonana	4	0.0004	IG	51		
		Pseudokirchneriella subcapitata	4	0.008	IG	47		
	Molluscs	*Lymnaea stagnalis*	41	0.0002	R	59.4	pH = 5.6–6.5, DOC = 35-40	Lewis et al. (2016)
		Mytilus galloprovincialis	28	0.05	IG	14	T = 18, pH = 8.0, S = 30	Coppola et al. (2019)
		Mytilus galloprovincialis	2	0.008	IG	60	T = 16, pH = 6–7, DOC = 0.08	Kadar et al. (2010)
	Crustaceans	*Daphnia Magna*	28	0.0004	M, IG and R	60, 58 and n.e.	RT	Keller et al. (2012) Jaafar et al. (2018)
	Fishes	*Oryzias latipes*	10	0.0002	IG	30	T = 25, pH = 7–7.6, H = 200	Li et al. (2009)
				0.05	M	90	T = 26, pH = 7	Chen et al. (2011)
A, Z and OC	Bacteria	*Escherichia coli*	0.5*	0.004	M and IG	52.9 and 60	T = 37, pH = 7.2	Rieger et al. (2016)
	Crustaceans	*Hyalella azteca*		0.1	M	67.5	T = 25	Paller and Knox (2010)
		Leptocheirus plumulosus		0.1	M	21.3		
		Americamysis bahia	2	0.5	M	47	T = 15	Burgess et al. (2004)
		Ampelisca abdita	2	0.5	M	48		

(*continued on next page*)

Table 1 (*continued*)

Sediment treatment	Group of organisms	Species	Expusure Time (days; *h; +min)	Concentrations (g/L)	Endpoints	Effects (%) of amendments/ elutriates	Water quality parameters (T = °C, S = ppt, TOC-DOC-COD-TAN-H = mg/ L)	References
		Paranephrops planifrons	10	350	IG	33.3	T = 15, pH = 7, TAN = 2	Parkyn et al. (2011)
	Fishes	*Danio rerio*	3	0.0001	IG and M	96.5 and 66	pH = 7, RT	Palcic et al. (2020)
		Oncorhynchus	0.5	1	BR	86.6	T = 14, pH = 8	Aly et al. (2016)
		mykiss	28	0.1	M	83.3	T = 14, pH = 7.4	Ukar et al. (2017)
		Dicentrarchus labrax	28	0.1	IG	42.2	T = 21.8, pH = 8.0 S = 37, TAN = 0.18	Aly et al. (2016)
		Gambusia affinis	10	0.005	M	42.8	T = 20	Casini et al. (2006)
		Oreochromis mossambicus	28	2	BR	41.7	T = 29, pH = 7.8, H = 58	James et al. (2000)
		Oryzias latipes	10	0.005	IG	30	T = 26, pH = 7	Chen et al. (2011)

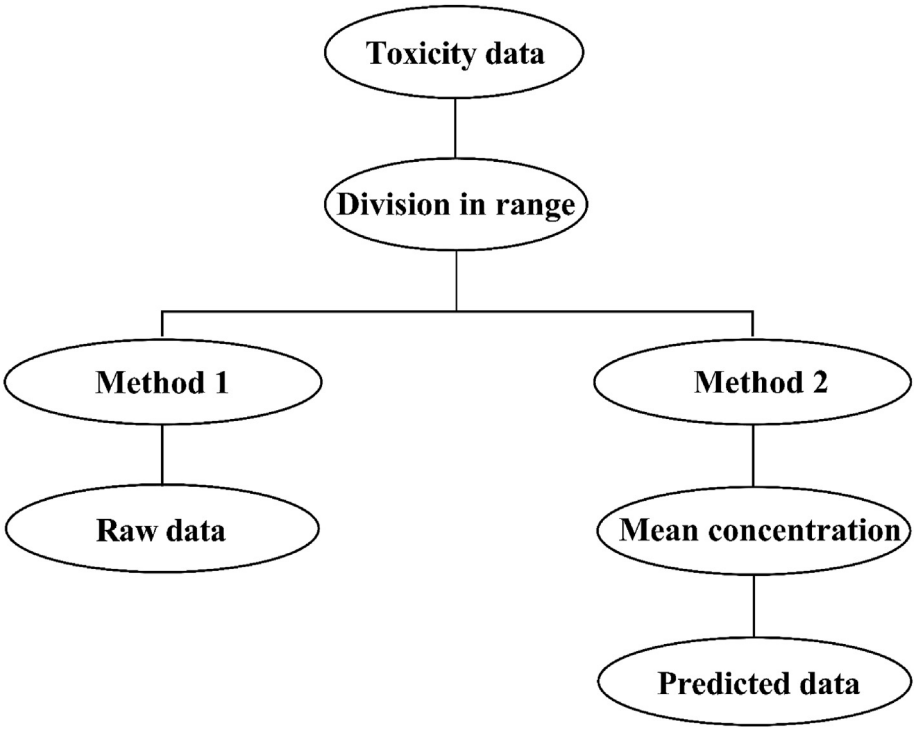

Fig. 1. Flowchart of data-processing.

$$Log\ (HCp) = \mu - \mathscr{K}p * \sigma \quad (3)$$

where HC_p is hazardous concentration for percentage of the species population, $\mathscr{K}_p$ is Aldenberg extrapolation factor that directly depends of the number of the studied species, μ and σ are the mean and the standard deviation of distribution, respectively.

Data were analyzed by Shapiro-Wilk's (S–W) test for normality and F-test for homoscedasticity (p-value <0.05). For each amendment, statistical significance between different groups of organisms was performed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons ($p < 0.05$). Two fixed factors (groups of organisms *vs* remediation methods) were crossed by a two-way ANOVA. All statistical analyses were performed using GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, La Jolla, California, USA, www.gra phpad.com).

3. Results and discussion

As reported in Table 2, RD and PD are normally distributed for AC (p values = >0.9999 and 0.1218, respectively), nZVI (p values = 0.34 and 0.18, respectively), OC, A, and Z (p values = 0.14 and 0.08, respectively). The value of RD and PD show variance homogeneity (homoscedasticity) for AC (p values = 0.11 and 0.06, respectively), for nZVI (p values = 0.98 and 0.49, respectively) and

Table 2
Goodness-fit, Shapiro-Wilk's (S–W) test for normality and F-test for homoscedasticity of total species for three remediation methods. p is the significance level. WR = whole range, MC = mean concentration.

Sediment treatment	Concentrations (g/L)	Adj-R2	S–W	*F*	*p*
AC	4.8–36.0 (WR)	0.97	>0.9999	0.11	<0.05
	14.9 (MC)	0.97	0.12	0.06	<0.05
nZVI	0.0002–0.5 (WR)	0.93	0.34	0.98	<0.05
	93.3 (MC)	0.96	0.18	0.49	<0.05
OC, A and Z	0.0001–350 (WR)	0.97	0.14	0.26	<0.05
	44.2	0.97	0.08	0.13	<0.05

for OC, A and Z (p values = 0.26 and 0.13). The results indicated that the lognormal distribution fits with most of the groups data points, with Adj-R^2 ranging from 0.79 to 0.98 (p-value <0.01) (as shown in Table 2 and Table S4).

3.1. The SSDs of AC

Taking into consideration both the entire selection (4.8–36.0 g/L, Fig. 2A) and its mean concentration (14.9 g/L, Fig. 2D), Bacteria were largely more susceptible than other organisms. Specifically, at the 14.9 g/L concentration (Fig. 2D), Bacteria group has shown a significant increase of sensitivity to AC respect to Annelids ($p < 0.05$), Molluscs ($p < 0.05$) and Crustaceans ($p < 0.01$) (see also Table S5). Crustaceans, in particular *D. magna*, exhibited the lowest susceptibility.

Considering the range 4.8–9.6 g/L (Fig. S1A), Bacteria, especially with *E. coli*, the Annelid *Limnodrillus* spp. and the Mollusc *C. fulminea* were the most sensitive species. Furthermore, regarding the respective mean concentration (7.2 g/L, Fig. S1D) Bacteria, with *E. coli* and *R. terrigena*, showed the highest significant sensitivity to AC respect to Molluscs ($p < 0.05$) (Table S5).

Given the range 12.0–16.3 g/L (Figura S1B), Annelids group, as reported in Table S2, has displayed a significant sensitivity respect to Molluscs ($p < 0.05$), that in turn were statistically significant respect to Crustaceans ($p < 0.01$). However, when the 13.4 g/L mean concentration has been viewed, Molluscs group were the highest susceptible respect to other groups of species, but not statistically significant (Fig. S1E).

Analyzing both the range 19.2–36.0 g/L (Fig. S1C) and its respective mean concentrations (26.4 g/L, Fig. S1E) the Fish *G. affinis* has shown a high sensitivity despite the p-values were greater than 0.05.

As displayed in Fig. 2A, some species within the same taxonomic group responded differently showing a variable sensitivity. The toxicity data of AC showed that the polychaetae *N. diversicolor* and *Limnodrillus* spp. were the least and most sensitive species among the Annelids, respectively. In particular, cumulative probability of *Limnodrillus* spp. exceeded 90% in SSD curves, whereas that of *N. diversicolor* resulted to be of 2%. Among Molluscs group, *M. meretrix* and *L. stagnalis* were the least sensitive species (with a cumulative probability of approximately 37%), whereas *C. fluminea* with a cumulative probability of 95% resulted the most sensitive species. Furthermore, *D. magna* and *T. battagliai* were respectively the least and most sensitive species among the Crustaceans group. Specifically, cumulative probability of *T. battagliai* was 60% in SSD curves, whereas that of *D. magna* resulted to be of 4%. Probably, considering that the diameter of the feeding chaetoceros was generally 7–9 mm, this substantial variability was due to possible differences in digestive biology of species (Cornelissen et al., 2006; Jonker et al., 2004, 2009; Millward et al., 2005; Zheng et al., 2018).

3.2. The SSDs of nZVI

Considering the raw data, also for the nZVI methods the Bacteria, specifically *P. fluorescens*, were largely susceptible than others organisms (Fig. S2). Given the whole range (0.0002–0.5 g/L, Fig. 2B), Bacteria, specifically *P. fluorescens* and *V. fischeri*, were statistically significant compared to Algae ($p < 0.0001$), Custaceans ($p < 0.001$), Molluscs ($p < 0.001$) and Fishes ($p < 0.001$) (Table S6). When taking into consideration the predicted data (Figs. S2C–D;

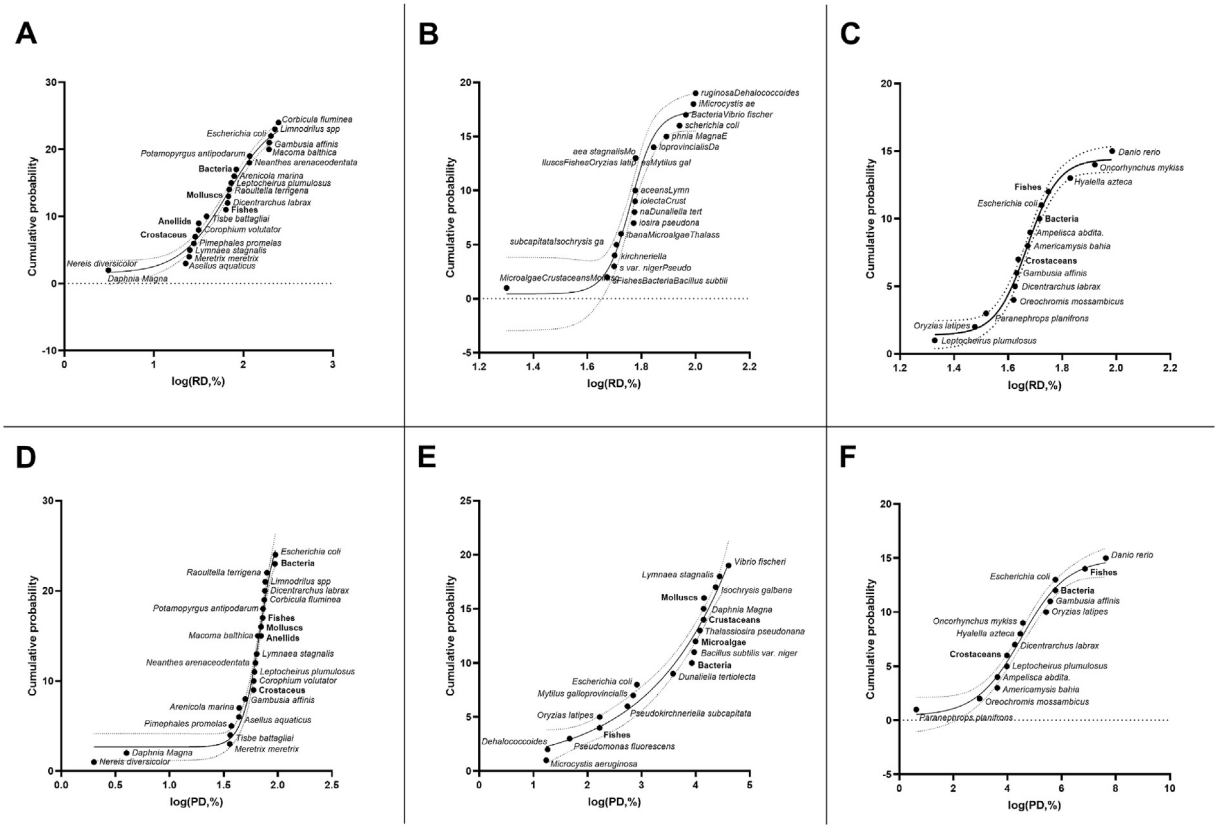

Fig. 2. Species sensitivity distribution of different groups species to AC, nZVI, OC, A and Z. The data in (A-B-C) are represented as raw data (RD) collected respectively from 4.8 to 36.0 g/L (AC), 0.0002–0.5 g/L (nZVI) and 0.0001–350 g/L (OC, A and Z); the data in (D-E-F) are reported as predicted data (PD) calculated respectively 14.9 (AC), 0.0933 (nZVI) and 44.2 g/L (OC, A and Z).

Fig. 2E), Bacteria, with *E. coli*, *P. fluorescens* and *V. fischeri,* and the Molluscs, in particular *L. stagnalis*, were among the most affected species despite the p-values were >0.05; whereas the Fishes and Algae were the less impacted class of organisms. Furthermore, Bacteria group showed a variable sensitivity. *Bacillus subtilis var. niger* was the least sensitive, whereas *P. fluorescens* and *V. fischeri* were the most sensitive species among Bacteria. Their cumulative probability in SSD curves were 20%, 100% and 87.2%, respectively. *P. fluorescens* and *V. fischeri* are gram-negative bacteria, which are more sensitive to environmental stress respect to *B. subtilis,* which is a gram-positive bacterium (Diao and Yao, 2009). Moreover, probably *A. fischeri* and *P. fluorescens* showed similar effects because they were equally sensitive to metal ions (Abbas et al., 2018; Abbondanzi et al., 2003).

Analysing the range 0.008–0.5 g/L (Fig. S2B), Bacteria group showed the highest significant sensitivity to nZVI when compared to Algae ($p < 0.001$) and Molluscs ($p < 0.05$) (see Table S6). Algae species were statistically significant respect to Fishes ($p < 0.001$), that in turn displayed high susceptibility respect to Molluscs ($p < 0.01$, Table S6).

3.3. The SSDs of OC, A and Z

Finally, for the OC, A and Z methods the Fishes, in particular *D. rerio* and *O. mykiss* species, were resulted the most susceptible considering the raw data (Figs. S3A–B), but not statistically significant (Table S7). When also given the predicted data (Figs. S3C–D), the Fish species, especially *D. rerio,* displayed a highest sensitivity to OC, A and Z remediation methods. Moreover, as shown in Table S7, when analyzing 44.2 g/L mean concentration (Fig. 2F) Fishes group were statistically significant respect to Crustaceans ($p < 0.05$) and Bacteria ($p < 0.01$), that in turn displayed high susceptibility respect to Crustaceans ($p < 0.0001$) (see also Table S7). Moreover, Fishes group showed a variable sensitivity. *O. latipes* was the least sensitive species (with a cumulative probability of about ~30%), but *D. rerio* displayed the highest sensitivity with a cumulative probability of 96.5%. Probably, the triazoles leaching from the zeolite channels can cause the Z toxicity. De La Paz et al. (2017) demonstrated that triazoles can inhibit hatching through affecting the hatching enzyme or impairing the release of ZHE1 enzyme.

Furthermore, taking into consideration both raw and predicted data, Crustaceans, in particular *L. plumulosus* and *P. planifrons*, have proven to be the less sensitive species (Fig. 2C; F).

3.4. Whole comparison of SSDs

As shown in Fig. 3, SSDs of different remediation methods based on the total species were constructed and the relationship of sensitivity between individual group species and total species was investigated for all amendments considering both raw and predicted data (Fig. 3).

Considering the raw data (Fig. 3A), the curves of remediation methods were almost overlapping with exception of Bacteria that showed the significant increase of sensitivity to nZVI respect to OC, A and Z ($p < 0.05$) and Crustaceans group that were statistically significant to AC respect to nZVI ($p < 0.01$) (see Table S8).

At intermediate concentrations and considering predicted data, the AC curve method shifted to the left (Fig. 3B). The adverse effect of nZVI was intermediary between all amendments, whereas the OC, A and Z curve shifted on the right showing higher toxicity for all studied species. Only Fishes group displayed a significant increase of sensitivity to OC, A and Z respect to AC ($p < 0.05$) and nZVI ($p < 0.05$) (Table S8).

The AC is more toxic to Fishes than the other four taxonomic groups of analyzed species (with the HC5 calculated at 3.44 g/L, CI = 0.18–8.97; Table 3), followed by Annelids, Molluscs and Crustaceans. The HC5 value of Crustaceans was found to be more than 3 times higher (11.16 g/L, CI = 5.92–14.81, Table 3) than that measured in Fishes. Similarly, it occurred for HC50. The decreasing sensitivity is: Fishes > Annelids > Molluscs > Crustaceans. Only for Bacteria, the HC5 and HC50 values were not calculated because just one concentration was available. nZVI method has a highest impact on Algae (8.58E-02 g/L, CI = 2.49E-03 - 0.35, Table 3) respect to Molluscs and Bacteria. The HC5 value of Molluscs was found to be more than 2 times higher than that of Algae. For Crustaceans and Fishes, HC5 and HC50 were not calculated because just one concentration was available. The decreasing sensitivity is: Algae > Bacteria > Molluscs.

About OC, A and Z methods, Fishes were the most affected species with HC5 of 9.46E-05 (CI = 3.78E-05 - 1.4E-03, Table 3). HC5 of Crustaceans group results to be more than 59 times higher than

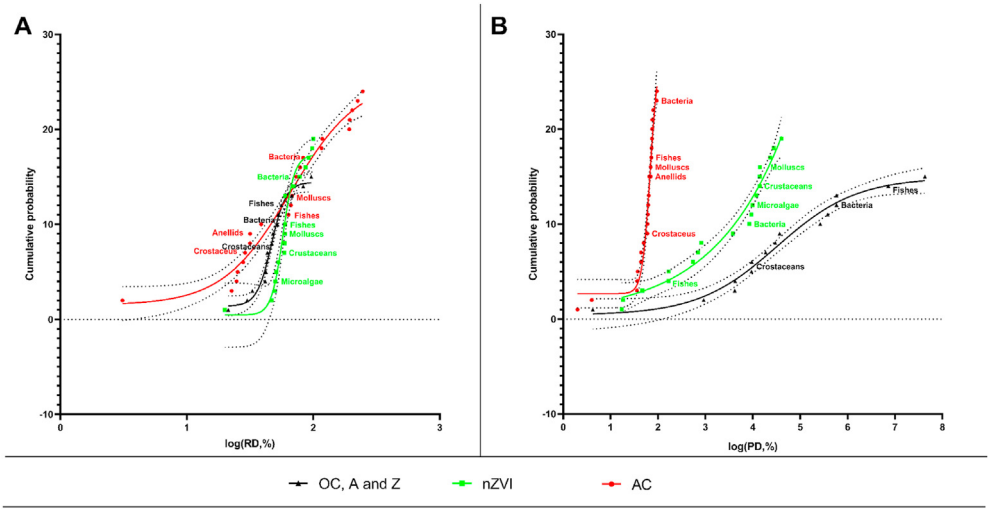

Fig. 3. SSD curves for total species exposed to different remediation methods. The data in (A) are represented as raw data (RD) and the findings in (B) are reported as predicted data (PD) for AC (red line), for nZVI (green line) and for OC, A and Z (black line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
The calculated hazard concentration at 5% (HC5) and 50% (HC50) of species (including their CI (Confidence interval)) of Bacteria, Annelids, Molluscs, Crustaceans, Algae, Fishes and total species for three remediation methods. n.a. = not available.

		HC5 (g/L)	CI	HC50 (g/L)	CI
AC	Bacteria	n.a.	n.a.	n.a.	n.a.
	Annelids	4.88	1.09–8.82	14.59	8.11–26.23
	Molluscs	6.56	2.76–9.64	14.19	9.87–20.39
	Crustaceans	11.16	5.92–14.81	19.66	15.06–25.65
	Fishes	3.44	0.18–8.97	17.30	6.12–48.87
	Total	4.74	2.96–6.53	14.00	10.89–17.98
nZVI	Bacteria	1.3E-02	2.43E-05 - 0.26	8.29	0.53–130.1
	Algae	8.58E-02	2.49E-03 - 0.35	1.15	0.29–4.65
	Molluscs	4.15E-02	5.46E-07 - 0.53	1.87	0.07–51.44
	Crustaceans	n.a.	n.a.	n.a.	n.a.
	Fishes	n.a.	n.a.	n.a.	n.a.
	Total	0.02	2.9E-03 - 0.08	1.92	0.67–5.46
OC, A and Z	Bacteria	n.a.	n.a.	n.a.	n.a.
	Crustaceans	5.59E-03	1.18E-05 - 0.08	1.38	0.10–18.44
	Fishes	9.46E-05	3.78E-05 - 1.4E-03	2.93E-02	2.6E-03 - 0.33
	Total	1.77E-04	5.69E-06 - 1.6E-03	1.21E-01	0.02–0.71

that of Fishes (5.59E-03, CI = 1.18E-05 - 0.08, see Table 3). Moreover, also in this case, for Bacteria, the HC5 and HC50 values were not calculated because just one concentration was available. As reported in Table 3, when considered the impact of both AC, nZVI, OC, A and Z methods on total species, OC, A and Z was the most toxic with the HC5 value of 1.77E-04 (CI = 5.69E-06 - 1.6E-03), followed by nZVI and AC. Specifically, the HC5 value of AC is much greater than that measured for OC, A and Z. In this case, the decreasing risk is: OC, A and Z> nZVI > AC (Table 3).

Focusing on HC5 and HC50 of remediation methods for each taxonomic group of species and total species, the toxicity profiles have been established on the basis of OECD criteria (2006) OECD, 2006. According to the United Nations Globally Harmonised System for Classification and Labelling (UNECE, 2003), AC and nZVI methods have been identified as "no-hazardous" to aquatic environment. However, OC, A and Z remediation displayed higher toxicity levels (i.e. acute and chronic) for species belonging to both saltwater and freshwater environments representing a potential risk to the aquatic life (i.e. class 3). Scarce information exists on the toxicity mechanisms associated with the use of OC, A and Z. The active biomonitoring studies indicated that the biopolymers used in some capping bound sand grains and other particles in a viscous matrix that appeared to entrap and possibly suffocate burrowing organisms (Paller and Knox, 2010). Janer et al. (2013) demonstrated that six types of nanosized clays, specially organoclay, were also able to induce apoptosis and to spread in cytoplasmic vesicles of the exposed cells at low concentrations. The toxicity impact of Z can be probably due to substances leaching from the zeolite channels, which are able to cause an increase of the specific enzymatic activities (Casini et al., 2006; Aly et al., 2016).

4. Conclusions

The present study investigated the toxicity of different remediation methods towards saltwater and freshwater species according to the species sensitivity distribution approach. When RD values were considered Bacteria group showed the higher sensitivity to nZVI respect to OC, A and Z, and Crustaceans to AC compared to nZVI. Taking into consideration the PD, Fishes group were ranked as more vulnerable to OC, A and Z compared to AC and nZVI. On the basis of HC5 and HC50, AC, OC, A and Z presented the higher adverse effects on Fishes. nZVI was more at risk for Algae, followed by Bacteria and Molluscs. In general, AC and nZVI were ranked as safer (i.e. at low risk) than all other amendments on the basis of GHS criteria. OC, A and Z proved to significantly present both acute and chronic toxicity. The risk of the considered amendments listed in a descending order are: (OC, A and Z) > nZVI > AC. Further investigations are necessary to understand the long-term effects of AC and nZVI after *in situ* application on the potential exposed biota.

Credit author statement

Luisa Albarano: Conceptualization, Methodology, Software. Giusy Lofrano: Data curation, Writing- Original draft preparation. Maria Costantini: Conceptualization, Investigation. Valerio Zupo: Methodology, Software. Federica Carraturo: Data curation. Marco Guida: Conceptualization, Supervision. Giovanni Libralato: Conceptualization, Supervision, Writing- Reviewing and 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115995.

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