Universidade de Aveiro
Ano 2020

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Effects of Trace elements and UV filters on *Ficopomatus enigmaticus*: larval development and biochemical responses

Efeito de Elementos vestigiais e Filtros UV em *Ficopomatus enigmaticus*: desenvolvimento larvar e respostas bioquímicas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica da Doutora Adília Pires, Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar, da Universidade de Aveiro, e do Doutor Carlo Pretti, Professor Associado no Departamento de Ciências Veterinárias, da Universidade de Pisa.

o júri

Presidente

Prof. Doutora Maria Marina Pais Ribeiro da Cunha
Directora do Mestrado de Biologia Marinha Aplicada, professora Auxiliar na Universidade de Aveiro.

Prof. Doutor Giovanni Libralato
Professor Auxiliar no Departamento de Biologia, Universidade de Nápoles

Doutora Adília da Conceição Marques de Oliveira Pires
Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar, da Universidade de Aveiro.

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Soundtrack da tese: The Smiths e Pink turns Blue (em loop).

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palavras-chave

Elementos vestigiais; Filtros UV; *Ficopomatus enigmaticus*; Embriotoxicidade; Biomarcadores; Ensaios ecotoxicológicos; *Vibrio fischeri*; *Phaeodactylum tricornutum*

resumo

Estudos anteriores sugeriram o serpulídeo de água salobra *Ficopomatus enigmaticus* como um bom organismo modelo para monitorização de águas costeiras e estuarinas, através da execução de testes de toxicidade em células espermáticas e no desenvolvimento larvar. Nesta tese, o impacto da exposição a elementos vestigiais (cobre, mercúrio, arsénio, cádmio e chumbo), a diferentes concentrações, foi estudado no desenvolvimento larvar de duas populações de *F. enigmaticus* (Mar Mediterrâneo e Oceano Atlântico, amostrados em Itália e Portugal, respectivamente). Inicialmente, uma análise molecular baseada na amplificação e sequenciação do gene *Cyt b* foi feita para confirmar que as duas populações pertenciam à mesma espécie, permitindo validar a comparação dos resultados de embriotoxicidade. O efeito, medido em termos de EC_{50}, mostrou que o mercúrio foi o metal mais tóxico para as larvas das duas populações. Os elementos vestigiais testados mostraram a seguinte ordem, da toxicidade mais elevada para a mais baixa: população Mediterrânica: mercúrio > cobre > chumbo > arsénio > cádmio; Atlântica: mercúrio > cobre > cádmio > arsénio > chumbo. Respostas das duas populações foram semelhantes para o arsénio. O chumbo foi o menos tóxico para a população Atlântica, enquanto que o cádmio foi o menos tóxico para a população Mediterrânica. Análises químicas no tecido e tubos calcários dos organismos e água recolhida nos locais de amostragem mostraram que as populações exibiram menor sensibilidade a um certo elemento, em conjunto com concentrações mais altas desse mesmo elemento nos tecidos. Estes resultados podem indicar resistência a efeitos tóxicos de um contaminante em particular, por organismos que tendem a acumular esse mesmo elemento. Estes resultados também sublinham a potencial correlação entre a resposta de organismos teste selvagens e a caracterização dos locais de amostragem para identificar possíveis erros ou diferenças na resposta de organismos modelo, durante a execução do ensaio.

Outro dos trabalhos desta tese tratou de filtros UV como 'Contaminants of Emerging Concern' e teve como objectivo avaliar a toxicidade de 7 filtros UV: (Óxido de Zinco (ZnO) e Dióxido de Titânio (inorgânicos), Benzofenona-3, Avobenzona, Octocrileno, Etilhexil metoxicinamato (EHMC) e 4-metilbenzilideno canfôra (orgânicos)) em três espécies: inibição da bioluminescência em *Vibrio fischeri*, inibição do crescimento em *Phaeodactylum tricornutum*, e no correcto desenvolvimento larvar de *F. enigmaticus*. Os resultados mostraram o dióxido de titânio como sendo o composto mais tóxico para *P. tricornutum* (EC_{50} de 0.04368 mg/L), enquanto que não induziu toxicidade para *V. fischeri* ou *F. enigmaticus*. O Etilhexil metoxicinamato mostrou-se muito tóxico para *V. fischeri* e o segundo mais tóxico para *P. tricornutum*. Para *F. enigmaticus*, as percentagens de desenvolvimento correcto mais baixas foram observadas para o 4-metilbenzilideno cânfora, com um EC_{50} de 0.836 mg/L, seguido pelo óxido de zinco (1.088 mg/L). No geral, a avobenzona induziu pouca toxicidade e o octocrileno foi o composto menos tóxico para as larvas de *F. enigmaticus*. Sobre o ensaio de desenvolvimento larvar em diferentes condições de luz e temperatura, as percentagens de desenvolvimento correcto mais altas foram obtidas a 25 °C, independentemente do tratamento de incubação. De acordo com a avaliação preliminar de toxicidade, o ZnO foi mais tóxico que o EHMC. Este estudo torna-se uma contribuição importante para fornecer dados ecotoxicológicos sobre a potencial toxicidade de filtros UV (tanto em relação a efeitos como ao comportamento sob factores ambientais), apresentando resultados gerados por espécies de três níveis tróficos, afectadas diversamente pela exposição a filtros UV.

A última tarefa desta dissertação refere-se ao estudo de parâmetros bioquímicos após a exposição crónica de adultos de *F. enigmaticus* a ZnO e EHMC. Foi observado que o EHMC induziu os níveis mais altos de peroxidação lipídica, a 0.5 mg/L, indicando a capacidade deste composto para, a concentrações elevadas, induzir dano oxidativo.

A capacidade de biotransformação (avaliada através da actividade das enzimas glutationa S transferases), mostrou que tanto o ZnO como o EHMC produziram uma resposta dependente da concentração. Ambos os filtros UV diminuíram significativamente os níveis de atctividade da acetilcolinesterase, o que aponta um efeito neurotóxico importante.

Os resultados obtidos nesta dissertação ajudam a reafirmar a sensibilidade e adequabilidade do serpulídeo *F. enigmaticus* para uso como organismo modelo em ensaios ecotoxicológicos e avaliações de risco ecológico.

Keywords

Trace elements; UV filters; *Ficopomatus enigmaticus*; Embryotoxicity; Biomarkers; Ecotoxicological assays; *Vibrio fischeri*; *Phaeodactylum tricornutum*

abstract

Previous studies suggested the suitability of the brackish-water serpulid *Ficopomatus enigmaticus* to be used as a model organism for both marine and brackish waters monitoring, by the performance of sperm toxicity and larval development assays. In this thesis, the impact of trace elements exposure (copper, mercury, arsenic, cadmium, and lead), at different concentrations, was studied on the larval development of two different populations of *F. enigmaticus* (Mediterranean Sea and Atlantic Ocean, collected in Italy and Portugal, respectively). Firstly, a molecular analysis based on *Cyt b* amplification and sequencing was carried out and confirmed that both populations belonged to the same species, allowing to validate the comparison of embryotoxicity results. The effect, measured in terms of EC_{50}, showed that mercury was the most toxic metal for larvae of both populations. The tested trace elements may be racked in the following order from the highest to the lowest toxicity: Mediterranean population: mercury > copper > lead > arsenic > cadmium; Atlantic: mercury > copper > cadmium > arsenic > lead. Responses of both populations were similar for arsenic. The lead was the least toxic element for the Atlantic population, while cadmium showed the least toxicity for the Mediterranean population. Chemical analyses on soft tissues and calcareous tubes of collected test organisms and their sampling site water showed that populations exhibited less sensitivity to a certain element, together with a relevantly higher concentration of the same element in soft tissues. These results may indicate a certain resistance to particular contaminant toxic effects by organisms that tend to accumulate that same toxicant. Moreover, these results also highlight the potential correlation between wild-caught test organisms' responses and deep characterization of the sampling site to identify putative abnormalities or differences in model organism response during bioassay execution.

Another task of this thesis tackled UV filters as Contaminants of Emerging concern and aimed to assess the toxicity of a set of seven UV filters (Zinc oxide (ZnO) and Titanium dioxide (inorganics), Benzophenone-3, Avobenzone, Octocrylene, Ethylhexyl methoxycinnamate (EHMC) and 4-methylbenzylidene camphor (organics)) on three species: bioluminescence inhibition in *Vibrio fischeri*, growth inhibition of *Phaeodactylum tricornutum*, and on the correct larval development of *F. enigmaticus*. Results showed titanium dioxide to be the most toxic compound for *P. tricornutum* (EC_{50} of 0.04368 mg/L), while it did not induce toxicity for *V. fischeri* or *F. enigmaticus*. Ethylhexyl methoxycinnamate was very toxic to *V. fischeri* and the second most toxic to *P. tricornutum*. For *F. enigmaticus*, the lowest percentages of correct development were observed when exposed to 4-methylbenzylidene camphor, with an EC_{50} of 0.836 mg/L, followed by Zinc oxide (1.088 mg/L). Overall, Avobenzone has induced low toxicity, and Octocrylene was the least toxic for *F. enigmaticus* larvae. Regarding the larval development assay under different light and temperature conditions, higher percentages of correct development were obtained at 25 °C, independently of the incubation treatment. According to preliminary toxicity assessment, ZnO was more toxic than EHMC. This study becomes a relevant contribution to provide ecotoxicological data on the potential toxicity of UV filters (concerning both effects and behaviour under environmental factors), presenting data generated by organisms belonging to three trophic levels, being differently affected by UV filters exposure.

The last task of this dissertation, concerns a biochemical parameters study after chronic exposure of adults of *F. enigmaticus* to ZnO and EHMC. It was observed that EHMC induced the highest levels of lipid peroxidation at 0.5 mg/L, pointing out the ability of this compound to, at high concentrations, produce considerable oxidative damage.

Biotransformation capacity (assessed by the activity of glutathione S transferases), showed that both ZnO and EHMC have displayed a concentration dependent response. Both UV filters have significantly decreased the levels of acetylcholinesterase activity, such meaning a huge neurotoxic effect.

The results obtained in this dissertation further help to reassure the sensitivity and suitability of the serpulid *F. enigmaticus* to be used as a model organism in ecotoxicological and ecological risk assessment assays.

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CHAPTER 1. GENERAL INTRODUCTION

1. General Introduction

1.1 Trace elements as classic pollutants

Coastal areas have been subjected to different threats derived from anthropogenic actions. These areas become vulnerable as they represent the main sink of man produced polluted effluents (Storelli et al., 2001). Industrial discharges and associated contamination by inorganic compounds are listed among the major drivers of aquatic systems deterioration (Kaur et al., 2010). Different industries produce effluents whose toxicity can affect the functioning of microbial communities' activity in wastewater treatment plants (WWTPs) and, therefore, weaken the degradation and elimination processes (Gómez et al., 2001). These effluents, not efficiently treated, will still contain a significant amount of trace elements that will pose an important risk to the aquatic environments, where they will reach, given that they are persistent and prone to negatively impact living organisms (Gopalakrishnan et al., 2008). Besides industrial activities, other two relevant contamination sources are agricultural and urban sewage effluents (Gupta and Singh, 2011; Bhuyan et al., 2017). Giving the increasing population around coastal areas, associated with industries and agriculture development as well as climatic events that will facilitate pollutants release into the aquatic systems, pollution by trace elements has become a global scale issue (Fernandes et al., 2008).

Trace elements refer to chemical elements that are, by definition, present in vestigial concentrations both in the environment and/or biological systems. Although detected in minimum amounts, such low concentrations can be enough to exert effects on a living organism (from genomic to physiological alterations) (Calevro et al., 1998; Monteiro et al., 2018; Marques et al., 2018). Studies point out Cadmium (Cd), Mercury (Hg) and Lead (Pb) among the most toxic trace elements (ATSDR, 2019). Arsenic (As) is also very harmful, mainly in its inorganic forms (Sattar et al., 2016). Nickel (Ni), Copper (Cu), Zinc (Zn) and Chromium (Cr) are required micronutrients, but they become toxic when up taken in large quantities (Cid et al., 2001).

In marine and brackish water ecosystems trace elements are usually trapped in the sediment, which already contains natural background levels (depending on the area's geological properties), notwithstanding anthropogenic activities being a major contributor to the increase of their load in the environment (Acevedo-Figueroa et al., 2006; Oliva et al., 2018; Ramachandra et al., 2018). Their concentrations in sediments are higher than those measured in the water column (Bryan and Langston, 1992; Fernandes et al., 2008). Planktonic and reef forming benthic organisms can become exposed to trace elements after

washout and sediment re-suspension events, both natural (bioturbation) and man induced (dredging), which increase the toxicants concentrations in water (Acevedo-Figueroa et al., 2006; Moreira et al., 2018).

In several sites along the Portuguese coast Santos-Echeandía et al. (2012) reported values of dissolved trace elements ranging from 1.12 to 100.04 ng/L for Cd, 0.59 to 198.6 ng/L for Cobalt (Co), 57.19 ng/L to 2884.98 ng/L for Cu, 0.66 to 28.08 ng/L for Hg, 110.33 to 886.21 ng/L for Ni, 2.07 to 31.08 ng/L for Pb and 91.54 to 4054.18 ng/L for Zn. For Mediterranean seawaters, dissolved trace elements were found by Schintu et al. (2008) at concentrations of 1 to 35 ng/L for Cd; 5 to 80 ng/L for Cu; 33 to 120 ng/L for Ni and 4 to 147 ng/L for Pb.

Previous studies, performed under laboratory conditions (both using water samples from the study site or a range of environmentally relevant concentrations) reported that trace elements pose a risk to both coastal and estuarine organisms. Waters contaminated with Zn, Manganese (Mn), Pb, Cd, Cr, Ni, Iron (Fe) and Cu caused development impairment in sea urchins (Kobayashi and Okamura, 2004); exposure to As caused oxidative stress in clams (Chiesa et al., 2017) and polychaetes (0.0, 0.05, 0.25 and 1.25 mg/L) (Coppola et al., 2016), as well as metabolic changes in mussels (at 1 mg/L) (Coppola et al., 2018). Pires et al. (2017) observed that exposure to As, Cd, Cu, Cr, Pb, Hg, Ni induced oxidative stress in polychaetes and retarded their regenerative capacity. The elements Cu, Pb, Zn, Cd and Hg have been found to be bioaccumulated and induce hepatotoxicity in fish (Metwally and Fouad, 2008).

1.2 Ultraviolet filters as emerging pollutants

'Emerging contaminants' is a term often applied broadly to designate aspects which should be defined with more accuracy. In a review by Sauvé and Desrosiers (2014) authors introduce the idea that the designation of 'emerging' would be better substituted by 'Contaminants of emerging concern (CECs)', a term comprising three definitions: a) true emerging contaminants – as new materials or compounds that were not previously known and start to be reported in scientific literature (e.g. nanoparticles, parabens, flame retardants and personal care products); b) contaminants of emerging interest – regarding toxicants whose environmental presence was recognized, although the extent of contamination and/or hazard potential was not completely understood (e.g. plastics, pharmaceuticals, endocrine disruptors and biological contaminants); iii) emerging issues arising from 'old contaminants' – nowadays having a link to climate change or other events (natural or man-induced), related to new insights on human health and environmental risk deriving from

'legacy' contaminants' behaviours/occurrences (e.g. salinity/pH variations, heat-waves, dissolved versus particulate Pb in drinking water). Contaminants of emerging concern regard compounds or substances that, to the present, have not yet been featured or regulated in health and environment protection guidelines or policies.

In recent years, pharmaceuticals, cosmetics and other Personal Care Products (PCPs) have been raising concern given their high frequency of usage, in large amounts, and, as a consequence, high rate of release into the environment, some being characterized by a persistent behaviour, bioactivity and potential to bioaccumulate (Juliano and Magrini, 2017). Contrary to pharmaceuticals, PCPs are intended for external application (Brausch and Rand, 2011). For this reason, their constituents do not undergo metabolic transformation and reach the environment in an unaltered state, a fact of concern even if metabolization might not always mean a reduction in toxicity (Ternes at al., 2004; Guengerich, 2006). Households and the industrial sector are major contributors to the enrichment of effluents with PCPs ingredients, that can reach coastal environments because elimination processes at WWTPs are inefficient (low degradation rates). Moreover, these compounds can also wash-off directly from the human skin during leisure and swimming activities (Kupper et al., 2006; Myers et al., 2015).

Despite the common detection in surface waters and perceived risk, ecotoxicological data on PCPs (e.g. shampoos, makeup, body lotions) is limited (Brausch and Rand, 2011; Torres et al., 2016). Recent studies reported the toxicity of PCPs' ingredients in several species: Triclosan and methyl-triclosan (antibacterial and antifungal agents incorporated in several toothpaste, lotion and detergent formulations) showed to be toxic for embryos of zebrafish (*Danio rerio*) and of the sea urchin *Paracentrotus lividus* (Macedo et al., 2017); parabens (a class of preservatives used in cosmetic products and pharmaceuticals) were responsible for reproductive/developmental impairment in the marine copepod *Tigriopus japonicus* (Kang et al., 2019); regarding polycyclic musks (fragrances) Ehiguese et al. (2020) observed that, in the estuarine clam *Ruditapes philippinarum,* galaxolide and tonalide induced oxidative stress and DNA damage.

Ultraviolet (UV) filters, given their protective properties against damage by UV radiation, can be found in a vast selection of sunscreens and cosmetic products. Their usage has increased along with growing awareness about the risks of sun exposure and, to effectively protect the skin from both UVA (320 - 400 nm) and UVB (290 – 320 nm), filters are usually combined in a broad-spectrum absorbance formulation (Brausch and Rand, 2011; Sánchez Rodríguez et al., 2015). Despite the recognized protection, some compounds are still able to induce sensitization and/or phototoxicity (Giokas et al., 2007).

To avoid this kind of occurrences, the European Regulation on Cosmetic Products currently approves 29 UV-filters for use in cosmetic products (list on Annex VI) (Regulation (EC) No 2019/680, European Parliament, 2019). This protection conferred by UV-filters is dependent on the type of active ingredients: organic filters (ex: 4-methylbenzyliden camphor (4-MBC)) will work on absorbing and dissipating radiation via photochemical and photophysical pathways, while inorganic filters (ex: Titanium dioxide (TiO_2); Zinc oxide (ZnO)) will mainly reflect and disperse UVA/UVB rays (Serpone et al., 2007; Díaz-Cruz et al., 2008).

Organic filters, mainly, are also applied in paints and other materials (e.g. coatings, lacquers, lenses) used to protect products from photodegradation (Tsui et al., 2014). Organic UV filters are not volatile, given their high boiling points, approximately around 400 °C (EPA, 2012). These compounds can be hydrophilic (high solubility in water, e.g. benzophenones) or lipophilic (high values of log K_{ow}, e.g. cinnamates).

Titanium dioxide and ZnO, two inorganic filters considered as safe for human health (due to lack of percutaneous absorption), are metal oxide particles added to sunscreens in the form of nanoparticles (150-300 nm and 200-400 nm, respectively), thus being more suitable for incorporation in skincare and cosmetic products (Osmond and McCall, 2010; Schneider and Lim, 2018). Titanium dioxide and ZnO can be coated with silica and other materials (e.g. aluminium oxide and hydroxide) to prevent photocatalytic reactions and improve stability (Smijs and Pavel, 2011).

The diffused commercialization of skin care products with SPF (Sun Protection Factor) is expected to keep rising considering the foreseen increase in population inhabiting coastal areas, a growth from 1.2 to 5.2 billion people by 2080 (Sánchez-Quiles and Tovar-Sánchez, 2015). Worldwide, the consumption of UV filters is estimated in 10.000 tons/year (Jurado et al., 2014). For the year of 2012, and regarding a per capita consumption of sunscreens, in Europe, Spain, Switzerland and Denmark were the countries with the highest values (~190, 150 and 140 mL, respectively); while the lowest consumption was observed in Poland and Russia (≤ 25 mL). Outside Europe, consumption per capita was the highest in the United States (~100 mL), followed by Australia (~65 mL) and Canada/Brazil (~50 mL) (Osterwalder et al., 2014).

In coastal environments, benzophenones are the most diffused group, being benzophenone-3 (BP-3) the most detected compound (up to µg/L) (Ramos et al., 2015; Mao et al., 2018). Seawater samples from Hawaii have displayed the highest BP-3 concentrations (1.395 mg/L) (Downs et al., 2016), with lower values being observed in Spain (up to 3317 ng/L, with 2.46 ng/g Dry Weight (DW) for marine sediments) (Sánchez Rodríguez et al., 2015; Martín et al., 2017). In Italy, measured concentrations of BP-3 in

seawaters were of 216 ng/L (Nguyen et al., 2011). The occurrence of benzophenones, represented by the previous values, is gaining attention as coastal tourism is in fast development (Sánchez-Quiles and Tovar-Sánchez, 2015). Other organic UV filters, as p-aminobenzoic acid, crylene, cinnamate and benzoyl methane derivatives, have also been detected in coastal waters (in the ng to µg/L range) (Ramos et al., 2015). Information concerning inorganic UV filters is more limited.

In this context, relevant ecological concerns have rose, especially related to the potential activity of UV filters, namely organics, as endocrine disruptors (estrogenic activity). In particular, this risk can be linked to compounds with high values of log K_{ow} (such being translated into lipophilicity) together with higher stability in the environment, which can lead to bioaccumulation (Schlumpf et al., 2001; Brausch and Rand, 2011). Sunscreens have also been associated with important coral bleaching events in areas where human recreational activities occur (Danovaro et al., 2008). Despite the mentioned above, a proper environmental risk assessment regarding these compounds has not yet been fully conducted (Schneider and Lim, 2018).

1.3 *Ficopomatus enigmaticus* as a new model organism

The use of bioindicator species and/or model organisms is an essential aspect in environmental monitoring studies. A selected test species must display sensitivity to low concentrations of a wide variety of chemicals and organisms must be available throughout the year, whether being collected from the field or cultured in laboratory (Oliva et al., 2018). Assays with these organisms must be reproducible and cost-effective, with generated data allowing the interpretation of a biological effect induced by a pollution event (Chiarelli and Roccheri, 2014). Bivalves are widely used as model and bioindicator species due to their wide distribution and abundance, sedentarism and consequent ease to sample, physiology, ability to accumulate pollutants and, lastly, ecological and economical relevance (Luna-Acosta et al. 2015; Laitano and Fernández-Gimenez, 2016). In terms of embryotoxicity tests, sperm toxicity assays with sea urchins have become the most widespread standardized bioassays for both seawater quality and sediments toxicity (concerning coastal and estuarine areas) (United States Environmental Protection Agency, 1994). Regarding polychaetes, this group has been mainly used for pollutants bioaccumulation and mortality/growth acute toxicity assays (ASTM, 1997; Díaz-Castañeda et al., 2009). However, recent studies also demonstrated the capacity of this group of organisms as good bioindicator species to a wide diversity of pollutants (including metals, nanoparticles,

pharmaceuticals), with alterations from cellular to individual levels (Freitas et al., 2015; Pires et al., 2017; De Marchi et al., 2018; Barbosa et al., 2020).

Ficopomatus enigmaticus (Fauvel, 1923) is a sessile polychaete from the Serpulidae family. It is thought to originate from the subtropical austral region, the one correspondent to Australia, and is now found in temperate areas, as a result of fouling to ships' hulls (Allen, 1953; Dixon, 1981; Styan et al., 2017). In brackish water estuaries and lagoons it forms reefs of calcareous tubes, with 5 cm or more in size, which encrust on hard substrates such as rocks, wood, shells, poles and piers (Oliva et al., 2018; Styan et al., 2017) (Figure 1). These organisms can endure a wide range of temperature and salinity conditions, being characterized by a high growth rate and abundance, which makes them a good choice for ecotoxicity testing (Fornós et al., 1997). They are brown in colour, exhibit branching gill plumes on the anterior portion of the body and are suspension feeders. These polychaetes reproduce sexually, releasing their gametes into the water, where the fertilization occurs (external fertilization), originating a trochophore larvae. The larvae drifts in the water column and, after 20 to 25 days, it settles to become the adult form (Fornós et al., 1997). The segregation of the calcareous tubes, by collar glands, occurs soon after the settlement (Casu et al., 2019). Recruitment rates are high and adult worms may show lasting longevity (up to several years) (Bianchi and Morri, 2001).

Figure 1– Reef portions of *Ficopomatus enigmaticus*. The darker part (the oldest) corresponds to the base of the reef while the clearer one is the newest part.

Coastal zones in the south-western Atlantic are inhabited by many marine exotic species, being *F. enigmaticus* one that produces a substantial ecological impact (Schwindt et al., 2004). It is a highly invasive, ecosystem engineer and habitat modifying species (Çinar, 2013; McQuaid and Griffiths, 2014). Reefs are of fast growth in Mediterranean areas, especially under eutrophic conditions (Zaouali and Baeten, 1983). As physical structures, they will provide shelter for many benthic species (Obenat and Pezzani, 1994; Schwindt et al., 2004) and will influence hydrodynamics and the geomorphology of the system, by altering sediments transport and deposition (Shumka et al., 2014). Besides these important ecological impacts, the fouling of reefs onto man-made hard substrates (e.g. tide gates, pipes) can also result in economic damage (Peria and Pernet, 2019).

As *F. enigmaticus* is an abundant species, which is spread in temperate waters in the Northern and Southern hemispheres (Straughan 1972; Schwindt and Iribarne 1998; Hove and Kupriyanova 2009), is of easy collection and sampling and allows the effortless manipulation of its physiological features, it presents itself as a good organism for toxicity testing (Oliva et al., 2018). Besides the contamination history, the presence of considerable reefs of *F. enigmaticus* motivated the selection of the two study areas described below.

1.4 Study areas

1.4.1 Ria de Aveiro (Aveiro, Portugal)

The Ria de Aveiro is an estuarine ecosystem with approximately 47 km^2 of area, located in the north-west coast of Portugal (40°38′ N, 8°45′ W). It is characterized by a complex system of channels and bays, composed by large extensions of sand and mud flats (Dias et al., 2000) (Figure 2). Such features grant its high relevance in terms of conservation value, being characterized by high productivity levels and making the habitat suitable for several invertebrate and fish species (Cid et al., 2001; Bueno-Pardo et al., 2018). The Ria de Aveiro is an historical example of trace elements enriched environment, as a result of the industrial production that has taken place in its surroundings (Martins et al., 2013). Ceramics, metallurgy, fertilizers, chlorine and soda and plastics were the main industries established in the region. Currently, a large part of those industries has shut down or left the area, although leaving contaminated sediments and waters behind (Martins et al., 2010). As an important improvement, industrial effluents produced by several facilities, that are still operating in the region at the present moment, do undergo treatment at WWTPs before being discharged into the inter-municipal wastewater network (SIMRIA) (CUF-QI Sustainability Report, 2015), thus helping to reduce ecological and human health risks.

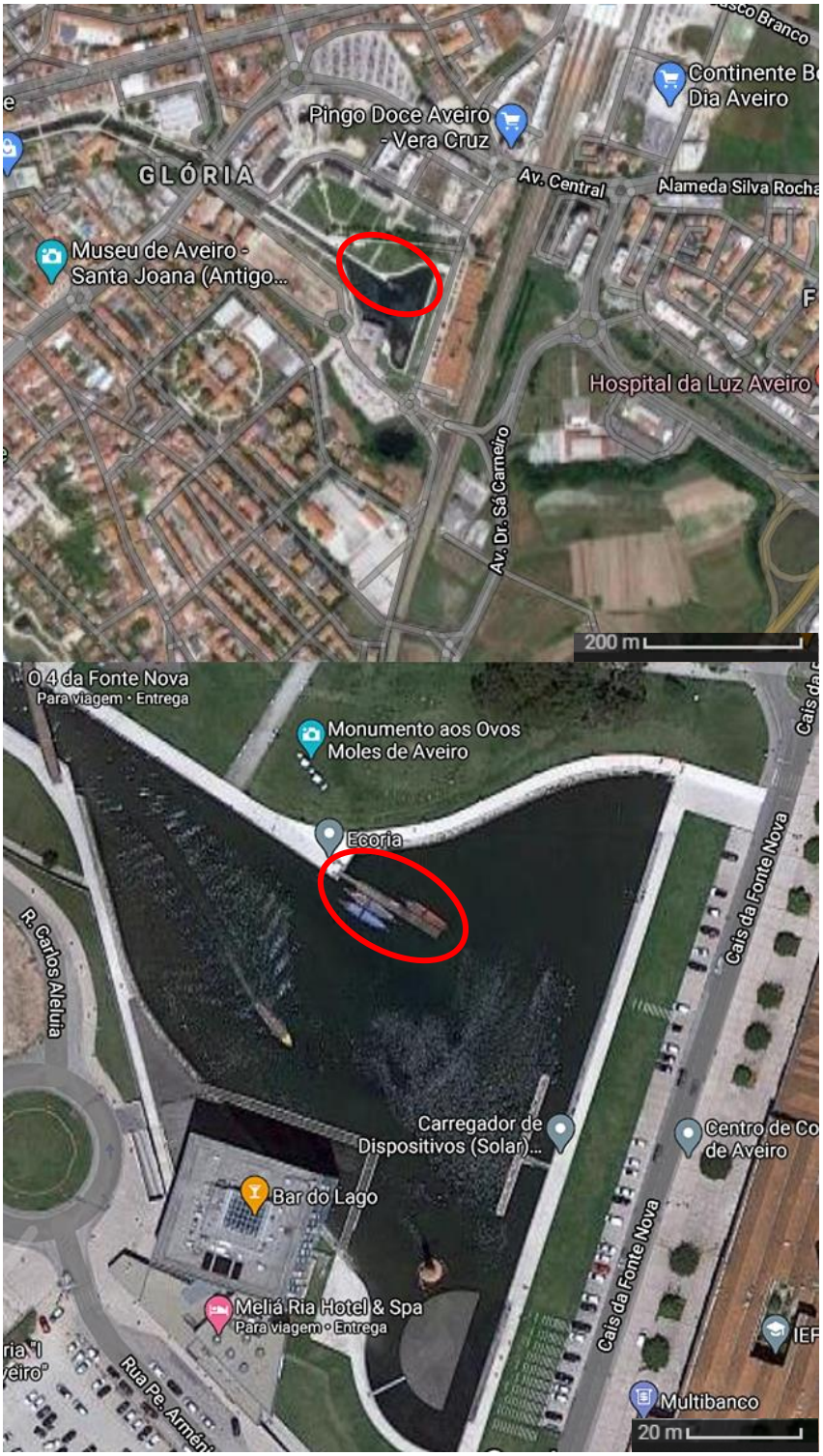

Figure 2 –Ria de Aveiro study area (Portugal) (Images from Google maps).

1.4.2 Fiume Morto, S. Rossore - Migliarino Regional Park (Pisa, Italy)

Fiume Morto, at S. Rossore - Migliarino Regional Park (Pisa, Italy) (43°44′ N,10°16′ E) is a channel that flows into Mar Tirreno, at 5 km south of Serchio river's mouth (Figure 3). Due to the presence of recent and fossil dune systems, important natural habitats, the area where it is located is considered heritage of UNESCO (Zucchelli, 2020). Fiume Morto is the collection point of non-treated urban effluents from Pisa and neighbouring regions, being also characterized by a low water circulation flow (Betti et al., 1985). These two aspects promote trace elements load and storage to increase in this brackish water system (Betti et al., 1985).

Figure 3– Fiume Morto at S. Rossore - Migliarino Regional Park study area (Italy) (Images from Google maps).

1.5 Thesis objectives

The following three chapters (Chapter 2, Chapter 3 and Chapter 4) here presented aimed to explore the suitability of *F. enigmaticus* to be used as model organism in ecotoxicological and ecological risk assessment assays and, to fulfil that objective toxicity assays were performed, based on *F. enigmaticus* larval development and adults' biochemical performance. Chapter 2, entitled '*Ficopomatus enigmaticus* early-development comparison between two distinct populations after trace element exposure', focused on assessing and comparing the response of developing embryos of an Atlantic and Mediterranean population of *F. enigmaticus* to Hg, Cd, Cu, As and Pb exposure, with effects being evaluated in terms of the percentage of badly developed larvae. A preliminary molecular analysis was performed in order to confirm the belonging of the two populations to the same species. Chapter 3, entitled 'Ecotoxicological screening of UV filters by a marine bioassays battery', concerned the toxicity assessment of a set of seven UV filters on the bioluminescence inhibition in *Vibrio fischeri*, growth inhibition of *Phaeodactylum tricornutum* and larval development of *F. enigmaticus,* aiming to compare the performance of three model species when exposed to UV filters. Based on obtained results, ZnO (inorganic filter) and Ethylhexyl methoxycinnamate (EHMC) (organic filter) were selected to perform a *F. enigmaticus* larval development assay under different conditions of light and temperature, to understand the toxic behaviour and potential degradation under environmental factors. Lastly, Chapter 4, entitled 'Biochemical response of *Ficopomatus enigmaticus* adults after organic and inorganic UV filter chronic exposure', aimed to perform a broad biomarkers study in adult polychaetes exposed to both ZnO and EHMC, assessing cellular damage, antioxidant defences, biotransformation mechanisms and neurotoxicity.

CHAPTER 2. *Ficopomatus enigmaticus* EARLY-DEVELOPMENT COMPARISON BETWEEN TWO DISTINCT POPULATIONS AFTER TRACE ELEMENT EXPOSURE

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2. *Ficopomatus enigmaticus* early-development comparison between two distinct populations after trace element exposure

2.1 Introduction

Recently, embryotoxicity tests have gained considerable relevance along with other standard biological assays, being included in environmental monitoring and management programs (Schirling et al., 2006; Chiarelli and Roccheri, 2014). The efficiency of toxicity testing using living material relies on organisms' sensitivity and their ability to respond to low pollutant levels. In this context, several studies have demonstrated how early life stages of marine invertebrate's present higher sensitivity than adults of the same species in contaminated environments (Ross and Bidwell, 2001; Pineda et al., 2012). Besides high sensitivity of early developmental stages (Azad et al., 2009; Azad, 2013), they are also economically and ecologically relevant (His et al., 1999). Disturbances at this development level may result in future population imbalances and impaired ecosystem functioning (Caswell, 2000; Baurand et al., 2014).

For ecologically relevant toxicology tests, suitable model organisms are represented by specimens that are both ready to use during most of the year (e.g. spawn of gametes), and geographically widespread (Richardson and Martin, 1994). Furthermore, to be considered as good models, organisms have to be abundant, easy to collect, and culture (Ross and Bidwell, 2001; Oliva et al., 2018). Despite such potential limitations, an increasing use of embryotoxicity tests has been observed, mainly using bivalves and sea urchins. As examples, Mai et al. (2012) performed embryotoxicity and genotoxicity tests to demonstrate that Copper (Cu), Metolachlor, and Irgarol® (pesticides) can cause larval abnormalities in the oyster *Crassostrea gigas*. With the sea urchin (*Paracentrotus lividus*), Morroni et al. (2018) explored the reversibility of effects caused on its development by toxicants such as Zinc (Zn), Lead (Pb), Cu, and Cadmium (Cd), revealing developmental plasticity of contaminated embryos. Although polychaete embryotoxicity tests have increased popularity, there is still limited information available regarding the effects of contaminants in the larval stages of this class of organisms (Lewis and Watson, 2012). Furthermore, standardized bioassay protocols for marine and brackish waters monitoring are very limited (Oliva et al., 2018). Among polychaetes, the species *Ficopomatus enigmaticus*, a sessile polychaete of the Family Serpulidae, has been already considered a good bioindicator for both marine and brackish water monitoring (Oliva et al., 2018, 2019). *Ficopomatus enigmaticus* is an abundant species with a wide spatial distribution, as well as being easy

to collect, and reproductively active year-round (Oliva et al., 2018, 2019). These organisms can endure a wide range of temperature and salinity conditions and are characterized by high growth rates and abundance, making this species a good candidate for ecotoxicity tests (Fornós et al., 1997).

Previous studies showed that polychaetes are severely affected by trace element bioaccumulation, resulting into oxidative stress and loss of redox homeostasis (Freitas et al., 2012; Coppola et al., 2016; Pires et al., 2017). Effects at a cellular level may impair organisms' embryogenesis, fertilization, larval settlement, and general physiological performance, including survival and growth (Gopalakrishnan et al., 2008; Hudspith et al., 2017).

Considering the facts mentioned above and the widespread of trace elements (see section 1.1), their persistence in aquatic systems and impacts on wildlife, the present study aimed to: i) evaluate the effects of five trace elements (Mercury (Hg), Arsenic (As), Cu, Cd, and Pb) on the larval development of the polychaete *F. enigmaticus*; ii) compare the responses to the aforementioned trace elements between two different populations (Mediterranean and Atlantic). These two goals aim to strengthen the ecological relevance of the *F. enigmaticus* larval development assay, focusing on the reproducibility of results between different populations collected from spatially distant areas. We tested the hypothesis that organisms from the same species would respond similarly to contaminants, even when collected from different populations.

2.2 Material and methods

2.2.1 Ecological characteristics

The polychaete *Ficopomatus enigmaticus* (Fauvel, 1923) is a filter-feeding serpulid tubeworm throughout the temperate waters of the Northern and Southern hemispheres. Colony structures of *F. enigmaticus* are characterized by massive calcareous reefs (Schwindt et al., 2004) as a consequence of their high fecundity and larval dispersal behaviour (Dittmann et al., 2009). Considering its habitat, brackish water with vertical hard structures such as concrete walls, pylons and ship hulls represent the selective substrate of the species.

2.2.2 Organisms collection and maintenance

Adult organisms of *F. enigmaticus* were collected in S. Rossore-Migliarino Regional Park – Fiume Morto (Pisa, Italy) and in the Ria de Aveiro (Cais da Fonte Nova Channel, Western Portugal), both for genetic analyses and to perform the larval development assay. Polychaetes from the two populations were collected during late spring period (end of May-beginning of June) and transferred to the laboratory together with water from the sampling sites. The correct identification of the individuals used in ecotoxicological tests was performed by a genetic analysis following the protocols described in Oliva et al. (2020). All specimens used in this study were correctly identified and attributed to the same taxonomic *F. enigmaticus* group.

Aquaria salinity was the same as in the sampling areas: 25 at the Ria de Aveiro and 15 at S. Rossore-Migliarino Regional Park. Salinity was increased daily up to a maximum of 5 values, until 30, as it is within the optimum salinity range for larval development (Oliva et al., 2018). Organisms were fed daily with an *Isochrysis galbana* suspension (3×10^5 cells/mL). At the end of the acclimation period (1 day for organisms from the Ria de Aveiro and 3 days for the organisms from S. Rossore-Migliarino Regional Park) aquaria conditions were: temperature 22 ± 1 °C, oxygen saturation >90 %, salinity 30 and pH 8.1 ± 0.1. Aquaria were maintained under a photoperiod of 10 h light: 14 h darkness.

2.2.3 Molecular analysis

Since cryptic species were discovered in the taxon *F. enigmaticus* (Styan et al., 2017), a molecular analysis was carried out to confirm the exact taxonomic attribution of the individuals used in the ecotoxicological tests. Samples collected at the Ria de Aveiro, Cais da Fonte Nova Channel (Western Portugal) (cf. Figure 3) (n = 39) and at San Rossore-Migliarino Regional Park (Italy) (cf. Figure 4) (n = 36), were preserved in 96 % ethanol. Total genomic DNA was extracted with NZY Tissue gDNA Isolation Kit (Nzytech) according to the manufacturer's instructions. DNA quality and concentration were tested by both agarose gel electrophoresis and spectrophotometrically at 260–280 nm. A gene portion (340 bp) of the mitochondrial cytochrome oxidase b (*Cyt b*) was analysed, following PCR protocols used by Styan et al. (2017) and Yee et al. (2019) for the same species. Chromatograms obtained from forward and reversal sequences were visualized by CHROMAS software v. 2.01 (Technelysium Pty. Ltd.). The obtained sequences (GenBank Accession Numbers: MH271215-20; MK934527-30, MK934533-34) were compared with those available in GenBank: *F. enigmaticus* (KP863736-KP863777), *F. miamiensis* (KP863779) and *F.*

macrodon (KP863778). Bayesian (BI) and maximum likelihood (ML) phylogenetic analyses were performed. The species *Hydroides cruciger*, belonging to the same family Serpulidae (KP178715; Straughan, 1967) was used as outgroup. The BI analysis was conducted in MrBayes v.3.12 (Huelsenbeck and Ronquist, 2001) (TrN + G model selected by MrModeltest (Nylander, 2004) according to the hierarchical likelihood ratio tests (hLRTs), four Markov Chain Monte Carlo (MCMC) chains, sampled every 1000 generations for a total of 4 million generations and 1000 sampled trees from each run discarded as burn-in). A ML tree was inferred using PhyML 3.1 algorithm implemented in Seaview v4 (Gouy et al., 2010) (1000 bootstrap replicates).

2.2.4 Contaminants

A set of trace element ions was chosen for the exposure assay, in the following nominal concentration: Cu^{2+} - 11.25-22.5-45-90 µg/L; Hg^{2+} - 0.45–0.9-4.5–9 µg/L; As^{3+} - 90-180-360-720 µg/L; Cd^{2+} - 90-450-900-1800 µg/L; Pb^{2+} - 45-225-450-1800 µg/L. These concentration ranges were based on those reported by Gopalakrishnan et al. (2008), who compared the toxicity of Hg, Cd, Ni, Pb and Zn in early life stages of the serpulid polychaete *Hydroides elegans*.

Dilutions of all assessed contaminants were prepared by dissolving the respective salt (copper chloride, mercury chloride, sodium arsenate, cadmium chloride and lead nitrate) in artificial seawater (ASW, salinity 30), prepared following the ASTM (2013) standard formulation. Copper sulphate pentahydrate was used as reference toxicant (Manfra et al., 2016) at concentrations of 11.25–22.5-45-90-180 µg/L of Cu^{2+}.

All reported exposure concentrations represent 90 % of each prepared contaminant dilution. The reduction in concentration was due to the fact that 1 mL of fertilized egg suspension (in clean filtered seawater) was added to 9 mL of each concentration replicate, as reported in the section below (2.2.5). All chemicals were purchased from Merck/Sigma-Aldrich (Milan, Italy).

2.2.5 Gametes emission and collection

The embryotoxicity tests followed the protocol reported in Oliva et al. (2019). Gamete release was induced by de-tubing serpulids (Hadfield et al., 1994). Each polychaete was collected individually, quickly rinsed in tap water and then placed in 1 mL of 30 ASW. After gametes emission (5–10 min), single female eggs were selected with a fertilization pre-test using an inverted microscope (Leica DMIL) for division synchronization check. Eggs of selected females were transferred together in a 300 mL beaker filled with ASW.

2.2.6 Gametes preparation and fertilization

The egg suspension was rinsed three times with fresh ASW to remove immature and damaged eggs (supernatant). Then the suspension was diluted to reach a concentration of 300 cells/mL. Sperm from 4 to 5 males was freshly collected, mixed and pipetted in the rinsed egg suspension at a concentration of about 3×10^5 cells/mL. The obtained suspension was gently stirred and then placed at 22 ± 1 °C for 40 min to allow fertilization.

2.2.7 Larval development assay

One mL of fertilized homogeneous egg suspension was pipetted into each dilution replicate, making up to 10 mL of final volume (9 mL of testing substance). Three replicates were set for each treatment and control (ASW). Then, all replicates were incubated at 20 ± 2 °C for 48 h, with a photoperiod of 10 h light: 14 h darkness. After the incubation the assay was stopped by adding few drops of buffered 37 % formaldehyde. The number of correctly developed and abnormal larvae (Figure 4) was counted for each condition and a percentage of poorly developed larvae was calculated as in Oliva et al. (2020). A 20 % of poorly developed larvae in controls was set as the threshold for the acceptability of the assay.

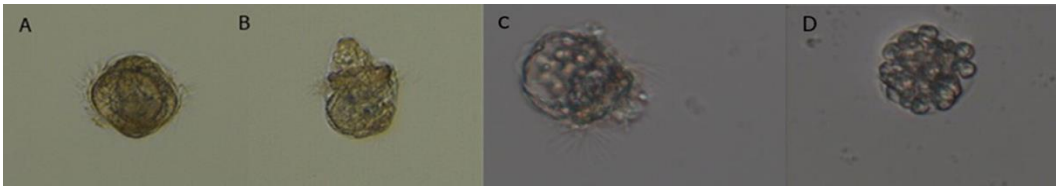

Figure 4 - (A) and (B) well developed larvae, (C) badly developed, (D) undeveloped larvae of *Ficopomatus enigmaticus*.

2.2.8 Trace element quantification

Trace element analyses were performed on the three different relevant matrices: water, whole organism tissue and calcareous tubes of *F. enigmaticus*, all collected at the same sampling site (see section 2.2.2). Salinity, pH, and oxygen saturation of water samples were measured. Samples were then stabilized with 2 % concentrated, ultrapure, HNO_3 for further analyses. Regarding organisms, they were collected and transported as reported in section 2.2.2. In laboratory, worms were gently removed from their tubes and about 5 g of fresh whole-body tissue and 5 g of calcareous tubes were collected, weighed, and then dried at 40 °C for 48 h. The dried samples were directly analysed for evaluating the concentration of Hg by using a DMA-80 Tri Cell (Milestone, FKV) according to the EPA 7473 (2007) method. As regards other trace element analyses, both dried tubes and tissues of *F. enigmaticus* were digested, following EPA, 2007A (2007), by using an Ethos 1 Microwave Digestion System (Milestone, FKV). Copper analysis was performed with an ICP Varian 720-ES (Agilent), according to EPA 6010D (2014), while As, Cd and Pb were quantified with an AAS Varian SpectrAA240Z following EPA 7010 (2007). Mercury and Cu standard, as well as a multi-element standard with As, Cd and Pb were 2 % HNO_3 solution of certified reference material quality. All reference solutions were purchased from Titolchimica, Italia.

2.2.9 Data analysis

After 48 h of exposure, samples were evaluated as the percentage of poorly developed larvae in comparison to controls. EC_{50} calculations were normalized to the control mean percentage of success using Abbott's formula (Volpi-Ghirardini and Arizzi-Novelli, 2001):

$$P = \left(\frac{P_e \times P_c}{100 - P_c}\right) \times 100$$

where Pc and Pe are control and experimental percentage response, respectively. EC_{50} and their 95 % confidence intervals were calculated according to PROBIT analysis (Finney, 1971). Each experiment was run in triplicate and a mean EC_{50} with relative 95% confidence limits was calculated. Moreover, both populations' responses were compared for each element at each concentration via a Student's t-test, to underline statistically significant differences in terms of concentration-response relationship.

2.3 Results

2.3.1 Molecular analysis

Twelve *Cyt b* haplotypes were identified out of the 75 *F. enigmaticus* sequences obtained from the Ria de Aveiro (Portugal) and San Rossore-Migliarino Regional Park (Italy). The percentage of nucleotide divergence observed in both populations of *F. enigmaticus* varied between 0.3 and 1.1 % (average 0.7 %), comparable to that one observed by Styan et al. (2017) (0.7 %) and by Yee et al. (2019) (0.4 %) and in other serpulid polychaetes (Halt et al., 2009). Furthermore, haplotypes described in this study showed 19 % of divergence from group 2 described by Styan et al. (2017) as a putative cryptic species. Both BI and ML analyses of *Cyt b* sequences showed all haplotypes identified at the Ria de Aveiro and San Rossore-Migliarino Regional Park constituting a single group, genetically similar to group 1 described by Styan et al. (2017) (Figure 5). The molecular analyses reported here are the first data known for Europe, beginning to fill the gap for this part of the world (Yee et al., 2019). Notwithstanding the use of molecular analyses must be considered in all ecotoxicological studies (as frequently in invertebrate species), the chance to find “monospecific” populations is common as demonstrated in the present study and in previous ones (Styan et al., 2017; Yee et al., 2019).

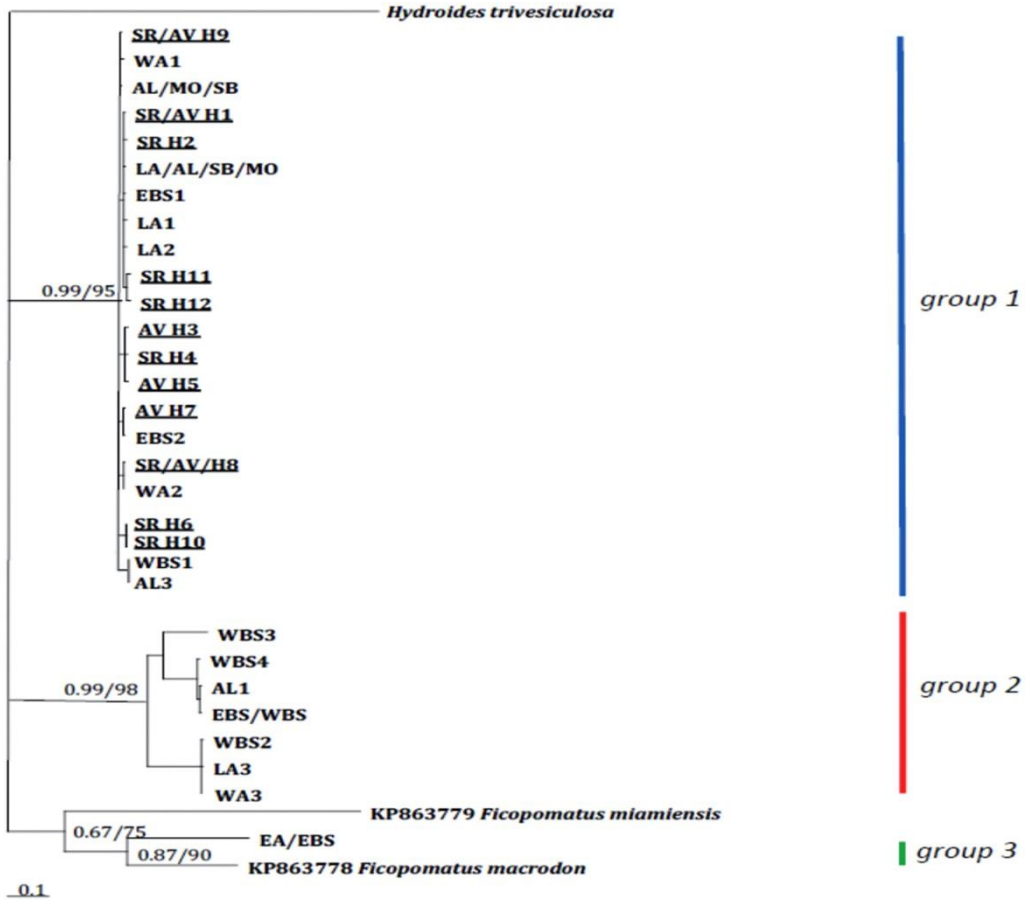

Figure 5 – Phylogenetic tree of the cytochrome b haplotypes of *Ficopomatus enigmaticus* from this paper (H1–H12 underlined) and from Australia (Styan et al., 2017) and California (Yee et al., 2019). Letter codes indicate the region of the site in either Italy (SR, San Rossore Park); Portugal (Av, Aveiro lagoon); Australia (WA, Western Australia; EBS, East of Bass Straight; WBS, West of Bass Strait) or California (AL, Alameda County; MO, Monterey County; SB, Santa Barbara County; LA, Los Angeles County). Clades are named as in Styan et al. (2017). Numbers at main nodes indicate the posterior probability of Bayesian analysis (BI) and bootstrap values by maximum likelihood (ML).

2.3.2 Ecotoxicology evaluation

Concentration-response curves for each trace element used in the present study showed a percentage of well-developed larvae decrease along the toxicant concentration increase (Figure 6). Copper and As concentration-response curves showed similar behaviour. The curves for each trace element in both populations (Mediterranean vs Atlantic) decreased in a linear and gradual way. With respect to Hg and Cd good larval development was seen in the Mediterranean population as it decreased more gradually, while the Atlantic population showed an abrupt drop of values. An opposite pattern was reported for Pb. The proportion of well-developed larvae in the Mediterranean population showed a sharp decrease, against the proportion of the Atlantic one, which reached a very low percentage, though in a more progressive way.

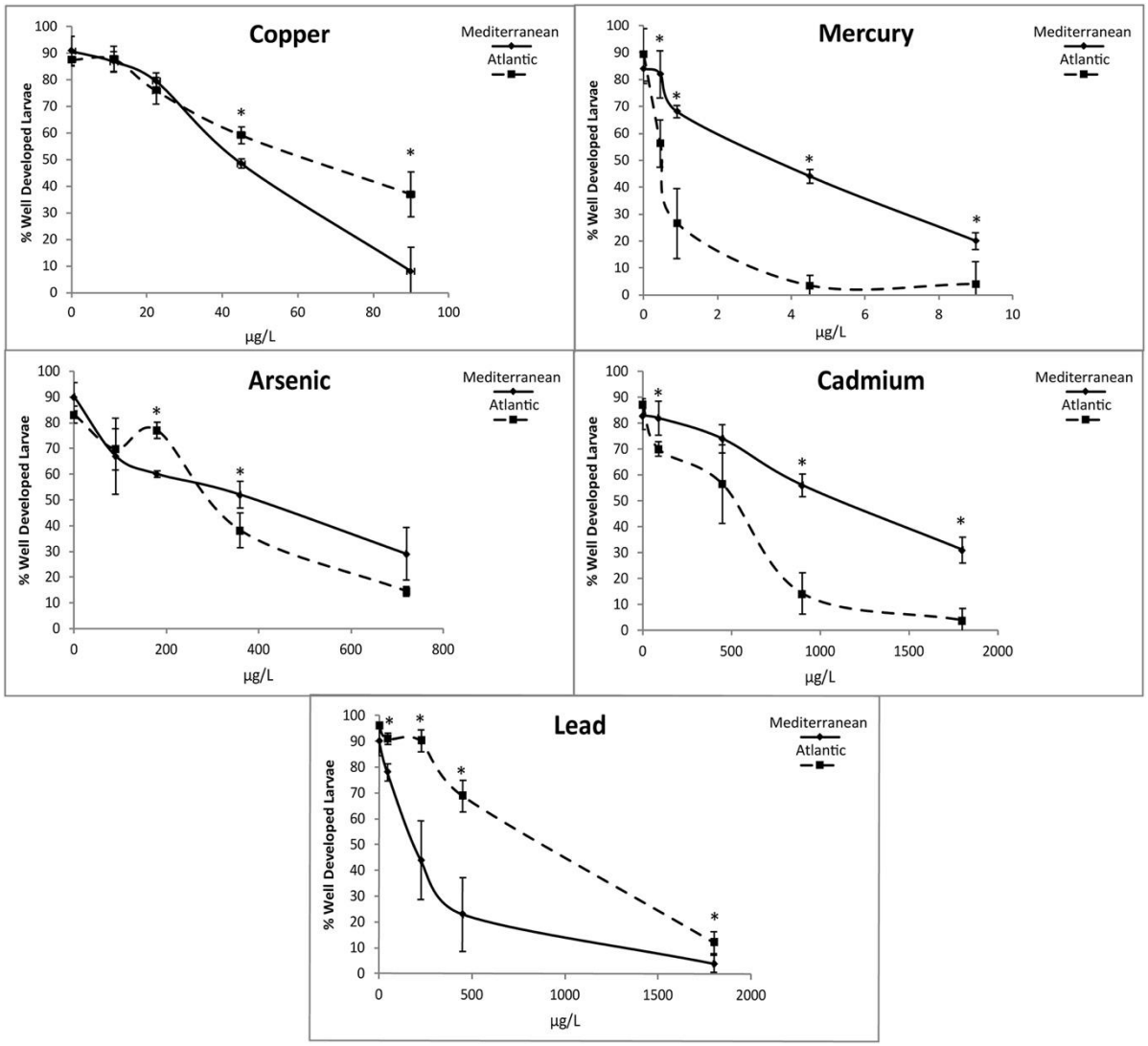

Figure 6 – Concentration-effect curves, calculated for all assessed elements, after 48 h exposure of *Ficopomatus enigmaticus* embryos. Each graph reports the curves for both assessed populations. For each concentration the mean percentage of well-developed larvae ± standard deviation was reported, n = 3. Differences between populations at each concentration were compared via Student's t-test, * = statistically significant differences, $p < 0.05$.

The percentages of effect in terms of poorly developed larvae for each concentration of each assessed element were used to calculate different Effect Concentration (EC_{10}-EC_{50}). In particular, the value for EC_{50} was adopted as the main term of comparison between the two populations. All the EC_{50} values, together with 95 % confidence limits, are reported in Table 1. The highest similarities between Mediterranean and Atlantic populations were obtained for As, with an EC_{50} value of 460.26 µg/L for the Mediterranean population and 411.40 µg/L for the Atlantic one. Also, with Cu the results obtained revealed values of EC_{50} of 110.02 µg/L and 67.91 µg/L for Mediterranean and Atlantic population, respectively.

Looking at Hg and Cd EC_{50} values it was possible to observe that the Mediterranean population was less sensitive, for both elements, with values of 3.97 µg/L (Hg) and 1563.28 µg/L (Cd). The same elements assessed with the Atlantic population, showed EC_{50} values of 0.27 µg/L and 249.75 µg/L for Hg and Cd, respectively. On the contrary, lead resulted in an EC_{50} value for the Atlantic population more than the double that of the Mediterranean one.

Table 1 - Values of EC_{50} (expressed in µg/L, with relative 95 % confidence limits (C.L.)) calculated for each trace element and both Mediterranean (Italy) and Atlantic (Portugal) populations. Results were obtained with a PROBIT model for the estimation of median effect concentration.

Ion	Italy		Portugal	
	EC_{50}	C.L. 95%	EC_{50}	C.L. 95%
Hg^{2+}	3.97	2.64 - 5.33	0.27	0.08 - 0.57
Cd^{2+}	1563.28	1206.47 - 2082.25	249.75	145.52 - 360.63
Cu^{2+}	110.02	63.51 - 309.57	67.91	n.c.
As^{3+}	460.26	328.27 - 681.13	411.40	169.99 - 648.02
Pb^{2+}	255.82	185.89 - 320.59	566.85	525.77 - 676.64

Table 2 reports results in terms of EC_{10} of all assessed elements for both populations. Atlantic organisms were more sensitive to cadmium (62.65 µg/L) in comparison to those from the Mediterranean region (393.57 µg/L). The opposite behaviour was observed for Pb: 66.65 µg/L and 252.61 µg/L of EC_{10} for the Mediterranean and Atlantic population, respectively. For arsenic and copper, the Atlantic population was more tolerant given that the EC_{10} values were higher than those listed for the Mediterranean one. The Mediterranean population showed values of 4.08 µg/L for Cu and 41.61 µg/L for As, while these were of 30.46 µg/L Cu and 123.39 µg/L As for the Atlantic organisms. Lastly, regarding Hg, the Mediterranean population displayed higher tolerance, with an EC_{10} of 0.72 µg/L, while this value was of 0.019 µg/L for the Atlantic population.

Table 2 - Values of EC_{10} (expressed in µg/L, with relative 95 % confidence limits (C.L.)) calculated for each trace element and both Mediterranean (Italy) and Atlantic (Portugal) populations. Results were obtained with a PROBIT model for the estimation of the effect concentration.

Ion	Italy		Portugal	
	EC_{10}	C.L. 95%	EC_{10}	C.L. 95%
Hg^{2+}	0.72	0.22 - 1.31	0.019	0.001 - 0.067
Cd^{2+}	393.57	163.05 - 600.22	62.65	16.91 - 115.45
Cu^{2+}	4.08	0.60 - 9.28	30.46	n.c.
As^{3+}	41.61	10.07 - 81.84	123.39	9.73 - 247.69
Pb^{2+}	66.65	31.87 - 104.28	252.61	188.21 - 309.69

2.3.3 Trace elements determination

Pollutant quantifications were performed on water, calcareous tubes, and organism whole tissues (Table 3, Table 4). Concentrations of selected elements, measured in water samples, were close to the instrumental limit of quantification (LOQ) and comparable between the two sampling sites, Fiume Morto (Pisa, Italy) and the Ria de Aveiro (Aveiro, Portugal) (Table 3). Similar results were obtained for quantified element concentrations in calcareous tubes (Table 4). Furthermore, concentrations were comparable between the two populations and deposition of the measured contaminants into tube composition appeared to be minimal. Element concentrations in serpulid whole tissues were similar between the two sampling sites for copper and arsenic (Table 4). In particular, tissues from the Mediterranean organisms had values of 25.36 mg/kg dry weight (DW) for Cu and 8.30 mg/kg DW for As, compared to 35.52 mg/kg DW Cu and 9.65 mg/kg DW As in tissues from those from the Atlantic population. Values for Hg were eight times higher in the Mediterranean population than the Atlantic populations (0.56 mg/kg DW vs 0.07 mg/kg DW), and Cd was almost double that of the Atlantic populations (1.57 mg/kg DW vs 0.72 mg/kg DW). Lead concentration in the Mediterranean population tissues was eight times lower than the one obtained for Atlantic organisms (1.07 mg/kg DW compared to 8.79 mg/kg DW).

Table 3 - Trace element concentration in water samples (µg/L) collected in both sampling areas: Fiume Morto (Pisa, Italy) and Ria de Aveiro (Aveiro, Portugal). LOQ in µg/L.

Element	Limit of Quantification (LOQ)	Sample Concentration	
		Fiume Morto	Ria de Aveiro
Hg	0.05	< LOQ	< LOQ
Cd	0.1	0.22	0.21
Cu	2.5	5.17	3.40
As	2.5	< LOQ	< LOQ
Pb	2.5	4.65	< 2.5

Table 4 - Trace element concentration in organisms' tissue and calcareous tubes samples collected in both sampling areas: Fiume Morto (Pisa, Italy – Mediterranean population) and Ria de Aveiro (Aveiro, Portugal – Atlantic population). Measure unit for each quantified element was mg/kg dry weight. LOQ in µg/L

Element	Limit of Quantification (LOQ)	Whole Tissue		Calcareous Tubes	
		Fiume Morto	Ria de Aveiro	Fiume Morto	Ria de Aveiro
Hg	0.005	0.56	0.07	0.06	0.01
Cd	0.2	1.57	0.72	0.06	0.05
Cu	0.6	25.36	35.52	5.71	3.79
As	0.6	8.30	9.65	0.84	0.95
Pb	0.3	1.07	8.79	2.76	2.66

2.4 Discussion

2.4.1 Embryotoxicity tests: comparison between Ficopomatus enigmaticus data with other serpulid species results

The effect, in terms of EC_{50}, of the larval exposure to environmentally relevant trace element pollutants, was evaluated with *F. enigmaticus* trochophores, comparing an Atlantic and a Mediterranean population of the same species. Among the few embryotoxicity studies using serpulids, Gopalakrishnan et al. (2008) compared the toxicity of Hg, Cd, Ni, Pb and Zn in the early life stages of *H. elegans*. Authors observed that the EC_{50} values, based on the percentage of poorly developed larvae, were dose-dependent, confirming the sensitivity of this species to the used trace elements. Comparing these results with those obtained in the current study, the Mediterranean population was more tolerant to Cd in comparison to *H. elegans* (EC_{50}: 86.66 µg/L), but similar sensitivity to this trace element was detected for

the Atlantic population. Considering Pb, while the Atlantic population of *F. enigmaticus* was more sensitive to this element if compared to *H. elegans* (EC_{50}: 260.64 µg/L), the Mediterranean population displayed a comparable response with this serpulid species (Gopalakrishnan et al., 2008). On the contrary, both populations of *F. enigmaticus* showed higher sensitivity to mercury when compared with *H. elegans* (EC_{50}: 9.33 µg/L). Regarding Cu, Atlantic *F. enigmaticus* showed higher sensitivity if compared with *H. elegans* (exposed to 100 µg/L of Cu) (Gopalakrishnan et al., 2007). In particular, *H. elegans* had 40 % well-developed larvae, while the percentage of *F. enigmaticus* from the Atlantic population, was only approximately 10 % at the same concentration. However, the Mediterranean specimens, under the same conditions, showed a percentage of well-developed larvae similar to *H. elegans*, confirmed by comparable EC_{50} values (122 µg/L). Effects of Cu were also compared with another study conducted by Ross and Bidwell (2001) that used another serpulid species, *Galeolaria caespitosa*. The authors tested an endpoint similar to that of the present work and had an EC_{50} value lower (between 16 and 40 µg/L) than the one obtained for *F. enigmaticus*.

Overall, for the Mediterranean population, trace element toxicity varied from Hg to Cd in a decreasing order, according to the EC_{50} values: Hg (3.97 µg/L) > Cu (110.02 µg/L) > Pb (255.82 µg/L) > As (460.26 µg/L) > Cd (1563.28 µg/L). For the Atlantic population, contaminants were ranked in the following order, also of decreasing toxicity, again in terms of EC_{50}: Hg (0.27 µg/L) > Cu (67.91 µg/L) > Cd (249.75 µg/L) > As (411.40 µg/L) > Pb (566.85 µg/L). Comparing the two populations, lead switched with Cd in the toxicity order for the lowest toxic element, while Hg in both populations, was the most toxic element for early development.

2.4.2 Embryotoxicity tests: comparison between Ficopomatus enigmaticus data and other model species

Due to the lack of information concerning serpulids in ecotoxicological bioassays, the findings of the present study were compared with more consistent literature relative to other widely used model species in ecotoxicology: the echinoid *Paracentrotus lividus* and the oyster *Crassostrea gigas*. Relative to *P. lividus*, Arizzi-Novelli et al. (2003) found EC_{50} values for Cd and Cu (of 230 µg/L and 62 µg/L, respectively), which are both similar to those calculated for the Atlantic *F. enigmaticus*. Mai et al. (2012) working with *C. gigas*, reported an EC_{50} of 212.30 µg/L for Cd, which was similar to the response of the Atlantic population. For the same species, Moreira et al. (2018) obtained 215.20 µg/L as EC_{50} for As, which was two times lower than the one of the Atlantic populations (411.40 µg/L).

2.4.3 Chemical analyses on tissues, calcareous tubes and water

Considering all the obtained results, the detected differences between the assessed populations of *F. enigmaticus* could be related to genetic inference (Harding et al., 2019) and/or other different environmental issues. This assumption has already been demonstrated to be one of the most diffused mechanisms of resistance in free spawning organisms (Galletly et al., 2007; Pease et al., 2010). Indeed, sensitivity could be related to diverse population with varying exposure histories to different trace element, which is site specific and can lead to different bioaccumulation levels (Rainbow et al., 2009).

It was expected that the trace element concentrations in the environment would also be found in tissues and organisms' calcareous tubes. The element quantification in polychaete tubes was based on the idea that they could act as a stocking matrix for xenobiotics and toxic concentrations of micronutrient. Due to the lack of this type of information in the literature, a similar hypothesis was assumed with peculiar behaviour of some aquatic arthropods that accumulate toxic compounds in their exoskeleton and discard them as they molt. For example, Auffan et al. (2013) observed that on *Daphnia pulex* the shedding of the chitinous exoskeleton (ecdysis) was a pivotal mechanism for the release of CeO_2 nanoparticles (ingested or adsorbed). Bergami et al. (2016) observed that larvae of *Artemia franciscana* exposed to toxic PS-NH_2 nanoparticles underwent multiple molts, hypothesizing that this occurrence was a physiological mechanism for toxicants release from the body. However, contrarily to what was expected, results of the present study showed trace element concentrations in calcareous tubes close to instrumental detection limits for both populations, indicating that these structures may not prevent metal accumulation in polychaete soft tissues. Also, the water collected at both sampling areas did not present relevant trace element concentrations. On the other hand, analysis of the whole tissues revealed significant concentrations of the trace elements of interest, and important differences between populations, pointing out bioaccumulation could be controlled by biotic factors such as gender, maturity, and size rather than by environmental contamination (Ray, 1984). In fact, trace elements are reported to be very prone to accumulate in invertebrate organism tissues (Radomyski et al., 2018).

Considering and interpreting the obtained results, and given that quantifications were performed once, this bioaccumulation may be due to other matrices not evaluated in the present study, such as suspended organic matter and suspended inorganic particles. This hypothesis could be related to the poorly selective filter-feeding activity of serpulids.

Moreover, to better establish the link between adults and larvae responsiveness to contaminants, some studies tested for inherited resistance to trace elements using marine oligo- and polychaetes (Langdon et al., 2003). For example, Grant et al. (1989) observed in *Hediste diversicolor* that the tolerance to Cu and Zn was inherited, having seen that the offspring from the contaminated site was more resistant to both contaminants when compared to the one from the clean site. This genetic event might also happen for *F. enigmaticus*, likely explaining part of the sensitivity displayed by the larvae. Therefore, polychaete eggs when in contact with high concentrations of contaminants may produce low quality abnormal embryos (Gopalakrishnan et al., 2008).

2.4.4 Relevance in ecotoxicological monitoring

In the present study EC_{10} values representing no-effect concentrations, instead of NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration), agreed with Warm and van Dam (2008). The Italian (Lgs. D. 173, 2016) and Portuguese (Portaria 1450, 2007) laws on Materials Classification, relative to threshold contamination levels for marine sediments, report concentrations much higher than the contaminants assessed in the present study, if compared to obtained EC_{10} values in this study. Moreover, considering that standardized tests for whole sediment assessment are lacking, there is a mandatory need to work with manipulated matrices such as elutriates (Haring et al., 2010), which usually hold only a portion of all hydrophilic contaminants present in whole sediment samples. For this reason, it is advantageous to work with a species able to show sensitivity to those lower concentrations, as it is the case of the polychaete *F. enigmaticus*.

2.5 Conclusion

Genetic analysis confirmed that all individuals from both tested populations belong to the species *F. enigmaticus*. Experimental data from the present work showed the toxic effects of selected trace elements on the larval development of this species, further confirming the sensitivity of *F. enigmaticus* early life stages to trace elements exposure. Demonstrated sensitivity is comparable between the two populations tested, thus strengthening the ecological relevance of this *F. enigmaticus* larval development assay. However, it is necessary to underline how specific sensitivity of bioassay endpoints with wild-caught organisms to specific contaminants can be affected by the presence of the contaminants in the organisms' sampling site, coupled with the bioaccumulation ability of

the test organism. Overall, these results contribute to the scarce understanding of the effects of contaminants on larval stages of polychaete species and might aid in the standardization of bioassay protocols for marine and brackish waters monitoring. Considering the initial hypothesis, results showed that test organisms collected in different areas can be used in wide-range monitoring plans. However, it is necessary to well characterize sampling areas in order to select organisms coming from environments with similar chemical status to avoid misinterpretations and over- or under-estimation of effects. For that, next-generation tests with embryos, adults, and multigenerational experiments on different species could be a possible the next research step.

CHAPTER 3. ECOTOXICOLOGICAL SCREENING OF ULTRAVIOLET FILTERS BY A MARINE BIOASSAYS BATTERY

This chapter will be submitted as:

Matilde Vieira Sanches, Matteo Oliva, Lucia De Marchi, Alessia Cuccaro, Rosa Freitas, Carlo Pretti. Ecotoxicological screening of UV filters by a marine bioassays battery.

3. Ecotoxicological screening of ultraviolet filters by a marine bioassays battery

3.1 Introduction

The potential action of organic UV filters as endocrine disruptors constitutes actually an important ecological concern. Threats imposed by these substances are mainly associated with their high lipophilicity, together with considerable stability in the environment (Schlumpf et al., 2001; Brausch and Rand, 2011). Recent studies have found significant concentrations (ng/g) of UV filter compounds in fish and mussels' tissues, mainly, but also in crustaceans, birds and mammals (Ramos et al., 2015). The toxic effects of better-known UV filters have been reported in *Danio rerio*: regarding Benzophenone-3 (BP-3), Blüthgen et al. (2012) found it to induce anti-androgenic activity in this fish species; Li et al. (2016) reported the compound 4-methylbenzyli-dine camphor (4-MBC) as capable of inhibiting acetylcholinesterase (AChE) activity and, thus, of impairing early neuronal and muscular development embryos of *Danio rerio*. Octocrylene affected the expression of genes related to development and metabolism, a result described by Blüthgen et al. (2014) also in zebrafish. Sunscreens and isolated ingredients (e.g. BP-3) have also been pointed out as the cause of important coral bleaching events (Danovaro et al., 2008; Ruszkiewicz et al., 2017). Despite the risks for aquatic biota, specially related to endocrine activity, neurotoxicity and development/physiology impairment, a proper environmental risk assessment regarding these compounds has not yet been conducted (Schneider and Lim, 2018), and very few studies are known on the effects of UV filters towards marine/estuarine polychaete species.

The aim of the present work was to perform a toxicity evaluation assessing marine and brackish water organisms belonging to different trophic levels. Seven UV filters (nanoparticulate Zinc oxide (nZnO), nanoparticulate Titanium dioxide ($nTiO_2$), BP-3, Avobenzone (AVO), Octocrylene (OCTO), Ethylhexyl methoxycinnamate (EHMC) and 4-MBC) were tested on the bioluminescence inhibition of *Vibrio fischeri* (marine microorganism), growth inhibition of *Phaeodactylum tricornutum* (primary producer) and, lastly, on the larval development of *Ficopomatus enigmaticus* (consumer). After single exposure to all seven UV filters and considering the obtained concentration-dependent response, nZnO and EHMC were selected for a second larval development assay, with different light and temperature conditions, to evaluate the influence of these environmental parameters on the acute toxicity induced by the two compounds.

3.2 Material and methods

3.2.1 Chemicals

Seven UV filters were selected for the ecotoxicological screening: nZnO (particle size < 100 nm), $nTiO_2$, (primary particle size 21 nm), EHMC, 4-MBC, AVO, OCTO and BP-3. Each chemical was firstly dissolved in methanol (< 1 %) due to reported low solubility in water. Stock solutions were prepared in artificial seawater (ASW) (prepared according to ISO 10253 (2016)). Suspensions of nZnO and $nTiO_2$ were sonicated for 1 min before to use.

Stock solutions were prepared as described previously, in order to obtain the following nominal concentrations: EHMC, 4-MBC and nZnO – 0.3125-0.625-1.25-2.5-5 mg/L; AVO and OCTO - 5-7.5-10-12.5-15 mg/L; BP-3 – 0.625-1.25-2.5-5-10 mg/L; $nTiO_2$ – 0.00625-0.0125-0.025-0.050-0.075 mg/L. Concentration ranges were based on the studies of Fairbairn et al. (2011), Paredes et al. (2014), Paredes and Bellas (2015) and Wehmas et al. (2015). All used UV filters were purchased from Merck - Sigma Aldrich (Milan, Italy).

3.2.2 Ecotoxicological screening

The ecotoxicological screening was performed by the use a battery of three different organisms: the marine bacterium *V. fischeri* (endpoint: inhibition of bioluminescence), the marine diatom *P. tricornutum* (endpoint: inhibition of growth) and the serpulid *F. enigmaticus* (endpoint: larval development).

3.2.2.1 *Vibrio fischeri* - bioluminescence inhibition

The luminescent bacteria test was performed in accordance with the methodology of ISO 11348, 2007. *V. fischeri* strain n. 19A4002A (expiry date 01/2021) was purchased as freeze-lyophilized bacterial cells (separated vials and always resuspended in NSW), from Ecotox LDS, Pregnana Milanese, MI, Italy. Lyophilized bacteria were resuspended in 1 mL of *V. fischeri* Rec Solution, purchased together with bacteria vials, in order to reactivate them. To assess the maximum % of effect (I %) induced by the maximum concentrations (maximum concentration for each compound was reported in section 3.2.1), a screening test was performed before determining ecotoxicological parameters ($EC_{20/50}$). Only UV filters showing an I % > 20 % were selected for the full test. *V. fischeri* bacteria were exposed to a dilution series of each sample and light emission was determined following incubation.

Light emission measurements, of bacteria in the samples, were registered after 15 and 30 min, after being compared to the ASW control. Tests were carried out at 15 °C, pH 6-8 (operative range) and four controls plus three replicates per sample were set. All measurements were performed making use of a M500 luminometer (with appropriate cells), which was PC interfaced. Acquisition and data processing ($EC_{10/20/50}$ calculated by Least Square method) were performed with the Microtox® Omni 1.16 software. Mean $EC_{10/20/50}$ resulting from each three replicates were calculated and expressed as mg/L (with respective 95 % confidence limits). The reference toxicant was zinc sulfate eptahydrate and results obtained for this assay fell into the laboratory Control Chart (6.88-12.45 mg/L Zn^{2+}).

3.2.2.2 *Phaeodactylum tricornutum* - growth inhibition

The assessment of growth inhibition on *P. tricornutum* was performed following ISO procedures, with slight changes to the base protocol (ISO 10253, 2016). *P. tricornutum* Bholin (CCAP 1052/1A) was the tested strain and was purchased from the reference center CCAP (Culture Collection of Algae and Protozoa—Scottish Association for Marine Science/SAMS Research Services Ltd). ASTM Enriched Saltwater Medium (ASTM-ESM, NSW supplemented with a salt mix and a vitamin mix, as described in ASTM E1218, 2012) was used for culturing *P. tricornutum* algae. The test started by inoculating the late logarithmic phase algae in 2 mL of fresh medium (24-well multiwall plates). Initial concentration was of 10^4 cells/mL and inoculates were left to grow at 20±2 °C, under cool white fluorescent continuous light (7000 lx) and slow shaking (80 rpm) for 72 h. Experiments ran in triplicate and the medium (ASTM-ESM) acted as control. Growth inhibition (cells/mL) after 72 h was the evaluated endpoint. Algal concentration was measured making use of a spectrophotometer (λ=670 nm) and samples were read in 1 cm optic-path cells (adaptation of ISO 10253:2016). Values of $EC_{10/20/50}$ for all seven UV filters were calculated by the Linear Interpolation Method for Sublethal Toxicity software (U.S.EPA, 1993). The reference toxicant was potassium dichromate and results obtained for this assay fell into the laboratory Control Chart (1.94-4.36 mg/L Cr^{2+}).

3.2.2.3 *Ficopomatus enigmaticus* – larval development assay

Ficopomatus enigmaticus (Fauvel, 1923) is a sessile polychaete from the Serpulidae family. In shallow brackish water estuaries and lagoons, it forms extensive reefs of calcareous tubes which encrust on hard substrates such as rocks, wood, shells, poles and piers (Oliva et al., 2018; Styan et al., 2017). Organisms can endure a wide range of temperature and salinity conditions, are characterized by high growth rate and abundance, and their larvae display dispersal behaviour, after fertilization has occurred externally (Fornós et al., 1997; Pernet et al., 2016).

Ficopomatus enigmaticus' reef pieces were collected in S. Rossore-Migliarino Regional Park – Fiume Morto (Pisa, Italy). Organisms were collected during the late summer period (September) and transferred to laboratory in wet conditions. Aliquots of water from the sampling site were also collected and used to setup the aquaria. Environmental salinity during sampling was 21. In laboratory it was increased, up to a maximum of 5 points/day, until reaching 30, value defined as within the optimum range for *F. enigmaticus* larval development (Oliva et al., 2018). During acclimation period, organisms were daily fed with an *Isochrysis galbana* algal suspension (final concentration in aquaria = 1×10^4 cells/mL). After 3 days, conditions in the aquaria were considered good for the bioassay execution (T 22 ± 1 °C, oxygen saturation > 90 %, salinity 30, pH 8.1 ± 0.1, photoperiod – 10 h light: 14 h darkness).

Larval-development assay was performed according to the protocol described in Oliva et al. (2019). Briefly, polychaetes were gently detubed in order to induce gametes emission (Hadfield et al., 1994; Nedved and Hadfield, 2009). Each organism was collected separately, rinsed few seconds in tap water to prevent any spermatic contamination, and then placed in a microplate well containing 1 mL of ASW (artificial sea water; ASTM, 2013), at salinity 30. After gametes release (5-10 min), eggs from each female were subjected to a fertilization pre-test, in order to select viable suspensions for the assay. The criterion was cell-division synchrony, which was checked with an inverted microscope (Leica DMIL). Selected egg suspensions were pooled in 300 mL ASW.

The egg suspension was rinsed three times with fresh ASW. This step aimed to remove damaged and immature eggs. Then eggs were counted and the clean suspension was diluted to obtain a concentration of 300 cells/mL. To proceed with fertilization, freshly collected sperms (from 4-5 males) were pipetted into the clean egg suspension, at a final concentration of 3×10^5 cells/mL. The suspension was gently stirred and left at 22 ± 1 °C for about 40 min (time needed for fertilization to occur).

One mL of fertilized-egg suspension was pipetted into each treatment replicate in order to make up to a final volume of 10 mL. All replicates were incubated for 48 h at 20 ± 2 °C, under a photoperiod of 10 h light: 14 h darkness. After the incubation time has passed, the assay was stopped by adding few drops of buffered 37 % formaldehyde in each replicate tube. As reported in Oliva et al. (2020), observing the shape displayed by the larvae, all appendices and size, the number of correctly developed and abnormal larvae was counted so it could be possible to calculate a percentage of poorly developed larvae. The acceptability threshold of the assay was set at 20 % of poorly developed larvae in controls. The reference toxicant was copper sulphate pentahydrate, results obtained for this assay fell into the laboratory Control Chart (40.05-71.02 µg/L Cu^{2+}). The mean percentage of poorly developed larvae was calculated for each assessed concentration of all compounds. $EC_{10/50}$ values and their 95 % confidence intervals were calculated via a PROBIT analysis (Finney, 1971). EC_{50} values were compared with a One-way ANOVA followed by a post-test (Tukey test for multiple comparison).

3.2.2.4 *Ficopomatus enigmaticus* larval development assay under different light and temperature conditions

The larval-development assay was repeated for 2 selected UV filters (nZnO and EHMC), using the same concentrations and procedures described before but testing the influence of temperature and light. For this, two different conditions of light (photoperiod and darkness) and temperature (20 and 25 °C) were selected, considering the potential occurrence of photo/thermo degradation. The experiment was run in triplicate and 4 sets of dilution and their replicates were prepared. Each set was incubated in a different combination of temperature and photoperiod, as following:

1) 20 °C, photoperiod of 10 h light: 14h darkness
2) 20 °C in darkness
3) 25 °C, photoperiod of 10 h light: 14h darkness
4) 25 °C in darkness.

The assay was stopped after 48 h of incubation. Percentage of poorly developed larvae of each treatment was calculated as reported before.

Percentage of effect as poorly developed larvae at each tested concentration in different light and temperature conditions were compared with a Two-way ANOVA (variables: concentration vs exposure conditions) followed by a post-test (Bonferroni's test).

3.3 Results

3.3.1 Vibrio fischeri - bioluminescence inhibition

Concerning the *V. fischeri* bioluminescence inhibition test, the screening test (not shown) indicated that only nZnO, EHMC, AVO and BP3 revealed an I% > 20 %. For this reason, only these compounds were submitted to a full test for the ECx calculation (Table 5). EHMC was the most toxic compound (EC_{50}=1.06 mg/L) with the lowest EC_{10}=0.06 mg/L. On the contrary AVO and BP-3 did not exhibit any toxicity within the test conditions, while nZnO showed an EC_{50}=8.57 mg/L and an EC_{10}= 2.43 mg/L.

Table 5 - *Vibrio fischeri*: inhibition of bioluminescence-full test (30 min of exposure). Results are expressed as $EC_{10/20/50}$ (mg/L) together with 95 % confidence limits (C.L.). ECs were obtained by the Least Square method (n.c: not-calculable).

UV filter	**EC_{10}**	**(C.L. 95 %)**	**EC_{20}**	**(C.L. 95 %)**	**EC_{50}**	**(C.L. 95 %)**
ZnO	2.43	n.c	3.87	n.c	8.57	n.c
EHMC	0.07	(0.05-0.09)	0.19	(0.16-0.23)	1.06	0.99-1.13
AVO	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
BP-3	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.

3.3.2 Phaeodactylum tricornutum - growth inhibition

For the *P. tricornutum* growth inhibition test, obtained values of EC_{50} and respective 95 % confidence limits are presented in Table 6. Nanoparticulate TiO_2 showed the lowest EC_{50} (0.043 mg/L), followed by EHMC (2.33 mg/L) and BP-3 (2.42 mg/L). The ones inducing lower effect were 4-MBC (3.90 mg/L), nZnO (4.55 mg/L) and AVO (9.45 mg/L). The least toxic compound was OCTO, with a not calculable EC_{50}. Calculated values almost matched the order displayed above (in decreasing order of toxicity: $nTiO_2$ > BP-3 > EHMC > 4-MBC > nZnO > AVO > OCTO).

Table 6 - *Phaeodactylum tricornutum*: inhibition of growth. Values of $EC_{10/20/50}$ (mg/L) together with 95 % confidence limits (C.L.), were calculated for each UV filter. ECs were obtained by a linear interpolation method. (n.c: not calculable).

UV filter	EC_{10}	C.L. 95 %	EC_{20}	C.L. 95 %	EC_{50}	C.L. 95 %
TiO_2	0.021	(0.011-0.027)	0.028	(0.024-0.031)	0.043	(00.42-0.044)
ZnO	0.79	(0.55-0.99)	1.34	(0.11-1.54)	4.55	n.c
EHMC	0.64	(0.45-0.71)	0.84	(0.76-0.90)	2.33	(1.93-3.75)
BP-3	0.31	(0.29-0.34)	0.62	(0.59-0.67)	2.42	(2.17-3.09)
4-MBC	0.70	(0.48-0.87)	1.06	(0.91-1.21)	3.90	(3.40-4.49)
AVO	2.01	(1.87-2.16)	4.02	(3.80-4.31)	9.45	(8.96-10.15)
OCTO	10.54	(9.30-11.83)	13.95	(13.41-14.91)	n.c	n.c

3.3.3 Ficopomatus enigmaticus – larval development assay

Different percentages of effect (as the proportion of poorly developed larvae), induced by each concentration of each assessed UV filter, were used to calculate EC_{50} values. Percentages of bad development produced by $nTiO_2$, at the highest concentrations, were less than 20 % (the threshold set for control acceptability) and, thus, EC_{50} values were not calculated for this compound. All obtained EC_{50} values, with their 95 % confidence limits, are reported in Table 7, them being 0.836 mg/L for 4-MBC, 1.088 mg/L for nZnO, 2.806 mg/L for EHMC, 4.035 mg/L for BP-3, 9.89 mg/L for AVO, and 14.736 mg/L for OCTO. Calculated EC_{10}s, intended as no-effect concentration, are also listed in Table 7. These results influenced the selection of nZnO and EHMC for a further larval development assay, where the influence of temperature and photoperiod on the toxic effect of those two compounds was evaluated.

Table 7 - *Ficopomatus enigmaticus*: larval development assay. Values of $EC_{10/50}$ (mg/L) together with 95 % confidence limits (C.L.) were calculated for each UV filter by the use of a PROBIT model; n.c: not calculable because badly developed larvae were always under 20 %.

UV filter	EC_{10}	C.L 95%	EC_{50}	C.L 95%
TiO_2	n.c.	n.c.	n.c.	n.c.
ZnO	0.054	(0.010-0.130)	1.088	(0.697-1.588)
EHMC	0.083	(0.008-0.224)	2.806	(1.690-5.692)
BP-3	2.322	(1.612-2.858)	4.035	(3.418-4.556)
4-MBC	0.132	(0.061-0.216)	0.836	(0.616-1.070)
AVO	1.418	(0.169-2.792)	9.89	(7.538-13.762)
OCTO	3.878	(1.414-5.653)	14.736	(11.849-22.355)

3.3.4 Ficopomatus enigmaticus larval-development assay under different light and temperature conditions

Concentration-response curves resulting from the larval development assay under different conditions of light and temperature, with EHMC (organic) and nZnO (inorganic), showed decreasing percentages of well-developed larvae with the increase of contaminant concentrations (Figure 7). When exposed to different light and temperature conditions, concentration-response curves of developed larvae showed different trends, even if the resulting EC_{50} values of each compound were not significantly different among the different conditions (Table 8).

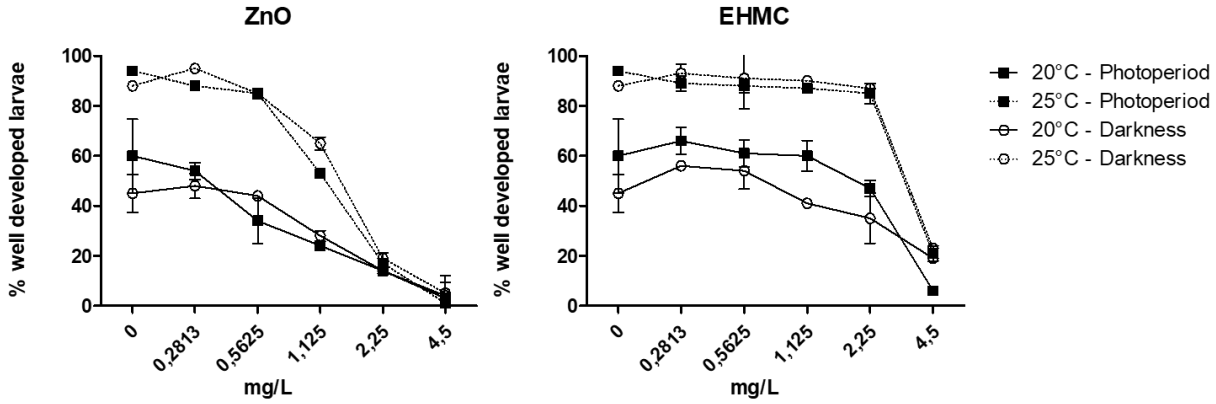

Figure 7 – Concentration-response curves of larval development assay, performed on both ZnO and EHMC, under different Temperature (20 - 25 °C) and Light (Photoperiod – Darkness) conditions. Values are reported as Mean ± SD, n=3. Solid line = 20 °C; dotted line = 25 °C; full squares = Photoperiod (10 h light: 14 h darkess); empty circles = Darkness.

Table 8 - *Ficopomatus enigmaticus*: larval development assay under conditions of photoperiod/dark, at T 20 °C/ 25 °C. Values of $EC_{10/50}$ (mg/L) together with 95 % confidence limits (C.L.) were calculated for ZnO and EHMC UV filters by the use of a PROBIT model. No statistically significant differences were observed.

	Photoperiod - EC_{50} C.L. 95 %		**Dark - EC_{50} C.L. 95 %**	
UV filter	**20 °C**	**25 °C**	**20 °C**	**25 °C**
ZnO	0.830 (0.523-1.134)	1.287 (1.122-1.448)	1.463 (0.912-1.920)	1.498 (1.300-1.691)
EHMC	2.870 (2.418-3.244)	3.64 (3.210-3.958)	3.301 (1.999-4.847)	3.725 (3.258-4.043)

For all treatments, except for darkness at 20 °C, the reduction of successful development was more considerable for nZnO where percentages decreased from the lowest concentration to the highest one, while EHMC presented a more gradual reduction (Figure 8). In fact, nZnO has produced lower EC_{50} values than EHMC (0.83 and 2.87 mg/L - photoperiod (20 °C); 1.287 and 3.64 mg/L - photoperiod (25 °C); 1.49 and 3.72 mg/L - dark (25 °C), respectively). The lowest percentages of well-developed larvae were observed for nZnO and EHMC in the 20 °C darkness treatment (EC_{50}s of 1.463 and 3.301 mg/L for nZnO and EHMC; respectively), and for nZnO under photoperiod at 20 °C (EC_{50} - 0.830 mg/L), with highest values reaching 50-56 %. Overall, for both nZnO and EHMC, the percentage of correct development was higher when exposure occurred at 25 °C (under the two treatments of photoperiod and darkness). The two-way ANOVA analysis indicated that the main source of variation is represented by the concentrations of both compounds [% of total variation higher for nZnO (70.96) than EHMC (55.32)], followed by the treatment combination (temperature and photoperiod). Regarding the temperature and photoperiod, the Bonferroni's post-test underlined that 25 °C positively affected the development of nZnO-exposed larvae within all tested concentrations except the higher ones. Higher temperature also positively affected the development of EHMC-exposed larvae within all tested concentrations except the highest one in darkness condition.

3.4 Discussion

The marine bioassays battery showed that all assessed UV filters were able to produce toxic effects in almost one or more of the selected species through different endpoints: inhibition of bioluminescence in *V. fischeri*, growth inhibition of *P. tricornutum* and impacts on larval development in *F. enigmaticus*.

Regarding *V. fischeri*, the toxic effects of nZnO have been strongly associated with its dissolution into zinc ions (Wehmas et al., 2015). This process was suggested to be induced by the close contact between the particle and the bacterial cell wall (Heinlaan et

al., 2008), which might be the explanation for differences between the present work outcomes and the ones obtained by Schiavo et al. (2018), due to different dissolution/interaction rates. Nanoparticulate ZnO was the fifth most toxic to *P. tricornutum* and the second most toxic UV filter for the developing larvae of *F. enigmaticus*, the last result in accordance with Fairbairn et al. (2011), reporting that nZnO had a strong toxic effect on the early development of the white sea urchin *Lytechinus pictus*. Toxicity was, once more, linked to dissolved Zn^{2+} ions, and discrepancies between *F. enigmaticus* and *L. pictus* sensitivities may be justified by physiological aspects, such as the duration of the developmental phase and distinct times for element uptake. In fact, the sea-urchin skeletogenesis begins at an early stage of the development (O'Donnell et al., 2010), while calcareous tubes production by *F. enigmaticus* starts later, after larval settlement (Casu et al., 2019)). Regarding results of nZnO, *F. enigmaticus* larvae exposed at different conditions, for both assessed light conditions (photoperiod and dark), the highest temperature generally improved the percentage of successful development. A possible explanation might be that *F. enigmaticus* larvae follow the pattern observed for almost all other invertebrates where low temperatures generally increase the time needed for development (Kupriyanova et al. 2001). Another explanation of reported results may be found in chemical behaviour of nZnO in solution, which can produce higher toxicity due to dissolution rates and photocatalytic reactions (Reed et al., 2012; Lee and An, 2013). Considering these observations, nZnO would not be stable in the environment but rather dissociate into its ionic form and thus represent a risk for marine and brackish water biota. Moreover, no statistically significant differences were observed between calculated EC_{50}s.

Relatively to the other assessed inorganic filter, $nTiO_2$, this was the compound that mostly affected *P. tricornutum* growth. Contrarily to the present work findings, Wang et al. (2016) reported the low toxicity of this UV filter, attributing results to the formation of considerably large $nTiO_2$ aggregates. However, tested exposure concentrations were higher than those assessed in the present work, with consequent higher sedimentation rate, fact that would diminish interaction between particles and cell walls and/or organisms. The same low toxic effect of $nTiO_2$ was observed, in the present study, for the larval development of *F. enigmaticus*. Due to lack of statistically relevant effect at all assessed $nTiO_2$ concentration, coupled with the fact that they were higher than those predicted for aquatic environments (Mueller and Novack, 2008; Gottschalk et al., 2009; Gondikas et al., 2014), results were not reported.

Regarding BP-3, this filter was the third most toxic to *P. tricornutum*. Results on *F. enigmaticus* showed an EC_{50} value comparable to findings of Paredes et al. (2014) for *M.*

galloprovincialis (4.035 mg/L versus 3.472 mg/L, respectively). Moreover, the same authors found 4-MBC, EC_{50} value of 0.854 mg/L for *M. galloprovincialis*, again comparable with *F. enigmaticus* (0.836 mg/L), confirming the sensitivity and suitability of using *F. enigmaticus* in ecotoxicological testing.

Both *P. tricornutum* and *F. enigmaticus* showed a high value of EC_{50} for AVO, not indicating great toxicity being induced by this compound.

While OCTO was the UV filter that least affected the correct development of *F. enigmaticus* larvae, Giraldo et al. (2017) have reported considerable toxicity of this compound to the early developmental stages of *P. lividus*.

The UV filter EHMC was the most toxic for *V. fischeri* and the second most toxic for *P. tricornutum*, right after $nTiO_2$ and followed by BP-3, 4-MBC, nZnO and AVO (an EC_{50} could not be calculated for OCTO). This UV filter was identified as an endocrine disruptor, able to impair the early development of fish (Balázs et al., 2016). Among the relative literature on invertebrates, Paredes et al. (2014) obtained an EC_{50} value with *M. galloprovincialis* embryotoxicity similar to the one calculated for *F. enigmaticus* in the present study (3.118 mg/L and 2.806 mg/L, respectively). For the larval development assay performed at different incubation conditions, percentages of correct development were again higher at 25 °C, while maximum toxicity was at 20 °C under photoperiod, similarly to nZnO. Moreover, comparing the two light exposure treatment outcomes, concentration-effect distribution was the same between them, indicating that toxicity was not influenced by photoperiod. Differences could only be perceived between the two selected temperatures, with samples exposed to 20 °C displaying lower percentage of well-developed larvae. These findings may indicate that experimental light intensity does not affect EHMC structure and/or its toxicity, according to Zhou et al. (2019), which observed few toxicity differences between EHMC and its degradation by-products. Overall, independently from its chemical status, EHMC or its by-products represent a hazard in coastal and marine systems (corroborated by the observation of no statistically significant differences between treatments).

Lastly, 4-MBC was the most toxic for *F. enigmaticus* larvae while being the fourth most toxic to *P. tricornutum*. The result obtained for *F. enigmaticus* agrees with the study of Torres et al. (2016), that has observed great toxicity of 4-MBC towards *D. rerio* and *P. lividus* embryos development.

In order to investigate ecotoxicological relevance, EC_{10} values (as no-effect concentration, according to Warm and van Dam, (2008)) were calculated for all three tested species. Concerning *P. tricornutum*, only $nTiO_2$ could be closer to represent an

environmental risk given that its value can be expressed as µg/L. Predicted environmental concentrations for nZnO are being set as values ≤ 2 mg/L (Luo et al., 2015). For EHMC and BP-3, concentrations already measured in the environment were found to be in the range of ng to µg/L (e.g. 216 ng/L of BP-3 were detected in Mediterranean waters) (Ramos et al., 2015; Mao et al., 2018; Nguyen et al., 2011). Since all other obtained EC_{10}s were expressed as mg/L, a real risk cannot be deduced from observed toxicity, at selected concentrations. Nonetheless, these values may become environmentally relevant when considering that EHMC and BP-3 are among the most used filters and that their consumption and usage frequency are predicted to increase (resulting in higher environmental concentrations). The same projection is suitable for nZnO.

3.5 Conclusion

The present study constitutes an important contribution to providing ecotoxicological data on the potential toxicity of UV filters (effects and behaviour under environmental factors) reporting a complete dataset of ecotoxicological parameters (EC_{50} and EC_{10}, as no-effect concentration) on two organisms with standardized assay protocols (*V. fischeri* and *P. tricornutum*) and on a promising-model one (*F. enigmaticus*). The results obtained identified 4-MBC as the most toxic compound to *F. enigmaticus* while TiO_2 and EHMC were the most toxic UV filters to *P. tricornutum* and *V. fischeri*, respectively. The increase of temperature revealed higher percentages of correct development. Furthermore, the present study revealed that the toxicity of both filters was not influenced by exposure to a photoperiod. However, further experiments are mandatory to describe both UV-filter effects at different organization scales and possible effects of by-products which may derive from UV-filters complexation/degradation phenomenon in natural environments.

CHAPTER 4. BIOCHEMICAL RESPONSE OF *Ficopomatus enigmaticus* ADULTS AFTER ORGANIC AND INORGANIC ULTRAVIOLET FILTERS CHRONIC EXPOSURE

This chapter will be submitted as:

Matilde Vieira Sanches, Lucia De Marchi, Matteo Oliva, Alessia Cuccaro, Rosa Freitas, Carlo Pretti. Biochemical responses of *Ficopomatus enigmaticus* after exposure to organic and inorganic UV filters.

4. Biochemical response of *Ficopomatus enigmaticus* adults after organic and inorganic ultraviolet filters chronic exposure

4.1 Introduction

Sunscreen consumption and commercialization have increased with growing awareness about the risks of UV radiation. At present, they are expected to keep rising with coastal tourism in fast development and the growth of population inhabiting coastal areas (Sánchez-Quiles and Tovar-Sánchez, 2015). These compounds, already found in surface waters and sediments, are entering the aquatic environment both directly – via wash-off during swimming and recreational activities - and indirectly – as surface runoff, industrial and urban effluents (bathing and clothes washing) (Poiger et al., 2004; Tovar-Sánchez et al., 2013; Tsui et al., 2014). Benzophenone-3 (BP-3) is now the most detected compound in coastal environments (Ramos et al., 2015; Mao et al., 2018). Nevertheless, for the two UV filters selected in this study, environmental concentrations of Zinc Oxide (ZnO) are being predicted as relatively high values as ≤ 2 mg/L (Luo et al., 2015) and Ethylhexyl methoxycinnamate (EHMC) concentrations, already measured in the environment, were values ranging from ng to µg/L (Ramos et al., 2015; Mao et al., 2018).

As a result of the increasing awareness of their potential threats to aquatic organisms, recent studies have been evaluating the impacts of ZnO and EHMC towards different marine species. Zhao et al. (2013) found ZnO nanoparticles (NPs) to be genotoxic, responsible for oxidative stress and capable of inducing developmental impairment in zebrafish. For the marine algae *Chlorella vulgaris*, Suman et al. (2015) reported that high concentrations of ZnO NPs (200 and 300 mg/L) induced an increase in the antioxidant enzyme superoxide dismutase (SOD) activity and lipid peroxidation (LPO) levels. The UV filter EHMC is known as an endocrine disruptor and teratogenic compound (Kaiser et al., 2012; Balázs et al., 2016). Few studies described its capacity to induce neurotoxicity and oxidative stress. Among them, generational studies and exposure of zebrafish embryos to EHMC were performed by Zhou et al. (2019). These authors concluded that, for F0 generations at 120 days post-fertilization, EHMC inhibited acetylcholinesterase activity and increased the activity of SOD.

Ficopomatus enigmaticus polychaetes might be also a target for organic and inorganic (nanoparticulate) UV filters due to their filter-feeding strategy and sedentary disposition (Canesi et al., 2012; Trevisan et al., 2014; Oliva et al., 2018). Considering the facts above, it becomes relevant to generate ecotoxicological data regarding polychaete species and their biochemical responses to UV filters exposure.

In this context, the present study aimed to assess the effects of the UV-filters ZnO and EHMC on *F. enigmaticus*. Adults (reef pieces) were chronically exposed to both contaminants (0.00, 0.01, 0.1 and 0.5 mg/L), for 28 days. The effects of ZnO and EHMC on these organisms were assessed in terms of biochemical endpoints: energy reserve content (protein content (PROT)), cellular damage (protein carbonylation (PC) and LPO levels), antioxidant and biotransformation enzymes (SOD, glutathione peroxidase (GPx) and glutathione S-transferases (GSTs)), and neurotoxicity enzymes (acetylcholinesterase (AChE) and carboxylesterase (CE)) activity.

4.2 Materials and methods

4.2.1 UV filters

Two UV filters, ZnO and EHMC, were chosen for a chronic exposure assay, in the following nominal concentrations : 0.00, 0.01, 0.1, 0.5 mg/L. Contaminant selection, as well as concentrations, were determined following the previous study presented in Chapter 3, which has ecotoxicologically screened the effects of a set of seven UV filters, among which ZnO and EHMC, on the larval development of *F. enigmaticus*. The selection of both ZnO and EHMC was based on induced toxicity, with obtained results showing a clear decrease in the percentage of well-developed larvae with the increase of both contaminants' concentration.

Due to low solubility in water, both stock solutions were prepared in methanol (< 1 %). Moreover, the suspension of ZnO (as nanoparticles) was sonicated for 1 minute before to use.

EHMC and ZnO were purchased from Merck - Sigma Aldrich (Milan, Italy).

4.2.2 Sampling and chronic exposure assay

Adults of *F. enigmaticus* were collected at S. Rossore-Migliarino Regional Park – Fiume Morto (Pisa, Italy), at the end of November. Organisms were maintained in the laboratory in 10 L aquaria, filled with water from the sampling site (salinity = 20). Salinity was adjusted by increasing up to a maximum of 5 units per day until reaching 30, the value set for larval development assays (Oliva et al., 2018).

Polychaetes were fed twice with an *Isochrysis galbana* suspension (3×10^5 cells/ml) and left to acclimate (one week) to laboratory conditions: 22 ± 1 °C, oxygen saturation > 90

%, salinity 30 and pH 8.1 ± 0.1. Aquaria was kept under a photoperiod of 10 h light: 14 h darkness (Oliva et al., 2018).

After acclimation, adult individuals, as reef pieces of approximately 50 g, were chronically exposed to ZnO and EHMC, for 28 days. Each 3 L aquaria, except those set up as control (filtered FSW at 30 of salinity), was spiked with EHMC or ZnO, in the needed volumes to have the defined nominal concentrations. Three replicates were set for each condition, making up to a total of 21 aquaria for the whole experiment. Water and contaminants were renewed weekly. Organisms were fed with *Isochrysis galbana*, as during acclimation, three times per week for the whole exposure period (28 days).

4.2.3 Preparation of cellular fractions

At the end of the exposure, a pool of organisms, from all three replicates and each condition, was collected removing individuals from their tubes, and weighed (0.3 to 0.5 g). Tissues were homogenized in 0.1 M phosphate buffer (pH 8, 1:4 w/v) with Elvejem potter. Homogenates were then sonicated for 10 s and centrifuged at 9.00'×g, for 20 min at 4 °C. Prepared samples were stored at – 80 °C till the use.

4.2.4 Biochemical Parameters

Cellular fraction extracts were used for a biomarker battery, aiming to determine: energy reserve content (protein content (PROT)), cellular damage (protein carbonylation (PC), lipid peroxidation (LPO) levels), antioxidant activity (superoxide dismutase (SOD) and glutathione peroxidase (GPx)), biotransformation (glutathione S-transferases (GSTs)) and neurotoxicity (acetylcholinesterase (AChE) and carboxylesterase (CE)). All the analyses were performed with a BioTek Synergy HT micro-plate Reader.

4.2.4.1 Protein content

The PROT content was determined following the methodology of Lowry et al. (1951), with bovine serum albumin (BSA) as standard (100 µg/mL). Under alkaline conditions, copper ions react with the peptide bonds of proteins and the Folin reagent is reduced, resulting in a colour change that allows protein quantification. The reaction mixture consisted of 1 mL of H_2O (as blank), 1 mL of sample, another 1 mL of standard and 0.5 mL of NaOH, in all test vessels. Then, 2.5 mL of Cu reactive solution and 0.25 mL of Folin reagent were added to the previous mixture, after 25 and 10 min, respectively. The

absorbance was determined spectrophotometrically, after 30 min, and measured at a wavelength of 750 nm. Results were expressed in PROT/mg/mL.

4.2.4.2 Cellular damage

The quantification of PC levels was performed in accordance with Mesquita et al. (2014). The protocol foresees the addition of NaOH (200 µL at 6 M) after 2,4-dinitrophenylhydrazine (DNPH 400 µL, at 10 mM in 0.5 M H_3PO_4) has been added to the protein solution (400 µL). DNPH reacts with the carbonyl groups to form 2,4-dinitrophenylhydrazone (Levine et al., 1990). The step of adding NaOH allows to determine the concentration of carbonyl groups in oxidized proteins with minimal interference of DNPH. Absorbances were then measured at 450 nm, after 10 min of incubation (using a molar extinction coefficient (ε) = 0.022 $mM^{-1}cm^{-1}$), and results were expressed in nmol/mg PROT.

The levels of LPO were measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979) protocol. This methodology is based on the reaction of LPO by-products, namely malondialdehyde (MDA). The reaction mixture was prepared with 0.1 mL of sample, 0.2 mL of sodium dodecyl sulfate (SDS, 8.1%), 1.5 mL of 20 % acetic acid solution (various pHs, then adjusted with NaOH) and 1.5 mL of TBA aqueous solution. The mixture was heated at 95 °C, for 60 min, and, when cooled down, a solution of both n-butanol and pyridine was added. After centrifugation, the amount of MDA was quantified spectrophotometrically and measured at 532 nm, using ε = $1.56x10^5$ $M^{-1}cm^{-1}$. Results were expressed as nmol MDA/g fresh weight (FW).

4.2.4.3 Antioxidant defence and biotransformation mechanisms

Determination of SOD activity followed the protocols of Marklund and Marklund (1974), which allow measuring the inhibition level of pyrogallol autoxidation. In alkaline pH, SOD can inhibit the autoxidation of pyrogallol (reducing the conversion of pyrogallol into the yellow coloured purpurogallin). The higher the SOD activity, the less yellow the sample becomes (Mesa-Herrera et al., 2019). To microplate wells already containing 176 µL of the reaction buffer, 20 µL of sample, 1 mM DTPA and 50 mM cacodylic acid, 4 µL of pyrogallol, at 30 mM, were also added so the reaction could occur. Absorbances were measured at 420 nm, every 15 s for 5 min, and activity was expressed as U/mL (U – defined as the amount of enzyme required to induce 50 % inhibition of pyrogallol autoxidation).

GPx was quantified according to Carmagnol et al. (1983). Enzyme activity was determined with ε of NADPH = 6.22 $mM^{-1}cm^{-1}$ and measured after reduced glutathione (GSH) oxidation coupled to the oxidation of NADPH, the last being catalysed by glutathione reductase. Cumene hydroperoxide worked as substrate for total GPx activity. The reaction mixture consisted of 0.25 mM hydroperoxide, 1 mM GSH, 1.4 units of yeast glutathione reductase, 1.43 mM NADPH and 1.5 mM KCN (in a 100 mM potassium phosphate buffer pH 7.0). In the end, absorbance values were measured at 340 nm, and obtained results were expressed as U/mg PROT (U representing the amount of enzyme that catalyses the formation of 1 µmol of nicotinamide adenine dinucleotide phosphate (NADPH) per min).

The protocol of Habig et al. (1979) was followed for GSTs activity determination. The assay consisted in the addition of 200 µL of reaction solution (potassium phosphate buffer, GSH and CDNB) to 100 µL of sample. Absorbances were then measured at 340 nm and ε was 9.6 $mM^{-1}cm^{-1}$ for 1-chloro-2,4-dinitrobenzene (CDNB). GSTs catalyse the conjugation of the substrate (CDNB) with glutathione, resulting in a thioeter. Values were expressed as U/mg PROT, with U representing the concentration of enzyme that will catalyse the formation of 1 µmol of dinitrophenyl thioeter per min.

4.2.4.4 Neurotoxicity

AChE activity was determined according to the methods of Ellman et al. (1961), which were adapted for measurements in a microplate reader. Acetylthiocholine iodide (5 mM) was used as substrate and quantifications were performed at 20 °C. The reactions, which resulted in the formation of 5-thio-2-nitrobenzoate, a yellow coloured compound (Pohanka et al., 2011), was set to occur in 300 µL of 0.1 M phosphate buffer (pH 8) with 20 µL of 0.01 M DTNB (5,5'-dithio-(2-nitrobenzoic acid)), 20 µL of 0.075 M substrate and 10 µL of tissue extracted sample. In the end, absorbances were measured every minute for 5 min, at 412 nm, and enzymatic activity was expressed in nmol/min/mg PROT.

CE activity was measured through the production of 4-nitrophenolate, the result of nitrophenyl butyrate (pNPB) hydrolysis. The pNPB hydrolysis rate was determined according to Hosokawa and Satoh. (2005) and Solé et al. (2018) (with adaptations to a BioTek Synergy HT micro-plate reader). The mixture for the kinetic assay contained 100 mM phosphate buffer (pH 7.4), the substrate pNPB at 1 mM and S9 fraction. As last step, absorbances were measured at 405 nm, at 25 °C, and the value 18×10^3 $M^{-1}cm^{-1}$ was used as molar extinction coefficient. Obtained results were expressed in nmol/min/mg PROT.

4.2.5 Data analysis

Given the lack of normality of obtained data, PROT, PC, LPO, SOD, GPx, GSTs, AChE and CE have been individually submitted to a two-factor design non-parametric permutational analysis of variance (PERMANOVA, Primer 6.0) (factor 1 - exposure to ZnO or EHMC; factor 2 – all four nominal concentrations). To evaluate the effects of both exposure treatments, a PERMANOVA main test was performed and tests were considered significant if $p \leq 0.05$. It being the case, pair-wise tests were applied to understand the effect of concentration within each exposure treatment and the effect of exposure treatment within each concentration. Null hypotheses tested were: 1) for each UV filter, there were no significant differences among exposure treatments; 2) for a given concentration, no significant differences were observed between both UV filters.

4.3 Results

4.3.1 Biochemical parameters

The PROT content decreased with exposure to both ZnO and EHMC, being the two highest concentrations of both filters (0.1 and 0.5 mg/L) significantly lower than control values (Figure 8). Significant differences in PROT content between UV filters were only observed at 0.1 mg/L, with ZnO exposed polychaetes presenting the lowest value.

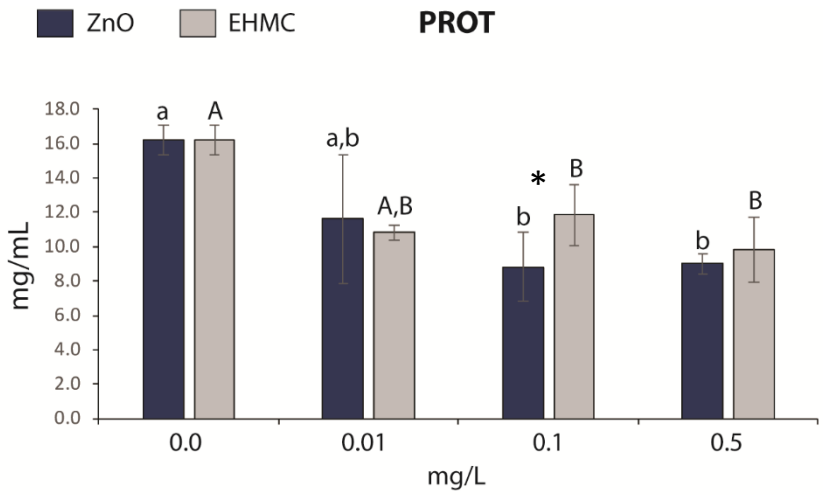

Figure 8 - Protein (PROT) content (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Different letters represent significant differences ($p \leq 0.05$) observed among concentrations. At each concentration, significant differences between contaminants are marked with an asterisk.

4.3.1.2 Oxidative damage

For ZnO, no significant differences in PC levels were observed among treatments (Figure 9). Similar results were also obtained for EHMC, with the exception of the concentration of 0.1 mg/L, which showed significantly lower levels than control organisms. No significant differences were observed between UV filters for a given tested concentration. The lowest value was produced by EHMC, at 0.5 mg/L (0.0075 nmol/mg PROT), and the highest was produced by ZnO, at 0.1 mg/L (0.0129 nmol/mg PROT).

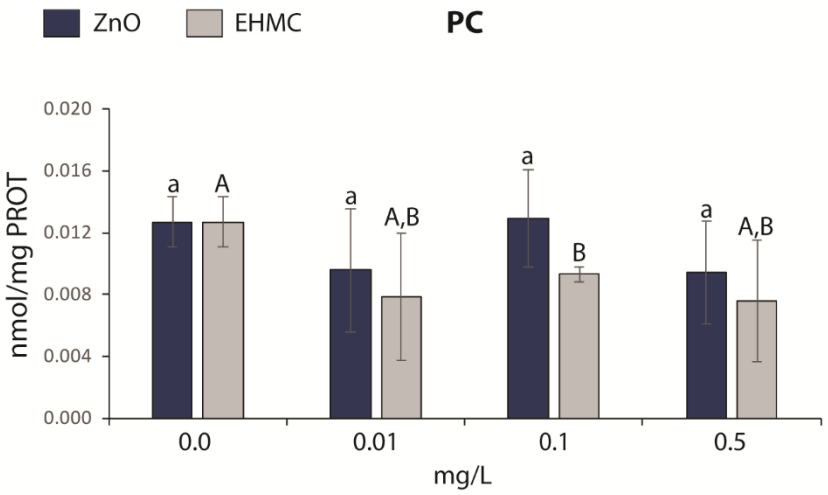

Figure 9 - Protein carbonylation (PC) (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Letters are representing significant differences ($p \leq 0.05$) observed among concentrations.

Regarding LPO levels, no significant differences were observed among control and ZnO treatments (Figure 10). A significant increase (of 185 %) in LPO was observed at the concentration of 0.5 mg/L for EHMC, in comparison to the remaining treatments. Significant differences between UV filters were obtained at the highest tested concentration, with higher LPO levels in polychaetes exposed to EHMC.

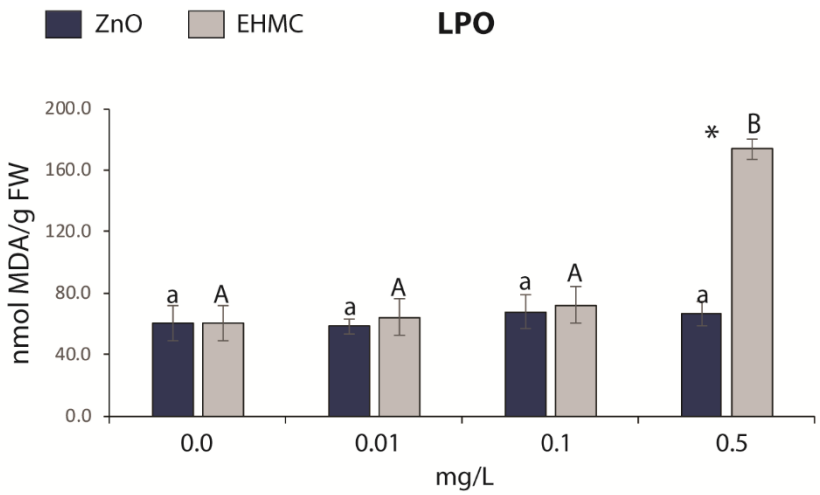

Figure 10 - Lipid peroxidation (LPO) (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Different letters represent significant differences ($p \leq 0.05$) observed among concentrations. At each concentration, significant differences between contaminants are marked with an asterisk.

4.3.1.3 Antioxidant defence and biotransformation mechanisms

Exposure to ZnO produced significant differences at 0.01 mg/L, when in comparison to the control (Figure 11). Superoxide dismutase activity significantly decreased after exposure to EHMC, at both 0.01 and 0.5 mg/L in comparison to the control (1.81 and 1.83 U/mL, respectively, against the value of the control), with no significant differences between 0.1 and 0.5 exposed polychaetes. Significant differences between UV filters were observed at the lowest tested concentration, with polychaetes exposed to ZnO presenting the highest SOD activity.

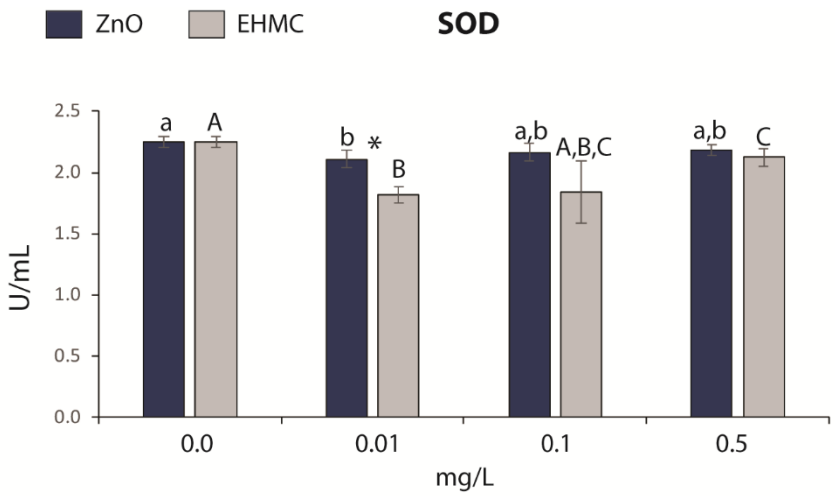

Figure 11 - Superoxide dismutase (SOD) activity (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Different letters represent significant differences ($p \leq 0.05$) observed among concentrations.

Concerning GPx activity, significant differences were observed between the control treatment and the concentration of 0.5 mg/L ZnO (Figure 12), with a decrease of approximately 24 % in GPx activity in organisms exposed to the highest ZnO exposure concentration. Organisms exposed to EHMC showed significantly lower GPx activity at the highest tested concentration (0.5 mg/L) compared with 0.1 mg/L.

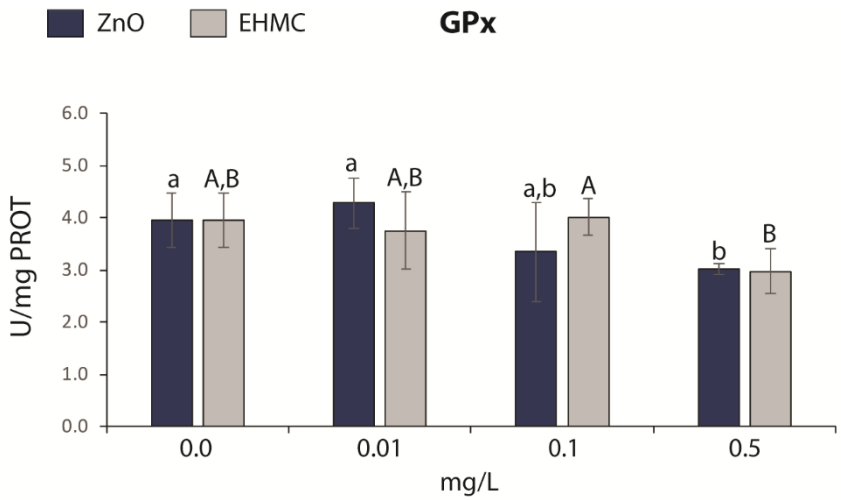

Figure 12 - Glutathione peroxidase (GPx) activity (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Letters are representing significant differences ($p \leq 0.05$) observed among concentrations.

In terms of GSTs activity, *F. enigmaticus* showed a significantly higher activity in organisms exposed to ZnO and EHMC in comparison to control ones (Figure 13). Highest values were found at 0.1 mg/L of ZnO and at the highest exposure concentration of EHMC (0.5 mg/L), corresponding to a 97 % and 95 % increase of the activity for ZnO and EHMC, respectively, in comparison to control treatments. No significant differences were observed between compounds at each tested concentration.

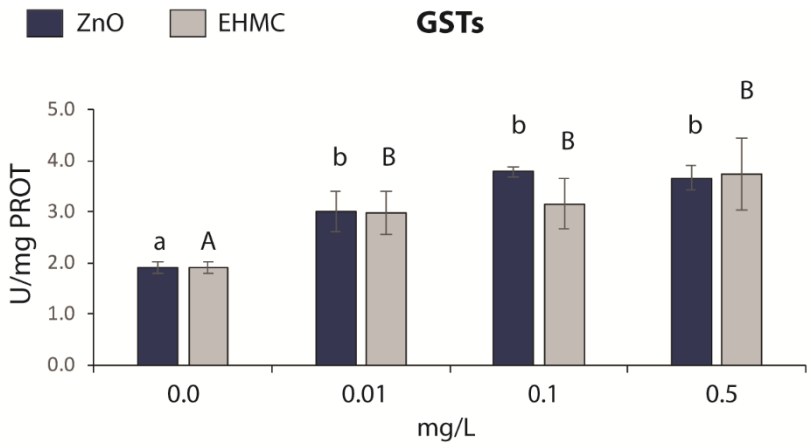

Figure 13 - Glutathione S-transferases (GSTs) activity (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Different letters represent significant differences ($p \leq 0.05$) observed among concentrations.

4.3.1.4 Neurotoxicity

All ZnO and EHMC exposure concentration significantly reduced AChE neurotransmission activity (Figure 14). Moreover, EHMC showed a clear concentration-dependant inhibition effect. The concentration of 0.01 mg/L showed significant differences between both contaminants, with higher AChE activity in polychaetes exposed to EHMC.

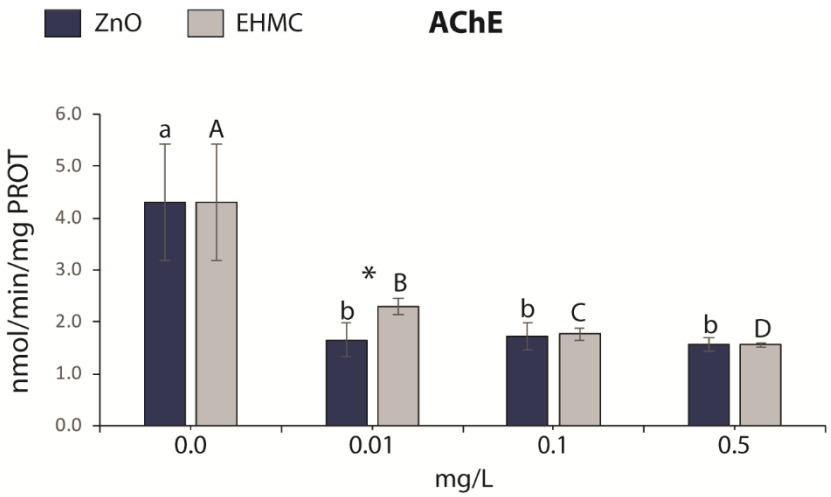

Figure 14 - Acetylcholinesterase (AChE) activity (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Letters are representing significant differences ($p \leq 0.05$) observed among concentrations. Significant differences between contaminants are marked with an asterisk.

Regarding CE activity, no significant differences were observed between ZnO exposed and control organisms (Figure 15). Although a slight decrease was observed at all exposure concentrations for both contaminants, only at 0.1 mg/L of EHMC the activity was significantly lower when compared to control.

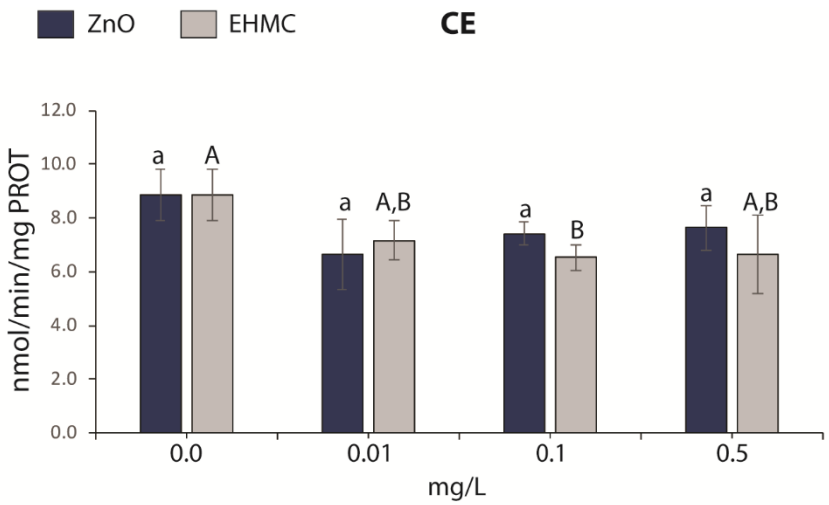

Figure 15 - Carboxylesterase (CE) activity (mean ± standard deviation) in *Ficopomatus enigmaticus* adults exposed to different concentrations of ZnO and EHMC (0.00; 0.01; 0.1; 0.5 mg/L). Letters are representing significant differences ($p \leq 0.05$) observed among concentrations.

4.4 Discussion

The present work aimed to evaluate the biochemical responses of *Ficopomatus enigmaticus* adults after chronic exposure to both ZnO and EHMC in order to gain insight on their toxicity mechanisms. Previous studies described that Nanoparticles (NPs) (e.g. inorganic UV filters – ZnO and TiO_2) induce oxidative stress in marine organisms (e.g. bivalves), whenever reactive oxygen species (ROS) are overwhelming or saturating antioxidant defence action (Al-Subiai et al. 2012; Rocha et al. 2015).

Oxidative damage in terms of lipid peroxidation (LPO) (oxidation of lipids) usually occurs when lipidic membranes are impaired by ROS (Catalá, 2009; Regoli and Giuliani, 2014). For all three concentrations, ZnO did not significantly induce LPO. Contrary to the observed in the present study, Ates et al. (2013) stated that exposure to Zn NPs induced oxidative stress to *Artemia salina* larvae, given that LPO levels were considerably high after 96 h of exposure. These authors tested concentrations of 10, 50 and 100 mg/L, values a hundred times higher than the ones used in this work with *F. enigmaticus* indicating that, even though organisms have undergone chronic exposure, concentrations used in the present study were probably too low to induce lipid peroxidation.

Protein carbonylation happens when ROS promote the oxidation of proteins (Suzuki et al., 2010). No significant differences were observed for PC, indicating that proteins are not being damaged, corroborating the low toxicity of this compound, at studied concentrations.

Superoxide dismutase works in detoxifying superoxide radicals. Its functioning needs the activation of GPx activity to remove the excess of H_2O_2, that has been produced by SOD, thus maintaining the H_2O_2 cellular balance (Halliwell and Gutteridge, 2007). Zinc oxide did not induce considerable changes in SOD activity, probably because selected concentrations might have been too low to influence enzyme activity, adding the fact that, although nanoparticulate ZnO has higher solubility in seawater, it also forms larger aggregates (Wong et al., 2010). Nanoparticle aggregates tend to settle to the bottom and become unavailable in the water column (Zhu et al., 2009). Although aquaria were equipped with an aeration system, it might have happened that ZnO NPs went back to their aggregate state. It might be the case that these aggregates are too large to enter the organisms' body via filter feeding. Given that interaction between nanoparticles and cells would not occur, there would also not be the need to activate antioxidant defence mechanisms mediated by SOD. Also LPO and PC behaviours can be explained by the stated above.

Glutathione peroxidase reduces lipid hydroperoxides by the oxidation of GSH to GSSG, thus performing a direct neutralization of ROS (Regoli and Giuliani, 2014). Our results showed that GPx activity slightly increased at the lowest concentration of ZnO, 0.01 mg/L. Toxicity of ZnO NPs has been largely attributed to dissociation into Zn^{2+} ions (Wehmas et al., 2015). It would be possible that zinc ions, at the lowest concentrations, were being filtered more easily, ending up in close contact with polychaetes' physiology and cells, thus inducing an increase in GPx activity. These results are in agreement with the overall lack of SOD activity and the levels of LPO and PC.

The amount of dissolved zinc ions, with their inherent toxicity, might have been enough to produce an almost concentration-dependent response for GSTs activity. In contrast with the present results, Marisa et al. (2016) found a decrease in GSTs activity when analysing the digestive gland of *Ruditapes philippinarum* exposed to ZnO NPs. Concentrations of ZnO used with *F. enigmaticus* could have produced a manageable amount of ROS, thus the substrate of GSTs would not be depleted. Such would explain the concentration-dependent increase in this enzyme activity, which detoxify cells when there has been exposure to reactive xenobiotic metabolites (Wright and Welbourn, 2002; Newman and Unger, 2003).

During impulse transmission across a cholinergic synapse, AChE is responsible for the breakdown of acetylcholine released into the synaptic cleft being, thus, a good biomarker for neurotoxicity studies (Taylor, 2001; Casu et al., 2019). In the present study, it was demonstrated that ZnO induced a neurotoxic effect to polychaetes chronically exposed to this contaminant. Similarly, it has been described that zinc has inhibitory effects

on AChE activity (Frasco et al. 2005). In addition, there are several studies reporting inhibition of cholinesterases activity in invertebrate organisms exposed to nanomaterials (Gomes et al., 2011; Marisa et al., 2016; De Marchi et al., 2017, 2018). The same might be happening for CE, although the decrease in activity was not so abrupt in comparison to the one observed for AChE. Thus, the obtained results may indicate that ZnO might be a stronger neurotoxicant than it is an oxidative stressor, unlike multi-walled carbon nanotubes (MWCNTs), with De Marchi et al. (2017) demonstrating that MWCNTs can induce both oxidative stress and neurotoxicity to *Diopatra neapolitana* and *Hediste diversicolor* polychaetes.

Biomarker studies are scarcer for EHMC. This UV filter has been associated with endocrine disrupting activity and teratogenicity, feature that might be related, to some extent, to oxidative stress events and neurotoxicity (Zhou et al., 2019). In fact, at the highest concentration of 0.5 mg/L EHMC has induced very high levels of LPO, in comparison to control and lower concentration treatments. Zhou et al. (2019) reported high content of malondialdehyde (MDA) and increased SOD activity in zebrafish that has been exposed to EHMC. This UV filter could be inducing an excessive production of ROS that would next activate antioxidant defence mechanisms. The increase in LPO would then be the consequence of ROS in excess and incapacity of SOD to maintain the balance. The present results demonstrated that SOD activity decreased in exposed polychaetes, which may explain high LPO levels. In fact, it might happen that, when exposed to considerable levels of contamination, antioxidant enzymes become overwhelmed and their activity undergoes progressive decrease, possibly leading to significant depletion (Regoli and Principato, 1995, Frenzilli et al., 2004). As for ZnO, also EHMC has produced a concentration-dependent response for GSTs. Again, such might be explained by the exposure to a manageable amount of ROS, induced by the concentrations used in this study. In the present study, EHMC decreased the levels of AChE, thus inducing neurotoxicity to adults of *F. enigmaticus*. Li et al. (2016), working with zebrafish embryos, reported a similar result with 4-methylbenzyli-dine camphor (4-MBC), a compound that has also inhibited AChE activity. Contrarily, Zhou et al. (2019) found AChE levels to be increased, again in zebrafish exposed to EHMC. Authors suggest that EHMC might have enhanced the production of acetylcholine without leading to its excessive accumulation and/or respective receptor saturation, factors that, if met, induce AChE inhibition.

EHMC followed the same trends produced by ZnO for all biochemical parameters, not being possible to state which one of the two contaminants was, overall, the most toxic to *F. enigmaticus*. In terms of oxidative stress and LPO, EHMC was clearly the most toxic

UV filter. Regarding neurotoxicity (AChE and CE activity), ZnO produced slightly lower values, in comparison to EHMC. These results reveal the importance of assessing a set of biochemical parameters instead of selecting just a few making it, thus, possible to understand better where and how contaminants exert their toxicity.

Confronting results from the larval development assay and the biomarkers study, a more significant effect was induced on the larvae of *F. enigmaticus* in comparison to the response displayed by adults, after exposure to both ZnO and EHMC. This observation goes in accordance with the statement that claims early life stages to be more sensitive than adult forms, when in contaminated environments (Rand et al., 1995; Ross and Bidwell, 2001; Pineda, 2012), further reassuring the relevance of using embryos and larvae in ecotoxicity testing.

CHAPTER 5. FINAL REMARKS

5. Final remarks

This thesis focused on providing a general overview of the issue that is the contamination of coastal areas by two different types of pollutants: trace elements (as Classic pollutants) and UV filters (as Contaminants of Emerging Concern). The contamination history and relevance of the two selected sampling sites (Ria de Aveiro (Portugal) and Fiume Morto, S. Rossore - Migliarino Regional Park (Italy)) was also explored in dedicated subtopics.

Ficopomatus enigmaticus selection as test species was based on a previous study that pointed out this serpulid polychaete as a suitable model organism for testing in Ecotoxicity (Oliva et al., 2018). In fact, results generated by all assays presented and discussed in this dissertation demonstrated *F. enigmaticus*' larvae, and their development as the endpoint, to be responsive and very sensitive to relatively low concentrations of all assessed contaminants. Such constitutes data supporting the proposal of Oliva et al. (2018) and becomes a contribution to the proper establishment of *F. enigmaticus* as a model organism to be used in marine and brackish waters monitoring.

Regarding the population's genetics study, to confirm that the two sampled populations (Mediterranean and Atlantic) belonged to the same species, it allowed to solidly compare and validate the different or similar responses, given as the percentage of correct development, generated by larvae of polychaetes from two distinct geographic locations. Molecular analyses here reported and new sequences uploaded to GenBank are the first data known for Europe, contributing to filling the knowledge gap there is for this part of the world (Yee et al., 2019). Further studies on *F. enigmaticus* genetic structure would be of interest to better understand the origin of this species.

In Chapter 2, the sensitivity demonstrated by larvae exposed to selected trace elements was comparable between the two populations tested, a result that can strengthen the ecological relevance of the used *F. enigmaticus* larval development assay. However, the sensitivity of a wild-caught organism or endpoint can be affected by the presence of the contaminants in the organisms' sampling site and/or biological tissues. Obtained results have contributed to the limited understanding on the effects of contaminants on larval stages of polychaete species and might become an aid in the standardization of bioassay protocols for marine and brackish waters monitoring. Values showed that test organisms collected in different areas could be used in wide-range monitoring plans, with the need to well characterize sampling areas in order to select organisms coming from environments with similar chemical status. To achieve such aims, next-generation tests with embryos,

adults, and multigenerational experiments on different species could be a possible next step.

About UV filters toxicity, discussed in Chapter 3 and 4, results showed that six of the seven selected UV filters induced an important toxic effect to the larval development of *F. enigmaticus*. As an exception, the weak toxicity displayed by TiO_2 was in agreement with studies by other authors. Regarding toxicity and photo/thermo stability of ZnO and EHMC, experimental data showed, again, that ZnO was more toxic than EHMC (for all assessed treatments). Higher temperature did play a role in increasing the percentage of well-developed larvae due to a physiological behaviour that is displayed by most invertebrate species. Considering such, temperature values on the original larval development assay protocols might be altered for better percentages on control treatments. It was described that ZnO does undergo environmental fate processes that enhance its toxicity and, in the case of EHMC, undergoing degradation or not, both the parent compound and/or its by-products will potentially impact the marine environment and biota.

This study could constitute an important contribution to strengthening ecotoxicological data on the potential toxicity of UV filters (effects and behaviour under environmental factors) and presents data generated by organisms of three trophic levels, differently affected under UV filters exposure.

Highlights from the last Chapter, about chronic exposure of adults to both ZnO and EHMC, followed by biochemical parameters analysis, were the considerable oxidative damage EHMC has induced at the highest concentration tested (0.5 mg/L), the clear concentration-dependent response GSTs has shown under exposure to both contaminants and the significant neurotoxicity induced by the two UV filters, mainly displayed by the great decrease in AChE activity. More biomarker studies regarding UV filters toxicity must be performed to strengthen literature on this topic and the test species range should be broadened for a better understanding of each different group of organisms is liable to be affected.

CHAPTER 6. REFERENCES

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