UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXVI CICLO DEL DOTTORATO DI RICERCA IN

AMBIENTE E VITA

EMERGING CONTAMINANTS:

ECOTOXICOLOGICAL EFFECTS AND RELATION WITH PARAMETERS ASSOCIATED TO GLOBAL CHANGE SCENARIO

Settore scientifico-disciplinare: **BIO/13**

DOTTORANDA
FRANCESCA PROVENZA

COORDINATORE
PROF. GIORGIO ALBERTI

SUPERVISORE DI TESI
PROF. ANTONIO TERLIZZI

CO-SUPERVISORE DI TESI
PROF.SSA MONIA RENZI

ANNO ACCADEMICO 2022/2023

Index

INDEX 1

INTRODUCTION 6

EMERGING CONTAMINANTS 6

References 13

MUSSEL WATCH PROGRAM FOR MICROPLASTICS IN THE MEDITERRANEAN SEA: IDENTIFICATION OF BIOMARKERS OF EXPOSURE USING *MYTILUS GALLOPROVINCIALIS* 17

Abstract 18

Keywords 18

Introduction 18

Plastics 20

Plastic degradation 21

Photodegradation of plastic 21

Thermal degradation of plastic 22

Biodegradation of plastic 22

Biofilm or biofouling 23

Plastic toxicity 24

Mytilus galloprovincialis as bioindicator of pollution 25

Biomarkers of microplastics in Mytilus galloprovincialis 26

Results from literature search 27

Conclusions and future perspectives 33

References 34

GLOBAL CHANGE 42

Causes and Effects of Climate Change 45

Cause 45

Effects 46

References 47

AIMS OF THE WORK 48

CHAPTER 1 49

IMPACTS OF PLASTIC-MADE PACKAGING ON MARINE KEY SPECIES: EFFECTS FOLLOWING WATER ACIDIFICATION AND ECOLOGICAL IMPLICATIONS 49

Abstract 50

Keywords 50

Introduction 50

Material and Methods 52
Experimental Design, Packaging Types and Leachates 52
µFT-IR Characterization of Plastic-Made Packaging 54
Ecotoxicological Tests: Exposure and Endpoints 54
pH Effects on Embryo Toxicity and Body-Size of P. lividus 55
Quality Assurance and Quality Control 55
EC_{50} Calculation and Statistical Analyses 56
Results 56
µFT-IR Characterization of Plastic-Made Packaging 56
Ecotoxicological Responses at pH Standard (8.1) 56
Water Acidification: Embryo Toxicity and Body-Size Reduction in P. lividus 58
Discussion 63
Conclusions 66
Supplementary Materials 66
References 68
CHAPTER 2 **74**
ECOTOXICOLOGICAL ASSESSMENT OF "GLITTER" LEACHATES IN AQUATIC ECOSYSTEMS: AN INTEGRATED APPROACH 74
Abstract 75
Keywords 75
Introduction 75
Materials and Methods 78
Glitters characterization 78
Leachates preparation 79
Toxicity tests 80
Test on bacteria 80
Test on algae 80
Test on crustacean 81
Test on echinoderms 81
Quality Assurance & Quality Control 81
Statistical analyses 81
Data integration 82
Results 84
Ecotoxicological responses 84
Impact on freshwater system 84
Impact on marine system 86
Species sensitivity 87
Color differences after the soaking time 88
Multivariate analysis 88
Discussion 90
Conclusions 93
Supplementary Materials 94

References 96

CHAPTER 3 **102**

SPARKLING PLASTIC: EFFECTS OF EXPOSURE TO GLITTER ON THE MEDITERRANEAN MUSSEL *MYTILUS GALLOPROVINCIALIS* 102

Abstract *103*

Keywords *103*

Introduction *103*

Material and methods *105*

Experimental design 105

Organisms 106

Glitter particles 106

Experimental condition 107

Glitter recovery 107

Biomarker analysis 107

Statistical analysis 109

Results *109*

Glitter recovery and mortality rate during the preliminary test 109

Glitter recovery and mortality rate during the confirmatory test 110

Biomarkers in the preliminary test 111

Biomarkers in the confirmatory test 112

Discussion *114*

Conclusions *116*

Supplementary Materials *117*

References *118*

CHAPTER 4 **123**

USE OF THE SEDIQUALSOFT® FOR DETERMINING THE TOXICITY OF CIGARETTE BUTTS FOR MARINE SPECIES: A WEATHER SIMULATION TEST 123

Abstract *124*

Keywords *124*

Introduction *124*

Materials and methods *127*

Experimental design 127

Production of the cigarette butts 128

Preparation of the leachates and elutriates 128

Eco-toxicological tests 130

Inhibition of bioluminescence, *Aliivibrio fischeri* (UNI EN ISO 11348-3:2019) 130

Algal growth inhibition, *Phaeodactylum tricornutum* (UNI EN ISO 10253:2017) 131

Fertilization efficiency, *Paracentrotus lividus* (EPA/600/R-95-136/Section 16) 131

Larval development (EPA/600/R-95-136/Section 15) and Larval body-size variations, *Paracentrotus lividus* 131

Quality Assurance and Quality Control 132

Data analyses 132

Results 134
Effect of "combustion" on classic and electronic-CBs toxicity 134
Effects of "combustion"- "time" and "atmospheric condition" on classic-CBs toxicity 136
Discussion and Conclusions 140
Supplementary Materials 143
S.1. Materials and Methods 143
Cigarette Composition 143
Artificial Sea Water (ASW) Composition 144
Composition of the Nutrients used for *P. tricornutum* Test 144
Preparation of Leachates and Elutriates 145
References 145

CHAPTER 5 **150**

ECOTOXICITY OF BASIL (*OCIMUM BASILICUM*) EXTRACT IN AQUACULTURE FEEDS: IS IT REALLY ECO-SAFE FOR THE AQUATIC ENVIRONMENT? 150
Abstract 151
Keywords 151
Introduction 151
Material and methods 154
Chemical profile of basil supercritical fluid extract (F1-BEO) 154
Ecotoxicity bioassay of substances 155
Toxicity bioassay: freshwater organisms 155
Toxicity bioassay: marine organisms 156
Statistical analysis and toxicological categorization 157
Results 158
Freshwater 158
Marine organisms 158
Principal component analysis 159
Toxicological categorization 160
Discussion 161
Conclusions 164
References 164

CONCLUSIONS AND FUTURE PERSPECTIVE **171**

Conclusions 171
Future Perspective 173

Introduction

Emerging contaminants

"Emerging contaminants (ECs) are defined as naturally occurring, manufactured or manmade chemicals or materials found in the environment whose toxicity or persistence are likely to affect the metabolism of a living being significantly."

(Tolboom et al., 2019)

Pharmaceuticals, pesticides, industrial chemicals, surfactants, and personal care products are among the new class of chemical substances now routinely detected in groundwater, surface water, municipal wastewater, drinking water and food. Endocrine disruptors, painkillers, antibiotics, hormones, and a wide range of other pharmaceutical substances, such as anti-inflammatory, anti-diabetic and anti-epileptic drugs, are among these "emerging contaminants."

Most of these compounds have not yet been studied for their environmental and human toxicology, and many of them are not or cannot be tested for their exposure in municipal water systems, making them a threat. In addition, by-products whose chemical composition is still unknown are produced when these toxins pass through drinking water treatment plants. According to the Handbook of Environmental Chemistry (2008), new potential emerging contaminants are discovered every day and new disinfection by-products are also created during treatment without their potential toxicity or impact on human health being known. This statement highlights the danger posed by our ignorance of these compounds.

In attempting to categorize the complicated web of new pollutants, the ecotoxicological viewpoints generally used to describe environmental pollutants resulted not satisfactory. Regarding new pollutants, the conventional toxicological strategy of classifying a substance according to its persistence, lipophilicity (preference for fatty tissue) and toxicity (both acute and chronic) has proven insufficient. As wastewater treatment plants continuously release contaminants into the environment, increasing contamination may exhibit a phenomenon known as "pseudo-persistence" Since many emerging contaminants are pharmaceutically manufactured to be actively absorbed

into cells and tissues, lipophilicity, a property generally used to assess how easily a contaminant would pass through cell membranes or enter tissues, is an insufficient characterization tool.

Instead, it is necessary to investigate and document the absorption, mechanism of action and biological endpoints of each developing contaminant to establish a link between the contaminant and the effect.

According to a strict definition by Smital (2008), emerging contaminants are defined as "any synthetic or naturally occurring chemical or microorganism that is not routinely monitored in the environment but has the potential to enter the environment and have known or suspected adverse effects on the environment and/or human health." It is important to remember that most emerging pollutants are not recently introduced or brand-new pollutants. Rather, most emerging pollutants are already known pollutants with recently discovered adverse effects or modes of action. Some of the naturally occurring emerging contaminants, for example, can be the water disinfection by products (DBPs), as reported by Miraji et al. in 2023. Therefore, the term "emerging" describes both the contamination and the growing concern associated with it. So-called "chemicals of emerging concern" or "pollutants of emerging concern" are other terms for such contaminants.

Emerging pollutants are currently a popular and topical subject of study. For regulators, the sheer volume of emerging pollutants presents a difficulty. How should we prioritize the study of emerging pollutants? How can we order the setting of quality standards or criteria for all these novel compounds about which we generally know very little about how they behave in the environment or how toxic they are to the ecosystem or human health? The popularity of novel pollutants is undoubtedly due to the need of academic researchers to generate interest in their work and obtain funding for their research efforts (Sauvé and Desrosiers, 2014).

Rachel Carson's 1962 book "Silent Spring" is probably responsible for the "emergence" of public awareness of new pollutants (Carson, 2002). She convinced readers that the widespread use of DDT to control mosquitoes and other pests had led to the death and disappearance of many birds, hence the title of the book. At the time, Carson was sharply criticized for daring to question all the societal benefits associated with the use of pesticides in general and DDT in particular.

History confirmed that she was right, and DDT was eventually banned. This is an excellent example of how an environmentalist sounded the alarm, which was followed by academic research to back up the claims with facts and expose the truth and dangers associated with DDT - which itself was first synthesized about a century before Carson's book and was used extensively during World War II. It gave us the revealing insight that pesticides and chemicals in general can be dangerous.

Once we focus on “emerging pollutants”, we need to better define what we are talking about. Since the classification of the term “emerging” is relative, what was a major pollution issue a decade or two ago may not be an emerging pollutant today. In a broader context, we might distinguish the focus on newly emerging pollutants (pollutants that have only recently emerged) from pollutants of concern (pollutants that have been present in the environment for some time but whose concern has only recently emerged).

Finally, we can include more recent concerns about traditional pollutants (new information or data that sheds new light on the concerns of known chemicals). If we consider these issues on a global scale: I) contaminants of emerging interest whose existence was already known but whose pollution problems were not fully recognized or appreciated, II) "real or truly new" emerging contaminants, new compounds or molecules that were not previously known or have only recently appeared in the scientific literature, and III) we also want to address new concerns about "legacy" contaminants, i.e. circumstances in which new information upends our perception of the dangers such legacy contaminants pose to the environment and human health.

Of course, there are many more contaminants that would fall into the category of "real or truly new" emerging contaminants, including pesticides, pharmaceuticals, personal care products, fragrances, plasticizers, hormones, flame retardants, nanoparticles, perfluoroalkyl compounds, chlorinated paraffins, siloxanes, algal toxins, various trace elements such as rare earths and radionuclides, etc. The list of potentially emerging pollutants is long, and these are just a few examples.

Pharmaceuticals have emerged as new pollutants in the last decade (even though they have been consumed for much longer). Naturally occurring hormones are also frequently analyzed and studied along with synthetic steroids and are potent endocrine disruptors. The emerging problem is that as we continue to improve our ability to detect various emerging contaminants (e.g., Anumol et al., 2013; Anderson et al., 2013), we have recognized that the presence of human or animal pharmaceuticals in surface waters is widespread (Benotti et al., 2013).

Concentrations of estrogenic hormones in wastewater and receiving surface waters are often well above the recognized threshold for fish feminization (Viglino et al., 2008) and traces of various pharmaceuticals can even be found in drinking water.

Pharmaceuticals and personal care products (PPCPs), surfactants, plasticizers, pesticides and flame retardants are the main groups of emerging pollutants (Yan et al., 2010). The following section discusses the fact that evolving biological pollutants are also a major concern.

Among the many chemicals that make up PPCPs are cosmetics and health items (such as vitamins, over-the-counter medicines and prescription drugs). After their release from wastewater treatment plants or their leaching from manure applied to fields, these chemicals find their way into the environment and drinking water supplies (Derksen et al., 2004). Although they are often present in high concentrations, like 1-10 g/L, so their potential for harm should not be ignored. PPCPs can have a more negative impact on non-target organisms in the environment than on humans.

Furthermore, the effects of prolonged exposure to these substances at low doses on animals have not been studied and cannot be inferred from acute exposure data. Finally, some PPCPs, such as synthetic estrogens, have been shown to be harmful even at the low levels at which they are found (Liu and Wong, 2013). In an extensive wastewater sampling in the Western Balkans region, analgesics, anti-inflammatories, antimicrobials, -blockers and lipid regulators were most frequently detected (Terzic et al., 2008).

The main antibacterial/antiseptic ingredient in liquid hand soaps, triclosan, is now also found in plastic toys, chopping boards, cutlery, and other products under the brand name Microbans. Although triclosan does not appear to pose a significant threat to human health on its own, it has been shown to be extremely toxic to some aquatic life and breaks down into several compounds that may pose a greater threat to human or wildlife health. In tap water, triclosan produces chlorinated by-products such as chloroform, which is believed to cause cancer in humans. In the environment, it can also photochemically or microbially degrade to certain dioxins, particularly 2,8-dichlorodibenzo-p-dioxin and 2,4-dichlorophenol, as well as a potentially bioaccumulative form of triclosan called methyl, according to Smital (2008).

In the USA and Europe, synthetic fragrances such as nitromusks and polycyclic musks are frequently detected in fish, lakes, and vapors, as well as in human breast milk and blood (Smital, 2008).

Some nitromusks have been linked to reproductive and fertility disorders and cancer. In addition, studies have shown that polycyclic musks have endocrine effects and that nitromusks have neurotoxic effects on animals (Taylor et al., 2014; Wang et al, 2023). The use of several nitromusks in cosmetics and personal care products has been banned in the EU, but polycyclic musks are still largely unregulated.

In the US, almost all musks are unregulated and there are no established exposure limits.

Plasticizers and their metabolites are measured in the environment (Horn et al., 2006) and in sewage treatment plant effluents (Barnabè et al., 2008). Plasticizers are additives used to increase flexibility or plasticity, such as bisphenol A or phthalates. They are particularly known as endocrine

disruptors (Ghisari and Bonefeld-Jorgensen, 2009) and are under scrutiny, with some of the plasticizers already banned or more strictly regulated.

Due to their environmental properties, several fluorinated chemicals, particularly perfluoroalkyl and polyfluoroalkyl (PPFA), have entered the market and are now the focus of stricter regulation.

The Organisation for Economic Co-operation and Development (OECD) in 2021 defined these compounds: "Per and polyfluoroalkyl substances (PFASs) are defined as fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it), i.e., with a few noted exceptions, any chemical with at least a perfluorinated methyl group (–CF3) or a perfluorinated methylene group (–CF2–) is a PFAS."

These compounds contain carbon-fluorine bonds, strong chemical bonds in organic chemistry, that means that they resist degradation when used in the environment. A lot of PFAS are easily transported in the environment and can covering long distances from the source of release (Buck et al., 2011).

Perfluorooctane sulfonic acid and its derivatives, or PFOS, have been banned since 2009 by the international Stockholm Convention. The EU's Persistent Organic Pollutants (POPs) Regulation has already placed restrictions on PFOS for over a decade.

The worldwide eradication of perfluorooctanoic acid (PFOA), its salts, and chemicals linked to PFOA is likewise governed by the Stockholm Convention. Since July 4, 2020, PFOA has been prohibited by the POPs Regulation.

The European Commission decided to impose restrictions on perfluorinated carboxylic acids (C9–14 PFCAs), their salts, and precursors in the EU/EEA starting in February 2023 in response to a proposal put up by the Swedish and German governments.

PFAS have been frequently observed to contaminate groundwater, surface water and soil. Cleaning up polluted sites is technically difficult and costly. If releases continue, they will continue to accumulate in the environment, drinking water and food.

Man-made nanoparticles and water treatment by-products are two other important types of pollutants currently commercialized. Treatment by-products occur when water treatment (drinking or wastewater) leads to the synthesis of new products from the interactions of reagents with the matrix, or when incomplete reactions of target pollutants lead to the production of some by-products that can still be toxic (Lajeunesse et al., 2011; 2013). For example, chlorination can produce trihalomethanes or haloacetic acids as by-products (Bond et al., 2012). Ozone is often proposed as an additional or alternative treatment that could reduce or eliminate such by-products, but because it is so reactive, ozone is also an important source of a variety of by-products.

Examples include the identification of the conversion products of antidepressants (Lajeunesse et al., 2013) or a natural estrogen (Segura et al., 2013).

The situation with nanoparticles is quite challenging. In this situation, it is necessary to rethink the paradigm of risk assessment. In fact, one of the problems with nanoparticles is the extraction from natural matrices and the measuring of it, this can cause an underestimation of the real presence of nanoparticles in environment. At least one of the dimensions of a nanoparticle must be smaller than 100 nanometers. Nanoparticles can be further divided into metal- and carbon-based nanoparticles, such as metal oxides or quantum dots, and carbon-based nanoparticles, such as carbon nanotubes or fullerenes.

Part of the challenge in assessing the risks of nanoparticles to the environment or human health is how to measure them: they cannot be filtered out by conventional means because the nanoparticles are smaller than the pores of the filter. Ultrafiltration is a possible option to separate these particles from their matrix, but it is not easy to implement (Quarato et al., 2021). Carbon-based nanoparticles behave partly like heavy organic compounds and partly like small particles. Therefore, the study has to take both aspects into account. Metal-based nanoparticles also need to be assessed in terms of toxicity to distinguish what proportion of toxicity is due to the metallic components of the nanoparticles dissolving in the media and to compare this with the proportion of toxicity due to the nanoparticles themselves (Pikula et al., 2020). The fate and hazards of nanoparticles in the environment are the subject of extensive research, although there are probably more questions than solutions at present (Tao et al., 2023; Yadav et al., 2023; Mahjoubian et al., 2023).

The advances in environmental chemistry, health, and environmental toxicology, will permit to better prevent harmful effects on environment and health. Emerging pollutants and problems with soil, air and water contamination have been around for millennia without us knowing about them.

Finally, if we wanted a clear definition of emerging contaminants, we might prefer to define contaminants of emerging concern (CEC) as naturally occurring, manufactured, or man-made chemicals or materials that have recently been identified or are suspected of existing in various environmental compartments and whose toxicity or persistence are likely to significantly alter the metabolism of a living being. Such potential CEC should be considered "emerging" if there is a lack of information about them in the scientific literature or there are questions with how poorly the problems they might create are understood (Sauvè and Desrosier, 2014).

In general, we expect CECs to be chemicals that show some potential to pose risks to human health or the environment and which are not yet subjected to regulatory criteria or norms for the protection of human health or the environment. Not all CECs will actually prove to be evil and

have some potential to cause tangible concerns; the focus is that the lack of pertinent environmental fate and ecotoxicological or toxicological data prevent the proper evaluation of associated risks (Qiu et al, 2016). An already regulated presumed well-known contaminant could certainly regain “emerging” status as new scientific information becomes available and thus force regulatory agencies to re-evaluate their norms and guidelines (Jeddi et al., 2022).

The challenges in the years to come will be to better understand contaminants of emerging concern, their concentrations in the environment as well as their toxic effects on organisms to achieve better manage risks to human health and the environment (Khan et al., 2022).

Increased water temperature, altered water currents, elevated salinity, increased erosion, and changes in freshwater runoff patterns in the case of estuarine habitats are just a few of the effects that global changes may have on marine ecosystems (Grabemann et al., 2001; Knowles and Cayan, 2002; DeLorenzo, 2015; McKinney et al., 2015). For improved environmental monitoring significance, research in marine ecotoxicology should currently consider the consequences of worldwide changes in organisms (Noyes and Lema, 2015; Cabral et al., 2019; Tlili and Mouneyrac, 2019). According to certain reports (Hooper et al., 2013; Nikinmaa, 2013; DeLorenzo, 2015; McKinney et al., 2015; Sampaio et al., 2018), there may be interactions between the availability and effects of toxicants and natural environmental reactions related to symptoms of global change.

It is still not enough to understand how toxins could interact with one another, how to assess toxicity, and how global changes affect society (Nikinmaa, 2013; McKinney et al., 2015; Noyes and Lema, 2015; Cabral et al., 2019; Tlili and Mouneyrac, 2019). It is well established that a warmer climate and more acidic and eutrophic marine ecosystems may increase combined stress, making organisms more sensitive to even small perturbations caused by chemical stressors, since organisms are pushed to the limits of their physiological tolerance range (Hooper et al., 2013; Kallenborn et al., 2011). This is supported by available data and simulation scenarios.

The lack of long-term experiments investigating the possible effects of newly emerging problematic issues, such as contamination by plastics (micro and nano), drug residues, nanomaterials, newly synthesized pesticides, or other emergent contaminants, is currently another gap in the literature concerning marine ecotoxicological studies (Tlili and Mouneyrac, 2019).

References

Allanou, R., Hansen, B. G., & Van der Bilt, Y. (1999). Public availability of data on EU high production volume chemicals. European Chemicals Bureau: European Commission.

Anderson, J. C., Carlson, J. C., Low, J. E., Challis, J. K., Wong, C. S., Knapp, C. W., & Hanson, M. L. (2013). Performance of a constructed wetland in Grand Marais, Manitoba, Canada: Removal of nutrients, pharmaceuticals, and antibiotic resistance genes from municipal wastewater. Chemistry Central Journal, 7(1), 1-15.

Anumol, T., Merel, S., Clarke, B. O., & Snyder, S. A. (2013). Ultra high-performance liquid chromatography tandem mass spectrometry for rapid analysis of trace organic contaminants in water. Chemistry Central Journal, 7, 1-14.

Barnabé, S., Beauchesne, I., Cooper, D. G., & Nicell, J. A. (2008). Plasticizers and their degradation products in the process streams of a large urban physicochemical sewage treatment plant. Water research, 42(1-2), 153-162.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D., & Snyder, S. A. (2009). Pharmaceuticals and endocrine disrupting compounds in US drinking water. Environmental science & technology, 43(3), 597-603.

Bond, T., Templeton, M. R., & Graham, N. (2012). Precursors of nitrogenous disinfection by-products in drinking water—a critical review and analysis. Journal of hazardous materials, 235, 1-16.

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., De Voogt, P., ... & van Leeuwen, S. P. (2011). Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. Integrated environmental assessment and management, 7(4), 513-541.

Cabral, H., Fonseca, V., Sousa, T., Costa Leal, M., (2019). Synergistic effects of climate change and marine pollution: an overlooked interaction in coastal and estuarine areas. Int. J. Environ. Res. Public Health 16, 2737. https://doi.org/10.3390/ ijerph16152737.

Carson, R., & Lear, L. J. Silent spring: pbk.

Delorenzo, M. E. (2015). Impacts of climate change on the ecotoxicology of chemical contaminants in estuarine organisms. Current Zoology, 61(4), 641-652.

Derksen, J. G. M., Rijs, G. B. J., & Jongbloed, R. H. (2004). Diffuse pollution of surface water by pharmaceutical products. *Water Science and Technology*, *49*(3), 213-221.

Ghisari, M., & Bonefeld-Jorgensen, E. C. (2009). Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicology letters, 189(1), 67-77.

Grabemann, H. J., Grabemann, I., Herbers, D., & Müller, A. (2001). Effects of a specific climate scenario on the hydrography and transport of conservative substances in the Weser estuary, Germany: a case study. Climate Research, 18(1-2), 77-87.

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., (2013). Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32 (1), 32–48. https://doi.org/10.1002/etc.2043.

Horn, O., Nalli, S., Cooper, D., & Nicell, J. (2004). Plasticizer metabolites in the environment. Water research, 38(17), 3693-3698.

Jeddi, M. Z., Hopf, N. B., Louro, H., Viegas, S., Galea, K. S., Pasanen-Kase, R., ... & Bessems, J. (2022). Developing human biomonitoring as a 21st century toolbox within the European exposure science strategy 2020–2030. Environment international, 168, 107476.

Kallenborn, R., Borgå, K., Christensen, J.H., Dowdall, M., Evenset, A., Odland, J.Ø., Knowles, N., Cayan, D.R., (2002). Potential effects of global warming on the Sacramento/ San Joaquin watershed and the San Francisco estuary. Geophys. Res. Lett. 29, 36–38.

Khan, S., Naushad, M., Govarthanan, M., Iqbal, J., & Alfadul, S. M. (2022). Emerging contaminants of high concern for the environment: Current trends and future research. Environmental Research, 207, 112609.

Lajeunesse, A., Blais, M., Barbeau, B., Sauvé, S., & Gagnon, C. (2013). Ozone oxidation of antidepressants in wastewater–Treatment evaluation and characterization of new by-products by LC-QToFMS. Chemistry Central Journal, 7, 1-11.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., & Sauvé, S. (2011). Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment–Evidence of biological effects. Chemosphere, 83(4), 564-571.

Liu, J. L., & Wong, M. H. (2013). Pharmaceuticals and personal care products (PPCPs): a review on environmental contamination in China. Environment international, 59, 208-224.

Mahjoubian, M., Naeemi, A. S., Moradi-Shoeili, Z., Tyler, C. R., & Mansouri, B. (2023). Toxicity of silver nanoparticles in the presence of zinc oxide nanoparticles differs for acute and chronic exposures in zebrafish. Archives of environmental contamination and toxicology, 84(1), 1-17.

McKinney, M., Pedro, S., Dietz, R., Sonne, C., Fisk, A.T., Roy, D., Jenssen, B.M., Letcher, B.R., (2015). A review of ecological impacts of global climate change on persistent organic pollutant and mercury pathways and exposures in arctic marine ecosystems. Curr. Zool. 61, 617–628.

Miraji, H., Eunice, M., Ripanda, A., Ngassapa, F., & Chande, O. (2023). Naturally occurring emerging contaminants: Where to hide?. HydroResearch.

Nikinmaa, M., (2013). Review climate change and ocean acidification—interactions with aquatic toxicology. Aquat. Toxicol. 126, 365–372. https://doi.org/10.1016/j. aquatox.2012.09.006.

Noyes, P.D., Lema, S.C., (2015). Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr. Zool. 61, 669–689.

OECD (2021). Reconciling Terminology of the Universe of Per- and Polyfluoroalkyl Substances: Recommendations and Practical Guidance. OECD Series on Risk Management. Paris: OECD Publishing. p. 23. Archived from the original on 13 July 2021.

Pikula, K., Chaika, V., Zakharenko, A., Savelyeva, A., Kirsanova, I., Anisimova, A., & Golokhvast, K. (2020). Toxicity of carbon, silicon, and metal-based nanoparticles to the hemocytes of three marine bivalves. Animals, 10(5), 827.

Quarato, M., Pinheiro, I., Vieira, A., Espiña, B., & Rodriguez-Lorenzo, L. (2021). Detection of silver nanoparticles in seawater using surface-enhanced raman scattering. Nanomaterials, 11(7), 1711.

Qiu, L., Dong, Z., Sun, H., Li, H., & Chang, C. C. (2016). Emerging pollutants–part i: occurrence, fate and transport. Water Environment Research, 88(10), 1855-1875.

Rosenfeld, P. E., & Feng, L. G. (2011). Emerging contaminants. Risks of hazardous wastes, 483, 484.

Sampaio, E., Lopes, A.R., Francisco, S., Paula, J.R., Pimentel, M., Maulvault, A.L., Repolho, T., Grilo, T.F., Pousao-Ferreira, P., Marques, A., Rosa, R., (2018). Ocean acidification dampens physiological stress response to warming and contamination in a commercially important fish (*Argyrosomus regius*). Sci. Total Environ. 618, 388–398. https://doi.org/10.1016/j.scitotenv.2017.11.059.

Sauvé, S., & Desrosiers, M. (2014). A review of what is an emerging contaminant. Chemistry Central Journal, 8, 1-7.

Segura, P. A., Kaplan, P., & Yargeau, V. (2013). Identification and structural elucidation of ozonation transformation products of estrone. Chemistry central journal, 7, 1-11.

Smital, T. (2008). Acute and chronic effects of emerging contaminants. *Emerging Contaminants from Industrial and Municipal Waste: Occurrence, Analysis and Effects*, 105-142.

Taylor, K. M., Weisskopf, M., & Shine, J. (2014). Human exposure to nitro musks and the evaluation of their potential toxicity: an overview. Environmental Health, 13(1), 1-7.

Tao, Z., Zhou, Q., Zheng, T., Mo, F., & Ouyang, S. (2023). Iron oxide nanoparticles in the soil environment: Adsorption, transformation, and environmental risk. Journal of Hazardous Materials, 132107.

Terzić, S., Senta, I., Ahel, M., Gros, M., Petrović, M., Barcelo, D., ... & Jabučar, D. (2008). Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. *Science of the total environment*, *399*(1-3), 66-77.

Tlili, S., Mouneyrac, C., (2019). The wedge clam *Donax trunculus* as sentinel organism for Mediterranean coastal monitoring in a global change context. Reg. Environ. Chang. https://doi.org/10.1007/s10113-018-1449-9.

Tlili, S., Mouneyrac, C. (2021). New challenges of marine ecotoxicology in a global change context. Marine pollution bulletin, 166, 112242.

Tolboom, S. N., Carrillo-Nieves, D., de Jesús Rostro-Alanis, M., de la Cruz Quiroz, R., Barceló, D., Iqbal, H. M., & Parra-Saldivar, R. (2019). Algal-based removal strategies for hazardous contaminants from the environment–a review. Science of the Total Environment, 665, 358-366.

Viglino, L., Aboulfadl, K., Prévost, M., & Sauvé, S. (2008). Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. Talanta, 76(5), 1088-1096.

Wang, T., Zou, H., Li, D., Gao, J., Bu, Q., & Wang, Z. (2023). Global distribution and ecological risk assessment of synthetic musks in the environment. Environmental Pollution, 121893.

Yadav, M., George, N., & Dwibedi, V. (2023). Emergence of toxic trace elements in plant environments: Insights into potential of silica nanoparticles for mitigation of metal toxicity in plants. Environmental Pollution, 122112.

Yan, S., Subramanian, S. B., Tyagi, R. D., Surampalli, R. Y., & Zhang, T. C. (2010). Emerging contaminants of environmental concern: Source, transport, fate, and treatment. *Practice periodical of hazardous, toxic, and radioactive waste management*, *14*(1), 2-20.

Mussel watch program for microplastics in the mediterranean sea: identification of biomarkers of exposure using *Mytilus galloprovincialis*

Francesca Provenza[1,2], Darian Rampih[3], Sara Pignattelli[4], Paolo Pastorino[5], Damià Barcelò[6,7], Marino Prearo[5], Antonietta Specchiulli[8], Monia Renzi[2,8]

[1] Bioscience Research Center, via Aurelia Vecchia, 32, 58015 Orbetello (GR), Italy

[2] Dipartimento di Scienze della Vita, Via L. Giorgieri, 10, Università degli Studi di Trieste, 34127 Trieste, Italy

[3] Univerza v Novi Gorici, Vipavska 13, 5000, Nova Gorica, Slovenia

[4] CNR-IBBR Institute of Bioscience and Bioresources, via Madonna del Piano, 10, 50019 Sesto Fiorentino, Italy

[5] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, 10154 Torino, Italy

[6] Catalan Institute for Water Research (ICRA-CERCA), 17003 Girona, Spain

[7] Institute of Environmental Assessment and Water Research (IDAEA-CSIC), 08034 Barcelona, Spain

[8] National Research Council-Institute for Biological Resources and Marine Biotechnologies (IRBIM), Via Pola 4, 71010, Lesina

Ecological Indicators

Volume 142, September 2022, 109212

https://doi.org/10.1016/j.ecolind.2022.109212

Abstract

Microplastics (MPs) are ubiquitous pollutants that have also been detected in the aquatic ecosystems at high concentrations. The use of shellfish as bioindicators is widespread for assessing and monitoring the environmental quality in both freshwater and marine environments. On this path, biomarkers represent an effective tool in monitoring programs. This minireview would broaden the existing knowledge on biomarkers of MPs in the Mediterranean mussel *Mytilus galloprovincialis*. This species was selected as it is widely distributed across the Mediterranean Sea and used as a bioindicator to monitor the presence of MPs in the marine environment. The literature search returned only 11 studies, mainly related to oxidative stress biomarkers. Although certain biomarkers were explored to estimate the effects of MPs on *M. galloprovincialis*, a battery of standardized and validated biomarkers as well as the inclusion of new ones are needed in future studies to obtain more comparable and robust findings across the Mediterranean Sea.

Keywords

Aquatic ecosystems; bioindicators; oxidative stress; pollution

Introduction

Plastic material is composed of long polymer chains and is used in many aspects of human life. According to its extremely favorable properties and worldwide use, its production has been continuously increasing since the 1950s (Zhang et al., 2021). Plastic is a general term for a material that can be formed into many different shapes and is mainly composed of carbon, hydrogen, oxygen, chloride, and silicon. In addition, there are many additives such as plasticizers, stabilizers, antioxidants, pigments, and flame retardants (Zhang et al., 2021) that change the properties of plastics and make them more durable, lightweight, and resistant to degradation (He et al., 2022; Manzoor et al., 2022). Plastic debris can be classified by size into mega- (> 100 mm), macro- (100-20 mm), meso- (20-5 mm), micro- (5000-10 μm), and nano- (< 100 nm) plastics (Renzi et al., 2018; Harris, 2020; Pignattelli et al., 2021a).

Microplastics (MPs) are considered emerging pollutants with adverse effects on various environmental compartments such as the atmosphere, aquatic ecosystems and biological resources and soil ecosystems (Ma et al., 2020; Renzi et al., 2019; Renzi et al., 2020; Pignattelli et al., 2021b). Microplastics can be divided into primary and secondary, according to different sources (Liu et al., 2021). Primary MPs refer to plastics manufactured as such and found in cosmetics, detergents,

beauty products, plastic beads, etc. Secondary MPs are plastics that originate from larger plastic parts and become MPs through various degradation processes (Liu et al., 2021). The degradation of secondary MPs can occur through different types of processes that can occur either independently or in collaboration. These processes are photooxidative degradation, thermal degradation, ozone degradation, catalytic degradation, mechanochemical degradation, and biodegradation (Manzoor et al., 2022). The toxicity of MPs is not only related to the toxics they release into the environment, but also to their size and shape; in particular, smaller MPs and nanoplastic particles are more toxic to organisms (Llorca et al., 2020). Higher size and shape of MPs determine higher toxicity, due to their longer residence time in organisms (Ma et al., 2020).

Microplastics in aquatic environment are the result of improper waste disposal (Zhang et al., 2021); they account for 93% of plastic waste in the oceans (Gedik and Eryaşar, 2020), entering the aquatic environment through a variety of pathways, primarily surface runoff, fishing, aquaculture, and other human activities (Pastorino et al., 2021). The fate of MPs in these ecosystems is influenced by ocean currents and hydrodynamic processes which potentially facilitate their transport long distances and accumulation in sediments, making them a global pollutant (He et al., 2022). In addition, the ubiquity of MPs can have adverse effects on the environment and biota, such as light scattering impairment, chemical alteration due to oxygen deprivation, intrusion, accumulation, and biomagnification across different trophic levels (Manzoor et al., 2022). Microplastics are capable of absorbing and accumulating pollutants from air, soil, and seawater, such as persistent organic pollutants, or attaching to other biological surfaces and forming biofilms (Kinjo et al., 2019; He et al., 2022). The attraction of microorganisms and the formation of biofilms inevitably change the properties of MPs and add new variables, which in turn can lead to more toxic MPs for aquatic organisms and environment. Biofilms are believed to have good adsorption properties and accumulate heavy metals, antibiotics, and other toxic substances that can easily spread through the aquatic food web (Llorca et al., 2020; Piccardo et al., 2021; He et al., 2022). Many aquatic organisms are used as promising tools for biomonitoring programs and assessing the environmental quality. Throughout time and evolution, organisms have had to adapt to environmental changes and develop defense strategies and mechanisms to survive; when the organism's threshold is crossed, it ceases to exist (Gadzała-Kopciuch et al., 2004). These adaptive mechanisms are used to fathom the amount, type, and effects of pollutants and/or contaminants on organisms, environment, and the impact on human society (Box et al., 2007). Bioindicators and biomonitoring are closely related, in fact, using it, is possible to obtain quantitative information about the health and quality of the environment (Parmar et al., 2016). For aquatic environments, bivalves as *Mytilus galloprovincialis* have been found to be the most suitable organisms for biomonitoring, becoming good bioindicators for their natural habitat (Li et al., 2019). Indeed, *M.*

galloprovincialis has a high ecological and commercial relevance in the Mediterranean Sea, where MPs contamination is also of particular concern (Grbin et al., 2019). Moreover, *M. galloprovincialis* was chosen for this minireview because it has been proposed by the International Council for the Exploration of the Sea to monitor MP pollution in the marine environment (Bråte et al., 2018; Davies and Vethaak, 2012).

Biomarkers are a useful tool for assessing and identifying stress and toxic effects in organisms, as they are normally produced during the life cycle of any organism. Their use in risk assessments is currently recommended to characterize the sub-lethal alterations and toxicological pathways at the root of major environmental impacts in aquatic ecosystems. They can be divided into specific and non-specific biomarkers related to the stress or pollutant/contaminant typology (Sarkar et al., 2006). A good biomarker must be measurable, have significant differentiation between noise and sign, and be responsive to the changes in the organism and its conditions (Aronson and Ferner, 2017). A lot of different compounds can be used as biomarkers in mussel depending on tissue, time of exposure and many other factors of investigation. Biomarkers are considered sensitive and could indicate early warning signals of environmental quality (González-Fernández et al., 2015). Biological responses are defined as changes that occur in different domains, from cellular to physiological and can be studied in different species such as body fluids, tissues, or organs (González-Fernández et al., 2015; Aronson and Ferner, 2017). This minireview provides a general overview of the status and effects of MPs on aquatic ecosystems, illustrating the usefulness of *M. galloprovincialis* as a good bioindicator for the marine ecosystems. In particular, the main aim is to highlight and relate to each other the biomarkers used to assess the impact of MPs on the bivalve *Mytilus galloprovincialis*. To do this, a literature search was performed in the Scopus (https://www.scopus.com/search/) and Web of Science (https://clarivate.com/) databases using the keywords: "*Mytilus galloprovincialis*" OR "bivalve" AND 'biomarkers' AND "microplastics" across all publication years until 5 June 2022, last day of research for the present study.

Plastics

In the context of sedimentology, to better understand plastic pollution, its effects, and its migration or transport through different media, numerous studies are being conducted that attempt to combine MP particles with normal sediment particles such as clay and silt. Once released in the environment, all plastic particles eventually settle to the seafloor, either through degradation, density differences, or a process known as biofouling, even via animal excretions (Harris, 2020; Llorca et al., 2020). Numerous considerations about MPs in organisms and sediments should be made before sampling an area. The sampled area must be identified as a net sink for sediment and

MPs or net erosion for sediment and MPs, or it may be in equilibrium (Harris, 2020). Wave energy, tidal range, sediment supply, and sea level rise are among the key processes that control the majority of sandy beaches, which are found along wave-dominated coasts and have a wide variety of geomorphic and sedimentological features (Komar, 1977; Davis and Hayes, 1984; Masselink and Hughes, 2014). Knowing if a beach is a net sediment sink (a depositional sedimentary environment), net erosional (a receding coast), or otherwise in equilibrium in in terms of sea level, sediment supply, and sediment removal mechanisms is relevant to the buildup of MP on beaches. This information could provide insight into the amount of MPs in the sediment and water column and provide insight into the extent of pollution to organisms, from sediment feeders up the trophic chain. If the above ideas are implemented over time (monitoring), this could provide clues to the improvement or degradation of the organism/area being studied for the past and future. Due to varying locations, pollution levels, and anthropogenic activities, sediment is saturated with MPs to varying degrees, ranging from 3 particles/kg to 11,600 particles/kg (Harris, 2020). Studies near Salina Island in the Aeolian Archipelago found values of 99-431 particles/kg dry weight (Renzi et al., 2018). Studies in the Barents Sea indicate that bivalves can accumulate significantly greater amounts of MPs than their environment; it is reported that bivalves had 3.7 x 10^4 elements/kg dry weight compared to sediment and seawater, which had 48 elements/kg dry weight and 27 elements/L, respectively (Li et al., 2019). One thing that is agreed upon by almost all researchers is that the predominant form of MPs particles is fibers (Li et al., 2019; Harris, 2020).

Plastic degradation

As mentioned earlier, MPs can be deformed or degraded by a variety of principles, from biodegradation to hydrolysis (Sharma and Chatterjee, 2017; Bhatt et al., 2021). Although plastic is resistant to degradation and long-lived in the environment, it degrades slowly over the years, during which time the plastic particles can change, release toxic substances, and alter the structure of the polymers (Zhang et al., 2021).

Photodegradation of plastic

The driving force behind the decomposition of plastics is light: UV light and visible light initiate the processes to reform various end groups of the polymer (Manzoor et al., 2022). Depending on the purity of the plastic, different degradation rates occur; typical for UV degradation is the reaction of chromophores with oxygen to form radicals (Zhang et al., 2021).

Thermal degradation of plastic

Like photodegradation, thermal degradation of plastics begins with the help of photodegradation, with it and oxygen it begins to weather, and radicals are released. Thermal degradation lasts only if the necessary energy is available for the reactions. This degradation depends on pressure, temperature, or energy differences and the substrate or degradation area, as it varies in water sediment or on the beach (Zhang et al., 202; Manzoor et al., 2022).

Biodegradation of plastic

This form of degradation occurs through the presence of microorganisms, either at the molecular, microscopic, or macroscopic level. It is assumed that the by-products produced by this degradation are not toxic (Manzoor et al., 2022). The process of biodegradation can also be considered biofouling, which can have negative effects on the ecosystem and organisms, as mentioned earlier (He et al., 2022). Many other processes are involved in plastic degradation that can interact and have positive or negative consequences for MP degradation or the environment and its biota (Fastelli et al., 2016; Ma et al., 2020; Zhang et al., 2021). Once plastic is discarded, degradation processes begin in different ways that can increase the number of particles in the medium. Studies show that heavily populated areas and areas with high industrial production correlate with more MP particles in the environment and in organisms. Figure 1 shows different mechanisms of plastic degradation and their partitioning (Sharma and Chatterjee, 2017).

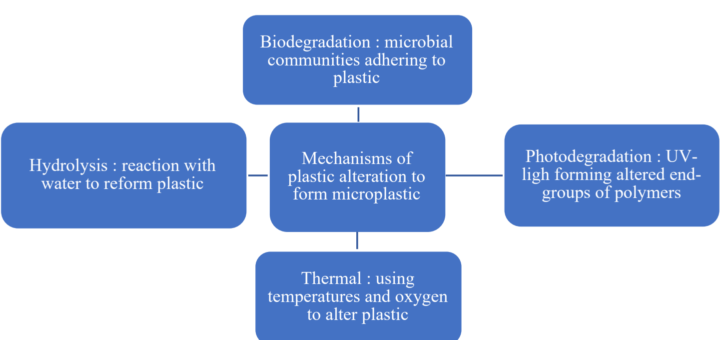


Figure 1 *Degradation of plastic particles by different mechanisms (Sharma and Chatterjee, 2017, modified).*

A holistic understanding of these processes can shed light on future MP pollution problems and ways to address them. Depending on the type of degradation and the process it undergoes, different methods can be used to determine various parameters and increase knowledge about the fate of MPs in the environment and organisms. These different methods are listed in Table 1.

Table 1 *Methods for identification of plastic features. FTIR: Fourier transform infrared spectroscopy; XRF: X-ray fluorescence spectrometry; GC/MS: gas chromatography coupled with mass spectrometry; XRD: X-ray diffraction; DSC: differential scanning calorimetry; TGA: thermogravimetric analysis; TOC: total organic carbon; LSC: liquid scintillation counting; GC: gas chromatography.*

Type	Technique	Method	Reference
Chemical composition	FTIR	FTIR measures the absolute value of IR-radiation absorbed by the microplastic particle	Gedik and Eryaşar, 2020
	Raman	Sample reflects radiation at different frequencies which Raman spectroscopy can measure	Vandermeersch et al., 2015
	XRF	Samples when exposed to outside energy their individual atoms emit X-ray photons which XRF can quantify	Zhang et al., 2021
	GC/MS	Degrading microplastic samples release additives which can be analysed quantitatively and qualitatively by comparing them with standard concentrations	Li et al., 2019; Liu et al., 2021
Shape and texture	Spectrophotometer	Spectrophotometer and Colorimeter are used to measure colour and Haze amount of scatter light that is reflected from the plastic particle	Sharma and Chatterjee, 2017
	Colorimeter		Zhang et al., 2021
	Microscope	Microscope use visible light and lenses to magnify the sample and to identify its shape, texture and colour but has limited range	Harris, 2020
Physicochemical properties	XRD	Analysing crystallinity in a sample XRD is used to measure order between molecules	Zhang et al., 2021
	DSC	DSC can analyse crystallinity by measuring heat required to melt the plastic particle	Zhang et al., 2021
	Potentiometric titration	Plastic can have different charges when exposed to time and weathering, with Potentiometric titration we can measure surface charge	Zhang et al., 2021
	TGA	TGA measures thermal stability of a plastic particle, it is used to monitor weight in relation of time and temperature changes	Liu et al., 2021
Mechanical properties	Instron universal materials testing machine	This technique follows standard methods by ASTM and ISO and is used to determine tensile properties, sheer strength, and other mechanical properties	Zhang et al., 2021
Mineralization	TOC analyser	TOC analyser can analyse carbon concentration by combusting the sample and measuring CO_2 released	Zhang et al., 2021
	LSC	LSC measures radioactive changes in sample when it is degrading	Zhang et al., 2021
	GC	GC can be used to measure CO_2 and CH_4 with and IR detector released from the sample	Liu et al., 2021
Microplastic identification	Pyrolysis	Pyrolysis is used to degrade microplastic particles with high temperature in inert atmosphere	Rebelein et al., 2021

Biofilm or biofouling

Biofouling is a process in which microorganisms attach to the substrate and/or MP particles and form communities. The most common biofilms are bacteria, algae, and fungi (He et al., 2022). Biofilm formation usually occurs according to the following processes: formation, reversible or irreversible adhesion, depending on the MP and the type of "glue" used by the microorganisms, which can be either covalent, ionic, hydrogen bonded, or formed by proteins, and production of biofilm formation and spreading of the organisms, as shown in Figure 2 (He et al., 2022). Due to the different density of plastics, MP particles can migrate vertically through the water column with the help of the biofilm and eventually accumulate in the sediment (Zhang et al., 2021).

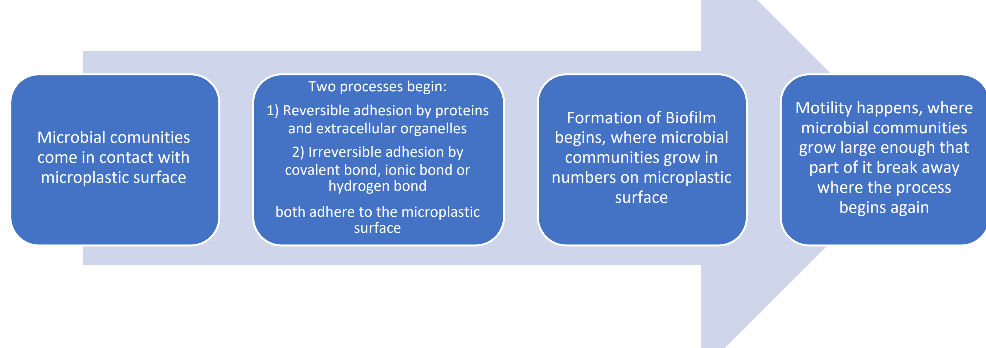


***Figure 2** Mechanisms of biofilm formation.*

Plastic toxicity

Microplastics enter the marine environment primarily through human activities (i.e., aquaculture, fishing, tourism, industrial and domestic wastewater systems) and their distribution is highly variable (Duis and Coors, 2016). A positive relationship has been established between the increase in human population density and the abundance of MPs, which could lead to an increase in plastic debris accumulating in the marine environment (Shahul Hamid et al., 2018). Physicochemical properties of MPs (i.e., size, chemical composition), hydrodynamic factors, and environmental characteristics (i.e., water currents, turbidity, temperature, wind) can influence their transport dynamics and, as a result, their distribution and accumulation in various marine areas (Ding et al., 2021).

There is little agreement on the biological consequences of MP pollution. Indeed, while large plastic particles (i.e., meso and macroplastics) can cause easily visible effects at the organism level, such as suffocation, entanglement, or intestinal blockage (O'Donovan et al., 2018), the direct and indirect physiological effects of small size particles (micro- and nano-plastics) on aquatic animals mainly remain unknown. Several studies have shown that once ingested, MPs can accumulate within the aquatic organisms and be translocated between body tissues, or that they can be eliminated for excretion or egestion via pseudofaeces (Graham et al., 2019). The accumulated MPs can have a variety of negative effects on the organism that ingests them. Internal and/or external injuries, mechanical damages, blockages of the gut tract resulting in pseudo-satiety sensation and physiological stress, alteration of feeding and retardation of growth, reduction in fertility, fecundity, and progeny survival rate are some of these (Graham et al., 2019). Furthermore, MPs can be used to introduce toxic compounds into marine organisms (Cole et al., 2011). Dark-colored, or black MP are reported to tend to adsorb PCBs (polychlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons) much more than other compounds (Ma et al., 2020). Chemical additives (i.e., flame retardants, nanoparticles) added to plastic products during manufacturing processes to

improve their final properties, as well as environmental contaminants (i.e., persistent organic pollutants, hydrocarbons and heavy metals) adsorbing on their surface by the marine environment can cause changes in metabolic and reproductive activity, a decrease in immune response, oxidative stress, cellular or sub-cellular toxicity and inflammation in marine biota (Padervand et al., 2020). Several additives are not covalently bonded to plastic polymers; therefore, they can migrate to the surface of the material and release chemicals into the environment (Do et al., 2022). In effluents from wastewater treatment plants, surface waters, and marine waters, additives have been detected (Do et al., 2022). Among them, bisphenol A and phthalates are two commonly plastic additives that are recognized as probable endocrine disruptors due to their capacity to interfere with hormone regulation in both human and wildlife (Hermabessiere et al., 2017). Finally, through bioaccumulation and biomagnification processes, MPs can be transferred to the food chain alone or in combination with other pollutants, with potential risks for human health (Lehel and Murphy, 2021). The accumulation of these particles could have effects on chromosomes and lead to infertility, obesity, and cancer (Sharma and Chatterjee, 2017).

Because MP are similar in size and shape to the natural prey of predators and filter feeders, mussels are thought to be most affected by the toxicity of MP pollution (Kinjo et al., 2019). Because these organisms constantly filtering a large volume of seawater (about 50 mL of seawater is pumped per minute, as reported by Li et al. in 2019), they can also consume MPs that are similar in size to their natural prey. For example, Paul-Pont et al. (2016) showed an increase in hemocyte mortality in *Mytilus* spp. Exposed to polystyrene MPs. Cole et al. (2020) reported that MP particles can penetrate fluids and tissues of *Mytilus* spp., which in turn translates into an immune response and the induction of cytotoxicity. Disorders in osmoregulation, energy and protein metabolism, and oxidative stress were found in *M. galloprovincialis* exposed to polystyrene MPs.

Mytilus galloprovincialis as bioindicator of pollution

Aquatic mollusks represent optimal and common model organisms for ecotoxicological risk assessment and ecosystem monitoring because they meet all the characteristics normally required, such as abundance, limited mobility, and longest (> 1 year) possible life span (Jong et al., 2022). Bivalves are among the largest taxa in the animal kingdom and are cosmopolitan in temperate intertidal zones (Cappello et al., 2018). The marine bivalve *Mytilus galloprovincialis* is a popular shellfish and a good bioindicator for a variety of toxicological and environmental studies. Properties as sensitivity to pollutants, wide geographic distribution and sedentary lifestyle, ease of sampling make organisms efficient tools for assessing the environmental quality. Mussels are sessile organisms and static filter-feeder (Cappello et al., 2018) and represent a biological resource

of high economic importance, but they are continuously exposed to many pollutants, due to their feeding activity. Intake of pollutants, through transfer and accumulation in tissues (Box et al., 2007), pose a health risk to them, resulting in an imbalance at the whole food chain, including humans (Li et al., 2015). Additionally, their hard shell and ease of handling in the laboratory minimize the risk of contamination during experiments (Li et al., 2019), making them excellent bioindicators. Furthermore, their low metabolic detoxification (Bolognesi et al., 2004) and close correlation with their habitat make mussels suitable for biomonitoring studies related to many toxicants, as well as MPs (Li et al., 2019). Ward et al. (2019) indicate that bivalves are selective particle eater, and that particle size, shape and surface characteristics affect rejection or ingestion of food and MPs. *Mytilus galloprovincialis* can select particles at the palps (Ward et al., 2019), which is important when laboratory experiments are performing with specific shapes and sizes of MPs, as they are very different and more complex from natural habitat, in terms of type, shape, size and composition, thought to be related to the degree of toxicity they cause (Zhang et al., 2021). An important observation that relates to the natural environment and the laboratory is the saturation of gills and labial palps, as this is likely to influence the uptake or rejection of particles such as MPs (Ward et al., 2019). Another selection of MP particles that should be considered is the width of the mouth of bivalves, which can be quite wide but still has an upper limit reported to be in the range of 600-900 µm (Ward et al., 2019).

Biomarkers of microplastics in *Mytilus galloprovincialis*

Biomarkers are a variety of biological indicators that are measurable at the quantitative level and indicate changes at the cellular, biochemical, molecular, and physiological levels (Lionetto et al., 2019). These indicators can be measured at the cellular level, body fluid level, tissue level or organ level. They can also provide us with information about the exposure or effect of xenobiotics (Gonzalez-Fernandez et al., 2015). In particular, the use of mussel gills for biochemical and biomonitoring studies could be useful because they are more sensitive to toxic pollutants and MP particles and increase the concentration of antioxidants to prevent oxidative damage (Bolognesi et al., 2004; Capó et al., 2021).

When an organism is exposed to stress or external pressures some responses are triggered, such as the production of reactive oxygen species (ROS), which in turn trigger the production of antioxidant compounds, both enzymatic and non-enzymatic, to prevent oxidative damage (Lam and Gray 2003; Pastorino et al., 2020). Many pollutants as MPs can disrupt redox homeostasis, forcing the antioxidant system to work overtime to restore balance (Trestrail et al., 2020). Conceptually, the interaction between pollutants and the antioxidant system can be divided into

three stages. An oxidative challenge (stage I) can be posed by a pollutant, necessitating an energetically demanding response from the antioxidant system (stage II). If the organism's response is insufficient to deal with the oxidative challenge, it will suffer from oxidative stress (stage III) (Trestrail et al., 2020).

The first stage of pollutant-induced redox homeostasis disruption occurs when the pollutant creates an oxidative challenge in the tissues of the exposed animal by rapidly increasing ROS concentrations (Pastorino et al., 2020). This increase in ROS concentration can occur when a pollutant initiates a physiological pathway that generates ROS (Magara et al., 2021), or when it increases the organism's aerobic respiration rate and, as a result, the levels of by-product ROS (Elia et al., 2020). ROS concentrations can act as a biomarker in this early stage, indicating whether a pollutant posed an oxidative challenge. The response of the antioxidant system is involved in the second stage of redox homeostasis disruption. If the homeostatic antioxidant capacity is sufficient, the first-stage ROS concentration spike will be immediately neutralized, and redox homeostasis will not be disrupted. If the homeostatic antioxidant capacity is insufficient to counteract the excess ROS, energy will be expended to synthesize antioxidant enzymes and molecules, increasing the organism's antioxidant capacity (Lushchak, 2011). Redox homeostasis is restored when the antioxidant capacity is sufficient to neutralize the excess ROS. The second stage antioxidant response is associated with a plethora of redox biomarkers. Since antioxidant capacity is influenced by a wide range of enzymes and molecules (Halliwell, 2007), and new antioxidant molecules are constantly being discovered, measuring all the antioxidant system's molecules and enzymes is quite challenging. Thus, only antioxidant biomarkers involved in critical pathways are commonly assessed.

Results from literature search

Results from literature search in Scopus (https://www.scopus.com/search/) and Web of Science (https://clarivate.com/) databases retrieved only 11 studies on biomarkers of MPs in *M. galloprovincialis* (Table 2).

Table 2 *Results from literature search (Scopus and Web of Science databases) on biomarkers of microplastics in Mytilus galloprovincialis. It is also indicated the type of study (E=experimental; F=field) and biomarker.*

Reference	Study type	Biomarker type
Abidli et al., 2021	E	Oxidative stress
Capó et al., 2020	F	Oxidative stress
Capolupo et al., 2021a	E	Oxidative stress; histopathology
Capolupo et al., 2021b	E	Oxidative stress; histopathology
Capolupo et al., 2021c	E	Oxidative stress; histopathology; immunological parametres
Cappello et al., 2021	E	Metabolomics
Choi et al., 2022	E	Reproductive; neurotoxic
González-Soto et al., 2019	E	Oxidative stress; histopathology
Pittura et al., 2018	E	Oxidative stress; histopathology; immunological parametres
Romdhani et al., 2022	E	Cytotoxic and genotoxic
Trestrail et al., 2021	E	Enzymatic digestive activity

In Table 3 are listed the biomarkers commonly used to detect toxicity in *M. galloprovincialis* as well as their locations and functions.

Table 3 *Biomarkers commonly used to detect toxicity in Mytilus galloprovincialis as well as their location and function.*

Biomarker	Location	Function	Reference
Activity of carbohydrases	Digestive gland	Catalyzes the breakdown of carbohydrates into simple sugar	Trestrail et al., 2021
Acetylcholinesterase inhibition, AChE	Gills	Servers as neurotransmitter	Pittura et al., 2018; Capolupo et al., 2021a, 2021b, 2021c Choi et al., 2022
Catalase (CAT)	Digestive gland and gills	Involved in removal of hydrogen peroxide	Pittura et al., 2018; González-Soto et al., 2019; Abidli et al., 2021; Capolupo et al., 2021a, 2021b, 2021c; Choi et al., 2022
DNA fragmentation rate	Haemolymph	Separation or breaking of DNA strands into pieces due to contaminant exposure	Pittura et al., 2018; Romdhani et al., 2022
Glutathione (GHS)	Digestive gland and gills	Serves in metabolism and detoxification processes	Pittura et al., 2018; Capò et al., 2021
Glutathione peroxidase (GPx)	Gills	Catalyses the reduction of hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxide radicals to alcohols and oxygen	Pittura et al., 2018; Capò et al., 2021
Glutathione reductase (GR)	Gills	Maintaining the supply of reduced glutathione	Pittura et al., 2018; Capò et al., 2021
Glutathione S-transferase (GST)	Digestive gland	Allows the conjugation of the reduced form of glutathione (GSH) to pollutant compounds	Pittura et al., 2018; Abidli et al., 2021; Capò et al., 2021; Capolupo et al., 2021a, 2021b, 2021c
Intra-lysosomal content of lipofuscin	Digestive gland	Oxidative alteration of macromolecules by oxygen-derived free radicals generated in reactions catalyzed by redox-active iron of low molecular weight	Capolupo et al., 2021a, 2021b, 2021c
Lysosomal β-hexosaminidase activity	Digestive gland	Breaking down toxic substances and act as recycling centre	González-Soto et al., 2019
Lysosomal Membrane Stability (LMS)	Haemolymph	Controls the passage of material into and out of lysosomes	Pittura et al., 2018; Capolupo et al., 2021a, 2021b, 2021c; Romdhani et al., 2022
Lysosome to cytoplasm volume ratio	Digestive gland	Advanced physio pathological condition in mussel hepatopancreas	Capolupo et al., 2021b
Lysozyme-specific activity	Haemolymph	Catalyzes the cleavage of β-1,4-glycosidic bonds between N-acetyl muramic acid and N-acetyl glucosamine in peptidoglycan	Capolupo et al., 2021c
Malondialdehyde	Digestive gland	Product of lipid peroxidation	Pittura et al., 2018; Abidli et al., 2021; Cole et al., 2020; Capò et al., 2021; Capolupo et al., 2021b, 2021c
Metallothionein	Digestive gland, gills	Cysteine (Cys)-rich protein that acts as a crucial antioxidant	Capolupo et al., 2021b
Micronuclei frequency	Haemolymph	Biomarker of chromosomal damage	Pittura et al., 2018; Romdhani et al., 2022
Necrosis of digestive tubule epithelium	Digestive gland	Damage to epithelial cells useful to adsorb substances into the body and restricting the entry of harmful substances	González-Soto et al., 2019;
Phagocytic activity	Haemolymph	Represents a vital facet of the innate immune response to pathogens, and plays an essential role in initiating the adaptive immune response	Capolupo et al., 2021c
Presence of brown cells in connective tissue and brown aggregates in epithelium	Digestive gland	Non-specific indicator of environmental pollution	González-Soto et al., 2019
Protease activity	Digestive gland	Catalyses hydrolysis of the dietary proteins	Trestrail et al., 2021
Protein carbonyls derivates	Gills	Marker of oxidative damage	Capò et al., 2021
Thiobarbituric Acid	Digestive gland, gills	By-product of lipid peroxidation	Cole et al., 2020
Transcriptional parameters	Mussel embryos	Transcripts encoding lysosomal enzymes	Capolupo et al., 2018
Steroid hormones	Haemolymph	Endogenous regulators of gametogenesis	Choi et al., 2022
Superoxide dismutase (SOD)	Digestive gland and gills	Antioxidant enzyme that serves as protection from toxic effect of reactive oxygen species and free radicals	Cole et al., 2020; Choi et al., 2022

Generally, almost all studies were performed in laboratory under controlled conditions, except the work of Capò et al. (2021) in which *M. galloprovincialis* were distributed in three areas with different anthropogenic impacts. This is due to the difficulty to set an experiment in the field controlling the number and the type of MPs. Indeed, in an open environment like marine ecosystem, it can be difficult put a fixed number of MPs and obtain certainly the contact between particles and organisms. Obviously, tests set in laboratory follow the indication of environmental dose of contaminant and ty to reproduce real environmental conditions. The biggest difference between experiments in laboratory and in field is the absence of dilution in laboratory and the absent or reduced recycle of the water. Also, the number of the organisms than can be exposed in laboratory is different by the number of organisms than can be exposed in a natural scenario.

Even though, results obtained from laboratory experiments are adaptable to natural ecosystem because the doses used in laboratory are usually realistic respect environmental level of contamination. Moreover, the quantity of organisms, the volume of water and the control of parameters (i.e., dissolved oxygen, salinity, pH, etc.) are proportioned to natural environmental.

It can be interesting and important try to create some experiment in natural field or in same similar location, also for simulate wave movement, change of temperature, and light condition during the day.

As regard the type of biomarker, most of published papers focused the attention on biomarkers of oxidative stress. Indeed, the measurement of antioxidant compounds involved in detoxification of ROS, such as glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione (GSH) and lipid peroxidation (LPO) has been shown to be an appropriate assessment tool in this framework for two reasons (Lam, 2009): a) the mechanisms that counteract ROS are well known and extensively studied; b) they can act as sentinel compounds to prevent ROS due to their immediate production.

Glutathione S-transferase plays a key role in the phase II of detoxification involved in the conjugation of GSH with phase I enzymes; its activity has been found to be increased in the gills of mussels when exposed to MPs (Capó et al., 2021) and PP leachates (Capolupo et al., 2021b). Contrary, polystyrene MPs did not induce any GST changes in *M. galloprovincialis* gills (Capolupo et al., 2021c) nor in the digestive gland of *M. galloprovincialis* exposed to chrysene-sorbed polystyrene MPs (Capolupo et al., 2021a). Superoxide dismutase is the main actor in the dismutation process to obtain hydrogen superoxide (H_2O_2) from the superoxide anion (O_2^-); its suitability as a biomarker is due to its rapid response to stress; normally, its activity is increased under stress conditions, especially in the gills, since they are the first to be exposed to toxins (Box et al., 2007). The study on oxidative stress biomarkers by Box et al. (2007) highlight that the levels

of GPx and SOD are significantly higher in the digestive glands than in the gills for the polluted area, further supporting these biomarkers as indicators of pollutants. In an experiment conducted under controlled conditions with *M. galloprovincialis*, SOD activity was found to be significantly increased in the first 24 h after exposure to 500 ng/mL of polystyrene microspheres (PS-MP) and polyamide microfibers (PA-MF), especially in the gills and digestive glands, but after 7 days of exposure, activity decreased and was similar to that of control organisms (Cole et al., 2020). On the other hand, other studies using larger plastics (100-1000 PE-MP/mL) for 4 days on *Mytilus* spp. showed no significant SOD activity after 24 hours (Wang et al., 2020), confirming that SOD activity is related and inversely proportional to plastic size. CAT is only involved in the reduction of H_2O_2 to H_2O at high H_2O_2 levels, while at low levels it is actively involved in the detoxification of other compounds, with H_2O_2 reduction always occurring (Gonzalez-Fernandez et al., 2015). In *in situ* experiments (aquaculture cages), a higher activity of CAT was found (Capó et al., 2021) in gills of *M. galloprovincialis* exposed to MPs. Abidliet al. (2020) showed how polyethylene microplastics (PE-MPs) can induce alteration in CAT activity. Moreover, *M. galloprovincialis* exposed to leachates from different polymers showed higher CAT activity in digestive gland than gills since peroxidation processes are frequently modulated in response to toxicant exposure (Capolupo et al., 2021b). Like the enzymes mentioned above, GPx is also involved in the removal of H_2O_2 (Capó et al., 2021); although GR is not directly involved in the detoxification of ROS, its function is equally important as it is involved in maintaining the ratio of GSSG (oxidized glutathione)/GSH, which in turn plays a key role in cellular homeostasis (Gonzalez-Fernandez et al., 2015); GSH, in turn, is actively involved in the detoxification of ROS (Box et al., 2007). The activity of GR was found to be higher in field experiments compared to the control group (Capó et al., 2021). Glutathione was detected in greater amounts in the digestive gland of *M. galloprovincialis*, which is a known organ for detoxification of pollutants, and higher than normal levels may indicate stress (Cappello et al., 2021).

Lipid peroxidation is considered a toxicity biomarker because it is used to assess lipid peroxidation of membranes, i.e., it reflects the damage caused by ROS (Gonzalez-Fernandez et al., 2015; Box et al., 2007). It is already expressed both *in situ* and under controlled environmental conditions, and in the latter, it was found particularly higher in the gills after 24 h of exposure (Cole et al., 2020; Capó et al., 2021).

Malondialdehyde (MDA) levels are commonly used to assess oxidative stress as a product of lipid peroxidation. For example, exposure of *M. galloprovincialis* to polystyrene microplastics (PS-MP, 3 μm) and nanoplastics (PS-NP, 50 nm) resulted in the up-regulation of MDA in the digestive

gland, indicating that lipid peroxidation phenomena might be induced by PS particles (Capolupo et al., 2021c).

Proton nuclear magnetic resonance (NMR) metabolomics has been also used in aquatic ecotoxicology since it allows simultaneous identification of changes in metabolic pathways after contaminants exposure (Cappello, 2020). However, only few studies have used NMR-based metabolomics to investigate the effects of MPs on aquatic biota (i.e., Lu et al., 2016, Qiao et al., 2019). The research performed on metabolites in *M. galloprovincialis* and their expression under stress conditions in relation to polystyrene MP particles by Cappello et al. (2021) could provide a basis for studying toxic responses to MPs and the results could be compared globally, at spatial and temporal level, providing a consistent protocol for monitoring baselines. Cappello et al. (2021) highlight some biomarkers that could provide clear signs of decreasing or increasing levels. On this path, glycine was a compound that steadily decreased while GHS dramatically increased in the first 24 hours after exposure to MPs. Lactate, glycogen, glucose, isoleucine, leucine, alanine, tyrosine, and valine increased in *M. galloprovincialis* exposed to MPs compared to control groups and could be used to determine ecotoxicity tests and MPs poisoning and responses (Cappello et al., 2021).

Genotoxicity biomarkers investigated in published literature mainly include DNA fragmentation rate and micronucleus frequency which provide information about the pollution taking place (Cole et al. 2020; Romdhani et al., 2022). Micronucleus test is used as a function of time and could be an indicator for monitoring purposes, while DNA strand breakage could be used for recent exposure to environmental stressors and contaminants in the organism (Bolognesi et al., 2004). Romdhani et al. (2022) assessed the impact of ecologically relevant MPs concentrations alone or combined with benzo[a]pyrene (MP-B[a]P) by means of micronucleus frequency and DNA fragmentation rate, highlighting an increase in groups exposed to MPs. The same findings were also shared by Cole et al. (2020) who exposed mussels to polystyrene MPs. Another important aspect that should not be underestimated is the difference between wild and caged mussels. Wild mussels have been shown to contain higher levels of accumulated heavy metals and persistent organic pollutants and therefore have higher micronuclei levels than caged mussels (Bolognesi et al., 2004). On the other hand, caged mussels have higher levels of DNA strand breaks, likely due to recent exposure to contaminants (Bolognesi et al., 2004).

Histological and cytological biomarkers are also a powerful tool for detecting and characterizing the biological endpoints of toxicants, and histopathology has been shown to be a sensitive indicator of health in aquatic organisms (Rašković and Berillis, 2022). For examples, neutral lipid (NL) accumulation, as well as the lysosome to cytoplasm volume ratio (LYS/CYT) significantly

enhanced in *M. galloprovincialis* exposed for 7 days to leachates from car tire rubber, polypropylene, polyethylene terephthalate, polystyrene, and polyvinyl chloride (Capolupo et al., 2021b). Increase in LYS/CYT is an advanced physio-pathological condition in mussels, and it is thought to precede highly hazardous processes to the viability of digestive cells and digestive gland functions (Capolupo et al., 2021b). Similarly, neutral lipid accumulation is a lipidosis biomarker that is thought to be caused by either an increase in cytosolic lipid content or a decrease in fatty acid processing (Capolupo et al., 2021b).

Several authors have already measured immune system impairment in marine organisms exposed to MPs (i.e., Paul-Pont et al., 2016). On this path, immunological effects on lysosomal membrane stability and phagocytosis were also observed in *M. galloprovincialis* exposed to MPs (Capolupo et al., 2021a, 2021b, 2021c; Romdhani et al., 2022).

Microplastics can also interfere with the activities of digestive enzymes, which convert carbohydrates, proteins, and fats into subunits that animals can absorb and use for energetic purpose (Karasov and Douglas, 2013). Because MPs were found in the digestive systems of aquatic invertebrates and can be retained for long periods of time (Fernández and Albentosa, 2019), there is ample opportunity for MPs to interfere with digestive enzyme functions. For such reason, Trestrail et al. (2021) determined the activities of seven key digestive enzymes in the digestive gland of *M. galloprovincialis*, highlighting how polymer type significantly affected the activities of carbohydrase enzymes.

Neurotoxicity biomarkers (i.e., acetylcholinesterase inhibition, AChE) were assessed by Capolupo et al. (2021a, 2021b, 2021c) and Choi et al. (2022). For example, Capolupo et al. (2021a) and Capolupo et al., (2021b) showed a decrease in acetylcholinesterase activity in *M. galloprovincialis* exposed to polystyrene MPs and PVC leachates, respectively.

In bivalves the steroid hormones estradiol and testosterone act as endogenous regulators of gametogenesis (Gauthier-Clerc et al., 2006). Estradiol is thought to regulate several reproductive processes in bivalves, including increasing oocyte diameter and ovarian protein content in female oysters and promoting vitellogenin protein accumulation (Li et al., 1998). Thus, the levels of estradiol and testosterone in bivalves influence the reproductive cycle and can be used as biomarkers of reproductive toxicity. Choi et al. (2022) found a decrease of both estradiol and testosterone in *M. galloprovincialis* after polyethylene terephthalate microfiber exposure, suggesting a disruption of the reproductive cycles of mussels.

Another important study, not illustrated in the Table 3, is represented by Cappello et al. (2021) which used for the first time a protonic Nuclear Magnetic Resonance (H NMR)-based metabolomic approach to examine the effects of short-term exposure of *M. galloprovincialis* to

polystyrene MPs. Although several metabolites were found, amino acids and osmolytes metabolites involved in the energy metabolism represented the major classes of compounds. Alanine serves as osmolyte and is involved in nitrogen metabolism, and it is present in gills, digestive gland, and posterior adductor muscle. Acetoacetate presents in posterior adductor muscle and digestive gland, servers as energy supply for cell activity and as signaling molecule. Betaine serves as osmolyte, but it is present only in digestive gland; the same thing for dimethylglycine and homarine. Instead, hypotaurine and taurine is present also in gills and posterior adductor muscle. Glutamate is a precursor for synthesis of glutamine and later glutathione which serves as protection against oxidative damage, and it is present in digestive gland and posterior adductor muscle. Glycine serves as osmolyte, carbon energy source for generation of ATP in digestive gland, gills, and posterior adductor muscle. Glycogen, useful for reproductive cycle, can be found in gills and digestive gland. Leucine and isoleucine, in digestive gland and posterior adductor muscle, serves as metabolite in immune system, energy reserves and is precursor for formation of lymphocytes. Finally, valine serves as proteinogenic amino acid as precursor for formation of lymphocytes and essential in immune system and we can find it in digestive gland, gills, and posterior adductor muscle. Thus, an integrated description of the perturbations induced by MPs in the metabolome of *M. galloprovincialis* was successfully reported by Cappello et al. (2021). However, there is a lack of information about metabolic pattern in *M. galloprovicnialis* that need to be filled in order to consider metabolites as useful biomarkers to reveal MPs toxicity.

Conclusions and future perspectives

The amount of information and knowledge about the serious but underappreciated problem of the toxic effects of MPs on organisms is growing. Although MPs affect the normal physiology and causing toxic effects on aquatic organisms, we know very little about biomarkers, which are indicator of normal and abnormal biological processes or responses at lower levels of biological organization (i.e., biochemical, cellular, or physiological levels). This minireview summarized the present situation in *M. galloprovincialis*, one of the most important bioindicators for the marine environment. The bibliometric analysis showed that several biomarkers were assessed. However, a battery of standardized and validated biomarkers is needed in future studies to obtain more comparable findings. Another challenge is the incorporation of new biomarkers (i.e., genetic, metabolomic, transcriptomic, histological, reproductive) at different levels of biological organization, as well as a multidisciplinary approach (adequate integration of multiple indicators) to perform a risk assessment, encouraging the use of chemometrics. Indeed, chemometric tools

(correlation and factor analyses) and several numerical models should be used to assess ecological and human (non-carcinogenic and carcinogenic) health risk due to MPs exposure.

Another aspect is that organism usually answer with a stress or an increment of some enzymes after an exposition to MPs, but it is not clear if organisms answer to plastic or to some components of this or to some substances related to plastic, as phthalates. Related to this, the acidification of the oceans and, in consequence, the change of pH, and the increasing of the temperature can change the relation between plastic and contaminant, but also the availability for organism and the possibility of absorbing it.

Furthermore, other interesting aspects should be represented by microbiota studies associated to MPs exposure. Finally, one of the major challenges ahead of us include determining the exact mechanisms of MPs toxicity, either alone or in combination with other environmental contaminants, as well as determining the exact mechanisms of MPs uptake by gill and gut cells and translocation of the ingested MP into the circulation and target tissues.

References

Abidli, S., Pinheiro, M., Lahbib, Y., Neuparth, T., Santos, M. M., & Trigui El Menif, N. (2021). Effects of environmentally relevant levels of polyethylene microplastic on *Mytilus galloprovinciali*s (Mollusca: Bivalvia): filtration rate and oxidative stress. Environmental Science and Pollution Research, 28, 26643-26652.

Aronson, J. K., & Ferner, R. E. (2017). Biomarkers—a general review. Current protocols in pharmacology, 76(1), 9-23.

Bhatt, P., Pathak, V. M., Bagheri, A. R., & Bilal, M. (2021). Microplastic contaminants in the aqueous environment, fate, toxicity consequences, and remediation strategies. Environmental Research, 200, 111762.

Bolognesi, C., Frenzilli, G., Lasagna, C., Perrone, E., & Roggieri, P. (2004). Genotoxicity biomarkers in *Mytilus galloprovincialis*: wild versus caged mussels. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 552(1-2), 153-162.

Box, A., Sureda, A., Galgani, F., Pons, A., & Deudero, S. (2007). Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 146(4), 531-539.

Bråte, I.L.N., Blázquez, M., Brooks, S.J., Thomas, K.V. (2018). Weathering impacts the uptake of polyethylene microparticles from toothpaste in Mediterranean mussels (*M. galloprovincialis*). Science of the Total Environment, 626, 1310-1318.

Capo, X., Rubio, M., Solomando, A., Alomar, C., Compa, M., Sureda, A., & Deudero, S. (2021). Microplastic intake and enzymatic responses in *Mytilus galloprovincialis* reared at the vicinities of an aquaculture station. Chemosphere, 280, 130575.

Capolupo, M., Gunaalan, K., Booth, A. M., Sørensen, L., Valbonesi, P., & Fabbri, E. (2021b). The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean mussel *Mytilus galloprovincialis*. Environmental Pollution, 283, 117081.

Capolupo, M., Rombolà, A. G., Sharmin, S., Valbonesi, P., Fabbri, D., & Fabbri, E. (2021a). Assessing the impact of chrysene-sorbed polystyrene microplastics on different life stages of the Mediterranean mussel *Mytilus galloprovincialis*. Applied Sciences, 11(19), 8924.

Capolupo, M., Valbonesi, P., & Fabbri, E. (2021). A comparative assessment of the chronic effects of micro-and nano-plastics on the physiology of the Mediterranean mussel *Mytilus galloprovincialis*. Nanomaterials, 11(3), 649.

Cappello, T., De Marco, G., Conti, G. O., Giannetto, A., Ferrante, M., Mauceri, A., & Maisano, M. (2021). Time-dependent metabolic disorders induced by short-term exposure to polystyrene microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. Ecotoxicology and Environmental Safety, 209, 111780.

Cappello, T., Giannetto, A., Parrino, V., Maisano, M., Oliva, S., De Marco, G., ... & Fasulo, S. (2018). Baseline levels of metabolites in different tissues of mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 26, 32-39.

Choi, J. S., Kim, K., Park, K., & Park, J. W. (2022). Long-term exposure of the Mediterranean mussels, *Mytilus galloprovincialis* to polyethylene terephthalate microfibers: Implication for reproductive and neurotoxic effects. Chemosphere, 299, 134317.

Cole, M., Liddle, C., Consolandi, G., Drago, C., Hird, C., Lindeque, P. K., & Galloway, T. S. (2020). Microplastics, microfibres and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.). Marine Pollution Bulletin, 160, 111552.

Cole, M., Lindeque, P., Halsband, C., & Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: a review. Marine pollution bulletin, 62(12), 2588-2597.

Davies, I. M., & Vethaak, D. (2012). Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Research Reports (CRR).

Davis Jr, R. A., & Hayes, M. O. (1984). What is a wave-dominated coast?. In Developments in Sedimentology (Vol. 39, pp. 313-329). Elsevier.

Ding, J., Sun, C., He, C., Li, J., Ju, P., & Li, F. (2021). Microplastics in four bivalve species and basis for using bivalves as bioindicators of microplastic pollution. Science of the Total Environment, 782, 146830.

Do, A. T. N., Ha, Y., & Kwon, J. H. (2022). Leaching of microplastic-associated additives in aquatic environments: a critical review. Environmental Pollution, 305, 119258.

Duis, K., & Coors, A. (2016). Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on personal care products), fate and effects. Environmental Sciences Europe, 28(1), 1-25.

Elia, A. C., Burioli, E., Magara, G., Pastorino, P., Caldaroni, B., Menconi, V., ... & Prearo, M. (2020). Oxidative stress ecology on Pacific oyster *Crassostrea gigas* from lagoon and offshore Italian sites. Science of The Total Environment, 739, 139886.

Fastelli, P., Blašković, A., Bernardi, G., Romeo, T., Čižmek, H., Andaloro, F., ... & Renzi, M. (2016). Plastic litter in sediments from a marine area likely to become protected (Aeolian Archipelago's islands, Tyrrhenian sea). Marine Pollution Bulletin, 113(1-2), 526-529.

Fernández, B., & Albentosa, M. (2019). Insights into the uptake, elimination and accumulation of microplastics in mussel. Environmental pollution, 249, 321-329.

Gadzała-Kopciuch, R., Berecka, B., Bartoszewicz, J., & Buszewski, B. (2004). Some considerations about bioindicators in environmental monitoring. Polish Journal of Environmental Studies, 13(5), 453-462.

Gedik, K., & Eryaşar, A. R. (2020). Microplastic pollution profile of Mediterranean mussels *(Mytilus galloprovincialis)* collected along the Turkish coasts. Chemosphere, 260, 127570.

González-Fernández, C., Albentosa, M., Campillo, J. A., Viñas, L., Fumega, J., Franco, A., ... & Bellas, J. (2015). Influence of mussel biological variability on pollution biomarkers. Environmental research, 137, 14-31.

González-Soto, N., Hatfield, J., Katsumiti, A., Duroudier, N., Lacave, J. M., Bilbao, E., ... & Cajaraville, M. P. (2019). Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo [a] pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*. Science of the Total Environment, 684, 548-566.

Graham, P., Palazzo, L., de Lucia, G. A., Telfer, T. C., Baroli, M., & Carboni, S. (2019). Microplastics uptake and egestion dynamics in Pacific oysters, Magallana gigas (Thunberg, 1793), under controlled conditions. Environmental pollution, 252, 742-748.

Grbin, D., Sabolić, I., Klobučar, G., Dennis, S. R., Šrut, M., Bakarić, R., ... & Štambuk, A. (2019). Biomarker response of Mediterranean mussels *Mytilus galloprovincialis* regarding environmental conditions, pollution impact and seasonal effects. Science of the Total Environment, 694, 133470.

Halliwell, B. (2007). Biochemistry of oxidative stress. Biochemical society transactions, 35(5), 1147-1150.

Harris, P. T. (2020). The fate of microplastic in marine sedimentary environments: a review and synthesis. Marine Pollution Bulletin, 158, 111398.

He, S., Jia, M., Xiang, Y., Song, B., Xiong, W., Cao, J., ... & Zeng, G. (2022). Biofilm on microplastics in aqueous environment: Physicochemical properties and environmental implications. Journal of Hazardous Materials, 424, 127286.

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., & Duflos, G. (2017). Occurrence and effects of plastic additives on marine environments and organisms: a review. Chemosphere, 182, 781-793.

Jong, M. C., Li, J., Noor, H. M., He, Y., & Gin, K. Y. H. (2022). Impacts of size-fractionation on toxicity of marine microplastics: Enhanced integrated biomarker assessment in the tropical mussels, *Perna viridis*. Science of The Total Environment, 835, 155459.

Kinjo, A., Mizukawa, K., Takada, H., & Inoue, K. (2019). Size-dependent elimination of ingested microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. Marine Pollution Bulletin, 149, 110512.

Komar, P. D. (1977). Beach processes and sedimentation.

Lam, P. K., & Gray, J. S. (2003). The use of biomarkers in environmental monitoring programmes. Marine Pollution Bulletin, 46(2), 182-186.

Lehel, J., & Murphy, S. (2021). Microplastics in the food chain: Food safety and environmental aspects. Reviews of Environmental Contamination and Toxicology Volume 259, 1-49.

Li, J., Lusher, A. L., Rotchell, J. M., Deudero, S., Turra, A., Bråte, I. L. N., ... & Shi, H. (2019). Using mussel as a global bioindicator of coastal microplastic pollution. Environmental pollution, 244, 522-533.

Li, J., Yang, D., Li, L., Jabeen, K., & Shi, H. (2015). Microplastics in commercial bivalves from China. Environmental pollution, 207, 190-195.

Li, Q. I., Osada, M., Suzuki, T., & MORI, K. (1998). Changes in vitellin during oogenesis and effect of estradiol-17β on vitellogenesis in the Pacific oyster *Crassostrea gigas*. Invertebrate Reproduction & Development, 33(1), 87-93.

Lionetto, M. G., Caricato, R., & Giordano, M. E. (2019). Pollution biomarkers in environmental and human biomonitoring. The Open Biomarkers Journal, 9(1).

Liu, Y., Li, R., Yu, J., Ni, F., Sheng, Y., Scircle, A., ... & Zhou, Y. (2021). Separation and identification of microplastics in marine organisms by TGA-FTIR-GC/MS: A case study of mussels from coastal China. Environmental Pollution, 272, 115946.

Llorca, M., Álvarez-Muñoz, D., Ábalos, M., Rodríguez-Mozaz, S., Santos, L. H., León, V. M., ... & Farré, M. (2020). Microplastics in Mediterranean coastal area: Toxicity and impact for the environment and human health. Trends in Environmental Analytical Chemistry, 27, e00090.

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., ... & Ren, H. (2016). Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. Environmental science & technology, 50(7), 4054-4060.

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. Aquatic toxicology, 101(1), 13-30.

Ma, H., Pu, S., Liu, S., Bai, Y., Mandal, S., & Xing, B. (2020). Microplastics in aquatic environments: Toxicity to trigger ecological consequences. Environmental Pollution, 261, 114089.

Magara, G., Sangsawang, A., Pastorino, P., Oddon, S. B., Caldaroni, B., Menconi, V., ... & Elia, A. C. (2021). First insights into oxidative stress and theoretical environmental risk of Bronopol and Detarox® AP, two biocides claimed to be ecofriendly for a sustainable aquaculture. Science of the Total Environment, 778, 146375.

Manzoor, S., Naqash, N., Rashid, G., & Singh, R. (2022). Plastic material degradation and formation of microplastic in the environment: a review. Materials Today: Proceedings, 56, 3254-3260.

Masselink, G., Hughes, M., & Knight, J. (2014). Introduction to coastal processes and geomorphology. Routledge.

O'Donovan, S., Mestre, N. C., Abel, S., Fonseca, T. G., Carteny, C. C., Cormier, B., ... & Bebianno, M. J. (2018). Ecotoxicological effects of chemical contaminants adsorbed to microplastics in the clam *Scrobicularia plana*. Frontiers in marine science, 5, 143.

Padervand, M., Lichtfouse, E., Robert, D., & Wang, C. (2020). Removal of microplastics from the environment. A review. Environmental Chemistry Letters, 18, 807-828.

Parmar, T. K., Rawtani, D., & Agrawal, Y. K. (2016). Bioindicators: the natural indicator of environmental pollution. Frontiers in life science, 9(2), 110-118.

Pastorino, P., Elia, A. C., Caldaroni, B., Menconi, V., Abete, M. C., Brizio, P., ... & Prearo, M. (2020). Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps). Science of the Total Environment, 715, 136946.

Pastorino, P., Pizzul, E., Bertoli, M., Anselmi, S., Kušće, M., Menconi, V., Renzi, M. (2021). First insights into plastic and microplastic occurrence in biotic and abiotic compartments, and snow from a high-mountain lake (Carnic Alps). Chemosphere, 265, 129121.

Paul-Pont, I., Lacroix, C., Fernández, C. G., Hégaret, H., Lambert, C., Le Goïc, N., ... & Soudant, P. (2016). Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. Environmental pollution, 216, 724-737.

Piccardo, M., Bertoli, M., Pastorino, P., Barceló, D., Provenza, F., Lesa, D., Renzi, M. (2021). Lethal and Sublethal Responses of *Hydropsyche pellucidula* (Insecta, Trichoptera) to Commercial Polypropylene Microplastics after Different Preconditioning Treatments. Toxics, 9(10), 256.

Pignattelli, S., Broccoli, A., Piccardo, M., Felline, S., Terlizzi, A., & Renzi, M. (2021a). Short-term physiological and biometrical responses of *Lepidium sativum* seedlings exposed to PET-made microplastics and acid rain. Ecotoxicology and Environmental Safety, 208, 111718.

Pignattelli, S., Broccoli, A., & Renzi, M. (2021). Stress Effect Induced by Microplastics Coupled with Acid Rain, on Garden Cress, During Short and Long Time: Two Exposures in Comparison. Annals of Agricultural & Crop Sciences, 6(6).

Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., ... & Regoli, F. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. Frontiers in marine science, 5, 103.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., & Lemos, B. (2019). Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Science of the Total Environment, 662, 246-253.

Rašković, B., & Berillis, P. (2022). Special Issue on the Histopathology of aquatic animals. Applied Sciences, 12(3), 971.

Rebelein, A., Int-Veen, I., Kammann, U., & Scharsack, J. P. (2021). Microplastic fibers—underestimated threat to aquatic organisms?. Science of the Total Environment, 777, 146045.

Renzi, M., Blašković, A., Bernardi, G., & Russo, G. F. (2018). Plastic litter transfer from sediments towards marine trophic webs: a case study on holothurians. Marine pollution bulletin, 135, 376-385.

Renzi, M., Blašković, A., Broccoli, A., Bernardi, G., Grazioli, E., & Russo, G. (2020). Chemical composition of microplastic in sediments and protected detritivores from different marine habitats (Salina Island). Marine Pollution Bulletin, 152, 110918.

Renzi, M., Specchiulli, A., Blašković, A., Manzo, C., Mancinelli, G., & Cilenti, L. (2019). Marine litter in stomach content of small pelagic fishes from the Adriatic Sea: sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*). Environmental Science and Pollution Research, 26, 2771-2781.

Romdhani, I., De Marco, G., Cappello, T., Ibala, S., Zitouni, N., Boughattas, I., & Banni, M. (2022). Impact of environmental microplastics alone and mixed with benzo [a] pyrene on cellular and molecular responses of *Mytilus galloprovincialis*. Journal of Hazardous Materials, 435, 128952.

Sarkar, A., Ray, D., Shrivastava, A. N., & Sarker, S. (2006). Molecular biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology, 15, 333-340.

Shahul Hamid, F., Bhatti, M. S., Anuar, N., Anuar, N., Mohan, P., & Periathamby, A. (2018). Worldwide distribution and abundance of microplastic: how dire is the situation?. Waste Management & Research, 36(10), 873-897.

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environmental Science and Pollution Research, 24, 21530-21547.

Trestrail, C., Nugegoda, D., & Shimeta, J. (2020). Invertebrate responses to microplastic ingestion: Reviewing the role of the antioxidant system. Science of The Total Environment, 734, 138559.

Trestrail, C., Walpitagama, M., Miranda, A., Nugegoda, D., & Shimeta, J. (2021). Microplastics alter digestive enzyme activities in the marine bivalve, *Mytilus galloprovincialis*. Science of the Total Environment, 779, 146418.

Vandermeersch, G., Van Cauwenberghe, L., Janssen, C. R., Marques, A., Granby, K., Fait, G., ... & Devriese, L. (2015). A critical view on microplastic quantification in aquatic organisms. Environmental Research, 143, 46-55.

Wang, X., Huang, W., Wei, S., Shang, Y., Gu, H., Wu, F., ... & Wang, Y. (2020). Microplastics impair digestive performance but show little effects on antioxidant activity in mussels under low pH conditions. Environmental Pollution, 258, 113691.

Ward, J. E., Rosa, M., & Shumway, S. E. (2019). Capture, ingestion, and egestion of microplastics by suspension-feeding bivalves: a 40-year history. Anthropocene Coasts, 2(1), 39-49.

Zhang, K., Hamidian, A. H., Tubić, A., Zhang, Y., Fang, J. K., Wu, C., & Lam, P. K. (2021). Understanding plastic degradation and microplastic formation in the environment: A review. Environmental Pollution, 274, 116554.

Global Change

Long-term changes in temperature and weather patterns are referred to as climate change. Such fluctuations may be brought on by significant volcanic eruptions or variations in the sun's activity. But since the 1800s, human activities—primarily the combustion of fossil fuels like coal, oil, and gas—have been the primary cause of climate change (Chu et al., 2017).

Fossil fuel combustion produces greenhouse gas emissions that serve as a blanket around the planet, trapping heat from the sun and increasing temperatures.

Methane and carbon dioxide are the primary greenhouse gases responsible for climate change. The first is produced by natural process, like methanogenesis and geological sources, or by human activities, like fossil fuel production, agricultural activities, waste management and biomass burning. These are produced, for instance, while burning coal or gasoline to heat a building. Carbon dioxide can also be released when woods and land are cleared. The main sources of methane emissions are oil and gas production and agriculture. The key industries that produce greenhouse gases include energy, industry, transport, buildings, agriculture, and land use (Walther et al., 2002).

The Intergovernmental Panel on Climate Change (IPCC) is the United Nations body for assessing the science related to climate change. The IPCC creates thorough Assessment Reports that detail the current level of scientific, technical, and socioeconomic understanding on climate change, its effects, potential dangers in the future, and strategies for slowing down its rate of occurrence.

The IPCC is a group of governments that are part of the WMO or UN and comprises 195 members. The work of the IPCC is supported by thousands of individuals from throughout the globe. It also releases Methodology Reports and Special Reports on subjects decided upon by its member nations. The IPCC establishes the current state of knowledge regarding climate change through its assessments. It indicates areas of agreement and gaps in the scientific community's understanding of climate change-related issues. Transparency and objectivity are ensured by the reports' multiple rounds of drafting and revision.

The most recent report is the Sixth Assessment Report, which includes a Synthesis Report and three contributions from Working Groups. In order to carry out its work program, the IPCC convenes meetings with government representatives, either as IPCC Working Groups or as plenary sessions of the Panel, to approve, adopt, and accept reports. The IPCC Plenary Sessions also decide on other matters, such as the organization's budget and report outlines, in addition to the work program.

The Synthesis Report Outline approved at the 52nd Panel Session of the IPCC is organized in three main sections: first section, ‘Current Status and Trends’; second section, ‘Long-term Climate and Development Futures’, explain project up to 2100 and beyond; final section is ‘Near-term Responses in a Changing Climate’, that considers international policy in interval between now and 2030-2040 (Lee et al., 2023).

The Earth's surface is currently around 1.1°C warmer on average than it was in the late 1800s (before to the industrial revolution) and warmer than it has ever been in the previous 100,000 years. The last four decades have been warmer than any decade since 1850, with the most recent decade (2011–2020) being the warmest on record.

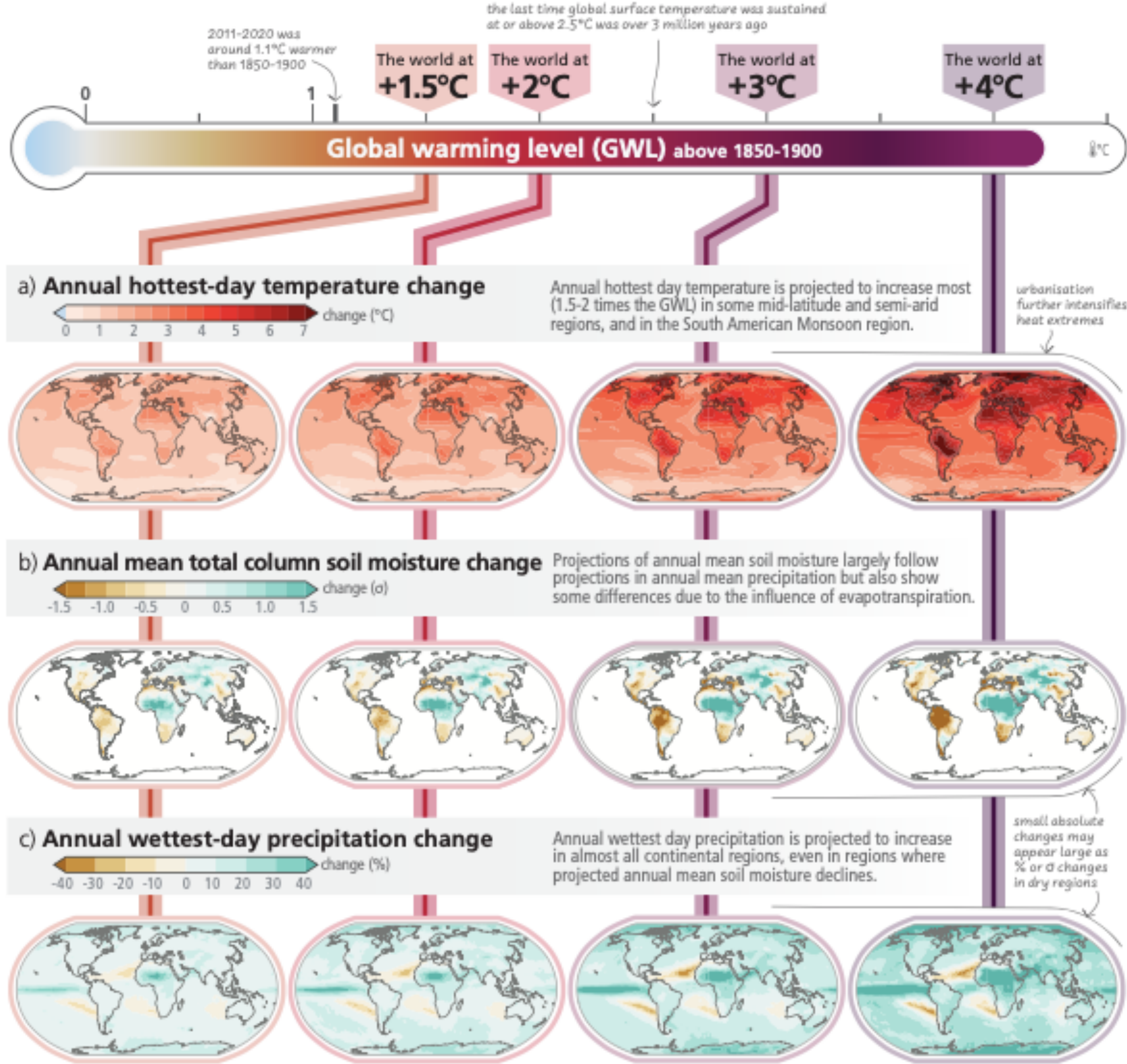


Figure 3 *Projected changes of annual maximum daily temperature, annual mean total column soil moisture CMIP and annual maximum daily precipitation at global warming levels of 1.5°C, 2°C, 3°C, and 4°C relative to 1850-1900. Simulated (a) annual maximum temperature change (°C), (b) annual mean total column soil moisture (standard deviation), (c) annual maximum daily precipitation change (%). In panels (b) and (c), large positive relative changes in dry regions may correspond to small absolute changes. In panel (b), the unit is the standard deviation of interannual variability in soil moisture during 1850-1900. (Lee et al., 2023)*

Intense droughts, water scarcity, destructive fires, rising sea levels, flooding, melting polar ice, catastrophic storms, and a decline in biodiversity are currently some of the effects of climate change (UN, 2023).

Causes and Effects of Climate Change

Fossil fuels, which include coal, oil, and gas, are by far the biggest cause of climate change, contributing more than 75% of all greenhouse gas emissions and almost 90% of all carbon dioxide emissions.

Global warming is the most important and analyzed aspect of the climate change. The heat from the sun gets trapped on Earth as a result of greenhouse gas production. The rate of global warming is presently higher than it has ever been. Weather patterns are shifting as a result of warming temperatures, which is also upsetting the natural order. This puts both us and all other kinds of life on Earth in grave danger.

Cause

Burning fossil fuels to provide power and heat accounts for a sizable portion of world emissions. Burning coal, oil, or gas still supplies the majority of the world's electricity, which produces carbon dioxide and nitrous oxide, two potent greenhouse gases that cover the planet and trap the sun's heat. A little over a quarter of the world's electricity is generated by renewable energy sources including wind, solar, and other natural resources, which, in contrast to fossil fuels, create very little to no greenhouse gases or other air pollutants (National Academies of Sciences, Engineering and Medicine, 2020).

Emissions from manufacturing and industry are mostly the result of burning fossil fuels to create energy for the production of items like textiles, electronics, plastics, cement, iron, and steel. Gases are also released during mining and other industrial activities, as well as during construction. Some products, including plastics, are manufactured from chemicals derived from fossil fuels, as are many of the machines used in manufacturing. These machines frequently run on coal, oil, or gas. One of the leading global producers of greenhouse gas emissions is the industrial sector.

Cutting down forests to make way for farms, pastures, or for other purposes increases emissions because when trees are felled, the stored carbon is released. An estimated 12 million hectares of forest are burned annually. Destruction of forests reduces nature's capacity to keep emissions out of the atmosphere because they absorb carbon dioxide. A percent of the world's greenhouse gas emissions are caused by deforestation, along with agriculture and other changes in land use (UN, 2023).

Fossil fuels are typically used to power cars, trucks, ships, and aircraft. As a result, emissions of greenhouse gases, particularly carbon dioxide, are greatly influenced by the transportation sector. Due to the internal combustion engines used in road cars, which burn petroleum-based fuels like

gasoline, they make up the majority. However, emissions from ships and aircraft are still rising. The majority of carbon dioxide emissions related to energy come from transportation.

In addition to deforestation and clearing land for agriculture and grazing, digestion by cows and sheep, production and use of fertilizers and manure for growing crops, and the use of energy to run farm machinery or fishing boats, typically with fossil fuels, all contribute to the production of food, which results in emissions of carbon dioxide, methane, and other greenhouse gases. Due to all of this, food production plays a significant role in climate change. Additionally, food distribution and packaging also contribute to greenhouse gas emissions.

Over half of all electricity used worldwide is consumed by residential and commercial structures. They continue to produce a sizable amount of greenhouse gas emissions since they use coal, oil, and natural gas for heating and cooling. Energy-related carbon dioxide emissions from buildings have increased over the past few years as a result of rising energy demand for heating and cooling, rising air conditioner ownership, and increased electricity use for lighting, appliances, and connected devices (Fahley et al., 2017).

Effects

The global surface temperature rises together with greenhouse gas concentrations. The warmest decade on record was from 2011 to 2020. Every decade since the 1980s has been warmer than the one before it.

Destructive storms have become more frequent and fiercer in many locations. As temperatures rise, more moisture evaporation occurs, exacerbating heavy rainfall and flooding and producing stronger storms. Tropical storm frequency and strength are influenced by the warming ocean. The main food supply for cyclones, hurricanes, and typhoons is warm ocean surface waters. These storms often cause fatalities and large-scale financial damages by destroying houses and towns.

Climate change is affecting the availability of water, making it increasingly limited in many areas. Global warming exacerbates water scarcity in regions that are already under water stress. Additionally, it raises the risk of agricultural and ecological droughts, which can damage crops and weaken ecosystems.

As a result of global warming, the ocean absorbs most heat. Over the past 20 years, there has been a noticeable increase in ocean warming at all ocean depths. Since water expands when it becomes warmer, the volume of the ocean increases along with it. People who live near coasts and islands are put in danger as sea levels rise due to ice sheets melting. Moreover, the water absorbs carbon dioxide, preventing it from entering the atmosphere. But increased carbon dioxide makes the water more acidic, which puts marine life and coral reefs at danger (Treweek, 2009).

Climate change puts marine and terrestrial creatures at risk. These risks increase with rising temperatures. One million species are in danger of being extinct in the next several decades. Extreme weather, forest fires, and exotic pests are among the threats posed by climate change.

Climate change is the biggest risk to human health. A few of the negative health implications of climate change include air pollution, illness, severe weather, forced relocation, mental health stress, rising rates of hunger, and poor nutrition in places where people cannot grow or receive enough food. An estimated 13 million people are murdered annually by environmental factors. Severe weather occurrences lead to a higher death toll and complicate health care systems' efforts to treat the increasing number of diseases brought on by altered weather patterns (Reidmiller et al., 2017).

References

Chu, E. W., & Karr, J. R. (2017). Environmental impact: Concept, consequences, measurement. Reference Module in Life Sciences.

Fahey, D., Doherty, S., Hibbard, K. A., Romanou, A., & Taylor, P. (2017). Physical drivers of climate change.

Lee, H., Calvin, K., Dasgupta, D., Krinner, G., Mukherji, A., Thorne, P., ... & Zommers, Z. (2023). Climate change 2023: synthesis report. Contribution of working groups I, II and III to the sixth assessment report of the intergovernmental panel on climate change.

National Academies of Sciences, Engineering, and Medicine. (2020). Climate change: evidence and causes Update 2020. The National Academies Press, Washington. https://doi. org/10, 17226, 25733.

Reidmiller, D. R., Avery, C. W., Easterling, D. R., Kunkel, K. E., Lewis, K. L., Maycock, T. K., & Stewart, B. C. (2017). Impacts, risks, and adaptation in the United States: Fourth national climate assessment, volume II.

Treweek, J. (2009). Ecological impact assessment.

United Nations Climate action. What Is Climate Change? (2023)

Walther, G. R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J., ... & Bairlein, F. (2002). Ecological responses to recent climate change. Nature, 416(6879), 389-395.

Aims of the work

Emerging contaminants can be materials, like plastics, microplastics and nanoparticles, chemical and green substances, personal care products and a lot of other kind of pollutants.

The principal aim of this study is to understand the environmental impact of different types of emerging contaminant.

The tools used to analyze this aspect are ecotoxicological assays, combined with biomarkers of oxidative stress.

Different bioindicators and different analysis, in addition to the use of innovative endpoint (like biometries in ecotoxicological assays) and the calculation of integrated index, can permit a more accurate evaluation of the impact of those contaminants on the environment.

In addition to evaluate the impact in standard condition, the second aim of the study is to understand the impact in a global change context.

Global change has a lot of aspect to investigate, the interest in this work is directed to know, in particular, the effect of pH on environmental impact of emerging contaminants.

The environmental impact of emerging contaminant in standard condition and in changed pH condition were evaluate setting some experiments that are reported in some published papers.

Chapter 1

Impacts of Plastic-Made Packaging on Marine Key Species: Effects Following Water Acidification and Ecological Implications

Manuela Piccardo [1,2], Francesca Provenza [1,3], Eleonora Grazioli [3], Serena Anselmi [3], Antonio Terlizzi [1,4] and Monia Renzi [1]

[1]Department of Life Sciences, University of Trieste, 34127 Trieste, Italy; manuela.piccardo@.phd.units.it (M.P.); f.provenza@bsrc.it (F.P.); aterlizzi@units.it (A.T.)

[2]CoNISMa, Piazzale Flaminio, 9 00192, Rome, Italy

[3]Bioscience Research Center, Via Aurelia Vecchia, 32; 58015 Orbetello, Italy; eleonora.grazioli@bsrc.it (E.G.); serena.anselmi@bsrc.it (S.A.)

[4]Stazione Zoologica Anton Dohrn, 80121 Naples, Italy

Journal of Marine Science and Engineering

J. Mar. Sci. Eng. 2021, 9(4), 432

https://doi.org/10.3390/jmse9040432

Abstract

This study evaluates the impacts of 16 different leachates of plastic-made packaging on marine species of different trophic levels (bacteria, algae, echinoderms). Standard ecotoxicological endpoints (inhibition of bioluminescence, inhibition of growth, embryo-toxicity) and alterations of ecologically significant parameters (i.e., echinoderms' body-size) were measured following exposure under different pH water conditions: marine standard (pH 8.1) and two increasingly acidic conditions (pH 7.8 and 7.5) in order to evaluate possible variations induced by ocean acidification. The results obtained in this study evidence that the tested doses are not able to significantly affect bacteria (*Vibrio fischeri)* and algae (*Phaeodactylum tricornutum*). On the contrary, *Paracentrotus lividus* larvae were significantly affected by several packaging types (13 out of 16) with meaningless differences between pH conditions.

Keywords

Biometric impairment; global changes; marine litter; *Vibrio fischeri; Phaeodactylum tricornutum; Paracentrotus lividus*

Introduction

In Europe, plastic production reached almost 58 million tonnes in 2019 and packaging represents the largest end-use market accounting for approximately 40% of the total demand, the growth of which has been accelerated by a global shift from reusable to single-use containers (Geyer et al., 2017; Plastic Europe, 2020). A wide variety of resin types are used for the production of packaging and food packaging: polystyrene (PS) and expanded-polystyrene (EPS) are widely present as containers of fish products; polyethylene terephthalate (PET) is used in water and juices bottles, as well as for the production of shopping bags; polyethylene (PE) is used for milk bottles and food packaging films; polypropylene (PP) is applied for microwave containers, sweets and snack wrappers (Plastic Europe, 2020). Considering the huge production of these type of products and their short lifetime (from production to disposal is about 0.5 years, Geyer et al., 2017), a proper waste management strategy is clearly necessary. According to Plastics Europe (2020), since 2006, the quantity of plastic post-consumer packaging waste sent to recycling sites has increased by 92%. The new Directive (EU) 2019/852 on Packaging and Packaging Waste set 50% as the recycling target for plastic packaging by 2025 and 55% by 2030, strengthening interest and commitment to the correct management of plastic materials. Meanwhile, many mistakes have been made and the evidence of such mistakes is clearly visible. In fact, food wrappers represent a

consistent slice of marine litter. In 2019, the International Coastal Clean-up (ICC) world campaign collected a total of 32,485,488 litter items, of which 4,771,602 were food containers, thus, representing the most abundant litter item (Ocean Conservancy, 2020). Plastic litter is widespread along the coasts of European Countries and in Italy, where waste is found at levels of 6.2 items per linear meter of beach and plastic accounts for 80% of the total waste recorded (Carpentieri et al., 2018). The situation is aggravated by the presence of waste dumps along the beaches, a phenomenon that is well documented and particularly frequent in Tripoli (3 ha), Beirut (Borj Hammoud, 15 ha), Normandy (10 ha), and Saida. Moreover, legal and illegal dumps (Guerranti et al., 2017) in coastal areas could represent a potential risk to the preservation of marine ecosystems. In these areas, the waste loss represents a significant and direct impact on marine ecosystems (AEA, 2006).

Large plastic packaging abandoned in the marine environment could affect wildlife via direct mechanical damage due to ingestion or trapping, and is also consistent source of microplastics (MPs). In fact, macro litter can be reduced in tiny particles by the action of wind, waves and solar radiation (UNEP, 2015; 2016). Meso- and micro-plastics represent the principal fractions of plastic litter that are found worldwide in abiotic matrices (Browne et al., 2011; Eerkes-Medrano et al., 2015; Mani et al., 2015; Alomar et al., 2016; Blašković et al., 2014; 2017) and can be transferred efficiently throughout the trophic web (Setälä et al., 2014; Romeo et al., 2015). This can lead to effects on detritivores (Renzi et al., 2018) and filter feeder species (Thompson et al., 2004; Graham et al., 2009; Murray and Cowie, 2011), while also affecting marine foodstuffs and humans (Renzi et al., 2018a; 2018b; 2018c; 2018d). Recent research highlighted that plastics can interact at different levels with feeding responses in tested species i.e., hard corals (Allen et al., 201, oysters (Li et al., 2018), sea anemones (Diana et al., 2020). Furthermore, other important biological functions could be impacted such as spore settlement and aggregation in *Ulva tepida* (Agusman et al., 2019) and settlement and growth in bryozoans (Li et al., 2016).

Plastic litter has the ability to release both microparticles and chemicals into marine water that able to affect marine species (Rochman et al., 2013; Pedà et al., 2016). Plastic leachates contain chemicals and environmental pollutants previously adsorbed by waste surfaces, such as plastic additives (i.e., phthalates and bisphenol A), as reported in the literature (Rochman et al., 2013; Pedà et al., 2016).

The recent literature has evidenced that global changes could not only affect temperatures, but also induce water acidification (Dupont et al., 2010) that will affect marine species and rocky subtidal communities (Asnaghi et al., 2013; 2014). Moreover, the occurrence of temporary sources of pH acidification in coastal marine ecosystems does not represent a rare phenomenon: temporary water

acidification is reported to originate from effluents of municipal or industrial waste-water treatment plants (Aniyikaiye et al., 2019), acidification from estuarine inputs (Sammut et al., 1995) and from intense activities by primary producers (Hinga, 2002). Coastal transitional ecosystems are naturally pH pulsating environments due to their fluctuating overall balance between a surplus of respiration and primary production (Specchiulli et al., 2008; Basset et al., 2013). Marine water acidification could impact juvenile and larval-stage development in calcifying organisms such as corals, molluscs, and echinoderms (Dupont et al., 2010a; 2010b; Kurihara et al., 2007; Suwa et al., 2009). Previous research has evidenced effects on fertilization and larval survival rates of echinoderms as a consequence of ocean acidification (Martin et al., 2011). Changes in the ecotoxicity of chemicals with marine water chemical features have been reported in the literature (Chou et al., 2018). Furthermore, the effects induced by water acidification on ecotoxicological responses of marine species exposed to chemicals under controlled pH and temperature conditions have only recently been highlighted (Serra-Compte et al., 2018; Fastelli et al., 2019).

This study aims to determine the effects of leachates of plastic-made packaging - (16 different types were tested) obtained from products bought in the supermarket - in three marine species belonging to different trophic levels. Classical ecotoxicological endpoints (inhibition of bioluminescence, inhibition of growth, embryo-toxicity) and innovative endpoint of ecological relevance (i.e., larval body size) were measured in order to collect information of ecological relevance. Packaging leachates differently affected the tested marine species at natural marine water pH (8.1). We also tested lower pH levels to evaluate the possible combined effects between packaging type and water acidification. In particular, two different acidified scenarios were prepared: A1 (pH 7.80), representative of a slight acidification (−0.3 pH units from standard water), and A2 (pH 7.50), representative of an extremely acidified context (−0.6 pH units from standard water).

Material and Methods

Experimental Design, Packaging Types and Leachates

Different plastic packaging types (i.e., 16) intended for food and drink products were bought in the supermarket and used to perform this study. The packaging was separated from the contents and cleaned of any food residues, if present, by careful washing with ultra-pure deionized water. Furthermore, to conduct the experimentation, care was taken to select the part of the packaging that was originally not in contact with the food.

Their detailed chemical composition and industrial use is reported in **Table 1.** Each plastic packaging type was cut into squares (2 x 2 cm) and 10 pieces were put in glass jars with 500 mL of filtered (0.45 μm) natural sea water (MW), opportunely corrected to pH 8.10 before starting the leaching test. A standard exposure plastic surface/water of 160 cm^2/L was obtained. This value was obtained by the exposure of square tiles of 2x2 cm dimensions on each side that were added to a litre of marine water.

Table 1 *Description of the packaging tested reported as "sample name", chemical composition (with abbreviations) and industrial use.*

Sample	Abbreviation	Chemical Composition	Industrial Use
Type_1	PP	Polypropylene	Packaging for Mozzarella cheese
Type_2	PDMS	Poly (dimethyl siloxane) Siloprene E3078	Baking paper
Type_3	PP	Polypropylene atactic	Pasta packaging
Type_4	PET	Polyethylene terephthalate	Water bottle
Type_5	PT - CX	Cellophane	Butter envelope
Type_6	PP	Polypropylene	Container of bread
Type_7	PS	Polystyrene atactic	Yogurt can
Type_8	PET	Polyethylene terephthalate	Shopping bag (recent type)
Type_9	PE+PET	Polyethylene + Polyethylene terephthalate	Bag
Type_10	PS	Polystyrene atactic	Meat tray
Type_11	PET	Polyethylene terephthalate	Cake tray
Type_12	PET + COLOUR	Polyethylene terephthalate + colour	Packaging for Mozzarella cheese
Type_13	PP+PE	Polypropylene + polyethylene copolymer	Cake envelope
Type_14	EPDM	Poly (ethylene:prophylene:diene)	Shopping bag (old type)
Type_15	PE	Polyethylene	Freezer bag
Type_16	PET	Polyethylene terephthalate	Shopping bag (new type)

Exposure doses, in terms of g/L, obtained by the standardization of the exposed surface are reported in **Table 2** and ranged from 0.03 to 2.42 g/L. Leaching time was fixed at T28 days, plastic pieces were maintained in agitation (100 rpm), under a natural light–dark cycle (16:8). At the end of the leaching time, the water was filtered at 0.45 μm and toxicity was tested in marine species belonging to different trophic levels. The packaging types that were found to be toxic in this first phase of the study were further tested at water pH 7.80 and 7.50 on the more sensitive organism (i.e., *P. lividus*).

Table 2 *Mean dose (± standard deviation; SD) of each packaging materials calculated from a standard exposure surface of 160 cm2 /L.*

Sample	Standard Exposed Surface 160 cm^2/L	
	Mean Dose (g/L)	SD (g/L)
Type_1	1.74	0.004
Type_2	0.25	0.018
Type_3	0.27	0.001
Type_4	1.13	0.007
Type_5	0.36	0.002
Type_6	2.42	0.011
Type_7	0.96	0.009
Type_8	0.08	0.001
Type_9	0.16	0.001
Type_10	0.78	0.003
Type_11	0.86	0.007
Type_12	0.24	0.001
Type_13	0.12	0.001
Type_14	0.03	< 0.001
Type_15	0.16	0.012
Type_16	0.06	< 0.001

μFT-IR Characterization of Plastic-Made Packaging

Chemical composition of plastic materials was determined by μFT-IR at the beginning and at the end of the test (T0 and T28) to evaluate plastic degradation during the experiments for each pH scenarios. Analyses were performed by μFT-IR (Thermo, i-10 Nicolet MX infrared imaging microscope, Thermo Fisher Scientific) equipped with standard detector for microscopy optimized to work under room temperature conditions (DTGS) operating in the spectral range 7600–450 cm^{-1} and with the liquid nitrogen cooled MCT-A operating within the spectral range 7800–650 cm^{-1}. Thermo Scientific™ OMNIC™ Picta™ user interface elaborated recorded data. Filters used to filtrate leaching water were also explored to determine the release of microfibers in water.

Ecotoxicological Tests: Exposure and Endpoints

Species from three taxonomic groups of ecological relevance in marine ecosystems were tested under standardized water conditions (pH 8.1): Bacteria (*Vibrio fischeri*), Algae (*Phaeodactylum tricornutum*), and Echinodermata (*Paracentrotus lividus*). Leachates were tested as such (100%) for *P. tricornutum* and *P. lividus*, and 90% for *V. fischeri*.

V. fischeri—A standardized protocol was used for the test on bacteria. Tests were performed according to UNI EN ISO 11348-3:2009 using a Microtox® photometer and lyophilised bacteria purchased by Microbiotests Inc. The percentage of inhibition of natural bioluminescence was calculated after 15 and 30 minutes of exposure to packaging leachates on two experimental replicates.

P. tricornutum—A standardized protocol was used for the test on algae (ISO 10253:2016 (E). An algal lot purchased by Ecotox® was tested after pre-enrichment in an ASW (Artificial Sea Water) culture medium. Illumination, temperature, salinity, and dark–light photo-cycles were set as reported in the protocol. Cell density measures were calculated from light absorbance by a spectrophotometer (Onda, mod. UV-30 scan; wavelength 670 nm, optical length 10 cm). The spectrophotometer response was calibrated using cell density versus an absorbance curve developed on tested algal stock performing counts by Burker chamber at each of the 10 points scalar dilution of 10^6 cell/mL stock. Percentage of growth inhibition (I %) after 72 h of exposure was calculated for three experimental replicates. Growth inhibition was calculated as detailed in the literature (Renzi et al., 2014).

P. lividus—Embryotoxicity after 72 h of exposure was tested following EPA 600/R-95-136/Section 15 adapted by Sartori et al. (2017). Mature specimens of sea urchin were caught in a natural marine areas (Tuscany) and maintained in captivity until the commencement of the experiment. Exposure tests were performed under fasting conditions as reported by the method. Percentages of abnormal

larvae were calculated on 100 *plutei*, randomly chosen, in each experimental replicate (n = 3). Larvae were considered abnormal if they showed developmental arrest, all arms were missing or of different lengths, additional arms with crossed lateral rods, an asymmetrical body width and other anomalies listed in the literature (Sartori et al., 2017). Results were normalized compared to controls according to Abbott (1987).

pH Effects on Embryo Toxicity and Body-Size of P. lividus

For *P. lividus*, three different packaging leachate types (Type_6 = PP; Type_13 = PP+PE; Type_16 = PET) were tested at different pH values (7.80 and 7.50, in addition to pH 8.10) and dilutions (100%; 50%; 25%) in order to evaluate changes in embryo-toxicity and body-size. pH is measured using a probe provided by Hanna Instrument. Sea water is acidified using HCl 0.1 M, and the stability of it is monitored twice a day. The plastic types were chosen on the basis of the severity of the induced effect (obtained in the first phase of the experiment at pH 8.10). Selected types were: Type_16, Type_13 and Type_6 corresponding to severe (89.29% of abnormal larvae), moderate (65.56%) and slight (34.18%) effects, respectively. Results were normalized compared to controls according to Abbott (1987). A series of 100-50-25% dilutions were tested to evaluate the effective concentration (EC_{50}). To better characterize the ecotoxicological responses of echinoderms, a further endpoint was used: the % reduction of arm length. Body-size of *plutei* was obtained measuring the mean arm lengths **(Figure S1)** by stereomicroscopy (Nikon, SMZ-800 N equipped with Nikon's software Nikon ACT-1). Measurements were performed on 15 normal and 15 anomalous animals.

Quality Assurance and Quality Control

Bioscience Research Center is a certified laboratory (ISO 9001:2015) and applies a severe control procedure under guidelines of the UNI EN ISO 17025:2005 to ensure the quality of produced data (ACCREDIA 1715L). QA/QC tests were performed as described by reference methods. Positive controls were performed by the direct exposure of tested species to standard toxicants. In particular, *V. fischeri* was tested with *3,5'*-dichlorophenol (I% 30 min = 42.26 ± 3.63); *P. tricornutum* responses were measured by $K_2Cr_2O_7$ (EC_{50} = 16.21 ± 1.72 mg/L); *P. lividus* was tested with $Cu(NO_3)_2*3H_2O$ (EC_{50} = 22.6-68.34 μg/L), yielding responses that were within the acceptability criteria defined by standard methods. Negative controls (n = 2 for *V. fischeri*, n = 3 for *P. tricornutum* and *P. lividus*) were performed on natural filtered (0.45 μm) marine water (MW) under different experimental conditions (pH = 8.20 ± 0.01; 7.82 ± 0.01; 7.52 ± 0.01). Recorded data were within the acceptability of tests under standard conditions (pH = 8.20).

EC_{50} Calculation and Statistical Analyses

Data were statistically analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Routines related to column statistics (mean, standard deviation, min–max ranges), t-test, and EC_{50} values were performed. Differences were considered significant at p-value < 0.05 (Sarni and Onorati, 2009).

Multivariate (ANOSIM two-way) tests were performed by Primer v6.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK) following the methods reported by Clarke and Warwick (2001) to evaluate the effect of water acidification and chemical composition of plastic packaging on biometrics of echinoderms. Analyses were performed on a Euclidean matrix of distance, calculated on normalized biometric data. A two factors nested experimental design was applied: "packaging type" (Control, Type_6, Type_13, Type_16; four levels, fixed) and "pH" (ST, A1, A2; three levels, fixed).

Results

µFT-IR Characterization of Plastic-Made Packaging

µFT-IR analysis of the plastic materials did not show significant changes in the superficial chemical fingerprint after 28 days of conditioning in marine water under different pH levels. In fact, compared to T0 spectra, T28 ones showed changes < 5% of the total matches (for further details refer to supplementary materials). No microfibers (> 0.45 micron) were found in filters of leachates.

Ecotoxicological Responses at pH Standard (8.1)

Ecotoxicological effects recorded in this study under standard pH conditions (ST) are reported in **Table 3** and summarized in **Figure 1**. A significant effect (> 20%) was recorded only in echinoderm larvae.

Table 3 *Synthesis of the ecotoxicological responses for each tested packaging leachate type. Data are expressed as mean effects (% ± standard deviation, SD) at the maximum dose tested (100% for P. tricornutum and P. lividus; 90% for V. fischeri). MW = marine water, i.e., negative control. White bars indicate no effect (< 20%); light grey, grey and dark grey bars show slight (20–39%), moderate (40–79%) and severe (80–100%) effects. Values in bold represent the sample type selected for the subsequent analysis.*

Sample Type	***V. fischeri*** I% (15 min)		I% (30 min)		***P. tricornutum*** I% (72 h)		***P. lividus*** % abnormal (72h)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MW	−3.52	2.56	−9	0.4	−.05	0.45	4.74	2.08
Type_1	−2.03	1.35	−9.08	1.61	−0.15	0.59	2.04	2.16
Type_2	−4.62	1.34	−8.66	1.73	1.59	0.81	2.04	1.83
Type_3	0.28	1.32	−7.51	2.12	−1.8	0.45	2.04	1.41
Type_4	−0.03	0.43	−1.15	0.12	−2.04	0.36	36.73	6.68
Type_5	2.15	3.97	−1.28	2.5	−0.4	0.8	52.3	2.36
Type_6	3.5	1.11	−2.68	1.61	0.7	0.61	34.18	2.89
Type_7	3.02	0.62	−3.53	0.72	1.3	0.44	45.15	3.3
Type_8	2.41	0.34	−2.28	0.62	0.97	0.36	63.01	2.75
Type_9	−7.43	2.33	−11.56	3.61	−0.29	0.5	84.69	2.16
Type_10	−6.58	1.11	−11.35	2.89	−0.64	0.18	72.19	2.22
Type_11	−6.96	1.19	−6.46	0.86	−4.42	0.92	97.45	1.29
Type_12	−7.19	8.35	−10.35	8.03	−0.47	1.27	73.72	1.26
Type_13	−10.41	3.37	−15.49	4.12	0.85	0.16	65.56	2.06
Type_14	−10.46	10.4	−14.32	11.37	0.96	0.81	78.06	1.29
Type_15	−11.12	6.27	−14.34	6.05	0.25	0.39	76.53	2.16
Type_16	−3.42	1.29	−3.48	2.28	−4.49	1.3	89.29	1.29

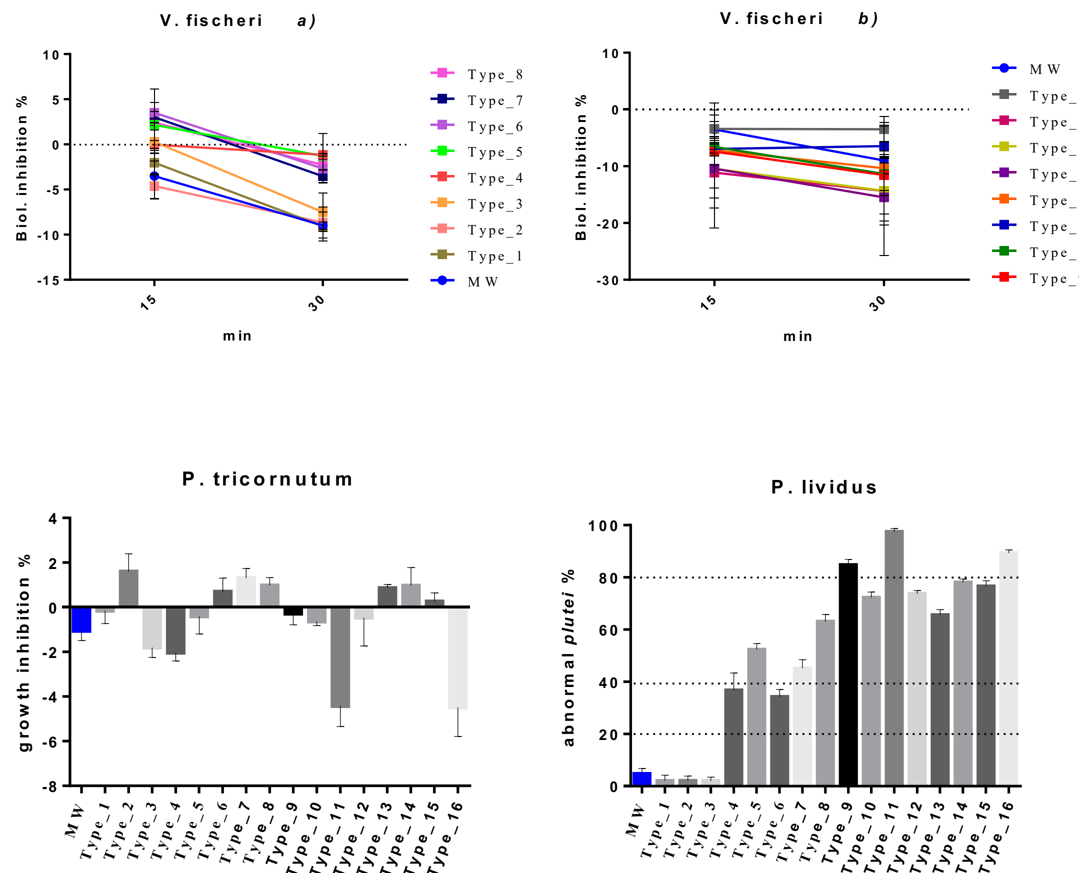


Figure 1 *Mean percentage of effects in tested species for each packaging type. Results are expressed in percentage (%). Standard deviation is represented. Negative values represent stimulation while positive values imply inhibition. MW = marine water (negative control, blue colour). Data are reported under standard pH conditions (8.10). Effect (> 20%) was recorded only in echinoderm larvae. Echinoderm data were corrected with Abbott's correction: (X-Y)/(100-Y)*100, where X is the effect cause by the sample and Y is the effect cause by the control. Leachate dilution: 100% for echinoderms and algae; 90% for bacteria.*

Concerning the species *V. fischeri*, inhibition of bioluminescence ranged between −11.12% and +3.50% after 15 min of exposure at 90% dilution. Negative values correspond to stimulation. Recorded effects were always lower than 20%. Consequently, tested leachates can be considered as not being toxic for this species. Longer exposure times (30 min) did not significantly change the percentage effect (between −15.49% and +3.50%) with it always remaining under 20%.

As regards *P. tricornutum*, the mean effect percentages ranged between −4.49% and +1.59%; in some cases, inducing algal growth inhibition (Type_2, 6, 7, 8, 13, 14, 15), in others, biostimulating (Type_1, 3, 4, 5, 9, 10, 11, 12, 16). Globally tested leachates can be considered as not being toxic at the tested doses.

Significant toxicity was recorded, on the contrary, in *P. lividus* exposed to almost all plastic materials. In particular, packaging types one to three (i.e., Type_1 = PP; Type_2 = PDMS; Type_3 = PP) were shown to not be toxic under the tested doses with mean effects of 2.04%. All other types reported effect > 20% (cut-off level of toxicity), inducing slight (20–39%; Type_4 = PET; Type_6 = PP), moderate (40–79%; Type_5 = PT-CX; Type_7 = PS; Type_8 = PET; Type_10 = PS; Type_12 = PET+COLOUR; Type_13 = PP+PE; Type_14 = EPDM; Type_15 = PE) and severe (80–100%; Type_9 = PE+PET; Type_11 = PET; Type_16 = PET) effects. Structural anomalies recorded on exposed embryos of *P. lividus* are represented in **Figure S2** and consist of cross lateral rods, split lateral rods, bended arms, crossed and exposed lateral rods, asymmetrical larval body growth, unequal antero-lateral arms, elongation of one of the post-oral arms, and broken or exposed lateral arms.

Water Acidification: Embryo Toxicity and Body-Size Reduction in P. lividus

Ecotoxicological responses of *P. lividus* exposed to plastic leachates (100% of dilution), under different pH conditions are reported in **Table 4**. Effects were evaluated by means of abnormal development and biometrics impairment at 72 h of exposure.

Table 4 *Ecotoxicological responses in P. lividus exposed to 3 pre-selected plastic packaging leachates under different pH conditions. Data reported are expressed as % of anomalous plutei, as mean length reduction in abnormal larvae compared to the corresponding control and normal plutei (at the maximum dose of exposure, 100%), and as* EC_{50}*. Data are grouped according to pH levels (ST = 8.1; A1= 7.80; A2 = 7.50 pH). NC = not calculable because the effect at maximum concentration was lower than 50%.*

Sample		Abnormal Larvae		Biometrics Reduction (Abnormal vs. Cnt)	Biometrics Reduction (Abnormal vs. Normal)	EC_{50}
		mean	SD			
Control_ST		4	2			NC
Type_6	PP	51.04	3.46	16.20	12.57	50.15
Type_13	PP+PE	63.54	2.00	17.57	15.12	52.67
Type_16	PET	67.01	3.06	30.51	16.73	88.71
Control_A1		4.00	2.00			NC
Type_6	PP	51.74	1.53	16.11	0.61	44.58
Type_13	PP+PE	57.99	1.53	22.24	12.02	51.08
Type_16	PET	73.61	4.51	32.39	18.47	60.91
Control_A2		4.00	2.00			NC
Type_6	PP	36.46	1.00	24.95	15.50	NC
Type_13	PP+PE	65.09	3.13	20.43	9.24	64.50
Type_16	PET	46.53	3.21	32.97	22.18	NC

As regards the percentage of abnormal larvae, controls reported the same values of anomalous larvae (4.00%), without significant differences between pH. At pH standard and A1 (weakly acid), the severity of polymer-based toxicity was: Type_16 > Type_13 > Type_6. In more acidified conditions (A2), Type_13 was the more toxic treatment. Type_6 (i.e., PP) induced less severe effects, corresponding to 51.04%, 51.74% and 36.46% in ST, A1 and A2 scenarios, respectively. Effects of water acidification on abnormalities (recorded effect at the maximum tested dose) were tested by t-test and are reported in **Table 5** and **Figure 2**.

Table 5 *Effect of water acidification in P. lividus ecotoxicological responses (100% of leachate dilution). T-test was performed within each plastic type comparing the different scenarios of acidification (ST vs. A1; ST vs. A2; and A1 vs. A2). "Weakly significant" corresponds to 0.05 < p-value < 0.01 and "Significant" to p-value < 0.01.*

Type	Comparison	***p-value***	Significance Level
	ST vs. A1	0.653	-
Type_6 (PP)	ST vs. A2	0.009	Significant
	A1 vs. A2	< 0.001	Significant
	ST vs. A1	0.020	Weakly significant
Type_13 (PP+PE)	ST vs. A2	0.213	-
	A1 vs. A2	0.015	Weakly significant
	ST vs. A1	0.831	-
Type_16 (PET)	ST vs. A2	< 0.001	Significant
	A1 vs. A2	0.001	Significant

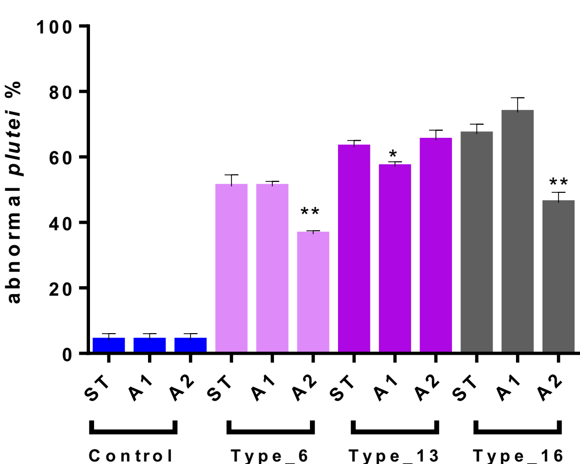


Figure 2 *Effect of water acidification in determining abnormal development of P. lividus larvae (100% of dilution). T-test was performed within each plastic type comparing the different scenarios of acidification (ST vs. A1; ST vs. A2; A1 vs. A2). * means weakly significant (0.05 < p-value < 0.01) and ** significant (p-value < 0.01) compared to both other pH conditions. ST means natural pH.*

Acidified conditions (A1, A2) often unexpectedly differed from standard (ST) pH, that is, decreasing the % of anomalous larvae. In particular, for Type_6 and Type_16, ST vs. A1 was not significantly different (*p*-value > 0.05), while significant differences (*p*-value < 0.01) were reported between ST vs. A2 and between A1 vs. A2. Type_13 corresponded to weak differences (0.01 < *p*-value < 0.05) in ST vs. A1 and A1 vs. A2. As regards the EC_{50} values, there was a trend reversal in polymer-based toxicity in ST and A1 scenarios: Type_6 > Type_13 > Type_16. The calculation of EC_{50} in A2 was possible only for Type_13 and corresponded to 64.5%.

The ability of Type_16 to induce more severe effects was confirmed by the mean length reduction (%) in abnormal larvae in respect to controls (up to 32.97%) and to normal *plutei* (up to 22.18%) (**Figure 3** and **Table 5)**. Biometrics revealed to be sensitive enough to also highlight differences between controls and normal larvae (at pH 8.1, **Figure 3**). **Figure 4** shows the effects on body-size of *P. lividus* larvae, after exposure to the three different plastic packaging types, water pH and dilution of leachates. Multivariate statistical analyses were performed on both normal and abnormal embryos to detect differences between factors. The ANOSIM test (two way) (**Table 6)** evidenced that, concerning abnormal embryos, pH is not effective to determine body-size differences (Global R = −0.034; *p* = 72.1%), while the type of plastic packaging material significantly affects this aspect (Global R = 0.287; *p* = 0.2%). In particular, larger differences were recorded between PP−PET (Global R = 0.142; *p* = 3.4%), while no differences were recorded between PP−Negative control (Global R = −0.327; *p* = 90%). A significant effect of packaging type was also recorded on the body-size of normal embryos (Global R = −0.270; p =1.7%). Also, in this case, larger differences are recorded between PP-PET (Global R = 0.142; *p* = 3.4%), while

no differences were recorded between PP−Negative control (p = 100%). In this case, pH showed a non-significant but higher effect than on abnormal embryos (Global R = 0.017; p = 33.8%).

Table 6 *ANOSIM test performed on factors affecting biometrics of echinoderms. Notes: Pairwise test performed between couples of considered levels of tested factors is reported (Sign. Couples). Only significant relationships are reported. * = slightly significant.*

.	Factors	Levels	Pairwise	Global R	Sign. Level %	Sign. Couples
NORMAL	pH	3	ST-A1; ST-A2; A1-A2	0.017	0.34	-
	packaging type	4	Type_6-Type_13; Type_6-Type_16; Type_13-Type_16; Cnt-Type_6; Cnt-Type_13; Cnt-Type_16	0.142	3.4 *	Type_6-Type_16 *
ABNORMAL	pH	3	ST-A1; ST-A2; A1-A2	0.034	72.1	-
	packaging type	4	Type_6-Type_13; Type_6-Type_16; Type_13-Type_16; Cnt-Type_6; Cnt-Type_13; Cnt-Type_16	0.287	0.2 *	Type_6-Type_16 *

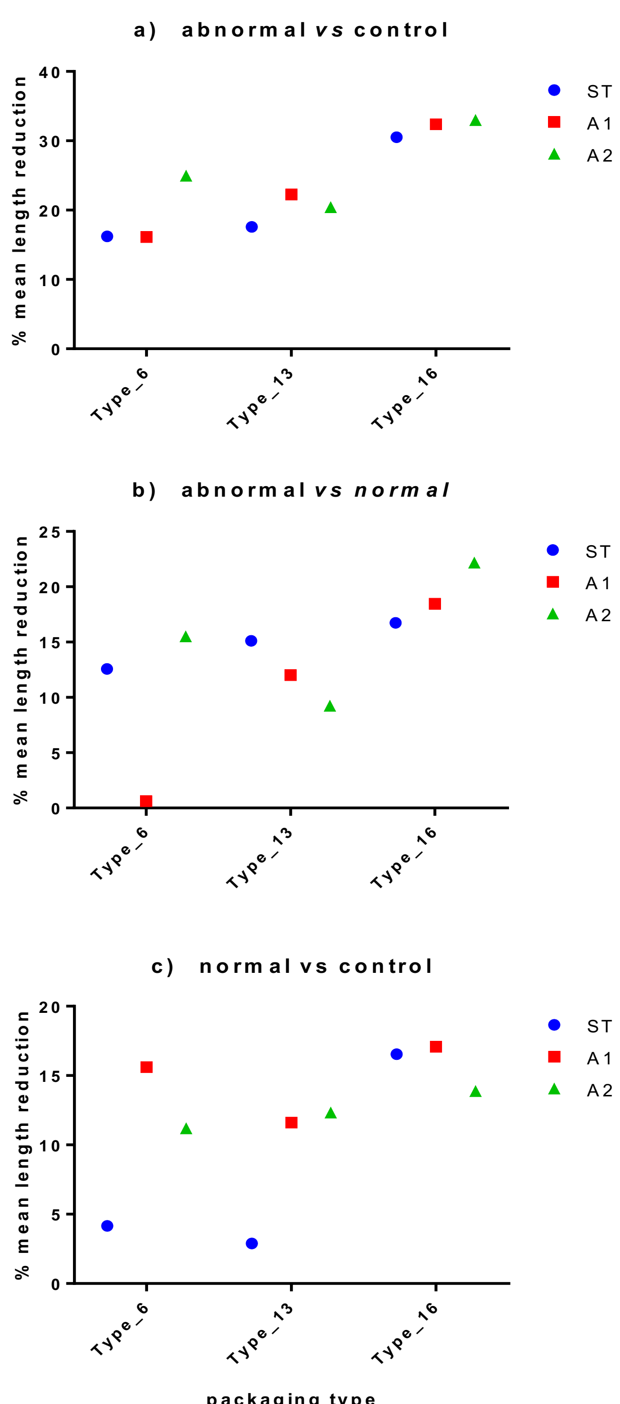


Figure 3 *Body-size reduction in P. lividus larvae exposed to different plastic types according to water pH (100% dilution). Mean arms lengths of anomalous larvae are compared respect to (a) controls and (b) normal larvae. (c) shows comparison between normal-developed larvae and controls. In all cases, Type_16 (i.e., PET) induced more evident effects.*

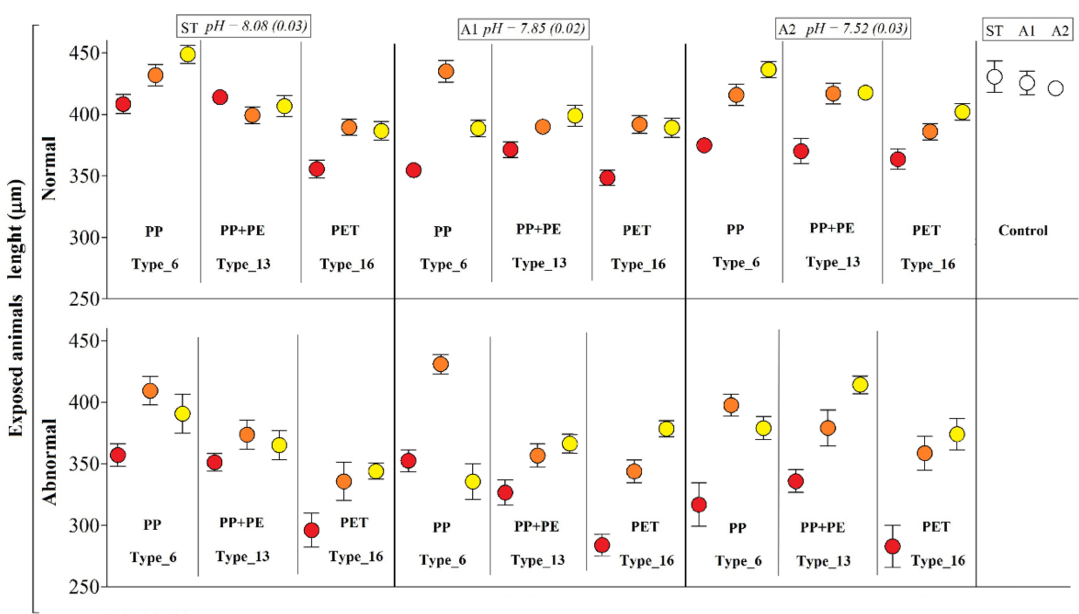


Figure 4 *Body-size of P. lividus larvae exposed to different plastic types according to water pH and leachate dilutions. Results are expressed as mean arms lengths (± SD) and calculated in both normal and abnormal larvae. Negative controls on the right. Tests were performed under three different pH scenarios: ST = standard marine water, pH 8.1; A1 = acidified (−0.3 pH units), pH 7.80; A2 = extremely acidified (-0.6 pH units), pH 7.50. Three different dilutions of the initial packaging leachate were used (red dots = 100%; orange dots = 50%; yellow dots = 25%) for PP (Type_6), PP+PE (Type_13), and PET (Type_16). Data present the Abbott's correction.*

Discussion

Plastic pollution in marine environments can severely affect ecosystems via different direct and indirect threats, causing physical damage, biological threats and chemical harm (Eerkes-Medrano et al., 2015; Basseling et al., 2018; Bucci et al., 2019). Only recently are we beginning to realize and study the chemical hazards associated with plastic litter known to be associated with a "cocktail of chemicals". In fact, leaching processes in marine habitats could determine significant releases of chemicals from plastic packaging and produce effects on marine species (Rochman et al., 2013; Pedà et al., 2016).

Results obtained in this study at pH standard (8.10 units) on leachates, evidenced that the tested doses are not able to significantly affect bacteria (*V. fischeri)* and algae (*P. tricornutum*). On the contrary, *P. lividus* larvae were significantly affected by several packaging leachates, meaning that this is a sensitive organism for testing plastic pollution. A total of 13 out of 16 packaging types were toxic. Specifically, Type_4 and 6 induced slight effects (< 40%), Type_5, 7, 8, 10, 12, 13, 14, 15 medium effects (40–80%), and Type_9, 11, 16 severe effects (> 80%). The first category is represented by PET and PP intended for water bottles and bread containers; the second one is composed of a great variety of single type (cellophane, PS, PET, PE, EPDM) and combined polymers (PE+PET, PP+PE, PET+COLOR) intended to contain butter, yogurt, meat, mozzarella,

cakes, as well as being generically used to produce “shopping bags” and “freezer bags”. The more hazardous category is represented by PET (alone or in combination with PE), intended for “bags”, “cake trays” and “new type shopping bags”. PET, together with PP and PE is the resin that is used more frequently for packaging purposes (Plastic Europe, 2020). Other studies have reported the toxicity of PET leachates (100 mg/L, 100% dilution) to echinoderm larvae (Piccardo et al., 2020): leachates prepared after only 72 h of conditioning in marine water induced abnormal development in 27.2% of cases, under fasting conditions. In the present study, the plastic concentration never exceeded 12 mg/L but a longer leaching time (28 days) evidently contributed to increased toxicity, inducing more severe consequences (up to 97.45%). The sensitivity of echinoderm larvae to plastic pollution is well documented in the literature. In 2012, Feng et al. (2012) reported that exposure to polysiloxane could affect the embryonic development of sea urchins (*Arbacia punctulata*). Oliviero et al. (2019) reported a drastic reduction in larval length (33%) in *plutei* exposed to PVC leachates, probably due to the presence of phthalates. PVC leachates (72 h, 10% dilution) containing polycyclic aromatic hydrocarbons and polychlorinated biphenyls affected sea urchin development, inducing developmental delays, malformation of skeletal structures and nervous and immune systems, as well as abnormal axis formation (Rendell-Bhatti et al., 2021).

The second part of this study was conducted focusing the attention on leachates of packaging which corresponded to slight (Type_6), moderate (Type_13) and severe (Type_16) toxicity, at different pH conditions. Different pH levels were prepared to simulate acidified (A1; −0.3 pH units = 7.80) and extremely acidified (A2; −0.6 pH units = 7.50) conditions in marine water. Ocean acidification is considered one of the principal consequences of global climate change (Raven, 2005) and this study wanted to understand the possible combined effect of plastic-made packaging leachates and water acidification on the ecotoxicological responses of *P. lividus* larvae. Effects on echinoderms consisted of both the percentage of abnormalities and biometrics variations. Acidification alone did not induce significant differences (as shown by controls) suggesting a low sensitivity to water acidification. However, echinoderms were capable of discerning chemical changes in the water medium that were not detectable by µFT-IR analysis, thus, providing a first alarm. In fact, on the one hand, no spectral variations were observed on plastic surfaces conditioned in standard and acidified marine water; on the other hand, echinoderms exposed to leachates at acidified conditions (A1-A2) highlighted statistically significant differences in respect to standard pH. In particular, acidified scenarios induced a lower percentage of anomalous larvae, suggesting a complex plastic–pH interaction that is difficult to understand. In this regard, a useful contribution could come from a more detailed chemical analysis. In fact, detailed information on leached chemical additives may require additional chemical analysis, such as adsorption chromatography coupled with GC–MS (not performed in this context).

The level of packaging-based severity shows Type_16 as the most toxic, and Type_6 as the least hazardous (confirming the results of the first part of the experiment). Type_16 is PET used for the production of new type of shopping bags, whereas Type_6 is PP intended to contain bread. Type_16 was also more toxic in terms of biometric impairment, resulting in animals being shorter than 30.51, 32.39 and 32.97% in ST, A1 and A2 conditions, respectively (abnormal vs. controls). Biometrics also demonstrated to be a sensitive endpoint in observing variations between abnormal and normal-formed embryos of treated animals. In fact, the first one highlighted arm lengths that were 16.73, 18.47 and 22.18% shorter. Biometrics can show a reduction in body-size when a stress condition is present in the environment: organisms under stress conditions activate metabolic patterns that cause energy consumption. If organisms have low levels of energy available, their development will be reduced. Thus, biometric variations could be considered a precocious marker of stress, as reported by other similar studies (Piccardo et al., 2020).

Another important implication of organisms' biomass reduction is the minor energy flow into the trophic web. Lindeman (Kontrick, 2018) was the first to demonstrate that ecosystem functioning can be represented by energy flow through a trophic pyramid or food web. The efficiency of energy transfer among higher trophic levels is often consistent with the hypothesis that trophic structure may control the fraction of energy consumed within each trophic level, rather than energetics controlling trophic structure (Wetzel, 1995). This energy transformation at each trophic level (as well as by each organism) represents the storage of potential energy that fuels metabolic processes and power output at each trophic level. Energy flow reflects the transfer of energy for productivity by all trophic levels (Schowalter, 2016). Each level of the trophic web cannot consume more matter than is available, and energy is lost during each transfer between trophic levels, moreover, a portion of the assimilated energy must be used to support metabolic work (e.g., for maintenance, food acquisition, and various other activities) and is lost through respiration (Schowalter, 2016). When organisms have less energy, one of the consequences is reduced reproductive success, involving minor organisms, thus, less matter and energy is available in the trophic web.

Analyzed in its entirety, the multivariate analysis highlighted that acid conditions were not relevant to induce important biometric impairment, and therefore, additional expenditure of energy was not required. In fact, the factor "acidification" influenced the occurrence of developmental anomalies in a positive way: larvae exposed to acid scenarios (A1, A2) statistically differed from standard water reporting a decreasing in the anomalous larvae percentage. Further studies are necessary to better elucidate this aspect.

Conclusions

This study aimed to determine the effects of leachates of plastic-made packaging (16 different types tested) in three marine species, belonging to different trophic levels. Tested doses were not able to significantly affect bacteria (*V. fischeri)* and algae (*P. tricornutum*). On the contrary, *P. lividus* larvae were significantly affected by several packaging leachates (13 out of 16), making this a sensitive organism for testing plastic pollution. The most hazardous polymer was the PET (alone or in a combination with PE) inducing up to 97.45% of abnormal larvae and *plutei*, with a mean arm length that was 30.51% shorter than the controls. This study also aimed to explore the combined effect between "packaging type" and "water acidification" (in the frame of global changes) on the ecotoxicological responses of *P. lividus*. Globally, water acidification was not able to induce biometric impairment, thus, additional expenditure of energy was not required. As a consequence, exposure to acidic water positively influenced the occurrence of developmental anomalies, decreasing the percentage of anomalous larvae. Further studies are necessary to better elucidate this aspect. Finally, biometric variations could be considered a precocious marker of stress in echinoderm larvae.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/jmse9040432/s1, Figure S1: Determination of body-size of P. lividus obtained by measuring the mean arms lengths (L1 + L2)/2; expressed in µm. Figure S2. Representation of recorded anomalies in P. lividus exposed to different plastic packaging types. (a) normal; (b–w) examples of different types of alterations: alteration of L1/L2 ratio, cross lateral rods, split lateral rods, bended arms, crossed and exposed lateral rods, asymmetrical larval body growth, unequal antero-lateral arms, elongation of one of the post-oral arms, broken lateral rods, exposed lateral rods, etc.

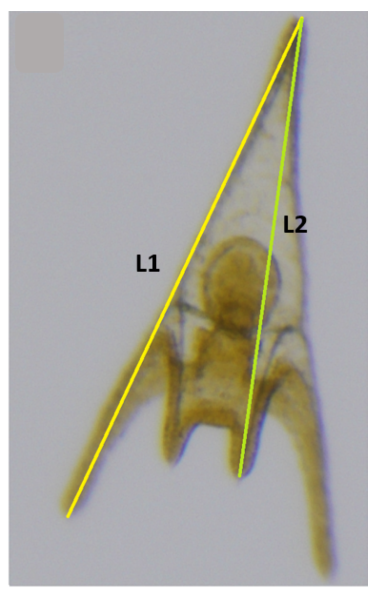


Figure S1 *Determination of body-size of P. lividus obtained by measuring the mean arms lengths (L1+L2)/2; expressed in µm.*

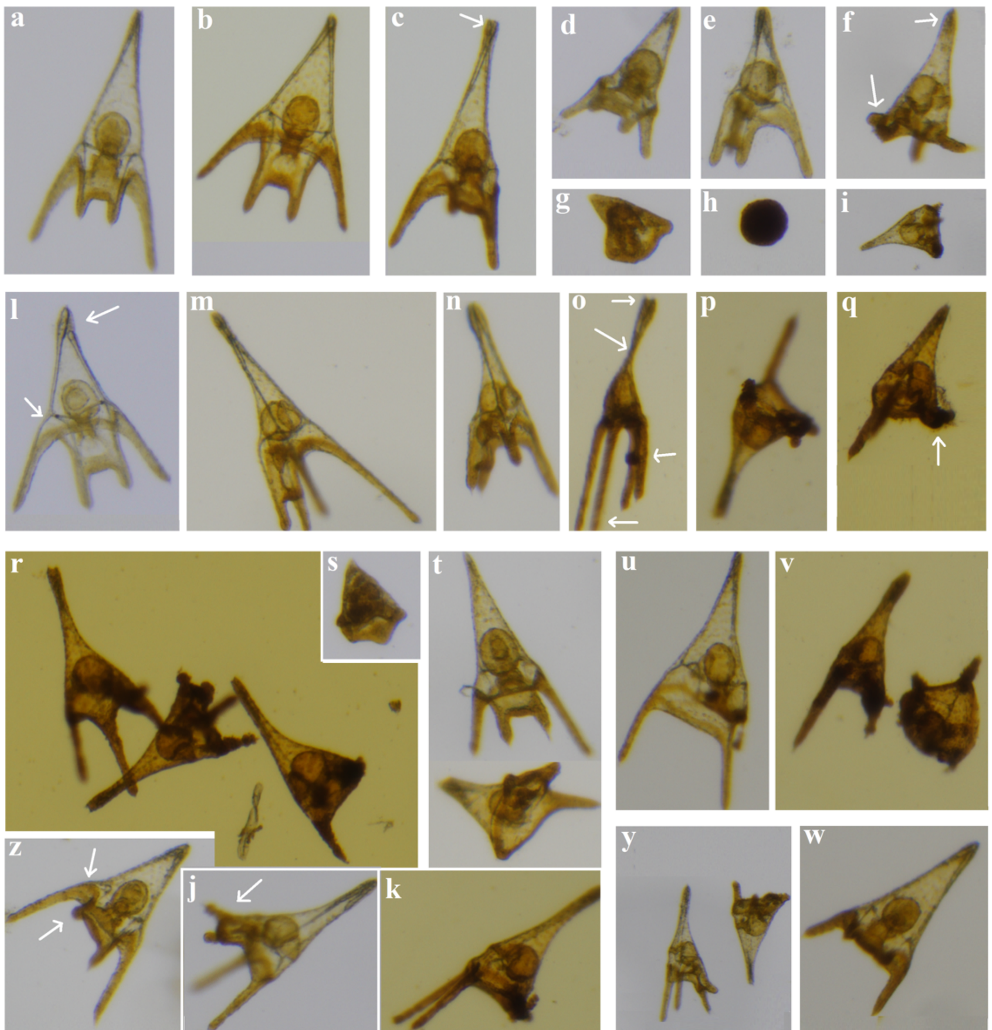


Figure S2 *Representation of recorded anomalies in P. lividus exposed to different plastic packaging types. a) normal; b-w) examples of different types of alterations: alteration of L1/L2 ratio, cross lateral rods, split lateral rods, bended arms, crossed and exposed lateral rods, asymmetrical larval body growth, unequal antero-lateral arms, elongation of one of the post-oral arms, broken lateral rods, exposed lateral rods, etc.*

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. J. econ. Entomol, 18(2), 265-267.

AEA. Problemi Prioritari per L'ambiente Mediterraneo; Report AEA 4/2006; AEA: Copenaghen, Denmark, 2006; p. 92, ISSN 1725–9177.

Allen, A. S., Seymour, A. C., & Rittschof, D. (2017). Chemoreception drives plastic consumption in a hard coral. Marine Pollution Bulletin, 124(1), 198-205.

Alomar, C., Estarellas, F., & Deudero, S. (2016). Microplastics in the Mediterranean Sea: deposition in coastal shallow sediments, spatial variation and preferential grain size. Marine environmental research, 115, 1-10.

Aniyikaiye, T. E., Oluseyi, T., Odiyo, J. O., & Edokpayi, J. N. (2019). Physico-chemical analysis of wastewater discharge from selected paint industries in Lagos, Nigeria. International journal of environmental research and public health, 16(7), 1235.

Asnaghi, V., Chiantore, M., Mangialajo, L., Gazeau, F., Francour, P., Alliouane, S., & Gattuso, J. P. (2013). Cascading effects of ocean acidification in a rocky subtidal community. PloS one, 8(4), e61978.

Asnaghi, V., Mangialajo, L., Gattuso, J. P., Francour, P., Privitera, D., & Chiantore, M. (2014). Effects of ocean acidification and diet on thickness and carbonate elemental composition of the test of juvenile sea urchins. Marine environmental research, 93, 78-84.

Basset, A., Barbone, E., Rosati, I., Vignes, F., Breber, P., Specchiulli, A., ... & Pinna, M. (2013). Resistance and resilience of ecosystem descriptors and properties to dystrophic events: a study case in a Mediterranean lagoon. Transitional Waters Bulletin, 7(1), 1-22.

Besseling, E., Redondo-Hasselerharm, P., Foekema, E. M., & Koelmans, A. A. (2019). Quantifying ecological risks of aquatic micro-and nanoplastic. Critical reviews in environmental science and technology, 49(1), 32-80.

Blašković, A., Fastelli, P., Čižmek, H., Guerranti, C., & Renzi, M. (2017). Plastic litter in sediments from the Croatian marine protected area of the natural park of Telaščica bay (Adriatic Sea). Marine pollution bulletin, 114(1), 583-586.

Blašković, A., Guerranti, C., Fastelli, P., Anselmi, S., & Renzi, M. (2018). Plastic levels in sediments closed to Cecina river estuary (Tuscany, Italy). Marine pollution bulletin, 135, 105-109.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., & Thompson, R. (2011). Accumulation of microplastic on shorelines woldwide: sources and sinks. Environmental science & technology, 45(21), 9175-9179.

Bucci, K., Tulio, M., & Rochman, C. M. (2020). What is known and unknown about the effects of plastic pollution: A meta-analysis and systematic review. Ecological Applications, 30(2), e02044.

Carpentieri, S., Di Vito, S., Zampetti, G., Scocchera, E., Nuglio, S., & Cugnata, F. (2018). Beach Litter, Indagine sui rifiuti nelle spiagge italiane. Legambiente, Maggio.

Chou, P. I., Ng, D. Q., Li, I. C., & Lin, Y. P. (2018). Effects of dissolved oxygen, pH, salinity and humic acid on the release of metal ions from PbS, CuS and ZnS during a simulated storm event. Science of the total environment, 624, 1401-1410.

Clarke, K. R., & Warwick, R. M. (2001). A further biodiversity index applicable to species lists: variation in taxonomic distinctness. Marine ecology Progress series, 216, 265-278.

Conservancy, O., & Conservation, A. (2020). Ocean Conservancy. The Problem with Plastics.

Diana, Z., Sawickij, N., Rivera Jr, N. A., Hsu-Kim, H., & Rittschof, D. (2020). Plastic pellets trigger feeding responses in sea anemones. Aquatic Toxicology, 222, 105447.

Dupont, S.; Lundve, B. and Thorndyke, M. Near future ocean acidification increases growth rate of the lecithotrophic larvae and juvenile of the seastar *Crossaster papposus*. J. Exp. Zool. Part B 2010a, 314, 382–359.

Dupont, S., Ortega-Martínez, O., & Thorndyke, M. (2010). Impact of near-future ocean acidification on echinoderms. Ecotoxicology, 19, 449-462.

Eerkes-Medrano, D., Thompson, R. C., & Aldridge, D. C. (2015). Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water research, 75, 63-82.

Fastelli, P., & Renzi, M. (2019). Exposure of key marine species to sunscreens: Changing ecotoxicity as a possible indirect effect of global warming. Marine pollution bulletin, 149, 110517.

Feng, D., Rittschof, D., Orihuela, B., Kwok, K. W. H., Stafslien, S., & Chisholm, B. (2012). The effects of model polysiloxane and fouling-release coatings on embryonic development of a sea urchin (*Arbacia punctulata*) and a fish (*Oryzias latipes*). Aquatic toxicology, 110, 162-169.

Geyer, J., Jambeck, J. R., & Lavender, K. L. (2017). Production, use, and fate of all plastics ever made, Law Sci. Adv, 3, e1700782.

Graham, E. R., & Thompson, J. T. (2009). Deposit-and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. Journal of experimental marine biology and ecology, 368(1), 22-29.

Guerranti, C., Cannas, S., Scopetani, C., Fastelli, P., Cincinelli, A., & Renzi, M. (2017). Plastic litter in aquatic environments of Maremma Regional Park (Tyrrhenian Sea, Italy): Contribution by the Ombrone river and levels in marine sediments. Marine Pollution Bulletin, 117(1-2), 366-370.

Hinga, K. R. (2002). Effects of pH on coastal marine phytoplankton. Marine ecology progress series, 238, 281-300.

Kontrick, A. V. (2018). Microplastics and human health: Our great future to think about now. Journal of Medical Toxicology, 14, 117-119.

Kurihara, H., Kato, S., & Ishimatsu, A. (2007). Effects of increased seawater pCO2 on early development of the oyster *Crassostrea gigas*. Aquatic Biology, 1(1), 91-98.

Li, H. X., Ma, L. S., Lin, L., Ni, Z. X., Xu, X. R., Shi, H. H., ... & Rittschof, D. (2018). Microplastics in oysters *Saccostrea cucullata* along the Pearl River estuary, China. Environmental Pollution, 236, 619-625.

Li, H. X., Orihuela, B., Zhu, M., & Rittschof, D. (2016). Recyclable plastics as substrata for settlement and growth of bryozoans Bugula neritina and barnacles *Amphibalanus amphitrite*. Environmental pollution, 218, 973-980.

Mani, T., Hauk, A., Walter, U., & Burkhardt-Holm, P. (2015). Microplastics profile along the Rhine River. Scientific reports, 5(1), 17988.

Martin, S., Richier, S., Pedrotti, M. L., Dupont, S., Castejon, C., Gerakis, Y., ... & Gattuso, J. P. (2011). Early development and molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to CO2-driven acidification. Journal of Experimental Biology, 214(8), 1357-1368.

Murray, F., & Cowie, P. R. (2011). Plastic contamination in the decapod crustacean *Nephrops norvegicus* (Linnaeus, 1758). Marine pollution bulletin, 62(6), 1207-1217.

Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., & Beiras, R. (2019). Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin *Paracentrotus lividus*. Environmental Pollution, 247, 706-715.

Peda, C., Caccamo, L., Fossi, M. C., Gai, F., Andaloro, F., Genovese, L., ... & Maricchiolo, G. (2016). Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: preliminary results. Environmental pollution, 212, 251-256.

Piccardo, M., Provenza, F., Grazioli, E., Cavallo, A., Terlizzi, A., & Renzi, M. (2020). PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations. Science of the Total Environment, 715, 136947.

Plastics Europe. Plastics—The Facts; PlasticEurope, Brussels, 2020.

Qi, Y., Wu, Z., He, J., Rittschof, D., Su, P., Ke, C., & Feng, D. (2019). Conspecific cues that induce spore settlement in the biofouling and green tide-forming alga *Ulva tepida* provide a potential aggregation mechanism. International Biodeterioration & Biodegradation, 145, 104807.

Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., ... & Watson, A. (2005). Ocean acidification due to increasing atmospheric carbon dioxide. The Royal Society.

Rendell-Bhatti, F., Paganos, P., Pouch, A., Mitchell, C., D'Aniello, S., Godley, B. J., ... & Jimenez-Guri, E. (2021). Developmental toxicity of plastic leachates on the sea urchin *Paracentrotus lividus*. Environmental Pollution, 269, 115744.

Renzi, M., & Blašković, A. (2018). Litter & microplastics features in table salts from marine origin: Italian versus Croatian brands. Marine pollution bulletin, 135, 62-68.

Renzi, M., Blašković, A., Bernardi, G., & Russo, G. F. (2018). Plastic litter transfer from sediments towards marine trophic webs: a case study on holothurians. Marine pollution bulletin, 135, 376-385.

Renzi, M., Blašković, A., Fastelli, P., Marcelli, M., Guerranti, C., Cannas, S., ... & Massara, F. (2018). Is the microplastic selective according to the habitat? Records in amphioxus sands, Mäerl bed habitats and *Cymodocea nodosa* habitats. Marine pollution bulletin, 130, 179-183.

Renzi, M., Guerranti, C., & Blašković, A. (2018). Microplastic contents from maricultured and natural mussels. Marine pollution bulletin, 131, 248-251.

Renzi, M., Roselli, L., Giovani, A., Focardi, S. E., & Basset, A. (2014). Early warning tools for ecotoxicity assessment based on *Phaeodactylum tricornutum*. Ecotoxicology, 23, 1055-1072.

Renzi, M., Specchiulli, A., Blašković, A., Manzo, C., Mancinelli, G., & Cilenti, L. (2019). Marine litter in stomach content of small pelagic fishes from the Adriatic Sea: sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*). Environmental Science and Pollution Research, 26, 2771-2781.

Rochman, C. M., Hoh, E., Kurobe, T., & Teh, S. J. (2013). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Scientific reports, 3(1), 1-7.

Romeo, T., Pietro, B., Pedà, C., Consoli, P., Andaloro, F., & Fossi, M. C. (2015). First evidence of presence of plastic debris in stomach of large pelagic fish in the Mediterranean Sea. Marine pollution bulletin, 95(1), 358-361.

Sammut, J., Melville, M. D., Callinan, R. B., & Fraser, G. C. (1995). Estuarine acidification: impacts on aquatic biota of draining acid sulphate soils. Australian Geographical Studies, 33(1), 89-100.

Sarni, A., & Onorati, F. (2009). Controls variability in algal growth inhibition test on *Dunaliella tertiolecta* as possible scale threshold. Biol. Mar. Medit, 14, 33-49.

Sartori, D.; Macchia, S.; Vitiello, V.; Morroni, L.; Onorati, F.; Pellegrini, D. ISPRA, Quaderni—Ricerca Marina n. 11/2017; Macchia, S., Sartori, D., Eds.; ISPRA, Roma, Italy, 2017; p. 60.

Schowalter, T. D. (2022). Insect ecology: an ecosystem approach. Academic press.

Serra-Compte, A., Maulvault, A. L., Camacho, C., Alvarez-Munoz, D., Barcelo, D., Rodriguez-Mozaz, S., & Marques, A. (2018). Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environmental Pollution, 236, 824-834.

Setälä, O., Fleming-Lehtinen, V., & Lehtiniemi, M. (2014). Ingestion and transfer of microplastics in the planktonic food web. Environmental pollution, 185, 77-83.

Specchiulli, A., Focardi, S., Renzi, M., Scirocco, T., Cilenti, L., Breber, P., & Bastianoni, S. (2008). Environmental heterogeneity patterns and assessment of trophic levels in two Mediterranean lagoons: Orbetello and Varano, Italy. Science of the Total Environment, 402(2-3), 285-298.

Suwa, R., Nakamura, M., Morita, M., Shimada, K., Iguchi, A., Sakai, K., & Suzuki, A. (2010). Effects of acidified seawater on early life stages of scleractinian corals (Genus Acropora). Fisheries Science, 76, 93-99.

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W., ... & Russell, A. E. (2004). Lost at sea: where is all the plastic?. Science, 304(5672), 838-838.

UNEP. Marine Litter Assessment in the Mediterranean; UNEP/MAP, Vassileos Konstantinou Ave.; 11635 Athens, Greece, 2015; p. 45, ISBN No: 978-92-807-3564-2, UNEP/MAP 48. Available online: www.unepmap.org (accessed on 15 April 2020).

UNEP. Marine Plastic Debris and Microplastics—Global Lessons and Research to Inspire Action and Guide Policy Change; United Nations Environment Programme: Nairobi, Kenya, 2016; p. 192. Available online: http://ec.europa.eu/environment/marine/good-environmental-

status/descriptor10/pdf/Marine_plastic_debris_and_microplastic_technical_report_advance_copy.pdf (accessed on 28 December 2018).

Wetzel, R. G. (1995). Death, detritus, and energy flow in aquatic ecosystems. Freshwater Biology, 33(1), 83-89.

Chapter 2

Ecotoxicological assessment of “glitter” leachates in aquatic ecosystems: an integrated approach

Manuela Piccardo[1], Francesca Provenza[1], Serena Anselmi[2] and Monia Renzi[1, 3]

[1]Dipartimento di Scienze della Vita, Università di Trieste, 34127 Trieste, Italy; manuela.piccardo@units.it; francesca.provenza@units.it

[2]Bioscience Research Center, via Aurelia Vecchia, 32, 58015, Orbetello, Italy; serena.anselmi@bsrc.it

[3]CoNISMa, Consorzio Interuniversitario per le Scienze del Mare, Piazzale Flaminio 4, 00196 Roma, Italy; mrenzi@units.it

Toxics

Toxics 2022, 10(11), 677

https://doi.org/10.3390/toxics10110677

Abstract

An alarming fraction within plastic pollution is that of microplastics (MP). A category of MP almost completely ignored is that of glitter. The objective of this study is to test the toxicity of nine types of glitter leachate (3 soak times: 3, 90 and 180 days) on model organisms in freshwater (*Allivibrio fischeri, Raphidocelis subcapitata, Daphnia magna*) and saltwater (*Allivibrio fischeri, Phaeodactylum tricornutum, Paracentrotus lividus*). An integrated approach was applied to obtain the percentage of ecotoxicological risk. The results show: i) photosynthesizing primary producers are the most sensitive trophic level; ii) algae transitioned from growth inhibition to biostimulation; iii) *D. magna* showed higher sensitivity after 48 hours compared to 24 hours; iv) *A. fischeri* responded more strongly in saltwater than in freshwater. The integrated data show a greater risk associated with the marine environment, with the highest risk for glitters that are hexagonal and composed of PMMA. Finally, the multivariate analysis shows that the toxicity of plastic leaching is a complex phenomenon that depends on the sensitivity of the species, in some cases on the soaking time and on the medium, and is not clearly linked to the polymer type, the contact area or the colours of the particles.

Keywords

Battery of bioassays; freshwater; marine environment; microplastics; chemical risk; integrated approach; *Allivibrio fischeri*; algae; *Daphnia magna; Paracentrotus lividus*

Introduction

In 2020, 367 million tons of plastic were produced worldwide, of which 15% produced in Europe (Plastic Europe, 2021). Most of the production (40.5%) is for the manufacture of packaging plastics, construction (20.4%) and motor vehicles (8.8%). 34.6% of waste produced in Europe is recycled, 42% is destined for energy production, and the remaining 23.4% is sent to landfills (Plastic Europe, 2021). The ever-increasing global production of plastics over the last seven decades has led to their widespread distribution in the environment, and they have become an important geological indicator of the Anthropocene (Haram et al., 2020; Sridharan et al., 2022). Due to poor waste management and without waste management infrastructure improvements, the cumulative amount of plastic waste available to enter the ocean is predicted to reach 250 million of metric tons by 2025 corresponding to an increase of an order of magnitude respect the level of 2015 (Jambeck et al., 2015). Plastic debris has been detected at all latitudes from the poles (Teuten

et al., 2009; Jones-Williams et al., 2020) to the equator (Tan et al., 2020), from surface waters (Moore et al., 2011; Suaria et al., 2016; Reynolds and Ryan, 2018) to the depths of the oceans (Watters et al., 2010; Woodall et al., 2014; Van Cauwenberghe et al., 2015), and represents a form of emerging pollution that may pose a threat to both freshwater (Blair et al., 2017; Horton et al., 2017; Bellasi et al., 2020) and marine environments (Li et al., 2016).

The most worrisome fraction within plastic waste is that of microplastics (MP), plastic particles ranging in size from 20 to 5000 µm (according to the definition given MSFD Technical Subgroup on Marine Litter, (MSFD Technical Subgroup on Marine Litter, 2013). The danger of MP lies in their bioavailability to a wide range of organisms and thus their toxicity through physical clogging, as well as their ability to act as a Trojan horse by transferring and mobilizing the cocktail of pollutants associated with plastic production or other co-pollutants or pathogens or alien species present in the surrounding medium (Bergmann et al., 2015; Koelmans et al., 2016; Hildebrandt et l., 2021; Sridharan et al., 2022). Various substances are used in the production of plastics: plasticizers (bisphenol A, BPA; phthalates such as diisobutyl phthalate, DIBP), catalysts (metals), flame retardants (hexabromocyclododecane, HBCD), pigments and dyes, etc. (Sridharan et al., 2022). Some of these substances have been shown to have harmful effects and are classified as carcinogenic or endocrine disruptors (e.g., phtalates, Liu et al., 2020). Because these substances are weakly associated with the surface of the microplastic, they can be released into the environment (Gunaalan et al., 2020) and, if ingested, can spread into organisms and have effects at different levels of biological organization (González-Soto et al., 2019).

MP can either result from the fragmentation of large objects (secondary microplastics), or they can enter the environment directly as pellets, beads, and fibers (secondary microplastics) (Bergmann et al., 2015). Some of these categories, such as microspheres in personal care products, have been withdrawn from the market in many European countries, including Italy (2018 budget law, https://www.gazzettaufficiale.it/eli/id/2018/12/31/18G00172/sg; accessed on 10 October 2022). However, there is another category of primary MP, which is almost completely ignored today: glitter. The term glitter refers to an assortment of small, flat, reflective particles made of a plastic polymer coated with a metal such as aluminum, titanium, iron, or bismuth which gives it a high reflectivity (Yurtsever, 2019). Commercially available glitter ranges in size from 50 to 6350 µm, with the most common sizes being around 200 µm (Blackledge and Jones, 2007). Glitter is produced by the ton each year worldwide and is used primarily in makeup and craft materials, but also in a variety of activities such as face washes, furniture, toys, clothing, and accessories (Tagg and Ivar do Sul, 2019). Plastic glitter comes in all colors and in various shapes (in precision-cut pieces of uniform size). Glitter can enter water bodies through sewage and landfill runoff (Tagg

and Ivar do Sul, 2019). Glitter particles have been found in surface waters and sediment samples from an Indian estuary (Nithin et al., 2022), in sediments from Lake Ontario (Ballent et al., 2016), and in urban dust from several Iranian cities (Dehghani et al., 2017; Abbasi et al., 2018; Silva et al., 2018; Abbasi et al., 2019). Even though many glitter particles are removed by wastewater treatment plants (Sun et al., 2019), a large amount of such particles still enter the ocean. Raju et al., 2020 estimated the daily input of glitter particles from a wastewater treatment plant to be 2.7-3.0 x 107.

Because it is waste in the environment, glitter would accumulate in the environment as a pollutant and interact with biota. Ecotoxicity studies on glitter particles are scarce. One of a kind is the study of Green et al., 2021 which reported the impacts of glitter manufactured of conventional (PET) or alternative materials (modified regenerated cellulose, mica or synthetic mica) on the biodiversity and ecosystem functioning of freshwater, lotic habitats. Overall, results indicate that both conventional and alternative glitters can cause ecological impacts in aquatic ecosystems causing a two-fold increase in the abundance of New Zealand mud snails (*Potamopyrgus antipodarum*) and reducing the root length of common duckweed (*Lemna minor*) and phytoplankton biomass. However, despite the multimaterial nature of the glitter particles, nothing is known about the chemical risk associated with the leachates of glitter on aquatic ecosystems.

The aim of this study is to test the toxicity of nine types of glitter purchased in the handicraft market, which differ by different shapes and sizes (and therefore different contact areas with the aqueous medium), colors (different dyes) and chemical compositions (poly-methyl-methacrylate PMMA, polyethylene PE, polyamide PA). Thus, for the first time, the chemical risk to model organisms in freshwater (bacteria *Aliivibrio fischeri*, algae *Raphidocelis subcapitata*, crustaceans *Daphnia magna*) and saltwater (*Aliivibrio fischeri*, algae *Phaeodactylum tricornutum*, sea urchin *Paracentrotus lividus*) was investigated, which helped to provide the first useful basis for ecotoxicological risk assessment. Specifically, glitter was used to prepare aqueous extracts obtained with three different soaking times (3, 90, and 180 days) to better investigate temporal dynamics. Finally, our results were used to calculate an integrated toxicity test battery index (hereafter TBI) and the corresponding ecotoxicological risk percentage (R%) listed in ISPRA, 2011 manual and previously calculated by Schiavo et al., 2019 for the ecotoxicological assessment of leachates from plastic pellets in freshwater matrices.

Materials and Methods

Glitters characterization

The glitters were purchased in physical stores, are in the form of powder, and were originally intended for the decorative arts (e.g., purpurine), home furnishings (e.g., additives for paints), and cosmetics (glitter for hair gel) industries. The leachates for the ecotoxicological test were prepared from nine types of glitter microparticles that differed in shape (hexagons, stars, and rectangles), size (from 221.64 to 3047.83 micrometers, mean maximum length), and color (silver, green, orange, purple, yellow, and pink) (see **Table 1**). The colors of the glitter were determined before and after the leaching test using the CLM-194 portable colorimeter (EOPTIS). The color values determined by the colorimeter are displayed in native CIELAB coordinates (L * a * b). The 1976 CIELAB color space is a color space defined in 1976 by the International Commission on Illumination (abbreviated CIE) and was intended to be a perceptually uniform color space in which a given numerical change corresponds to a similar perceived color change. In this color space, the distance between two points shows approximately how different the colors are in terms of luminance (L), chroma (a), and hue (b). In other words, luminance is brightness, chroma is saturation, and hue is tint (in terms of blue and yellow). Finally, the differences in color values were used as evidence of color output.

Table 1 *Characteristics of the 9 glitters used to prepare the leachates: sample code, precursor, stereomicroscopic images, shape, mean maximum length and area (expressed in µm and µm^2 ± standard deviation, respectively), number of particles tested, mean total area for each particle (face A+B), total surface area exposed to water for each type (number of particles * area face A+B, in µm^2 ± SD), and chemical composition of the outer layer. PMMA= polymethyl methacrylate; PE= polyethylene; PA= polyamide.*

Sample code	**Precursor**	**Photo**	**Color**	**Shape**	**Mean max length (µm ± SD)**	**Mean area for face (µm^2 ± SD)**	**# particles/L tested**	**Mean area face A+B (µm^2 ± SD)**	**Total surface exposed to water (cm^2 / L ± SD)**	**Chemical composition of the outer layer**
CA6/1	Purpurine for decorative arts		Green	Hexagonal	221.64 ± 13.48	3.94E+04 ± 3.44E+03	3.81E+04	78800	30.00	PMMA
CA6/2			Purple	Hexagonal			3.81E+04	78800	30.00	PMMA
CA6/3			Orange	Hexagonal			3.81E+04	78800	30.00	PMMA
CA6/4			Pink	Hexagonal			3.81E+04	78800	30.00	PMMA
CA6/5			Yellow	Hexagonal			3.81E+04	78800	30.00	PMMA
H1	Glitter for paint		Silver	Hexagonal	244.66 ± 34.39	4.12E+04 ± 7.84E+03	4.48E+04	82400	36.87	PE
C5/1	Glitter for hair gel		Silver	Hexagonal	954.09 ± 315.24	6.26E+05 ± 3.29E+05	5.65E+02	1252000	7.08	PMMA
C6/2	Glitter for hair gel		Silver	Star	3047.83 ± 63.08	3.91E+06 ± 1.44E+05	3.41E+02	7820000	26.70	PA
CA7/5	Purpurine for decorative arts		Silver	Rectangle	2504.22 ± 616.51	8.13E+05 ± 2.23E+05	1.92E+03	1626000	31.22	PE

Leachates preparation

The leachates were prepared by immersing 100 mg of each glitter in 1 L of artificial seawater (ASW) and ISO FRESHWATER (ISO FW) (see supplementary materials for the composition of these solutions). This concentration was chosen because it is the reference concentration in numerous regulations such as REACH EU Regulation No. 1907/2006, the OECD Guidelines for the Testing of Chemicals (Section 3, Ready Biodegradability Test, OECD/OCDE, 2006) and has been used in numerous studies with plastic particles (Wegner et al., 2012; Yang et al., 2020; Piccardo et al., 2020; Liu et al., 2021).

Because a large number of containers were needed (i.e., 66), the dispersions were prepared in plastic bottles. To account for a possible contribution from plastic, two different negative controls (ASW only or ISO FW) were considered: the control in glass bottles (hereafter referred to as CN/GL) and the control in plastic bottles (hereafter referred to as CN/PL). The experiment included three different soaking periods: 3, 90 and 180 days (hereafter referred to as TIME 1, 2, and 3, respectively). During this time, samples were shaken daily at 40 rpm for 1 hour and stored at room temperature (22-25°C) in a dark place.

At the end of the soaking periods, the dispersions were filtered with a vacuum pump at 0.45 micron (mixed cellulose ester filter membrane) and the liquid was used for the ecotoxicological tests. The glitters on the filters were otherwise observed under a stereomicroscope to detect any morphological changes and analyzed again with the colorimeter.

Toxicity tests

Two different batteries of standardized tests were used to test the toxicity of glitter leachates to freshwater and marine environments. For freshwater, the battery consisted of *Aliivibrio fischeri* (acute toxicity test; UNI EN ISO 11348-3:2019; endpoint: inhibition of bioluminescence), *Raphidocelis subcapitata* (chronic toxicity test; UNI EN ISO 8692:2012; endpoint: growth inhibition), and *Daphnia magna* (acute toxicity test; UNI EN ISO 6341:2013; endpoint: immobility). For marine environments, the battery consisted of *Aliivibrio fischeri* (acute toxicity test; UNI EN ISO 11348-3:2019; endpoint: inhibition of bioluminescence), *Phaeodactylum tricornutum* (chronic toxicity test; UNI EN ISO10253:2017; endpoint: growth inhibition), and *Paracentrotus lividus* (acute toxicity test; Chapman et al. 1995 + ISPRA Quaderni Ricerca Marina 11/2017; endpoint: abnormal larvae). For more information on the protocols used, see the Supplementary materials (Supplementary material, **Table S1**: Toxicity tests performed on glitter leachates).

Test on bacteria

Biological responses to bacteria were tested on the species *Aliivibrio fischeri* using the Microtox® (Ecotox) photometer and lyophilized bacteria from a monospecific population of the NRRL-B-11177 strain, purchased from Microbiotests Inc. Lyophilized bacteria are diluted using distilled water, as reported in UNI EN ISO 11348-3:2019, and are maintained at 4°C before the start of the toxicity test. The percentage of inhibition of natural bioluminescence was measured after 15 and 30 minutes of exposure to 90% of the concentration of the leachate in fresh and salt water, using two experimental replicates.

Test on algae

Biological responses to marine algae were tested on the microalga *Phaeodactylum tricornutum*. Biological responses to freshwater algae were tested on the microalga *Raphidocelis subcapitata*. Percent growth inhibition (I%) after 72 hours of exposure was performed on leachates. In both cases, cell density was measured spectrophotometrically and calculated by light absorption at a wavelength of 670 nm. The response of the spectrophotometer was calibrated using a curve of cell density versus absorbance generated with the algal material tested by counts in the Burker chamber at each of the 10 points of scalar dilution of 106 cells/mL of material. The tests were performed in triplicate at 20 ± 2 °C.

Test on crustacean

Biological responses to crustaceans were tested on *Daphnia magna*. Resting eggs of *Daphnia magna* were rinsed with ISO FW and transferred to a Petri dish with additional ISO FW. They were left to hatch in an incubator at 21 ± 1°C (18:6-hour light: dark) for 3 days. Exposure studies were performed in the dark at 20 ± 2°C. Exposure solutions (10 mL) were placed in 10-mL multi-wells, and 5 neonates (< 24 hours post hatching) were placed in each well and kept in the dark. The neonates were not fed for the duration of the experiment. After 24 and 48 hours, the immobility (mortality) of the animals in the container was recorded. Animals that were unable to swim within 15 seconds of gently shaking the test vessel were considered immobile. All exposure experiments were performed in quadruplicate.

Test on echinoderms

Biological responses to echinoderms were tested in the sea urchin *Paracentrotus lividus*. Mature sea urchin specimens were captured in a natural marine area (Tuscany) and kept in captivity until the start of the experiment. Exposure tests were performed under fasting conditions as indicated in the method. The percentage of abnormal larvae was calculated on 100 randomly selected *plutei* in each experimental series (n = 3). Larvae were considered abnormal if they exhibited arrested development, all arms were missing or of different lengths, extra arms with crossed lateral bars, asymmetric body width, and other anomalies listed in the literature (ISPRA, 2017).

Quality Assurance & Quality Control

Toxicity tests was performed by the authors in a certified laboratory (UNI EN ISO 9001:2015; UNI EN ISO 17025:2005) to ensure the quality of the data generated. QA/QC assays were performed as described in the previously mentioned reference methods. Positive controls were performed by direct exposure of the tested species to standard toxicants: *A. fischeri* was exposed to 3,5′-dichlorophenol; *P. tricornutum*, *R. subcapitata* and *D. magna* were exposed to $K_2Cr_2O_7$; *P. lividus* was exposed to $Cu(NO_3)_2 * 3H_2O$, which was within the acceptance criteria defined by the standard methods.

Statistical analyses

All data are expressed as mean effect percentage ± standard deviation. In cases where the negative control in plastic (CN/PL) exceeded the threshold set by the method, results were normalized relative to controls using the Abbott formula (X - Y) / (100 - Y) * 100, where X = % of effect in treatment sample; Y = % of effect in control sample (CN/PL).

2- way ANOVA (significance of observed differences between the factors “time” and “polymer” within treatments) were performed on ecotoxicity dataset. Differences were considered significant

at p- level < 0.05. Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com, accessed October 2022).

Multivariate analyzes were performed using Primer v7.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK) according to the methods described in Clarke and Warwick, 1999. The Euclidean distance matrix was calculated for effect data normalized as percentages. The factors tested for mean effects were: Time (three levels, fixed: 1, 2, and 3) and Polymer (three levels, fixed: PMMA, PA, PE). An environmental (normalized) data set composed of the variables "contact area", "ΔE", "ΔL", "Δa"¸"Δb" was used to determine their relative importance on the biological variables (DistLM function, namely distance-based linear models).

ΔE is a unit of measurement used to calculate and quantify the difference between two colors (one a reference color, the other a sample color trying to match it) based on L*a*b* coordinates. ΔE was calculated according to the following formula:

$$deltaE76 = \Delta E = \sqrt{(L_a - L_b)^2 + (a_a - a_b)^2 + (b_a - b_b)^2}$$

Where L is the luminance, a is the chromaticity, and b is the hue. Subscript a and b means after and before the leaching test, respectively. Delta E is measured on a scale from 0 to 100, where 0 represents a small color difference and 100 represents complete distortion.

Data integration

The data integration used in this study started from the quantitative assessment proposed by Hartwell and successively modified by ISPRA et al., 2011. It calculates the "ecotoxicological risk", weighting the type of endpoint observed, the type of environmental matrix analyzed, and the level of agreement of the test results. To calculate the TBI, the percentage of effect (%E) on each endpoint was corrected to obtain the score test endpoint (SEi) according to the following formula:

$$SEi = \%E\ (M * S)\ SCF$$

where: SCF (Statistical Correction Factor) = Student t-test differences between samples and control. Values 0, 1, 2, 3 and 4 were attributed to SCF, corresponding to no effect ($p > 0.05$), biostimulation ($p < 0.05$), high biostimulation ($p < 0.01$), toxicity ($p < 0.05$) and high toxicity ($p < 0.01$); matrix (M) was set as 2 for the leachate samples; severity (S) was set as 2 for bioluminescence, 3 for algal growth, 4 for development, and 5 for mortality.

SEi is expressed on a scale of 0-100 relative to the test battery used as follows:

$$SEi = Sei\ [(\%Em)/SEmax)]$$

where: %Em = maximum percentage of observed effect corresponding to the maximum achieved MS, and SEmax is the maximum calculated score test endpoint.

The toxicity test battery integrated index (TBI) is calculated using the following formula:

$$\%TBI = (\sum \%SEi)/\mathrm{N}$$

where: N = number of endpoints (=3).

The TBI can be used to calculate the percent ecotoxicological risk (%R) as follows:

$$\%R = [\%TBI * (\sum \mathrm{SEi} + \mathrm{C})]/\%\mathrm{Sei}$$

Where $C = (\frac{N}{2-X})^3$ with X = number of statistically non-significant endpoints.

Ecotoxicological risk is defined as follows: non-significant (R ≤ 5%), moderate (5 < R ≤ 20%), high (20 < R ≤ 50%), very high (R > 50%).

Results

Ecotoxicological responses

Impact on freshwater system

The effects on freshwater system were measured using a multispecies battery of *A. fischeri*, *D. magna*, and *R. subcapitata* and are shown graphically in **Figure 1**.

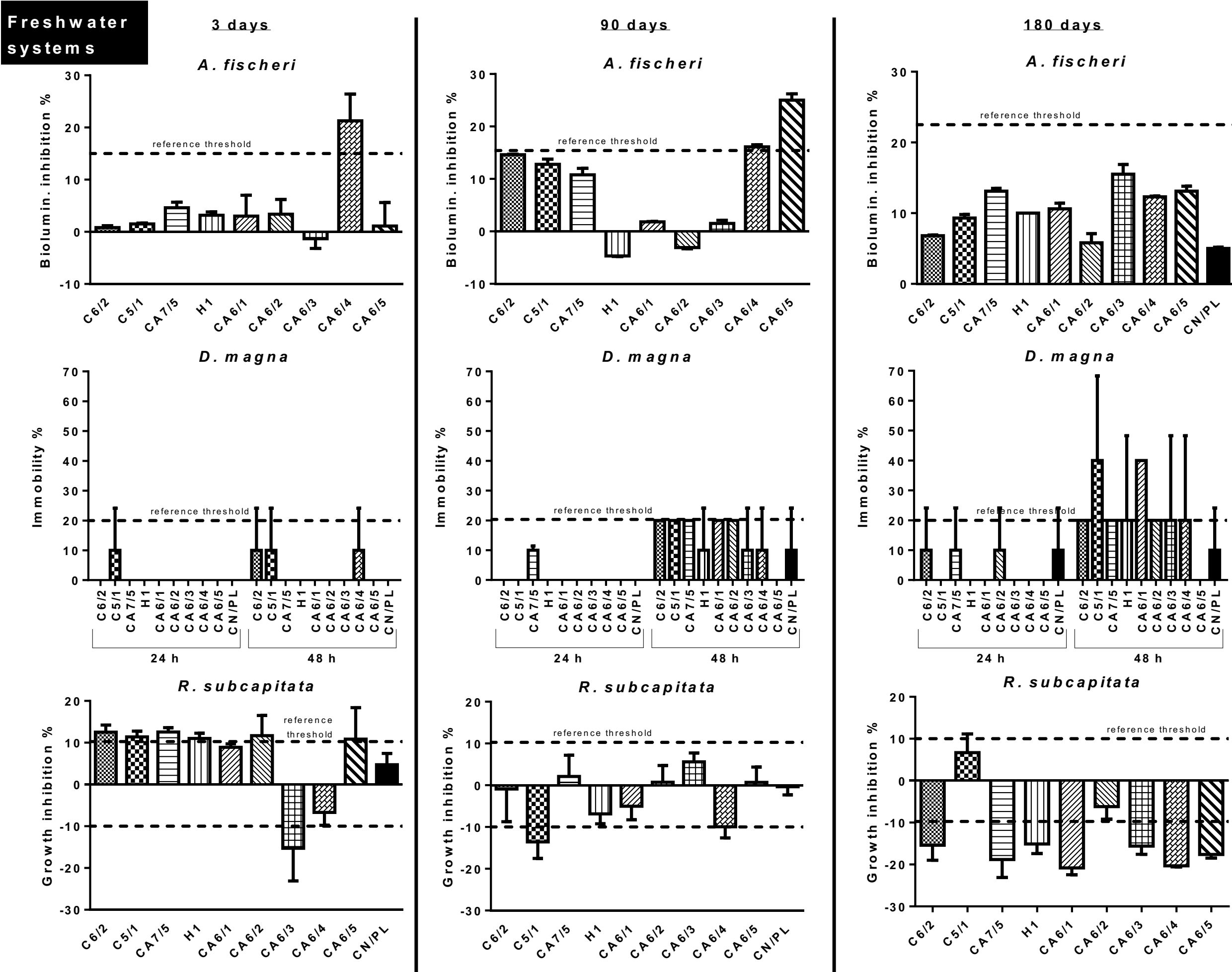


Figure 1 *Biological responses of the different model species exposed to glitter leachate in freshwater according to the different soaking times: 3, 90 and 180 days. The dashed line refers to the reference threshold established by the method (15% for A. fischeri; 20% for D. magna; 10% for R. subcapitata). Negative controls in plastic containers (CN/PL) are shown in black-fill pattern (if not present, data were normalized using the Abbott formula, see Section 2.5).*

Leachate with a soaking time of 3 days resulted in inhibition of bacterial bioluminescence above threshold in one case (i.e., CA6/4, 21.3%), inhibition of algal growth in most cases except CA6/4 (-6.67%), but also biostimulation of *R. subcapitata* in the case of CA6/3 (-15.26%). Inhibition of bacterial bioluminescence rarely exceeded the threshold after 90 and 180 days of soaking, as did the percentage of mortality in *D. magna*. If at time 2 the effect on the algae is within the limits, it worsens after 180 days, but in the opposite direction, i.e., of biostimulation with values ranging from -15.1% to 20.8%.

The data analysis through the TBI approach reveals an absent ecotoxicological risk in most of cases with the exception for CA6/4 and CA6/5 at TIME 1 and 2 (%R= 6% and 8.5%, respectively) (**Table 2**).

Table 2 *Results after integration of values from bioassays performed in freshwater: percentage of ecotoxicological risk (%) and the corresponding ecotoxicological risk level (from absent to very high).*

	3 days		90 days		180 days	
	R%	**Ecotoxicological risk**	**R%**	**Ecotoxicological risk**	**R%**	**Ecotoxicological risk**
C6/2	4.1	absent	4.9	absent	-2.8	absent
C5/1	4.2	absent	2.1	absent	3.1	absent
CA7/5	4.7	absent	3.6	absent	-1.9	absent
H1	3.6	absent	-1.9	absent	-1.7	absent
CA6/1	2.9	absent	0.2	absent	-4.2	absent
CA6/2	-0.4	absent	-0.6	absent	0.0	absent
CA6/3	-2.6	absent	1.8	absent	0.0	absent
CA6/4	6.0	moderate	3.9	absent	-2.6	absent
CA6/5	0.0	absent	8.5	moderate	-1.5	absent
		absent	moderate	high	very high	
		≤ 5%	5% - 20%	20% - 50%	>50%	

Impact on marine system

Impact on marine system were measured using a multispecies battery of *A. fischeri*, *P. lividus*, and *P. tricornutum* and are shown graphically in **Figure 2**.

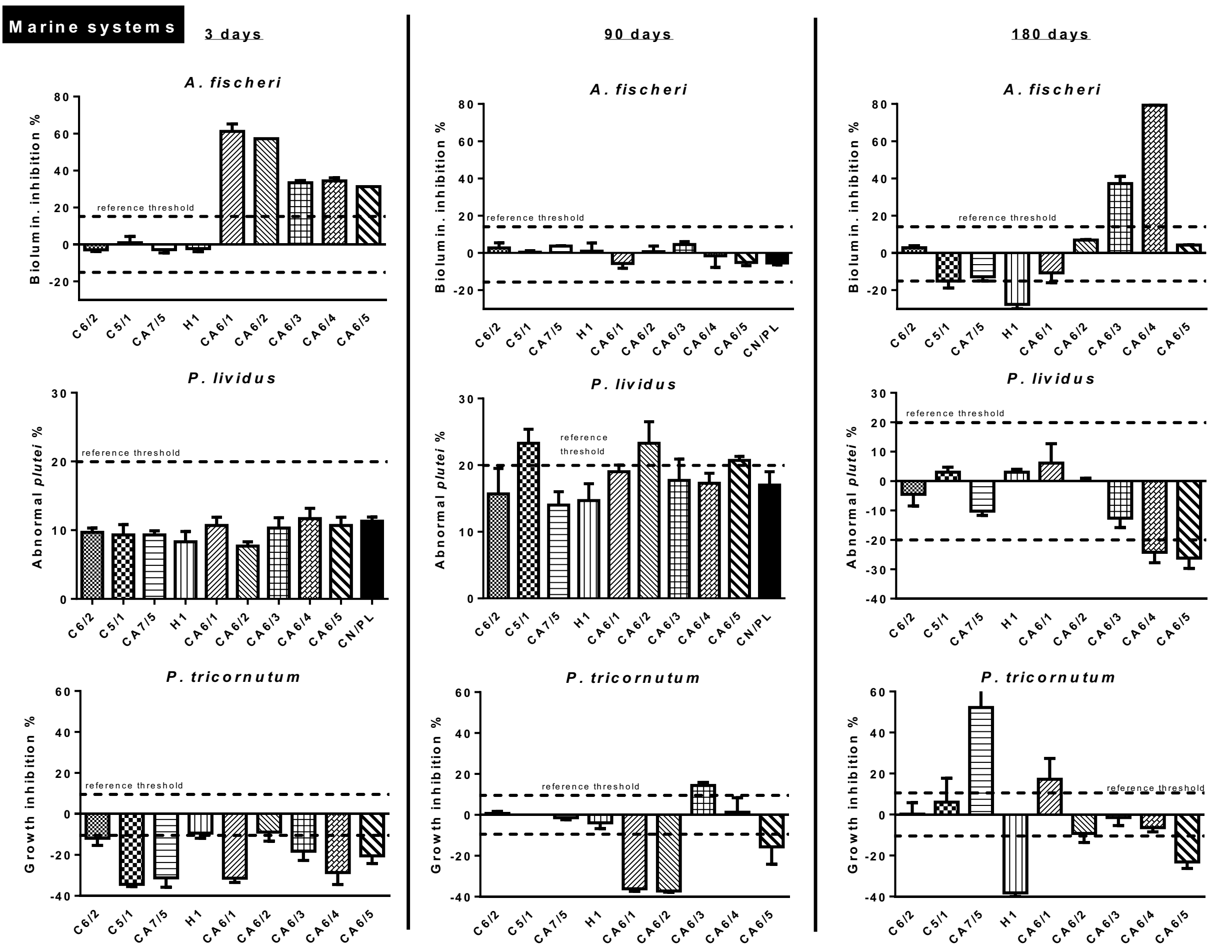


Figure 2 Biological responses of the different model species exposed to glitter leachate in saltwater according to the different soaking times: 3, 90 and 180 days. The dashed line refers to the reference threshold established by the method (15% for A. fischeri; 20% for P. lividus; 10% for P. tricornutum). Negative controls in plastic containers (CN/PL) are shown in black-fill pattern (if not present, data were normalized using the Abbott formula, see Section 2.5).

A considerable percentage of bioluminescence inhibition in *A. fischeri* was measured at TIME 1 for the group of glitters from CA6/1 to CA6/5 (values ranging from 31.3% to 61.2%). At time point 2, the response returned to the accepted values and then returned at time point 3 with a dual response: inhibition for CA6 / 3 and CA6 / 4 (37.2% and 79.3%, respectively) and biostimulation in C5 / 1 and H1 (-15.1% and -27.2%, respectively). In *P. lividus*, threshold exceedance was observed for C5/1, CA6/2 and CA6/5 at TIME 2 and for CA6/4 and CA6/5 at TIME 3. *P. tricornutum* exceeded the thresholds several times, with inhibition in some cases (up to 52.2%), but biostimulation in the majority of cases (maximum effect in H1, TIME 3, -38.2%).

The data analysis through the TBI approach reveals a moderate ecotoxicological risk for all type of glitter except C6/2 and H1 and a high risk (%R= 21.3) for CA6/2 (**Table 3**).

Table 3 *Results after integration of values from bioassays performed in saltwater: percentage of ecotoxicological risk (%) and the corresponding ecotoxicological risk level (from absent to very high).*

	3 days		90 days		180 days	
	R%	**Ecotoxicological risk**	**R%**	**Ecotoxicological risk**	**R%**	**Ecotoxicological risk**
C6/2	-0.7	absent	0.0	absent	0.0	absent
C5/1	-11.5	absent	7.7	moderate	0.0	absent
CA7/5	5.0	moderate	-1.7	absent	15.8	moderate
H1	-1.0	absent	0.0	absent	-8.6	absent
CA6/1	10.0	moderate	-6.1	absent	0.0	absent
CA6/2	21.3	high	1.6	absent	0.0	absent
CA6/3	5.1	moderate	3.3	absent	7.3	moderate
CA6/4	2.0	absent	0.0	absent	18.4	moderate
CA6/5	3.6	absent	5.6	moderate	-10.6	absent
		absent	Moderate	high	very high	
		≤ 5%	5% - 20%	20% - 50%	>50%	

Species sensitivity

The species utilized for toxicity tests responded differently to the investigated samples, showing different sensitivity. **Table 4** shows the number of times the stress thresholds were exceeded, specifically by species, with no internal distinction between time points. The score can vary from a minimum of 0 to a maximum of 3 (corresponding to the three time points). The most sensitive trophic level was that of photosynthesizing primary producers, which responded to the treatment in two ways: in some case by reducing the growth rate, in others by increasing it. The second most sensitive species was *A. fischeri* followed by the larval stages of the small planktonic crustacean *D. magna* and the *plutei* of the sea urchin *P. lividus*.

Table 4 *Exceeding thresholds for effect, broken down by species and treatment type, with no distinction between different time points. The score can range from a minimum of 0 (white) to a maximum of 3 (red). Green corresponds to a score of 1 and yellow to 2.*

	A. fischeri	***R. subcapitata***	***D. magna***	***A. fischeri***	***P. tricornutum***	***P. lividus***
C6/2					+	
C5/1		+		+	+	
CA7/5		+			+	
H1		+		+	+	
CA6/1		+			+	
CA6/2					+	
CA6/3		+			+	
CA6/4		+			+	
CA6/5		+			+	

Color differences after the soaking time

The total differences (ΔE) recorded in time 1 come from 2.6 to 42.4, the maximum being obtained for CA6/2 (**Table 5**). Focusing on the most hazardous type of glitter, a variation of -41.7 in chroma (Δb) was the most overall influential parameter of the total difference (E). Negative values in Δb are indicative of a bluer effect. The total differences observed in time 3 range from 2.9 to 30.9, peaking at CA6/2. Regarding the others most dangerous type of glitter, the brightness variations in CA7/5 were the parameter with the greatest influence on the total difference (ΔE), due to the leaching of the surface reflective layer, as shown by the microscopic observations (Supplementary materials, **Figure S1:** CA7/5 type glitter: one of this glitter has the typical outer reflective layer, another is completely uncolored). With respect to CA6/3 and CA6/4, hue variation was the most influential parameter overall. Further details on the measured color variations and how these variations are correlated with ecotoxicological responses are provided in more depth by the multivariable analysis (Section 3.4).

Table 5 *CIEL lab color differences (D65/2°) between glitter before and after the soaking in saltwater.*

	TIME 1				TIME 3			
samples	**Δ E**	**Δ L**	**Δ a**	**Δ b**	**Δ E**	Δ L	Δ a	Δ b
CA6/1	36.3	-8.3	7.2	-34.6	16.9	-8.8	-4.1	-13.9
CA6/2	42.4	7.2	-41.7	1.7	30.9	-5.9	-29.7	-6.3
CA6/3	7.5	-1.7	3.8	6.2	15.9	-5.1	8.9	12.2
CA6/4	16.6	1.4	13.3	9.9	14.5	5.7	-1.4	13.2
CA6/5	22.0	3.2	4.5	21.3	12.8	-1.5	11.5	5.5
C6/2	3.3	3.0	-0.8	-1.2	2.9	2.1	1.3	1.5
C5/1	8.9	5.6	-4.7	-5.1	5.7	5.1	1.9	1.5
CA7/5	10.6	10.6	-0.5	-1.0	15.8	15.7	-0.8	0.7
H1	2.6	2.5	-0.4	-0.6	17.0	-7.6	0.5	-15.2

Multivariate analysis

2-ways ANOVA (Supplementary materials, **Table S2:** P values of 2-way ANOVA) was performed on marine and fresh-datasets to determine any possible contribution of the factors "time" and "polymer" in the ecotoxicological responses. Multivariate analysis confirmed the importance of the factor TIME in determining the differences in the species *R. subcapitata* (p-value= 0.0018) and *D. magna* (p-value= 0.0296). In marine systems, the algal test shows an effect of growth biostimulation independent of the time factor in many cases (p-value= 0.4383). The factor "polymer" however, never resulted statistically significant.

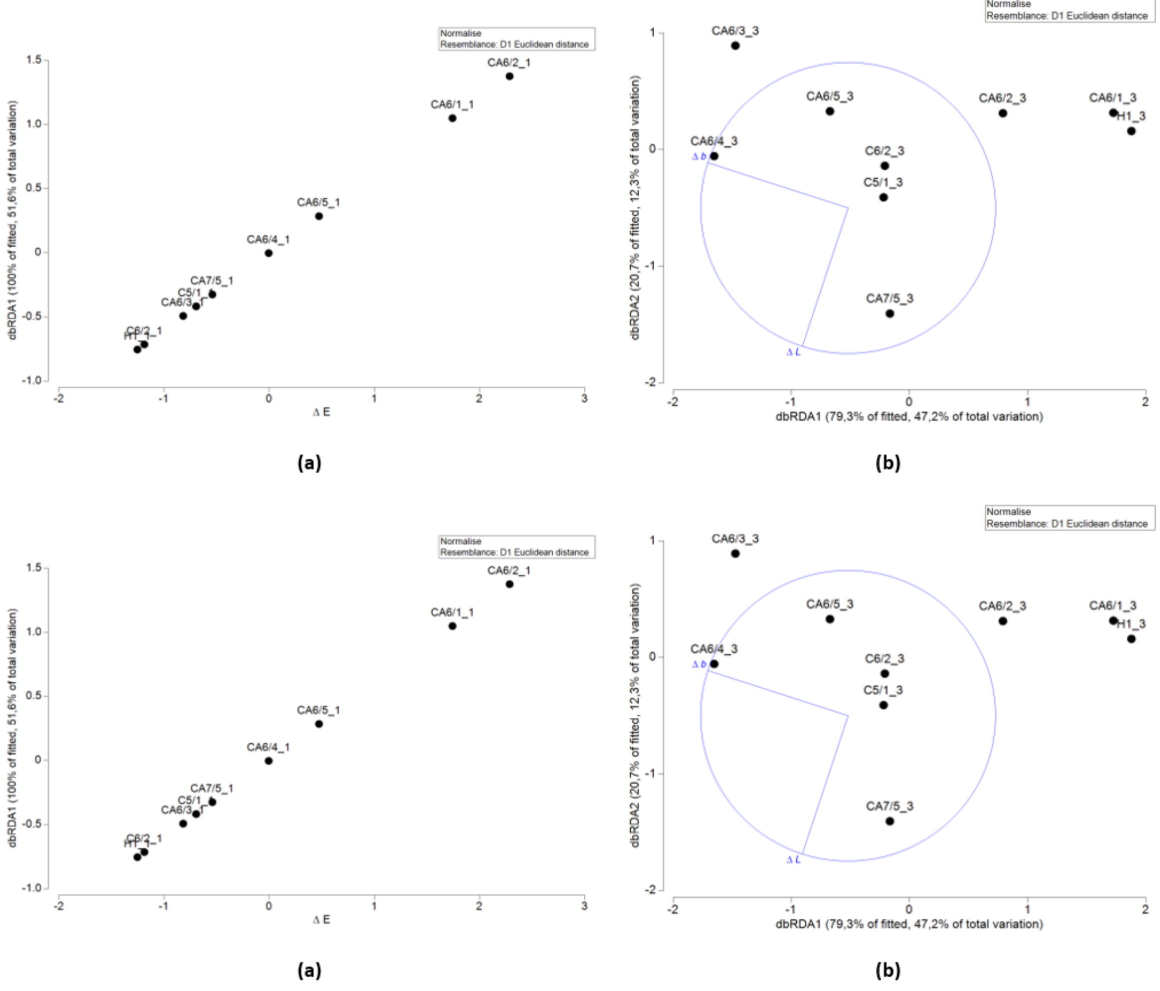


__Figure 3__ Multivariate analysis multivariate analysis performed with Primer7 to determine possible correlation between particle contact area and color variations on ecotoxicological responses in salt water: a) time 1 (i.e., 3 days), b) time 3 (i.e., 180 days).

The integrated TBI approach has shown that the most vulnerable context is that of the sea at time 1 and 3. Therefore, for these two scenarios, the multivariate analyzes were deepened to determine the possible importance of some variables such as particle contact area and color variations on the ecotoxicological responses. At time 1, the marginal test of the DistLM model showed that the ΔE value (p value = 0.002, R ^ 2 = 0.94) was statistically significant and could explain 51.6% of the total variability (**Fig. 3a**). As can be seen from the graph, the higher ΔE value is associated with the higher risk percentage: CA6/1 (R% = 10.0) and CA6/2 (R% = 21.3). At Time 3, the most influential variable was the "hue" component (Δb), consistent with a p-value = 0.006 (R ^ 2 = 0.79). The combination of Δb and ΔL shown in **Figure 3b** partially explains the greater risk associated with CA7 / 5 (R% = 15.8), CA6 / 3 (R% = 7.3), and CA6 / 4 (R% = 18.4) glitters. A greater toxicity of CA6 appears to be associated with variations in hue, with values tending toward yellow suggesting a possible release of dyes into the aqueous medium. The toxicity of CA7/5, on the other hand, is associated with large variations in brightness (ΔL), most likely due to the release of the reflective surface layer, as discussed earlier in Section 3.3.

Discussion

A double negative control (only water in plastic and glass bottles) has been performed in order to discriminate any possible contribution due to the composition of the container. The differences in the ecotoxicological responses of the negative control stored in glass and plastic bottles are shown in **Figure S2** (Supplementary materials, **Figure S2**: Comparison between the ecotoxicological responses recorded in the two different negative controls). In most cases, the responses are similar. Greater differences (> 100%) are reported by *A. fischeri* in saltwater at time 1, and by *P. lividus* at time 3 (> 25 %). Nevertheless, the data correction was performed with the Abbott formula using the values from the plastic control (CN/PL), thus any possible differences measured can be linked to the presence of glitters.

The glitter in freshwater behaved differently regardless of the polymer. However, a small temporal gradient was observed especially in the algal test and in *D. magna*. *R. subcapitata* passed from inhibition of algal growth to biostimulation. Therefore, it is suggested that the composition of elutriates changed between TIME 1 (3 days) and TIME 3 (180 days). Thus, after 180 days, trace elements (e.g., metals) may have been released to promote photosynthetic activity and thus increase cell growth. Chae et al., 2020 reported a biostimulatory effect on four different algal species after exposure to expanded polystyrene (EPS, 2 g/L, immersed for 28 days). Responses of *D. magna* after 48 hours also increase with time until the effect threshold is exceeded. Higher sensitivity at 48 hours compared to 24 hours was previously reported by Lithner et al., 2012, who also reported an EC_{50} value for high-density polyethylene (HDPE) elutriate ranging from 17 to 24 g/L. The study we conducted shows that a much lower concentration (100 mg/L) is able (for PE) to cause a mortality of 20% (glitter H1 and CA7/5) and 40% for another polymer (PMMA), i.e. CA6/1 and C5/1 glitter. Finally, the test for inhibition of bioluminescence of the bacterium *A. fischeri* showed a response above the effect threshold only in some cases (C5/1, CA6/3, CA6/4, CA6/5), without statistically significant differences in the time factor, but in each case attributable to the PMMA polymer. The low sensitivity to plastic elutriates in freshwater that we found is consistent with the report of Schiavo et al., 2018, which showed an inhibitory response < 25% after testing elutriates of PE, PP and PS at the same concentration as ours (100 mg /L).

The studies always performed with *A. fischeri* but with elutriates in seawater are rarer. For example, Piccardo et al., 2021 reported very little inhibition after 30 min (< 15%). The present study showed a more significant response (up to 79.3% at TIME 3) for PMMA (glitter CA6 / 4). This discrepancy could be due to the different chemical composition of the particles tested (PE vs. PMMA) as well as the different soaking times (28 vs. 180 days) and total area exposed (16 vs. 30 cm ^2/L). Similar to *R. subcapitata*, the saltwater counterpart *P. tricornutum* also showed good

sensitivity to elutriates and frequently exhibited a biostimulatory response. For example, a similar hormesis phenomenon was observed in the marine microalga *Dunaniella tertiolecta* with PE leachate (100 mg/L) (Schiavo et al., 2021).

The use of biological assays is a holistic approach that allows evaluation of toxicity and overall effects of all components, including potential additive, synergistic, and antagonistic effects (Wadhia and Thompson, 2007). Living organisms integrate the positive and negative effects of chemicals with which they come into contact with the environmental conditions to which they are exposed during the experiment and respond to the biologically active components present (Keddy et al., 1995). Given the varying sensitivity of organisms to chemicals (Van der Berg, 2021), as well as the overall toxicity of various chemicals released from plastics, the need for a series battery of bioassays covering a wide range of trophic levels to assess the toxicity and ecological risks of plastic leachates is evident (Barrick et al., 2021; Gao et al., 2022). Organisms from different trophic levels play a fundamental role in maintaining balance in ecosystems, and their characteristics have become an important index for assessing environmental quality (Brack et al., 2016). Our study supports this approach. Indeed, the species we used for toxicity tests responded differently to the samples we tested. The most sensitive trophic level was that of photosynthesizing primary producers, the second most sensitive species was *A. fischeri*, followed by larval stages of small planktonic crabs *D. magna* and plutei of sea urchin *P. lividus*. The information we collected will therefore support future studies aimed at determining the best test battery for assessing the ecotoxicological risk of plastic elutriates.

Once data have been collected from a series of bioassays, the need to integrate them even more arises when a strong contradiction in the responses has emerged. The importance of integration is that the data can be more easily interpreted in order to make a risk assessment. In this sense, the TBI approach has become widespread at the Italian level. The data integration used in this study is based on the quantitative assessment proposed by Hartwell, gradually modified by Baudo et al. (ISPRA, 2011). It calculates the "ecotoxicological risk" by weighting the type of endpoint observed, the type of environmental matrix analyzed, and the level of agreement of the *t-test* results. This integration confirmed that greater risk was associated with the marine environment, with the highest risk for glitter CA6. CA6 are hexagonal particles of PMMA. There is a significant knowledge gap regarding the impact of PMMA microplastics and nanoplastics (NP) on aquatic biota (Venâcio et al., 2019). Venâcio et al., 2019 used a series of standard bioassays with four marine microalgae and one marine rotifer species (*Brachionus plicatilis*) to test the toxicity of 40-nm nanoparticles. PMMA-NP was able to induce mortality in rotifers at concentrations greater than 4.69 mg/L with an estimated 48-h median lethal concentration of 13.27 mg/L. Results

collected by Brandts et al., 2021 show that PMMA-NPs activate the antioxidant defences of gilthead sea bream (*Sparus aurata*) and induce changes in lipid metabolic pathways and genotoxicity in blood cells (40 nm; 0-10 mg/L; 24 h and 96 h exposure). With increasing particle size (1-230 μm), Thomas et al., 2020 demonstrated mild toxicity of PMMA microplastics in early life stages of *Paracentrotus lividus* (no significant increase in developmental defects or in terms of reduced fertilisation rate at concentrations of 0.1-10 mg/L). Our study thus lays the first foundations for assessing the ecotoxicological risk of this particular type of plastic polymer, which, although not one of the most produced plastics in Europe (10.7%; Plastic Europe, 2021), has been shown to be one of the dominant polymers in glitter. A product survey conducted on 37 commercial products containing different glitters showed PMMA is the second most frequent polymer after PE (Piccardo et al., 2022).

Finally, multivariate analysis shows that plastic leachates toxicity is a complex phenomenon, dependent from the species-sensitivity, in some case by soaking time, by medium (*A. fischeri* in saltwater had greater responses compared to freshwater), and not clearly related with the polymer type. Focusing on the context which gives a worst ecotoxicological outcome (saltwater time 1 and 3), the possible role of contact surface and dyes release was explored. In particular, the possible release of dyes was indirectly described by differences in color expressed as ΔE and its components (Δa, Δb, ΔL). Colorimeter gives the color in the CIEL lab space characterize by L, a, and b. L is the luminance, a chromaticity, and b is the hue. Together are used to calculate the deltaE. ΔE says how much are differences in color (0 is not perceptible by human eyes, 100 is color totally different). In some case, this approach resulted useful. For example, at time 1, the marginal test of the DistLM model showed that the ΔE value was statistically significant and could explain 51.6% of the total variability. Such higher ΔE value was associated with the higher risk percentage: CA6/1 (R% = 10.0) and CA6/2 (R% = 21.3). Further, the brightness variations in CA7/5 (at time 3) were the parameter with the greatest influence on the total difference (ΔE). Such variation we supposed could be due to the leaching of the surface reflective layer, as shown by the microscopic observations.

According to a recent classification, additives can be divided into four main classes based on their functional and structural components: functional additives, colorants, fillers, and reinforcing agents (Hansen et al, 2013). Colorants include pigments and azo dyes, which are commonly used to treat textile products (Gunaalan et al., 2020). The limited knowledge on the effects of plastic additives often concerns the large category of functional additives, which include stabilizers, flame retardants, antistatic, plasticizers, lubricants, and biocides. Some of these classes of compounds (e.g., plasticizers and flame retardants) are covered by international legislation aimed at mitigating

their effects (Gunaalan et al., 2020). However, little or nothing is known about the ingredient in the dyes. In the context of glitter, color and shine are key to its success. In fact, glitter is often used in festive contexts to enhance the shine and color of accessories, clothing, and makeup. The optical properties that make glitter unique result from the combination between the color variability given by the dyes and the reflectivity given by the metal core. Andrady and Rajapakse, 2019 give a percentage between 1 and 4 percent by weight of the polymer for the category of colorants. However, the list of possible substances included in this category is not known. Consequently, it is also difficult to perform a chemical analysis of the elutriates that could complete the study we conducted. Future studies could focus on chemical characterization techniques of non-target species (since the exact composition of the dyes is not known). In this respect, a chemical screening of non-target species using Liquid or gas chromatography coupled with mass spectroscopy could be a good start (Tetu et al., 2019; Capolupo et al., 2020).

Conclusions

The chemical risk posed by a specific category of microparticles, namely glitter, was tested using two batteries of ecotoxicological tests (one each for the aquatic, freshwater, and marine environments). Different biological responses (stronger in seawater) and species-specific responses were found, which in some cases also depended on soaking time. The need to use test batteries for ecotoxicological risk assessment (ERA) of plastic elutriates is thus confirmed, as also highlighted by other authors Gao et al., 2022. The species most sensitive to this form of pollution were algae, followed by the bioluminescent bacterium *A. fischeri*, and then by the primary consumers *D. magna* and *P. lividus*. There is a significant lack of knowledge regarding the effects of micro- and nanoplastics on aquatic biota, particularly polymers such as poly-methyl-methacrylate (PMMA). Our study therefore lays the initial groundwork for assessing the ecotoxicological risk of this particular type of plastic polymer.

Supplementary Materials

The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/toxics10110677/s1.

ARTIFICIAL SEAWATER (ASW) recipe. Amount given in g/L:

1- NaCl = 22.0

2- $MgCl_2 * 6H_2O$ = 9.7

3- Na_2SO_4 = 3.7

4- $CaCl_2 * 2H_2O$ = 1.32

5- KCl = 0.65

6- $NaHCO_3$ = 0.2

7- H_3BO_3 = 0.023

ARTIFICIAL FRESHWATER (AFW) recipe. Preparation of 4 stock solutions (amount given in g/L):

1- $CaCl_2$ = 11.76

2- $MgSO_4 * 7H_2O$ = 4.93

3- $NaHCO_3$ = 2.59

4- KCl = 0.23

For the final AFW solution, mix 25 mL of each of the previous solutions and keep the volume to 1 L.

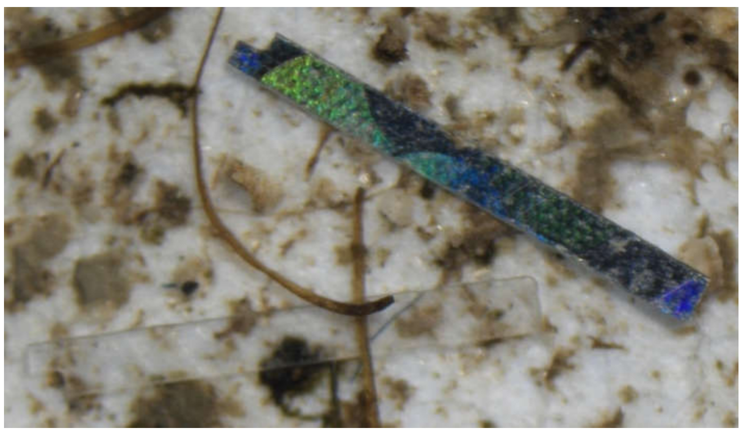


Figure S1 *CA7/5 type glitter: one of this glitter has the typical outer reflective layer, another is completely uncolored.*

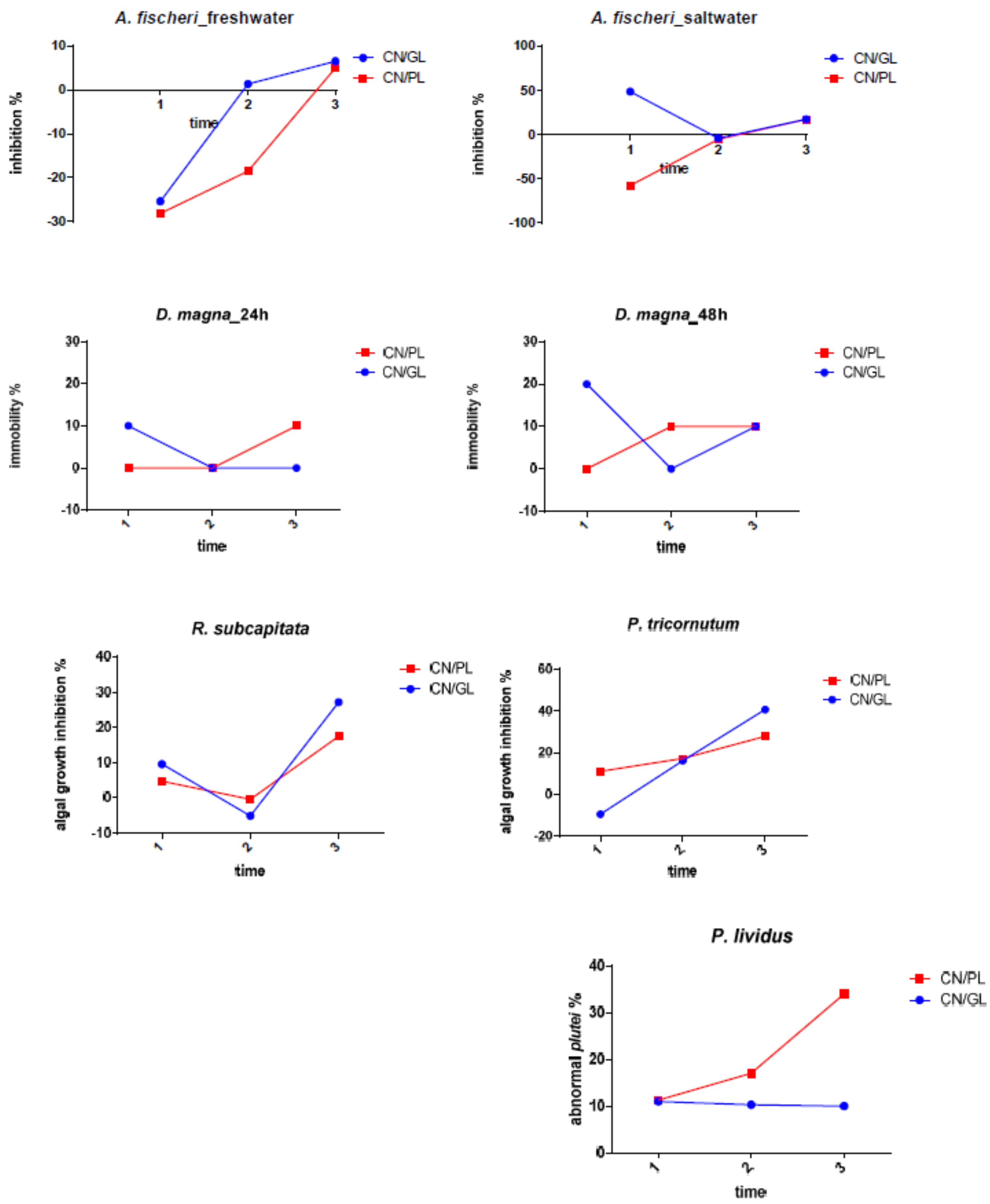


Figure S2 *Comparison between the ecotoxicological responses recorded in the two different negative controls: water in plastic bottles only (CN/PL) and in glass bottles (CN/GL).*

Table S1 Toxicity tests performed on glitter leachates.

Species		Endpoint	Type	Method	Test duration	Temperature (°C)	Illumination	Aquatic system
Aliivibrio fischeri	Bacteria	Inhibition of bioluminescence	Acute	UNI EN ISO 11348-3:2019	15 minutes 30 minutes	15 ± 1	-	Fresh saltwater
Paracentrotus lividus	Sea urchin	Larval development	Chronic	Chapman et al. 1995 ISPRA Quaderni Ricerca Marina 11/2017	72 hours	18 ± 1	dark	Saltwater
Raphidocelis subcapitata	Algae	Growth inhibition	Chronic	UNI EN ISO 8692:2012	72 hours	20 ± 2	6000-10000 lux	Freshwater
Phaeodactylum tricornutum	Algae	Growth inhibition	Chronic	UNI EN ISO 10253:2017	72 hours	20 ± 2	6000-10000 lux	Saltwater
Daphnia magna	Crustacean	Immobility	Acute	UNI EN ISO 6341:2013	24/48 hours	20 ± 2	dark	Freshwater

Table S2 P values of 2-way ANOVA, performed to highlight the possible role of the factors TIME and POLYMER in determining the differences in biological responses of saline and freshwater species. Values in bold are statistically significant (< 0.05).

	FRESHWATER			SALTWATER		
	A. fischeri	*R. subcapitata*	*D. magna*	*A. fischeri*	*P. tricornutum*	*P. lividus*
POLYMER	0.8978	0.8507	0.7644	0.0764	0.8063	0.9832
TIME	0.3371	**0.0018**	**0.0296**	0.7078	0.4383	**0.0004**
Interaction	0.8184	0.7591	0.9283	0.3726	0.9831	0.7505

References

Abbasi, S., Keshavarzi, B., Moore, F., Turner, A., Kelly, F. J., Dominguez, A. O., & Jaafarzadeh, N. (2019). Distribution and potential health impacts of microplastics and microrubbers in air and street dusts from Asaluyeh County, Iran. *Environmental pollution*, *244*, 153-164.

Abbasi, S., Soltani, N., Keshavarzi, B., Moore, F., Turner, A., & Hassanaghaei, M. (2018). Microplastics in different tissues of fish and prawn from the Musa Estuary, Persian Gulf. *Chemosphere*, *205*, 80-87.

Andrady, A. L., & Rajapakse, N. (2019). Additives and chemicals in plastics. *Hazardous Chemicals Associated with Plastics in the Marine Environment*, 1-17.

Ballent, A., Corcoran, P. L., Madden, O., Helm, P. A., & Longstaffe, F. J. (2016). Sources and sinks of microplastics in Canadian Lake Ontario nearshore, tributary and beach sediments. *Marine pollution bulletin*, *110*(1), 383-395.

Barrick, A., Champeau, O., Chatel, A., Manier, N., Northcott, G., & Tremblay, L. A. (2021). Plastic additives: challenges in ecotox hazard assessment. *PeerJ*, *9*, e11300.

Bellasi, A., Binda, G., Pozzi, A., Galafassi, S., Volta, P., & Bettinetti, R. (2020). Microplastic contamination in freshwater environments: A review, focusing on interactions with sediments and benthic organisms. *Environments*, *7*(4), 30.

Bergmann, M., Gutow, L., & Klages, M. (2015). *Marine anthropogenic litter* (p. 447). Springer Nature.

Blackledge, R. D. (Ed.). (2007). *Forensic analysis on the cutting edge: new methods for trace evidence analysis*. John Wiley & Sons.

Blair, R. M., Waldron, S., Phoenix, V., & Gauchotte-Lindsay, C. (2017). Micro-and nanoplastic pollution of freshwater and wastewater treatment systems. *Springer Science Reviews*, *5*, 19-30.

Brack, W., Ait-Aissa, S., Burgess, R. M., Busch, W., Creusot, N., Di Paolo, C., ... & Krauss, M. (2016). Effect-directed analysis supporting monitoring of aquatic environments—an in-depth overview. *Science of the Total Environment*, *544*, 1073-1118.

Brandts, I., Barría, C., Martins, M. A., Franco-Martínez, L., Barreto, A., Tvarijonaviciute, A., ... & Teles, M. (2021). Waterborne exposure of gilthead seabream (*Sparus aurata*) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels. *Journal of Hazardous Materials*, *403*, 123590.

Capolupo, M., Sørensen, L., Jayasena, K. D. R., Booth, A. M., & Fabbri, E. (2020). Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. *Water research*, *169*, 115270.

Chae, Y., Hong, S. H., & An, Y. J. (2020). Photosynthesis enhancement in four marine microalgal species exposed to expanded polystyrene leachate. *Ecotoxicology and Environmental Safety*, *189*, 109936.

Clarke, K. R., & Warwick, R. M. (1998). A taxonomic distinctness index and its statistical properties. *Journal of applied ecology*, *35*(4), 523-531.

Dehghani, S., Moore, F., & Akhbarizadeh, R. (2017). Microplastic pollution in deposited urban dust, Tehran metropolis, Iran. *Environmental Science and Pollution Research*, *24*, 20360-20371.

Gao, Z., Yu, H., Li, M., Li, X., Lei, J., He, D., ... & Shi, H. (2022). A battery of baseline toxicity bioassays directed evaluation of plastic leachates—Towards the establishment of bioanalytical monitoring tools for plastics. *Science of The Total Environment*, *828*, 154387.

González-Soto, N., Hatfield, J., Katsumiti, A., Duroudier, N., Lacave, J. M., Bilbao, E., ... & Cajaraville, M. P. (2019). Impacts of dietary exposure to different sized polystyrene microplastics

alone and with sorbed benzo [a] pyrene on biomarkers and whole organism responses in mussels Mytilus galloprovincialis. *Science of the Total Environment*, *684*, 548-566.

Green, D. S., Jefferson, M., Boots, B., & Stone, L. (2021). All that glitters is litter? Ecological impacts of conventional versus biodegradable glitter in a freshwater habitat. *Journal of Hazardous Materials*, *402*, 124070.

Gunaalan, K., Fabbri, E., & Capolupo, M. (2020). The hidden threat of plastic leachates: A critical review on their impacts on aquatic organisms. *Water Research*, *184*, 116170.

Hansen, E., Nilsson, N. H., Lithner, D., & Lassen, C. (2013). Hazardous substances in plastic materials. *COWI in cooperation with Danish Technological Institute*, 7-8.

Haram, L. E., Carlton, J. T., Ruiz, G. M., & Maximenko, N. A. (2020). A plasticene lexicon. *Marine pollution bulletin*, *150*, 110714.

Hildebrandt, L., Nack, F. L., Zimmermann, T., & Pröfrock, D. (2021). Microplastics as a Trojan horse for trace metals. *Journal of Hazardous Materials Letters*, *2*, 100035.

Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., & Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of the total environment*, *586*, 127-141.

ISPRA, *Batterie di saggi ecotossicologici per sedimenti di acque salate e salmastre*. 2011.

ISPRA, Saggio di fecondazione e saggio di sviluppo embrionale con il riccio di mare *Paracentrotus lividus* (Lamarck) (Echinodermata, Echinoidea). ISPRA, 2017.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., ... & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771.

Jones-Williams, K., Galloway, T., Cole, M., Stowasser, G., Waluda, C., & Manno, C. (2020). Close encounters-microplastic availability to pelagic amphipods in sub-antarctic and antarctic surface waters. *Environment International*, *140*, 105792.

Keddy, C. J., Greene, J. C., & Bonnell, M. A. (1995). Review of whole-organism bioassays: soil, freshwater sediment, and freshwater assessment in Canada. *Ecotoxicology and Environmental Safety*, *30*(3), 221-251.

Koelmans, A. A., Bakir, A., Burton, G. A., & Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environmental science & technology*, *50*(7), 3315-3326.

Li, W. C., Tse, H. F., & Fok, L. (2016). Plastic waste in the marine environment: A review of sources, occurrence and effects. *Science of the total environment*, *566*, 333-349.

Lithner, D., Nordensvan, I., & Dave, G. (2012). Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile–butadiene–styrene, and epoxy to Daphnia magna. *Environmental Science and Pollution Research*, *19*, 1763-1772.

Liu, C., Qiu, J., Tang, Z., Hu, H., Meng, F., & Li, A. (2021). Effects of polystyrene microplastics on growth and toxin production of alexandrium pacificum. *Toxins*, *13*(4), 293.

Liu, W., Zhao, Y., Shi, Z., Li, Z., & Liang, X. (2020). Ecotoxicoproteomic assessment of microplastics and plastic additives in aquatic organisms: a review. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *36*, 100713.

Moore, C. J., Lattin, G. L., & Zellers, A. F. (2011). Quantity and type of plastic debris flowing from two urban rivers to coastal waters and beaches of Southern California. *Revista de Gestão Costeira Integrada-Journal of Integrated Coastal Zone Management*, *11*(1), 65-73.

MSFD Technical Subgroup on Marine Litter, *Guidance on Monitoring of Marine Litter in European Seas*. 2013.

Nithin, A., Sundaramanickam, A., & Sathish, M. (2022). Seasonal distribution of microplastics in the surface water and sediments of the Vellar estuary, Parangipettai, southeast coast of India. *Marine Pollution Bulletin*, *174*, 113248.

OECD/OCDE, "OECD Guidelines for the Testing of Chemicals - Revised Introduction To the Oecd Guidelines for Testing of Chemicals, section 3," Paris: OECD Publishing, 2006, pp. 1–13.

Piccardo, M., Anselmi, S., & Renzi, M. (2022). Journey into the local market in search of "glitter" microparticles: Mini product investigation and relative chemical-physical characterization. *Environments*, *9*(9), 119.

Piccardo, M., Provenza, F., Grazioli, E., Anselmi, S., Terlizzi, A., & Renzi, M. (2021). Impacts of plastic-made packaging on marine key species: Effects following water acidification and ecological implications. *Journal of Marine Science and Engineering*, *9*(4), 432.

Piccardo, M., Provenza, F., Grazioli, E., Cavallo, A., Terlizzi, A., & Renzi, M. (2020). PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations. *Science of the Total Environment*, *715*, 136947.

Plastics Europe, "Plastics — The Facts 2021 An analysis of European Plastics Production, Demand and Waste Data," Belgium, 2021.

Raju, S., Carbery, M., Kuttykattil, A., Senthirajah, K., Lundmark, A., Rogers, Z., ... & Palanisami, T. (2020). Improved methodology to determine the fate and transport of microplastics in a secondary wastewater treatment plant. *Water research*, *173*, 115549.

Reynolds, C., & Ryan, P. G. (2018). Micro-plastic ingestion by waterbirds from contaminated wetlands in South Africa. *Marine pollution bulletin*, *126*, 330-333.

Schiavo, S., Oliviero, M., Chiavarini, S., Dumontet, S., & Manzo, S. (2021). Polyethylene, Polystyrene, and Polypropylene leachate impact upon marine microalgae *Dunaliella tertiolecta*. *Journal of Toxicology and Environmental Health, Part A*, *84*(6), 249-260.

Schiavo, S., Oliviero, M., Romano, V., Dumontet, S., & Manzo, S. (2018). Ecotoxicological assessment of virgin plastic pellet leachates in freshwater matrices. *Journal of Environmental Accounting and Management*, *6*(4), 345-353.

Silva, A. B., Bastos, A. S., Justino, C. I., da Costa, J. P., Duarte, A. C., & Rocha-Santos, T. A. (2018). Microplastics in the environment: Challenges in analytical chemistry-A review. *Analytica chimica acta*, *1017*, 1-19.

Sridharan, S., Kumar, M., Saha, M., Kirkham, M. B., Singh, L., & Bolan, N. S. (2022). The polymers and their additives in particulate plastics: what makes them hazardous to the fauna?. *Science of The Total Environment*, *824*, 153828.

Suaria, G., Avio, C. G., Mineo, A., Lattin, G. L., Magaldi, M. G., Belmonte, G., ... & Aliani, S. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. *Scientific reports*, *6*(1), 37551.

Sun, J., Dai, X., Wang, Q., Van Loosdrecht, M. C., & Ni, B. J. (2019). Microplastics in wastewater treatment plants: Detection, occurrence and removal. *Water research*, *152*, 21-37.

Tagg, A. S., & do Sul, J. A. I. (2019). Is this your glitter? An overlooked but potentially environmentally-valuable microplastic. *Marine pollution bulletin*, *146*, 50-53.

Tan, F., Yang, H., Xu, X., Fang, Z., Xu, H., Shi, Q., ... & Li, H. (2020). Microplastic pollution around remote uninhabited coral reefs of Nansha Islands, South China Sea. *Science of the Total Environment*, *725*, 138383.

Tetu, S. G., Sarker, I., Schrameyer, V., Pickford, R., Elbourne, L. D., Moore, L. R., & Paulsen, I. T. (2019). Plastic leachates impair growth and oxygen production in *Prochlorococcus*, the ocean's most abundant photosynthetic bacteria. *Communications biology*, *2*(1), 184.

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., ... & Takada, H. (2009). Transport and release of chemicals from plastics to the environment and to

wildlife. *Philosophical transactions of the royal society B: biological sciences*, *364*(1526), 2027-2045.

Thomas, P. J., Oral, R., Pagano, G., Tez, S., Toscanesi, M., Ranieri, P., ... & Lyons, D. M. (2020). Mild toxicity of polystyrene and polymethylmethacrylate microplastics in *Paracentrotus lividus* early life stages. *Marine Environmental Research*, *161*, 105132.

Van Cauwenberghe, L., Devriese, L., Galgani, F., Robbens, J., & Janssen, C. R. (2015). Microplastics in sediments: a review of techniques, occurrence and effects. *Marine environmental research*, *111*, 5-17.

van den Berg, S. J., Maltby, L., Sinclair, T., Liang, R., & van den Brink, P. J. (2021). Cross-species extrapolation of chemical sensitivity. *Science of the total environment*, *753*, 141800.

Venâncio, C., Ferreira, I., Martins, M. A., Soares, A. M., Lopes, I., & Oliveira, M. (2019). The effects of nanoplastics on marine plankton: A case study with polymethylmethacrylate. *Ecotoxicology and environmental safety*, *184*, 109632.

Wadhia, K., & Thompson, K. C. (2007). Low-cost ecotoxicity testing of environmental samples using microbiotests for potential implementation of the Water Framework Directive. *TrAC Trends in Analytical Chemistry*, *26*(4), 300-307.

Watters, D. L., Yoklavich, M. M., Love, M. S., & Schroeder, D. M. (2010). Assessing marine debris in deep seafloor habitats off California. *Marine Pollution Bulletin*, *60*(1), 131-138.

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., & Koelmans, A. A. (2012). Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environmental Toxicology and Chemistry*, *31*(11), 2490-2497.

Woodall, L. C., Sanchez-Vidal, A., Canals, M., Paterson, G. L., Coppock, R., Sleight, V., ... & Thompson, R. C. (2014). The deep sea is a major sink for microplastic debris. *Royal Society open science*, *1*(4), 140317.

Yang, Y., Guo, Y., O'Brien, A. M., Lins, T. F., Rochman, C. M., & Sinton, D. (2020). Biological responses to climate change and nanoplastics are altered in concert: full-factor screening reveals effects of multiple stressors on primary producers. *Environmental Science & Technology*, *54*(4), 2401-2410.

Yurtsever, M. (2019). Tiny, shiny, and colorful microplastics: are regular glitters a significant source of microplastics?. *Marine pollution bulletin*, *146*, 678-682.

Chapter 3

Sparkling plastic: effects of exposure to glitter on the mediterranean mussel *Mytilus galloprovincialis*

Francesca Provenza[1,2], Serena Anselmi[1], Antonietta Specchiulli[3], Manuela Piccardo[2], Damià Barcelò[4,5], Marino Prearo[6], Paolo Pastorino[6], Monia Renzi[2]

[1]Bioscience Research Center, 58015 Orbetello (GR), Italy

[2]Dipartimento di Scienze della Vita, Università degli Studi di Trieste, 34127 Trieste, Italy

[3]National Research Council—Institute for Biological Resources and Marine Biotechnologies (IRBIM), Lesina, Italy.

[4] Catalan Institute for Water Research (ICRA-CERCA), 17003 Girona, Spain

[5] Institute of Environmental Assessment and Water Research (IDAEA-CSIC), 08034 Barcelona, Spain

[6]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, 10154 Torino, Italy

Environmental Toxicology and Pharmacology

Volume 96, November 2022, 103994

https://doi.org/10.1016/j.etap.2022.103994

Abstract

Microbeads and fragments have been widely studied, while glitter remains neglected by the literature although found in a variety product (e.g., body paints, nail polish, cosmetics, craft products). The main aim of this study was to assess the effects of different types and concentrations of glitter particles on *Mytilus galloprovincialis* after 7 days of exposure. The experiment was divided into a preliminary test and a confirmatory test. Our findings support the hypothesis for a link between concentration and type of glitter particles, percentage of recovery and oxidative stress in *M. galloprovincialis*. There was a significant correlation between particle length and percentage of particles recovered in water, suggesting that the digestive tract of *M. galloprovincialis* retains smaller particles more. In addition, we noted an increase in antioxidant defense induced by smaller particles. Moreover, certain types of glitter crumbled and shortened in length, resulting in higher levels of oxidative stress biomarkers. Finally, the star-shaped glitter particles had a different effect on oxidative stress biomarkers. Further studies are needed to clarify the toxic effects of glitter on aquatic organisms and to quantify its proportion to other microplastics in the environment.

Keywords

Biomarkers; marine litter; mussels; oxidative stress; superoxide dismutase

Introduction

Plastic makes up the bulk of marine debris (Napper and Thompson, 2020). Because plastic pollution, especially microplastics (MPs), has a wide-ranging impact on ecosystems (Avio et al., 2015; Dehaut et al., 2016), much recent research has focused on its occurrence (Pauna et al., 2019; Wakkaf et al., 2020, Tan et al., 2022), transport dynamics (Enders et al., 2019; Uzun et al., 2022), and effects on the aquatic food web (Sharma and Chatterjee, 2017; Waring et al., 2018; Li et al., 2020; Bhutto and You, 2022).

Two broad categories of MPs are distinguished: primary and secondary plastics (Rocha-Santos and Duarte, 2015). Primary MPs are directly manufactured (microbeads, glitter, industrial granules <5 mm), while secondary MPs derive from the fragmentation of larger plastics in the environment (i.e., microfibers from synthetic fibers, particulate materials from municipal waste) (Yurtsever, 2019). The process by which secondary MPs form can be anthropogenic or natural in origin (air, wind, light, water, wave action).

Microbeads and fragments have been widely studied, while other types of MPs remain understudied (Tagg et al., 2019), such as glitter, a common MP albeit rarely mentioned in the literature (Yurtsever, 2019). Glitter particles are found in a variety of products (e.g., body paints, nail polish, cosmetics, craft products) and applications (Guerranti et al., 2019). The European Chemicals Agency (ECHA) has compiled a list of polymer-based materials including glitter among others that are intentionally added to such products (ECHA, 2019).

Glitter is sometimes used as a model for MPs (Elkhatib, 2020) because MPs is defined also by particle size. Furthermore, plastic glitter matches the definition of MPs in the environment in distribution, shape, and other characteristics (Hartmann et al., 2019). In brief, glitter can be considered a "symbol of microplastic" comprising all the hazardous characteristics of MPs (Yurtsever, 2019).

But unlike MPs, glitter particles are covered with potentially toxic molecules which may be released in the environment during the weathering process. Most glitter is made from polyethylene terephthalate (PET) (weight range, 1.32-1.41 g/cm) metallized with aluminum, titanium, iron, or bismuth (Yurtsever, 2019). The metallic coating produces high reflectivity (Locher et al., 2018). Commercially available glitter ranges in size from 50 to 6250 μm but the most common is 200 μm (Blackledge and Jones, 2007). It comes in a variety of shapes (i.e., hexagonal, square, triangular, striped, heart, star, moon, diamond, flower, snowflake, butterfly, irregular) that are uniformly large, precisely cut, and sometimes with numerous notches (Yurtsever, 2019). Based on characteristics, size, and shape, the definition of plastic glitter is consistent with the definition of MPs. A major hazard posed by glitter is that it is scattered on the ground, flows into sewers, and then dispersed. Hence, it is no coincidence that glitter is often found in wastewater treatment plants and the sediments of aquatic ecosystems (Ballent et al., 2016; Hurley et al., 2018; Yurtsever, 2019). But because it is environmental waste, accurate information on the interaction between organisms and glitter particles is lacking.

Currently, we are unable to estimate glitter emissions into the environment (Tagg and Ivar do Sul, 2019). Glitter particles can be discharged into the aquatic environment directly or indirectly (Lehel and Murphy, 2021). Direct releases occur if glitter particles are washed off during outdoor activities or when removing glitter body paint or makeup. Even if glitter can be retained by wastewater treatment plants in sludge, the application of biosolids to soil can result in almost 100% of the glitter being transported into aquatic habitats (Crossman et al., 2020). In comparison to a comprehensive coverage of other types of microplastic particles, a real quantification of glitter particles does not appear within any microplastic papers and is likely to be currently underestimated due to methodological constraints and incorrect categorization (Yurtsever, 2019).

Among the aquatic organisms that hold promise as biomonitoring tools for assessing the environmental quality of marine ecosystems (Prokić et al., 2021), the bivalve *Mytilus galloprovincialis* has proven highly suitable to assess the occurrence and impact of MP pollution (Li et al., 2019). *M. galloprovincialis* is a filter-feeding marine bivalve of ecological and commercial importance in the Mediterranean Sea, where MP pollution is of particular concern (Lusher, 2015).

With these points in mind, we wanted to investigate the effects of glitter particles on *M. galloprovincialis* and to determine whether glitter could cause oxidative stress when administered experimentally. Indeed, measurement of oxidative stress in aquatic organisms exposed to MPs yields information on their physiological status (Prokić et al., 2019). Our hypothesis was that glitter particle type and concentration would influence the percentage of particle recovery and the level of oxidative stress in *M. galloprovincialis.*

Material and methods

Experimental design

Mytilus galloprovincialis was exposed to different glitter concentrations and types over a period of 7 days. The experiment was divided into a preliminary test and a confirmatory test. In the preliminary test, *M. galloprovincialis* was exposed to two glitter types (Type 6_CA6/2 and Type 3_CA7/5) obtained from local commercial markets in Trieste (northeastern Italy) at two different doses (10 and 20 particles) for 7 days. Each experimental condition was performed in five replicates (*n*=5) and organisms (*n*=1 per replicate). The glitter was immersed in 800 mL of seawater (glitter concentration: 12.5 particles/L and 25 particles/L, respectively). The water was collected at a non-contaminated site (Talamone, Maremma Natural Park, Tuscany, Italy; 42°33'24.436" N; 11°7'34.664") and was not filtered prior to use (preliminary test: salinity 30.2 g/L; pH 8.1; confirmatory test: salinity 31.1 g/L; pH 8.0). The high standard of the water quality was confirmed by ecotoxicological tests performed on seawater collected from Talamone site. The tests were conducted with a battery of three species from different trophic levels: bacteria (*Aliivibrio fischeri*), primary producer (*Phaeodactylum tricornutum*) and primary consumer (*Paracentrotus lividus*), which always gave values within the recorded effects (<5%). At the end of the preliminary experiment, oxidative stress was measured to configure the confirmatory test design. During the confirmatory test, *M. galloprovincialis* was exposed to nine types of glitter for 7 days at a single dose of 50 particles. Each experimental condition consisted of ten replicates

(n=10) in which organisms (n=1 per replicate) and glitter particles were exposed to 800 mL of seawater (glitter concentration: 62.5 particles/L).

For each experiment, negative controls were prepared for the preliminary (n=5) and the confirmatory test (n=10). No glitter was added to the negative control container. A blank sample was prepared for each test and filtered to remove glitter or other MPs as described in Piccardo et al. (2021).

Organisms

Specimens of *Mytilus galloprovincialis* were collected in May 2022 at a pristine site (Talamone, Maremma Natural Park, Tuscany, Italy; 42°33'24.436" N; 11°7'34.664"). The organisms were measured with a caliper (size class 4.9 ±0.6 cm for the preliminary and 5.1 ±1.0 cm the confirmatory test). The organisms were examined to verify health status and to exclude the absence of glitter and other MPs (control analysis at time zero).

Glitter particles

Glitter of different colors, shapes, sizes, and polymer types was obtained from local commercial markets in Trieste (northeastern Italy). Nine types of glitter were used for the experiments (**Table 1**). Based on preliminary test results (experiment 1), nine types were selected for the confirmatory test: three gray types (Type 1, Type 2, Type 3) in different shapes and five types (Type 4 to Type 9) of the same shape but different in color. With this experimental arrangement we were able to observe which glitter shapes and colors *M. galloprovincialis* might preferentially filter. General characteristics (color, shape, size [mean ± standard deviation, SD]) were measured on the glitter particles selected from the glitter stock. Particle size was measured using a stereomicroscope (Nikon P-DSL32) connected to a camera (Nikon DSFi3) and software (NS-Elements D.4.60) calibrated with a micrometrical calibrated slide to correctly determine dimension. Chemical analysis of the plastic outer coating was performed by microscopy in conjunction with Fourier transformed infrared detection (μFT-IR), Nicolet iN10, Thermo Scientific, Waltham, MA, USA) using a reflectance detector cooled with liquid nitrogen. Spectra were collected at ten different points and compared to obtain the average spectrum for chemical identification. The spectra were identified when they matched the spectra collected in the Bioscience Research Center (BsRC, Orbetello, Italy) MPs library (threshold for matching set at 80%). Instrument performance was tested with standard plastic materials before chemical analysis.

Table 1 *Glitter types in the confirmatory test. Analysis of the plastic type was performed using μFT-IR on the outer coating. The length (μm) is given as mean ± standard deviation.*

Type	Color	Plastic Type	Shape	Initial length (μm)
Type 1	Gray	Polyamide	Star	3073.8 ± 21.7
Type 2	Gray	Polymethyl methacrylate	Hexagon	1860.1 ± 925.9
Type 3	Gray	Polyethylene	Rectangle	2345.5 ± 540.4
Type 4	Gray	Polyethylene	Small pentagon	241.3 ± 32.7
Type 5	Yellow	Polymethyl methacrylate	Small pentagon	280.5 ± 14.6
Type 6	Pink	Polymethyl methacrylate	Small pentagon	336.1 ± 17.5
Type 7	Orange	Polymethyl methacrylate	Small pentagon	276.0 ± 10.3
Type 8	Violet	Polymethyl methacrylate	Small pentagon	276.2 ± 9.9
Type 9	Green	Polymethyl methacrylate	Small pentagon	309.9 ± 39.2

Experimental condition

One organism per replicate was tested in 800 mL of natural seawater in a glass jar. After immersion, a counted amount of glitter particles was added to each replicate and the amount per liter of water was measured. Each glass jar was maintained under constant oxygenation during the experiment using an air pump (Newair1, Newa, Italy). The organisms were not fed; they were kept at controlled temperature (18 ± 2 °C) and subjected to a 16:8 h light-dark cycle. The organisms were checked daily; the mortality rate was also calculated for each day of exposure.

Glitter recovery

At the end of the experiment, each replicate was filtered with a 0.45 μm cell nitrate filter. The glass jar and mussels (outer surface) were carefully cleaned with seawater before filtration. The filters were stored in glass Petri dishes and observed under a stereomicroscope (Nikon P-DSL32) connected to a camera (Nikon DSFi3) and software (NS -Elements D.4.60) to calculate the percentage of glitter in each replicate. For each replicate, five glitter particles were measured with the software to calculate average particle length (±SD) and to determine changes over baseline.

Biomarker analysis

Following water filtration to determine the amount of glitter recovery, each specimen was processed for tissue analysis of biomarkers. The organism was opened, and the digestive gland and the gills were removed with a steel scalpel and forceps. The tissue was weighed and frozen with liquid nitrogen. Samples were stored at -80 °C till analysis of oxidative stress biomarkers.

Biomarker analyses were performed using protein fraction S9 (De Marchi et al., 2017). Each organism underwent a battery of biomarker analyses: malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST). A fraction was extracted in phosphate buffer 50 mM + EDTA 2 mM and then added to a test tube containing the organism's tissue. The ratio of tissue to buffer varied depending on the tissue: 1:4 (w/v) for the digestive glands and 1:2 (w/v) for the gills. The tissues were homogenized using an Ultraturrax

while keeping them cold, and then centrifuged (12,000 x g for 12 min at 4 °C) to extract the protein fraction. The supernatant was collected, placed in 2-mL Eppendorf tubes, and stored at -80 °C until analysis. The protein content in each sample and each tissue was quantified spectrophotometrically (Fulltech Peak, mod. UVVIS 7100 S, Fulltech Instruments, Rome, Italy) at 750 nm according to the colorimetric method of Lowry (Dubois, 1956) to normalize the data. The S9 protein fraction was placed in a test tube containing NaOH 0.5 M, Folin-Ciocalteau reagent and a mixture of reagents ($CuSO_4$ * 5 H_2O, Rochelle salt, and Na_2CO_3) and analyzed.

Lipid peroxidation was tested using 10% of the complex protein (Uchiyama and Mihara, 1978). The protein fraction was contacted with 1% phosphoric acid (v/v) and 0.6% thiobarbituric acid (w/v). After heating (96 °C for 25 min) and centrifugation (4000 RPM at room temperature for 5 min) with 1-buthanol, malondialdehyde (MDA) was quantified spectrophotometrically at a wavelength between 535 and 520 nm. The delta was calculated using the delta of absorbance at the beginning and at the end of measurement. The results are expressed as µmol of MDA/mg of protein.

For SOD quantification (Gao et al., 1998), the S9 protein fraction was mixed with Tris-EDTA buffer (pH 8.2) and pyrogallol. This method indirectly quantifies SOD based on the enzyme's ability to inhibit pyrogallol autoxidation. The enzyme was quantified spectrophotometrically at 420 nm after a reaction time of 3 min. The results are expressed as U/mg of protein.

GPx analysis (Badary et al., 2005) was performed using a fixed concentration of 0.5 mg/mL protein. Reaction of the S9 fraction was performed with a mixture of GSH (reduced glutathione) 10 mM, GSSG reductase 2.4 U/mL, and NADPH 1.5 mM. After incubation at 37 °C for 5 min in the presence of hydrogen peroxide, GPx kinetics were quantified spectrophotometrically at 340 nm for 2 min. The measurements were obtained by calculating a delta value and expressed in nmol/(mg*min).

GST analysis (Habig et al., 1974) was performed with the S9 protein fraction exposed to a reaction mixture of GSH (reduced glutathione) 10 mM and CDNB (1-chloro-2,4-dinitrobenzene) 60 mM. After mixing, GST was quantified spectrophotometrically at 340 nm for 5 mina and the delta was calculated. The results are expressed in mol/(g*min). Two organs were analyzed for each replicate and two analytical replicates were processed for each. The analytical replicates were averaged (±SD). Three replicates of each analytical condition were averaged (±SD). Dead organisms were not analyzed because the tissue could not be extracted from the animal. In such cases, replicates of the analyzed samples were the ones remaining.

Statistical analysis

Data normality and homoscedasticity were tested with the Shapiro-Wilk and the Levene test, respectively. Significant differences in percent recovery and glitter length before (T0) and after (T7) the experiments (preliminary and confirmatory tests) were assessed with the non-parametric Wilcoxon test. Two-way ANOVA with organ (digestive glands and gills), treatment group (glitter type), and organ × glitter type as independent variables was used to test for statistically significant differences in oxidative stress biomarkers. Dunnett's multiple comparison test was used to compare the treatment to the control group. One-way ANOVA was performed to determine differences in the percentage of recovery of glitter particles between the treatment groups. In addition, principal component analysis (PCA) was performed to summarize the effect of glitter particle (color and shape) on oxidative stress. Finally, the correlation between particle length and percentage of recovery was tested using Spearman's rank correlation coefficient (ρS). Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). PCA plots were generated using open-source data analysis software R Studio version 1.1.463 (RStudio, Inc., Boston, MA, USA).

Results

Glitter recovery and mortality rate during the preliminary test

The preliminary test revealed two important aspects, one related to the number of particles and the other to glitter length. The higher the number of particles added to the water, the lower the content of glitter particles found in water (higher the content of glitter particles in *M. galloprovincialis*). The results showed a lower, but not significant (one-way ANOVA; $p > 0.05$) percentage of glitter particles recovery after exposure to 20 Type 3 particles (larger particles) than after exposure to 10 and 20 Type 3 and 6 particles (**Table 2**). In addition, many Type 3 glitter particles were discolored. At the end of the experiment, no significant changes were observed in glitter particle length (Wilcoxon test; $p > 0.05$ for all glitter types). Exposure for 7 days in salt water and the presence of a living organism do not lead to structural damage or smoothing of the glitter (**Table 2**). The mortality rate was 20% for each of the treatment groups.

__Table 2__ Glitter recovery (filtered water) in the preliminary test. SD = standard deviation. NS indicates no significant changes (Wilcoxon test; p >0.05) in glitter length before (T0) and after the experiment (T7). N= number of glitter particles. Lowercase letter (a) indicates not significant percentage of glitter particles recovery after exposure (one-way ANOVA; p >0.05).

Glitter	Items	% Recovery		Length (µm)		Difference in length (T0 vs. T7)
	N	Mean	SD	Mean	SD	*p* <0.05
Type 3	10	75[a]	7.1	2681.15	528.5	NS
Type 6	10	75[a]	21.2	349.3	17.7	NS
Type 3	20	60[a]	14.1	2714.25	598.1	NS
Type 6	20	71[a]	3.5	354.05	49.2	NS

Glitter recovery and mortality rate during the confirmatory test

Table 3 presents the mortality rate recorded during the confirmatory test, which was assigned to the day of occurrence after the start of the experiment. Dead animals were removed from the experiment and from the analysis of stress biomarkers.

The confirmatory test showed that *M. galloprovincialis* preferentially filtered smaller particles (Type 4 to Type 9), since they were not recovered during the water filtration. Recovery rates were highest for Type 1, Type 2, and Type 3 glitter particles (**Table 4**). Significant changes in glitter length (Types 5, 6, 9) were observed at the end of the experiment (Wilcoxon test, p <0.05). Significant differences between recovery rates were observed for three glitter types (Type 4, 6, 7) (one-Way ANOVA, p <0.05; **Table 4**). A significant positive correlation was observed between the percentage of particles recovered and glitter length (ρS 0.75; p=0.025).

__Table 3__ Mortality rate (percent; %) of Mytilus galloprovincialis during the confirmatory test and days of exposure associated with mortality.

Glitter Type	% Mortality	Day of death
1	16.67	6
2	0	-
3	16.67	3
4	33.33	1 (16.67%) and 6 (16.67%)
5	16.67	1
6	16.67	3
7	0	-
8	16.67	1
9	16.67	2

Table 4 *Recovery of glitter during the confirmation test (50 items/800 mL). SD= standard deviation. Asterisks (*) indicate significant changes in glitter length before (T0) and after the experiment (T7) (Wilcoxon test) and in the percent recovery between glitte r types (one-way ANOVA). NS= not significant.*

Glitter	**% Recovery**		**Length (µm)**		**Difference in Length (T0 vs. T7)**
	Mean	SD	Mean	SD	p<0.05
Type 1	97.3	9	2997.3	73.6	NS
Type 2	96.7	5.8	1900.8	886.3	NS
Type 3	72.7	26	2600.3	569.6	NS
Type 4	46.0*	13.9	250.9	46.6	NS
Type 5	69.3	16.8	259.2	12.8	-7.4% (*)
Type 6	58.7*	24.1	258.9	18.4	-22.9% (*)
Type 7	55.3*	16.7	260.8	11.2	NS
Type 8	67.3	16.2	265.5	7.0	NS
Type 9	62.0	18	248.2	10.1	-19.7% (*)

Biomarkers in the preliminary test

Table S1 presents the results of two-way ANOVA for treatment, organ, and treatment x organ interaction on oxidative stress biomarkers. The analysis showed a significant effect (p <0.05) of treatment, organ, and interaction treatment x organ on SOD and MDA levels. There was also a significant effect (p <0.05) of treatment on GST and GPx levels (p <0.05) (**Table S1**).

There was a significant difference in SOD between the digestive gland of mussels exposed to a dose of 10 Type 6 (T6 10) and 20 Type 6 (T6 20) glitter particles compared to the control group (p <0.05), whereas SOD in the gills was similar after treatment than in the respective control groups (**Fig. 2**). There was a significant difference in MDA content in the digestive gland between the treatment and the control groups (p <0.05), whereas there was a significant difference in MDA content in the gills only between a dose of 10 Type 6 (T6 10) in the treatment and the control group. Although a significant treatment effect was detected, there was no significant difference in GST content in the digestive gland and the gills between the treatment and the control group. Finally, there was a significant increase in GPx concentration in the digestive gland and the gills at both doses (10 and 20 items/800 mL).

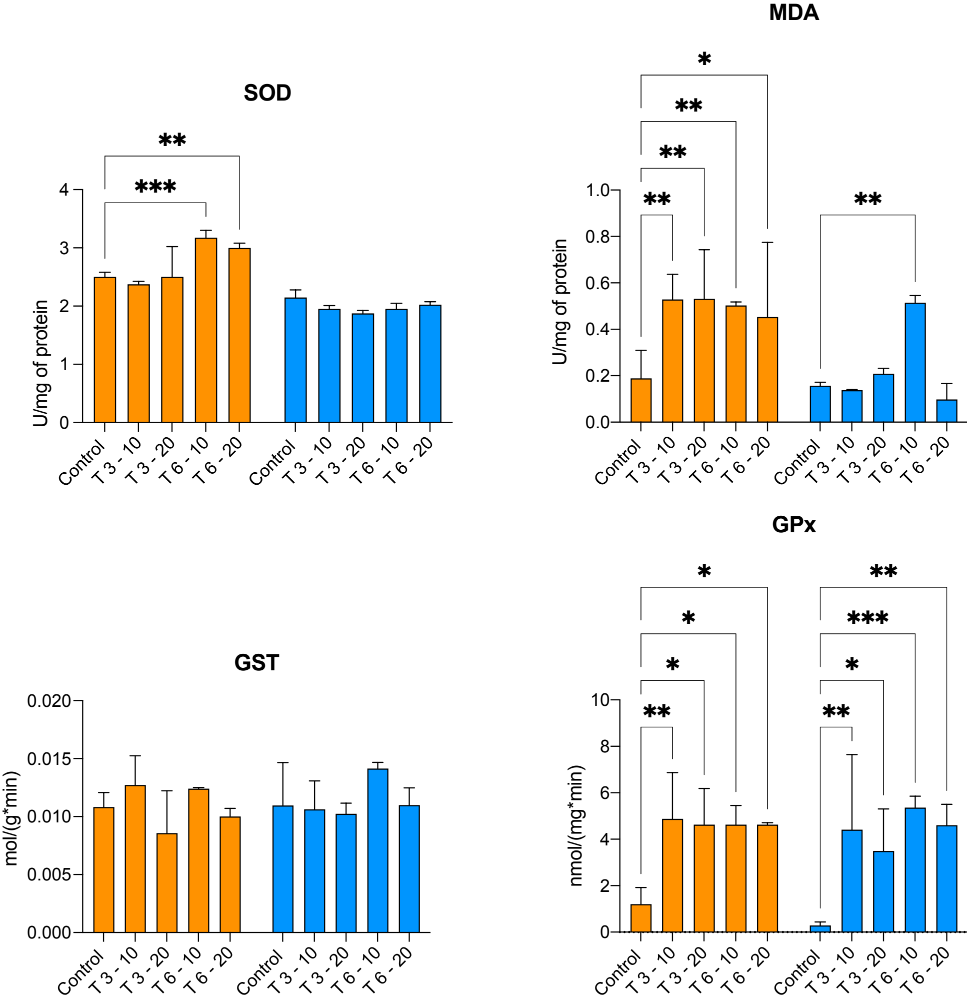


Figure 1 *Preliminary test. Oxidative stress biomarkers in the digestive glands (orange) and gills (blue) of Mytilus galloprovincialis exposed to glitter particles (Types 3 and 6) for 7 days. Asterisks denote a significant difference according to Dunnett's multip le comparison test (*p ≤ 0.05; **p ≤ 0.01; **p ≤ 0.001). T denotes the particle type (Types 3 and 6), 10 and 20 denotes the dose (number of glitter particles per 800 mL).*

Biomarkers in the confirmatory test

Table S2 presents the results of two-way ANOVA for treatment, organs, and treatment x organ interaction on oxidative stress biomarkers. The analysis showed a significant effect (p <0.05) of treatment, organs, and treatment x organ interaction on SOD and GST levels. There was a significant effect (p <0.05) of treatment and treatment x organ interaction on MDA content and a significant effect (p <0.05) of organ and treatment x organ interaction on GPx concentration (p <0.05). There was a significant increase in SOD activity in the digestive glands after treatment with glitter particle types 2, 3, 4, 5, 6, 7, and 9 but not in the gills (**Fig. 3**). MDA in the digestive glands was increased after treatment with glitter particle types 6, 7, and 9, and after treatment with types 6, 8, and 9 in the gills (**Fig. 3**).

Glutathione S-transferase (GST) was significantly increased in the digestive gland after treatment with glitter type 6 but not in the gills (**Fig. 3**).

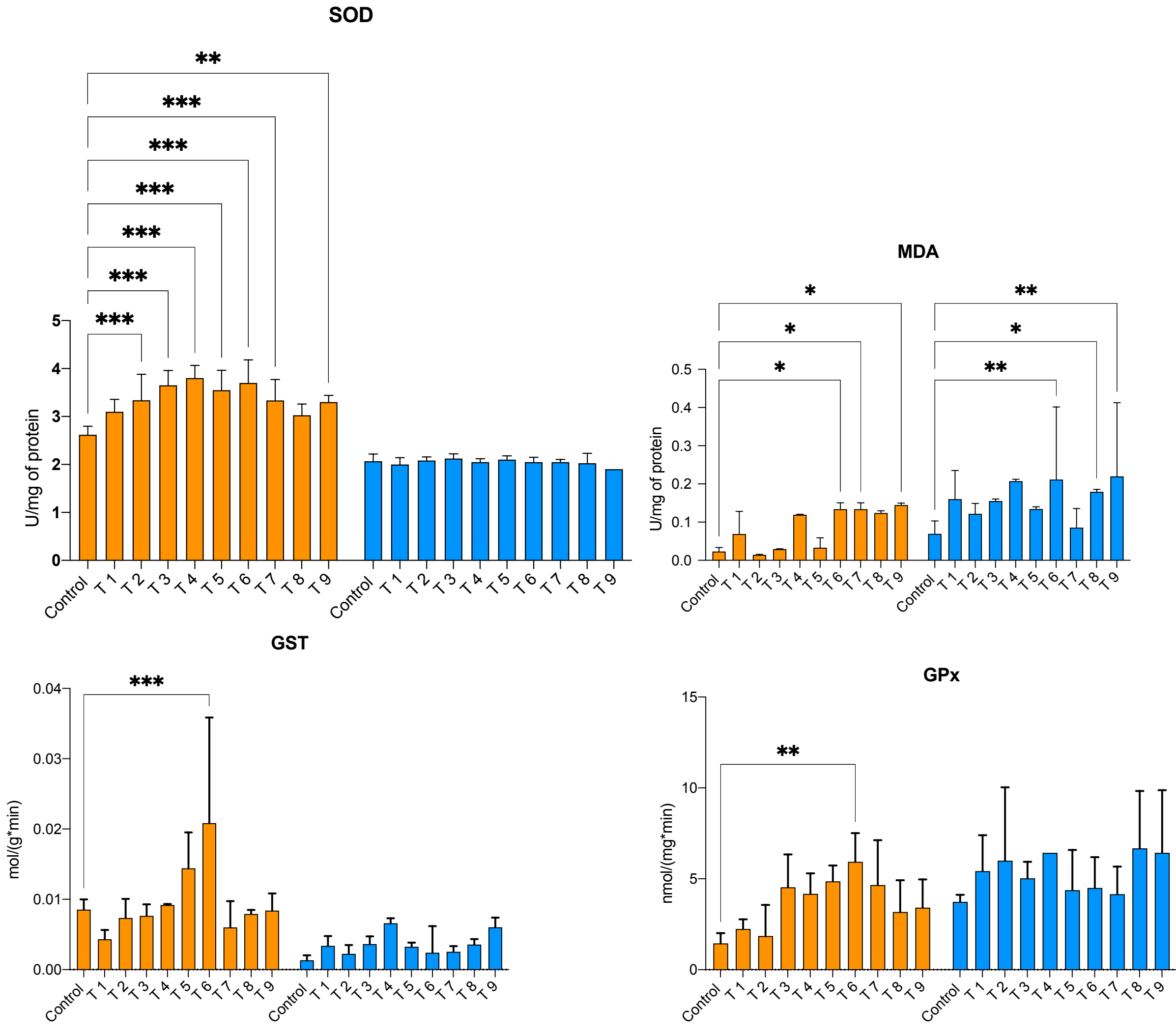


Figure 2 *Confirmatory test. Oxidative stress biomarkers in the digestive glands (orange) and gills (blue) of Mytilus galloprovincialis exposed to glitter particles (50 particles/800 mL) for 7 days. Asterisks denote significant differences according to Dunnett's multiple comparison test (*p ≤ 0.05; **p ≤ 0.01; **p ≤ 0.001). T denotes particle type (Type 1 to 9).*

Glutathione peroxidase (GPx) was significantly increased in the digestive gland after treatment with glitter type 6 and in the gills in the treatment and the control groups.

The first PCA (**Fig. 4a**) showed that the first (Dim1) and the second (Dim2) components accounted for meaningful amounts of total variance (40.7%): Dim1 explained 22.1% of the total variance and Dim2 18.6% (**Fig. 4a, b**). The overlap of the confidence ellipses (95%) of the colored particles suggests similar effects on oxidative stress biomarkers in *M. galloprovincialis* (**Fig. 4a**). In contrast, separation of the violet cluster (**Fig. 4b**) indicates a different effect of the star-shaped particles on oxidative stress in *M. galloprovincialis.*

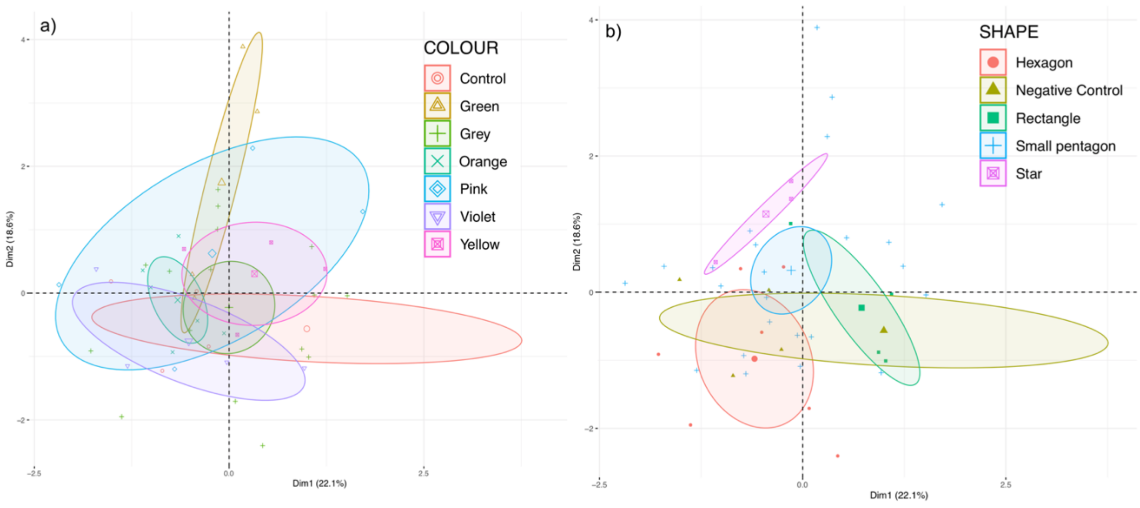


Figure 3 *Principal component analysis of oxidative stress biomarkers: a) clusters based on particle color; b) clusters based on particle shape. Confidence ellipses (95%) plot values for each group.*

Discussion

Glitter is a source of primary MPs and a new type of environmental pollutant (Yurtsever, 2019). With this study we tested the hypothesis that glitter particle type and dose would affect particle recovery and oxidative stress in *M. galloprovincialis*. The confirmatory test showed that more glitter accumulated in the digestive system of *M. galloprovincialis* (less in filtered water) as particle length decreased. This was confirmed by the significant positive correlation between the percentage of recovery of glitter in the filtered water and particle length. Fernàndez and Albentosa (2019) reported that larger MP particles were excreted faster than smaller ones contrary to our preliminary test results: lower recovery rates of larger particles in water on the preliminary test. The discrepancy in study findings stemmed most likely from the difference in particle size. Fernàndez and Albentosa (2019) used larger particles than we did (22 µm vs. 3073.8 µm). The glitter particles we administered, which were larger than the phytoplankton typically ingested by mussels (e.g., Cartier et al., 2004), might have been too large to immediately pass through the bivalve gut so that the lower recovery rates were due to the difficulty of *M. galloprovincialis* to clear the digestive tract of larger particles. It should be noted that mussels actively consume particles much larger than their normal diet (Kinjo et al., 2019).

We observed that exposure to glitter particles (Type 6), for which there was a low recovery rate and a significant change in length at the end of the confirmatory test, caused more oxidative stress than did exposure to the other types. Since glitter can fragment easily, it is more readily ingested by organisms and more likely to accumulate in the digestive tract of filter feeders (Shalom et al.,

2022). This finding draws attention to another problem associated with the production of glitter: the cutting process produces numerous notches that contain tiny particles referred to as waste (Yurtsever, 2019). Since the smaller glitter particles are more harmful to organisms, especially to filter feeders, the waste present in glitter packets could prove even more harmful since it is much smaller than the glitter itself.

Moreover, exposure to stress or environmental pollutants triggers a series of responses: production of reactive oxygen species (ROS) and of antioxidants to prevent oxidative damage (Lam and Gray, 2003; Pastorino et al., 2020). For the present study we used superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), and malondialdehyde (MDA) as biomarkers. Generally, it was found a significant effect of all glitter types on oxidative stress biomarkers. SOD activity provided a biomarker for a rapid oxidative response to glitter particles in both tests, with higher levels observed in the digestive gland of *M. galloprovincialis* exposed to smaller glitter particles.

Superoxide dismutase is the main player in dismutation to obtain hydrogen peroxide (H_2O_2) from superoxide anion (O_2^-). Previous studies on oxidative stress biomarkers in *M. galloprovincialis* exposed to different levels of pollution (Box et al., 2007) showed significantly higher SOD levels in the digestive glands than in the gills, further supporting our findings. In another study conducted under controlled conditions with *M. galloprovincialis* (Cole et al., 2020), SOD activity especially in the digestive glands was significantly increased in the first 24 h after exposure to 500 ng/mL of polystyrene microspheres and polyamide microfibers but was then decreased and similar to the control organisms after 7 days of exposure. Other studies using larger amounts of MPs (100-1000 particles/mL) for 4 days on *Mytilus* spp. found that SOD activity was inversely proportional to particle size (Wang et al., 2020). This observation is shared by our findings that SOD activity was significantly higher after exposure to smaller glitter particles.

Glutathione S-transferase plays a key role in phase II detoxification involved in the conjugation of GSH with phase I enzymes. Generally, GST activity was increased in the gills of mussels exposed to MPs (Capo et al., 2021) and PP leachate (Capolupo et al., 2021a), whereas exposure to polystyrene MPs did not induce changes in GST activity in the gills of *M. galloprovincialis* (Capolupo et al., 2021b) or in the digestive glands of *M. galloprovincialis* exposed to chrysene-sorbed polystyrene MPs (Capolupo et al., 2021c). We observed a significant increase after exposure to glitter Type 6 only in the confirmatory test. The absence of a change in GST is consistent with previous studies on mussels exposed to virgin and pyrene-contaminated MPs for 7 days (Avio et al., 2015) and may be related to a limited ROS-scavenging role of GST, as previously suggested (Capolupo et al., 2021a).

Like GST and SOD, GPx is also involved in the removal of hydrogen peroxide (H_2O_2). Its measurement provides for assessment of environmental pollution (Capo et al., 2021). Glutathione peroxidases are particularly sensitive for detecting the early onset of pro-oxidant stress, even at low levels of environmental pollution (Regoli and Giuliani, 2014). We noted an increase in GPx on both tests. These results are in line with a previous study (Ribeiro et al., 2017) that reported an increase in GPx activity after 3 days of exposure to polystyrene MPs (20 µm; 1000 µg/L; 14 days) in the gills and the digestive glands of the marine clam *Scrobicularia plana*.

Lipid peroxidation is a biomarker of toxicity and is used to assess membrane lipid peroxidation. The increase in MDA observed in both tests suggests that exposure to glitter particles causes cellular lipid peroxidation. Higher levels indicate an excess of ROS and oxidative substances not counterbalanced by antioxidant defense over time, which can lead to severe oxidative damage. Elevated levels were found after exposure to smaller glitter particles, as noted by studies on organisms exposed to MPs under controlled environmental conditions for 24 h (Capo et al., 2021).

Finally, the PCA biplot shows that compared to the other particles the star-shaped ones had a different effect on oxidative stress biomarkers. This shape was related to Type 1 glitter particles, in which the percentage of recovery (97.3%) was higher. This suggests that *M. galloprovincialis* exposed to Type 1 glitter was unable to retain these large star-shaped particles (3073.8 µm) in the digestive gland and that the antioxidant response differed (lower effects) from that after exposure to the other glitter types.

Conclusions

The present study provides preliminary data for what happens when a marine filter-feeding species such as *M. galloprovincialis* is exposed to different glitter particle types and concentrations for 7 days. The findings support our hypothesis that the concentration and the type of glitter particles can affect the percentage of recovery and oxidative stress in *M. galloprovincialis*. There was a significant relationship between particle length and the percentage of particles recovered in water, suggesting that the digestive tract of *M. galloprovincialis* retains smaller particles more. In addition, there was an increase in antioxidant defense response after exposure to smaller particles. Certain types of glitter also crumbled and shortened, resulting in higher levels of oxidative stress biomarkers. On this path, this study provides evidence that glitter particles can evoke negative effects as conventional microplastics in terrestrial (i.e., Baho et al., 2021), freshwater (i.e., Kukkola et al., 2021; Piccardo et al., 2021) and marine (i.e., Gola et al., 2021; Provenza et al., 2022) ecosystems. Studies demonstrating the presence and the amount of glitter in the environment are

still scant to date, so the extent of the problem is not fully known. Investigation of the effects of individual components of glitter on organisms is warranted to quantify the proportion of glitter in MPs pollution. A layer of Mylar^{TM} (a film of polyethylene terephthalate), poly-methyl methacrylate, polyvinyl chloride, or other resin blends, usually covered by a metal layer of aluminum, titanium, iron, or bismuth to give them their typical "sparkly" appearance makes up the composition of glitter, which is more complex than the commonly reported microplastic particles. Thus, further studies are needed to test the toxicity of phthalates and metals (alone or combined) on model aquatic organisms. A future area of focus is also to experiment with different types of glitter and different exposure times in other aquatic organisms.

Supplementary Materials

Table S1 *Preliminary test. Results of two-way ANOVA of organ (digestive gland; gill), treatment (glitter Types 3 and 6) and interaction (organ × treatment) on oxidative stress biomarkers in Mytilus galloprovincialis. Degrees of freedom (dfn = numerator, dfd = denominator) and F statistics (F) are provided. Asterisks (*) denote significant differences. MDA denotes malondialdehyde, SOD superoxide dismutase, GST glutathione S-transferase, GPx glutathione peroxidase.*

Biomarker	F - Organ (dfn, dfd)	Organ (F-value)	F - Treatment (dfn, dfd)	Treatment (F-value)	F- Organ-Treatment interaction (dfn, dfd)	Organ-Treatment interaction (F-value)
SOD	(1;6)	324*	(4;24)	6.95*	(4;24)	7.18*
MDA	(1;6)	14.9*	(4;24)	8.3*	(4;24)	4.9*
GST	(1;6)	3.59	(4;24)	3.06*	(4;24)	0.92
GPx	(1;6)	0.58	(4;24)	10*	(4;24)	0.41

Table S2 *Confirmatory test. Results of two-way ANOVA of organ (digestive gland; gill), treatment (Types 1 to 9) and interaction (organ × treatment) on oxidative stress biomarkers in Mytilus galloprovincialis. Degrees of freedom (dfn = numerator, dfd = denominator) and F statistics (F) are provided. Asterisks (*) denote significant differences. MDA denotes malondialdehyde, SOD superoxide dismutase, GST glutathione S-transferase, GPx glutathione peroxidase.*

Biomarker	F - Organ (dfn, dfd)	Organ (F-value)	F - Treatment (dfn, dfd)	Treatment (F-value)	F- Organ-Treatment interaction (dfn, dfd)	Organ-Treatment interaction (F-value)
SOD	(1;10)	342.1*	(9;55)	4.34*	(9;55)	3.74*
MDA	(1;10)	4.8	(9;55)	5.44*	(9;55)	3.02*
GST	(1;10)	49.52*	(9;55)	3.45*	(9;55)	3.8*
GPx	(1;10)	9.15*	(9;55)	1.51	(9;55)	2.24*

References

Avio, C. G., Gorbi, S., & Regoli, F. (2015). Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. Marine environmental research, 111, 18-26.

Badary, O. A., Abdel-Maksoud, S., Ahmed, W. A., & Owieda, G. H. (2005). Naringenin attenuates cisplatin nephrotoxicity in rats. Life sciences, 76(18), 2125-2135.

Baho, D. L., Bundschuh, M., & Futter, M. N. (2021). Microplastics in terrestrial ecosystems: Moving beyond the state of the art to minimize the risk of ecological surprise. Global Change Biology, 27(17), 3969-3986.

Ballent, A., Corcoran, P. L., Madden, O., Helm, P. A., & Longstaffe, F. J. (2016). Sources and sinks of microplastics in Canadian Lake Ontario nearshore, tributary and beach sediments. Marine pollution bulletin, 110(1), 383-395.

Bhutto, S. U. A., & You, X. (2022). Spatial distribution of microplastics in Chinese freshwater ecosystem and impacts on food webs. Environmental Pollution, 293, 118494.

Blackledge, R. D., & Jones Jr, E. L. (2007). All that glitters is gold!. Forensic analysis on the cutting edge: New methods for trace evidence analysis, 1-32.

Box, A., Sureda, A., Galgani, F., Pons, A., & Deudero, S. (2007). Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 146(4), 531-539.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., & Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environmental science & technology, 42(13), 5026-5031.

Capo, X., Rubio, M., Solomando, A., Alomar, C., Compa, M., Sureda, A., & Deudero, S. (2021). Microplastic intake and enzymatic responses in *Mytilus galloprovincialis* reared at the vicinities of an aquaculture station. Chemosphere, 280, 130575.

Capolupo, M., Gunaalan, K., Booth, A. M., Sørensen, L., Valbonesi, P., & Fabbri, E. (2021). The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean mussel *Mytilus galloprovincialis*. Environmental Pollution, 283, 117081.

Capolupo, M., Rombolà, A. G., Sharmin, S., Valbonesi, P., Fabbri, D., & Fabbri, E. (2021). Assessing the impact of chrysene-sorbed polystyrene microplastics on different life stages of the Mediterranean mussel *Mytilus galloprovincialis*. Applied Sciences, 11(19), 8924.

Capolupo, M., Valbonesi, P., & Fabbri, E. (2021). A comparative assessment of the chronic effects of micro-and nano-plastics on the physiology of the Mediterranean mussel *Mytilus galloprovincialis*. Nanomaterials, 11(3), 649.

Cartier, S., Pellerin, J., Fournier, M., Tamigneaux, E., Girault, L., & Lemaire, N. (2004). Use of an index based on the blue mussel (*Mytilus edulis* and *Mytilus trossulus*) digestive gland weight to assess the nutritional quality of mussel farm sites. Aquaculture, 241(1-4), 633-654.

Cole, M., Liddle, C., Consolandi, G., Drago, C., Hird, C., Lindeque, P. K., & Galloway, T. S. (2020). Microplastics, microfibres and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.). Marine Pollution Bulletin, 160, 111552.

Crossman, J., Hurley, R. R., Futter, M., & Nizzetto, L. (2020). Transfer and transport of microplastics from biosolids to agricultural soils and the wider environment. Science of The Total Environment, 724, 138334.

Dehaut, A., Cassone, A. L., Frère, L., Hermabessiere, L., Himber, C., Rinnert, E., ... & Paul-Pont, I. (2016). Microplastics in seafood: Benchmark protocol for their extraction and characterization. Environmental pollution, 215, 223-233.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Soares, A. M., & Freitas, R. (2017). The impacts of emergent pollutants on *Ruditapes philippinarum*: biochemical responses to carbon nanoparticles exposure. Aquatic toxicology, 187, 38-47.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28(3), 350-356.

ECHA. (2019). Note on substance identification and the potential scope of a restriction on uses of 'microplastics'. Available at: https://echa.europa.eu/ (Accessed June 23, 2022)

Elkhatib, D., 2020. Microplastic in water and wastewater (Doctoral dissertation, University of Rhode Island). Available at: https://digitalcommons.uri.edu/cgi/viewcontent.cgi?article=2194&context=oa_diss (Accessed June 23, 2022)

Enders, K., Käppler, A., Biniasch, O., Feldens, P., Stollberg, N., Lange, X., ... & Labrenz, M. (2019). Tracing microplastics in aquatic environments based on sediment analogies. Scientific Reports, 9(1), 15207.

Fernández, B., & Albentosa, M. (2019). Dynamic of small polyethylene microplastics (≤ 10 μm) in mussel's tissues. Marine pollution bulletin, 146, 493-501.

Gao, R., Yuan, Z., Zhao, Z., & Gao, X. (1998). Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. Bioelectrochemistry and Bioenergetics, 45(1), 41-45.

Gola, D., Tyagi, P. K., Arya, A., Chauhan, N., Agarwal, M., Singh, S. K., & Gola, S. (2021). The impact of microplastics on marine environment: A review. Environmental Nanotechnology, Monitoring & Management, 16, 100552.

Guerranti, C., Martellini, T., Perra, G., Scopetani, C., & Cincinelli, A. (2019). Microplastics in cosmetics: Environmental issues and needs for global bans. Environmental toxicology and pharmacology, 68, 75-79.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of biological Chemistry, 249(22), 7130-7139.

Hartmann, N. B., Huffer, T., Thompson, R. C., Hassellov, M., Verschoor, A., Daugaard, A. E., ... & Wagner, M. (2019). Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris.

Hurley, R., Woodward, J., & Rothwell, J. J. (2018). Microplastic contamination of river beds significantly reduced by catchment-wide flooding. Nature Geoscience, 11(4), 251-257.

Kinjo, A., Mizukawa, K., Takada, H., & Inoue, K. (2019). Size-dependent elimination of ingested microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. Marine Pollution Bulletin, 149, 110512.

Kukkola, A., Krause, S., Lynch, I., Smith, G. H. S., & Nel, H. (2021). Nano and microplastic interactions with freshwater biota–current knowledge, challenges and future solutions. Environment International, 152, 106504.

Lam, P. K., & Gray, J. S. (2003). The use of biomarkers in environmental monitoring programmes. Marine Pollution Bulletin, 46(2), 182-186.

Lehel, J., & Murphy, S. (2021). Microplastics in the food chain: Food safety and environmental aspects. Reviews of Environmental Contamination and Toxicology Volume 259, 1-49.

Li, C., Busquets, R., & Campos, L. C. (2020). Assessment of microplastics in freshwater systems: A review. Science of the Total Environment, 707, 135578.

Li, J., Lusher, A. L., Rotchell, J. M., Deudero, S., Turra, A., Bråte, I. L. N., ... & Shi, H. (2019). Using mussel as a global bioindicator of coastal microplastic pollution. Environmental pollution, 244, 522-533.

Locher, K., Borghardt, J. M., Wachtel, H., Schaefer, K. J., & Wagner, K. G. (2018). Mechanistic study on hydrodynamics in the mini-scale biphasic dissolution model and its influence on in vitro dissolution and partitioning. European Journal of Pharmaceutical Sciences, 124, 328-338.

Lusher, A. (2015). Microplastics in the marine environment: distribution, interactions and effects. Marine anthropogenic litter, 245-307.

Pastorino, P., Elia, A. C., Caldaroni, B., Menconi, V., Abete, M. C., Brizio, P., ... & Prearo, M. (2020). Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps). Science of the Total Environment, 715, 136946.

Pauna, V. H., Buonocore, E., Renzi, M., Russo, G. F., & Franzese, P. P. (2019). The issue of microplastics in marine ecosystems: A bibliometric network analysis. Marine Pollution Bulletin, 149, 110612.

Piccardo, M., Bertoli, M., Pastorino, P., Barceló, D., Provenza, F., Lesa, D., ... & Renzi, M. (2021). Lethal and sublethal responses of *Hydropsyche pellucidula* (Insecta, Trichoptera) to commercial polypropylene microplastics after different preconditioning treatments. Toxics, 9(10), 256.

Prokić, M. D., Gavrilović, B. R., Radovanović, T. B., Gavrić, J. P., Petrović, T. G., Despotović, S. G., & Faggio, C. (2021). Studying microplastics: Lessons from evaluated literature on animal model organisms and experimental approaches. Journal of Hazardous Materials, 414, 125476.

Prokić, M. D., Radovanović, T. B., Gavrić, J. P., & Faggio, C. (2019). Ecotoxicological effects of microplastics: Examination of biomarkers, current state and future perspectives. TrAC Trends in analytical chemistry, 111, 37-46.

Provenza, F., Rampih, D., Pignattelli, S., Pastorino, P., Barceló, D., Prearo, M., ... & Renzi, M. (2022). Mussel watch program for microplastics in the Mediterranean sea: Identification of biomarkers of exposure using *Mytilus galloprovincialis*. Ecological Indicators, 142, 109212.

Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine environmental research, 93, 106-117.

Ribeiro, F., Garcia, A. R., Pereira, B. P., Fonseca, M., Mestre, N. C., Fonseca, T. G., ... & Bebianno, M. J. (2017). Microplastics effects in *Scrobicularia plana*. Marine pollution bulletin, 122(1-2), 379-391.

Rocha-Santos, T., & Duarte, A. C. (2015). A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. TrAC Trends in analytical chemistry, 65, 47-53.

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environmental Science and Pollution Research, 24, 21530-21547.

Shalom, J., Mazlan, N., Jannah, M., & Najah, S. (2022). Microplastics in marine benthic filter feeder: a review on the occurrence, routes of ingestion, method of extraction and effects to the ecosystem. Journal of Sustainability Science and Management, 17(5), 179-199.

Sun, J., Dai, X., Wang, Q., Van Loosdrecht, M. C., & Ni, B. J. (2019). Microplastics in wastewater treatment plants: Detection, occurrence and removal. Water research, 152, 21-37.

Tagg, A. S., & do Sul, J. A. I. (2019). Is this your glitter? An overlooked but potentially environmentally-valuable microplastic. Marine pollution bulletin, 146, 50-53.

Tan, Y., Dai, J., Wu, X., Wu, S., & Zhang, J. (2022). Characteristics, occurrence and fate of non-point source microplastic pollution in aquatic environments. Journal of Cleaner Production, 341, 130766.

Uchiyama, M., & Mihara, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry, 86(1), 271-278.

Uzun, P., Farazande, S., & Guven, B. (2022). Mathematical modeling of microplastic abundance, distribution, and transport in water environments: a review. Chemosphere, 288, 132517.

Wakkaf, T., El Zrelli, R., Kedzierski, M., Balti, R., Shaiek, M., Mansour, L., ... & Rabaoui, L. (2020). Characterization of microplastics in the surface waters of an urban lagoon (Bizerte lagoon, Southern Mediterranean Sea): Composition, density, distribution, and influence of environmental factors. Marine Pollution Bulletin, 160, 111625.

Wang, X., Huang, W., Wei, S., Shang, Y., Gu, H., Wu, F., ... & Wang, Y. (2020). Microplastics impair digestive performance but show little effects on antioxidant activity in mussels under low pH conditions. Environmental Pollution, 258, 113691.

Waring, R. H., Harris, R. M., & Mitchell, S. C. (2018). Plastic contamination of the food chain: A threat to human health?. Maturitas, 115, 64-68.

Yurtsever, M. (2019). Glitters as a source of primary microplastics: an approach to environmental responsibility and ethics. Journal of Agricultural and Environmental Ethics, 32(3), 459-478.

Chapter 4

Use of the Sediqualsoft® for determining the toxicity of cigarette butts for marine species: a weather simulation test

Manuela Piccardo[1], Francesca Provenza[1,3], Serena Anselmi[3], Andrea Broccoli[3], Antonio Terlizzi[1,2,3], Monia Renzi[1,2]

[1]Dipartimento di Scienze della Vita (DSV) Università degli Studi di Trieste, Via Giorgeri,10; 34127, Trieste, Italia

[2] Consorzio Nazionale Interuniversitario per le Scienze del Mare (CoNISMa), Piazzale Flaminio, 9; 00196, Roma, Italia

[3] Bioscience Research Center, Via Aurelia Vecchia, 32; 58015, Orbetello, Italia

[4] Stazione Zoologica A. Dohrn, Villa Comunale; 80121, Napoli, Italia

Journal of Marine Science and Engineering

J. Mar. Sci. Eng. 2021, 9(7), 734

https://doi.org/10.3390/jmse9070734

Abstract

Cigarette butts (CB) are among the dominant components of marine and beach litter. Few studies are actually developed, and environmental effects of CB on marine species are still poorly known. This study aims to evaluate the ecotoxicological impact for marine organisms to both classic and electronic CB. Three representative species of different trophic levels in marine ecosystems (*Aliivibrio fischeri*, bacteria; *Phaeodactylum tricornutum*, algae, primary producers; *Paracentrotus lividus*, echinoderm, consumers) were tested. Effects were evaluated from natural aging of CBs due to exposure to atmospheric conditions (natural sunlight *versus* simulated rain), and different times (1 *vs* 2 weeks). Results were weighted together obtaining a synthetic risk for the environment (Class of Hazard) by Sediqualsoft®. Classic CB resulted the worst representing a slight-moderate risk compared to electronic CB (absent Class of Hazard). Smoked classic CB constituted a higher environmental risk than unsmoked ones. The highest risk was generated by classic CB after one week exposure under dry weather. We suggest the use of the Sediqualsoft® software for risk assessment studies of sediments polluted with contaminants of various kinds, especially in association with a weight of Evidence (WOE) approach.

Keywords

Cigarette butt-derived toxicity; Emerging pollutants; *Aliivibrio fischeri*; *Phaeodactylum tricornutum*; *Paracentrotus lividus*; Class of Hazard; Electronic-cigarette; Beach litter; Environmental risk assessment; Bioassay

Introduction

Marine litter is defined as "any persistent, manufactured or processed material discarded, disposed of or abandoned in the marine and coastal environment" (UNEP, 2009). Since the first record of entanglement and ingestion of plastic items in the 1960s (Kenyon and Kridler, 1969), marine litter went from being treated as a curiosity, to posing a risk to marine ecosystems (Marine Anthropogenic Litter, 2015) because of its ubiquity, persistence and ability to interact with biota. The monitoring campaigns of litter accumulated along the coasts (also known as beach litter) were originally designed to heighten public awareness. Over a thirty-year period, they have developed into a monitoring tool to make an assessment of the magnitude of the problem (Nelms et al., 2017). To date, several studies have analysed the problem from the point of view of its composition, density and possible sources.

Regardless of which category of litter fall (cigarette butts, CBs, are classified as “plastic”, “isolated category” or even how “paper/cardboard”, as in the case of the Italian protocol; Fortibuoni et al., 2021), CBs represent a consistent component of marine litter. In the 2019, the International Coastal Cleanup (ICC) world campaign collected a total of 32,485,488 litter items, of which 4,211,962 were cigarette butts (CBs) so representing the 2nd most abundant litter item after the category “food wrappers” (Ocean Conservancy, 2020). Cigarette butts earn the 1st place in the top 10 list of most frequently items collected during beach clean-ups realized by volunteers (Ocean Conservancy, 2019). Its relative abundance within the marine litter in continental coasts is extremely variable and capable of reaching values > 40 % (Beccherucci et al., 2014; Blickley et al., 2016; Hidalgo-Ruz et al., 2018; Oigman-Pszcol et al., 2007; Santos et al., 2005). Many factors contribute to the transport and presence of CB on beaches, including natural (prevalent wind, currents, rivers) and human aspects such as the behaviour of smokers in public places near and on the beach itself, the density and proximity to high population urban areas, in addition to the frequency and efficacy of public cleaning services (Ariza et al., 2012; Araùjo et al., 2019). In addition to this, local authority clean-up efforts are quite successful at collecting larger pieces of beach litter (as reported in the study performed on Cyprus island; Loizidou et al., 2018). On the contrary, smaller pieces, such as cigarette butts and other plastic items related to recreational activities, may remain on the beaches or become a potential source of marine litter. In the first case, they accumulate and become an integral part of the beach system: they can be buried, remain exposed to solar radiation, or come into contact with the seawater and rain, thus releasing the large number of chemical substances known to be present in cigarettes.

This study focused the attention on CB toxicity simulating the contamination of two different environmental matrices: the marine water and the beach sediment. In the first scenario the toxicity of cigarette butts’ leachates derived by the traditional (CBs) and electronic cigarette (ECs) was explored and compared. Experiment was conducted using both smoked (S-) and unsmoked (U-) cigarettes to investigate the role of combustion in determining the toxicity. From the point of view of traditional cigarettes, is known that a large number of chemicals (including fungicides, herbicides, insecticides, and pesticides) are used in the growing and processing of tobacco, and the manufacture of cigarettes (Glantz et al., 1998). As a consequence, there are over 5000 compounds present in cigarettes. Among these, at least 150 (of which 44 are found in large amounts) are highly toxic, mainly because of their carcinogenic and mutagenic potential (Slaughter et al., 2011). When burned, many of the chemicals present in cigarettes produce new compounds (Moriwaki et al., 2009): smoked cigarette butts contain nicotine, pinane, phenanthrene (Savino et al., 1989) and other chemicals such as polycyclic aromatic hydrocarbons. Regarding electronic cigarettes, many research gaps still exist (Spahn et al., 2021). Since the first prototype built by Hon Lik in 2003

(Dutra et al., 2016), electronic cigarettes, have surged in both popularity and device complexity. Electronic cigarettes operate using the evaporation–condensation principle of aerosolization to produce an inhaled vapour containing a combination of nicotine, excipients (essentially propylene glycol and glycerol), and flavouring agents (Etter et al., 2011). Very recently, among the various e-cig configurations that over the years have been designed, there are those that have a drip tip similar to classic cigarettes. That is, they have a real filter filmed on the outside in order to effectively emulate the filter of the classic cigarettes. The idea of the manufacturer, in fact, is precisely to give the consumer the feeling of having a traditional cigarette between his lips. The filters of e-cigs vary in composition: from 100 % cotton to poly(lactic) acid (PLA) the most produced biodegradable plastic obtained through the fermentation of sugar extracted from corn, cane molasses, potatoes, sugar beets, etc (Abraham et al., 2021). In 2020, about 26.6 % of the smoking population preferred e-cigs as tobacco product, in Italy (Kunst, 2020). The relative consumption of these 2nd-generation e-cigs (filter-equipped) is currently unknown; however, it is likely to be thought that electronic cigarette butts can enter the composition of the marine and beach litter alongside traditional cigarette butts.

The second experiment was intended to recreate a much more complex and, in our opinion, not yet explored interaction dynamic between butts, sandy sediments and seawater. Specifically, the attention was put on the typology of CB resulted to be more toxic by the previous step (i.e., CCB), exposing the different species not to leachates but to elutriates. In the context of this study, natural sandy sediments were manually, and under laboratory conditions, polluted with CB and then used for the preparation of the elutriates. It is known that cellulose acetate cigarette butts may persist for 18 months or more under normal environmental conditions (Ach, 1993). In this time frame, the exposure of butts to different weathering agents can affect the release dynamics of the contaminants present in them, influencing the final toxicity of elutriates. In order to explore this mechanism, the sediments previously polluted were subjected to different simulated atmospheric conditions (simulated rain, and natural sunlight) and time (1 and 2 weeks).

Although different studies have already proved the harmful to aquatic species of compounds associated to CCBs (Slaughter et al., 2011; Booth et al., 2015; Micevska et al., 2006; Savino et al., 1989; Wright et al., 2015), the ecological risk of CB-elutriates for coastal and marine environment remain scarce (Lee et al., 2015). Furthermore, to our knowledge, a comprehensive study evaluating their integrated effect on marine ecosystems has not yet been performed. In this study, results were collected using a battery of ecotoxicological bioassay (*Aliivibrio fischeri*, bacteria; *Phaeodactylum tricornutum*, algae, primary producers; *Paracentrotus lividus*, echinoderm, primary consumers) and integrated to estimate a synthetic ecotoxicological risk level by mean of the specific software

Sediqualsoft®. Sediqualsoft® is a computer tool developed by ISPRA (the Italian Higher Institute for Environmental Protection and Research) with the collaboration of the Università Politecnica delle Marche. Conceived in 2011 by the group of Piva and collaborators (Piva et al., 2011), over the years it has been validated and finally inserted as a useful tool for the implementation of the Ministerial Decree 173/2016, the Italian Regulation laying down detailed rules and technical criteria for the authorization of seabed waste materials. The ecotoxicological classification of each sediment sample is based on the use of weighted integration criteria and produces a 5-level ecotoxicological risk scale: absent, slight, moderate, major, severe.

In conclusion, the specific aims of the present study were the following: (i) to compare the toxicity of of leachates, derived from classic- and electronic-cigarette butt; (ii) to explore the role of combustion in determining the toxicity of leachates; (iii) to study the influence of different atmospheric conditions (rain *vs* dry) and time of weathering (1 *vs* 2 weeks) in determining the toxicity of elutriates derived from sediments previously polluted with cigarette butts; (iv) to assess the adequacy and utility of the Sediqualsoft® software in the development of the ecotoxicological class of hazard for sediments polluted under laboratory conditions.

Materials and methods

Experimental design

This study consisted in two separated multifactorial experiments (**Figure 1)**. The first one wanted to evaluate which type of butt, between the classics (CCB) and the electronic ones (ECB), was more toxic. To establish this, an experiment in the traditional and most immediate form, that of the preparation of leachates, was set up in artificial sea water (ASW). Toxicity was evaluated using both smoked (S-) and unsmoked (U-) cigarettes to investigate the role of combustion in determining the toxicity. A three factors nested experimental design was applied: "cigarette type" (ECB *versus* CCB, two levels fixed); "combustion" (Smoked *versus* unsmoked, two levels fixed) and "beaker" (i.e., replicates, three levels, random).

Classic cigarette butts were used for contaminating natural sediments during the second experiment. After the artificial contamination, sandy samples underwent a weather simulation test. The weather simulation test was conducted placing the contaminated sediments in a selected and protected area outside the lab in January 2020. The lab is located in Tuscany (Italy), 1.5 km from the sea. "Dry" condition was simulated simply exposing the samples outdoor to natural air and light, protected by natural rain. To reproduce the "rain", samples were wet with standard fresh water (SFW) by a drip sprayer every 2 days with a sand/water ratio of 1:1 (v/v). The trays in which the sediments were placed, were without holes on the bottom. The different species were so

exposed not to leachates but to elutriates. A four factors nested experimental design was so applied: "combustion" (Smoked *versus* unsmoked, two levels fixed); "atmospheric condition" (rain *versus* dry, two levels fixed); "time" (1 week *versus* 2 weeks, two levels fixed) and "tray" (i.e., replicates, two levels, random).

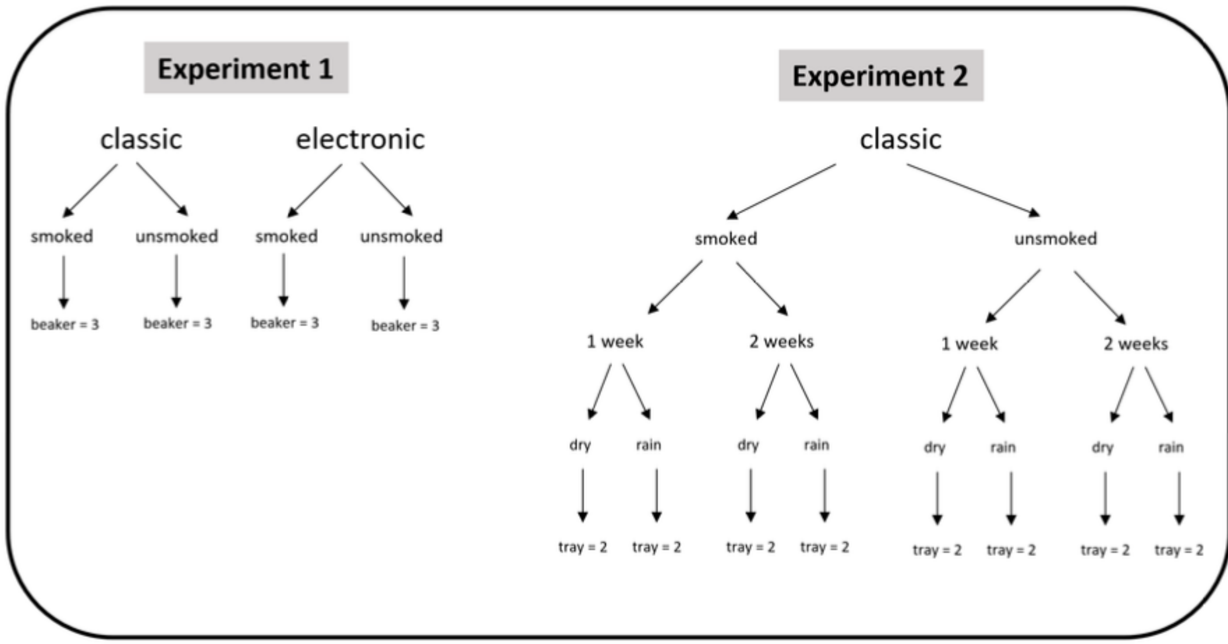


Figure 1 *Multifactorial experimental plans related to the experiment 1 and 2.*

Production of the cigarette butts

Classic cigarettes were artificially smoked by placing a cigarette against a vacuum, which was turned on and off to mimic a smoker's action. The cigarettes were smoked down to approximately 1 cm above the filter. Butts consisted of the filter plus the residual tobacco, paper and ashes.

Electronic cigarette used in the context of this experiment vaporizes a nicotine-rich liquid (0.5 mg of nicotine for ECB) and present the flavouring of citrusy. The production of electronic cigarette butts followed the same procedure adopted for the traditional cigarettes: a vacuum worked till complete combustion of the cigarette. According to the producer, filters are made of poly(lactic) acid.

Preparation of the leachates and elutriates

Experiment 1. Ten CBs of each cigarette type (classic or electronic) were placed in separate glass bottles each containing 1 L of ASW, agitated for 1 h at 100 rpm and then filtered using nitrocellulose membrane (with pore size of 0.45 μm) to remove particulate matter. Control samples consisted in ASW only. In the absence of scientific data relating to the toxicity of ECB, a rather high concentration, not environmentally relevant (10 CB/L), was selected. In fact, several studies demonstrated that a concentration of 10 classic CB/L is capable of clearly inducing toxicity in

marine organisms such as benthic foraminifera (Caridi et al., 2020), marine polychaetae (Wright et al., 2015) and brackish water fish (Lee et al., 2015).

Experiment 2. Natural sandy sediments (NSS) were collected from an unpolluted beach located in Tuscany in March 2020, by means of metal spoon, transported and stored at 5±1°C (in darkness) until the moment of the analysis. NSS were tested before starting experiments and resulted to have an "Absent" eco-toxicological risk according to the same battery of species and approach used in this study. The day of the experiment, NSS were first homogenized by hand and then distributed in trays of 25x25 cm of size creating a layer of 5 cm (with a total solid volume of 3125 cm^3 per tray). Each tray was first artificially polluted by the addition, on the surface or by burying, of classic CBs (0.11 CCB/L of natural sediments) and then exposed to the different atmospheric conditions according to the experimental plan previously described. The selection of the concentration to test was hard because of the insufficient data available in literature. On the base of this, two order of magnitude lower than the experiment 1 was selected, but still remain difficult to assess if it can be considered as environmentally relevant. Spent the time of weathering, CCBs were removed from the sediment and elutriates prepared following the protocol suggested by the Italian Minister Decree n. 173/2016 for marine sediments (D.M. 173/2016): ASW (salinity of 35 ± 1 PSU) in 1:4 ratio of sediment - ASW, weight/volume (1 part of sediment: 3 parts of ASW) was added and immediately agitated for 1 hour at 100 rpm. After that, elutriates were filtered using nitrocellulose membrane (with pore size of 0.45 µm) and checked for chemical-physical parameters (dissolved oxygen, salinity and pH, **Tab. 1**). Before exposure to target species, in some case pH were corrected to comply with the standardized requirements of the eco-toxicological tests (refer to supplementary materials, **Table S1**). Consequently, the effects detected in this study are attributable solely to the chemical composition of the solutions and not to possible variations in the chemical-physical parameters of the same due to the presence of CBs. Control samples consisted in elutriates obtained by sediments that never were in contact with CB.

Table 1 *Parameters recorded on leachates (CBs in water) and elutriates (CBs in sediments) before corrections. Corrections were made only if necessary (*), in order to comply with the standard of the protocols (for details, refer to supplementary materials, Table S1). ECB = electronic cigarette butts; CCB = classic cigarette butts.*

Experiment 1

Matrix	Type	Sample	pH (pH Unit)	Salinity (PSU)	Dissolved Oxygen (mg/L)
ASW	ECB	Unsmoked	7.78	35.81	5.20
		Smoked	7.90	35.41	5.20
	CCB	Unsmoked	7.77	35.40	5.50
		Smoked	7.92	36.20	5.30

Experiment 2

Matrix	Time	Atmospheric Condition	Sample	pH (pH Unit)	Salinity (PSU)	Dissolved Oxygen (mg/L)
Beach sediment	1 week	Rain	Smoked	8.01 *	36.45	11.30
			Unsmoked	8.00 *	36.66	12.60
			Negative control	8.02 *	36.86	12.50
				8.00 *	36.93	13.30
		Dry	Smoked	8.00 *	36.16	11.70
			Unsmoked	8.01 *	38.48	10.40
			Negative control	7.96 *	35.71	11.60
				8.03 *	37.70	11.90
	2 weeks	Rain	Smoked	7.98 *	36.58	5.40
			Unsmoked	7.98 *	36.78	5.60
			Negative control	7.97 *	38.50	6.02
				7.96 *	36.56	6.80
		Dry	Smoked	7.98 *	37.35	6.00
			Unsmoked	7.99 *	37.44	5.70
			Negative control	8.02 *	37.75	11.50
				7.97 *	38.04	8.01

Eco-toxicological tests

Leachates and elutriates were tested using a standard battery of eco-toxicological assays on three species considered representative of different trophic levels in marine ecosystems and having different sensitivity toward toxicants: *i)* inhibition of bioluminescence in the marine bacterium *Aliivibrio fischeri* (acute; 15 and 30 minutes); *ii)* algal growth inhibition of the diatom *Phaeodactylum tricornutum* (chronic; 72 hours); *iii)* spermiotoxicity (acute; 20 minutes) in sea urchin *Paracentrotus lividus* and finally, *iv)* embryo-toxicity (chronic; 72 hours) of sea urchin *Paracentrotus lividus*. Bioassays complied with the Italian Law (D. Lgs. 173/2016).

Inhibition of bioluminescence, *Aliivibrio fischeri* (UNI EN ISO 11348-3:2019)

The endpoint selected was represented by the inhibition of bioluminescence emitted by the marine bacteria *A. fischeri* when exposed to sample. The bioluminescence was measured using a luminometer set at 430 nm. The test was conducted in duplicate at 15 ± 1 °C for 15 and 30 minutes. The initial concentration of bacteria was 10^6 cells and the maximum testable concentration of sample corresponded to 90%. During the test, negative control (ASW) and positive control (3.4 mg/L of 3,5-dichlorophenol) were set up in duplicate. The test was considered valid if the inhibition of the positive controls resulted between 20 - 80%.

Algal growth inhibition, *Phaeodactylum tricornutum* (UNI EN ISO 10253:2017)

The endpoint selected was the growth inhibition of the marine diatom *P. tricornutum*. The algae, in exponential growth phase, was exposed to the different experimental conditions and put at continuous light for 72 h to allow a rapid growth. At time intervals of 24 hours, samples were shaken and measured using a spectrophotometer at 670 nm wavelength. Tests were performed in triplicate at 20 ± 2 °C. The initial concentration of tested specimens was 10^4 cells and sample concentration tested was 100%. Specific nutrients (S1 + S2 +S3) were added to each sample, in accordance with ISO (2016), with the exception of the negative controls because the algal culture medium was already nutrient enriched. Positive controls with potassium dichromate (n=3) were set up. The test was considered valid if the algal concentration in the negative controls after 72h, was >16 times than the starting concentration and if the EC_{50} of the positive controls was 20.1 ± 5.3 mg/L.

Fertilization efficiency, *Paracentrotus lividus* (EPA/600/R-95-136/Section 16)

This method evaluates the spermiotoxicity in the sea urchin *P. lividus*. Male and female gametes emission were obtained by intraoral injection of 1 mL of 1 M potassium chloride from adults of homogeneous size. Sperms were before exposed to the leachates/elutriates for 20 minutes, and then put in contact with eggs in order to allow fertilization. The ratio of sperms and eggs, for mL of solution, was 15000:1. After other 20 minutes, 2-3 drops of Lugol fixative were added in every samples to stop the cellular division and allow results lectures by microscopy. 100 eggs per replicate were counted to determine the number of eggs correctly fertilized. The sample concentration tested was 50% according to ISPRA Guideline n. 11 (2017) (ISPRA, 2017) and the test was performed in triplicate. Negative control (ASW) and positive control (copper (II) nitrate) were tested to evaluate the quality of obtained results. The test was considered valid if the negative control reported > 80% of normal fertilized eggs and if EC_{50} of the positive control was included between 21.69 and 68.18 µg/L Cu^{2+}.

Larval development (EPA/600/R-95-136/Section 15) and Larval body-size variations, *Paracentrotus lividus*

The first endpoint of this analysis was the larval development in sea urchin, *P. lividus*, after 72 hours of exposure. Male and female gametes were obtained as reported above. Eggs were fertilized by sperm and after 20 minutes, correctly fertilized eggs were exposed to the testing leachates/elutriates. After 72 hours of development, 2-3 drops of Lugol fixative were put in every samples to stop the cellular division and allow results lectures by microscopy. After 72 hours of normal development, fertilized eggs reach the larval stage of *pluteus*: to determine the % of abnormal larvae, 100 *plutei* were counted per each replicate. Larvae were considered abnormal if

showed developmental arrest, all arms missing or with different length, additional arms cross lateral rods, asymmetrical body width and other anomalies listed by literature (ISPRA, 2017). The sample concentration tested was 50% according to ISPRA Guideline n. 11 (2017) (ISPRA, 2017) and the test was performed in triplicate. Negative control (ASW) and positive control (copper (II) nitrate) were set up. The test was considered valid if the negative control reported > 80 % of normal developed larvae and if EC_{50} of the positive control was included between 22.60 and 68.34 µg/L Cu^{2+}.

Recent research recorded significant differences among body-size (i.e., maximum arm lengths) of normo-formed larvae in population of *P. lividus* exposed to chemicals compared to body-size of natural population at the same developmental age (Messinetti et al., 2018; Piccardo et al., 2020). The second endpoint of this analysis used the same samples previously analysed for the detection of anomalies in embryos development, to evaluate the effects of the leachates/elutriates on larval body size of *P. lividus* by means of stereomicroscopic measurement of their mean arm lengths (Nikon, SMZ-800 N equipped with Nikon's software Nikon ACT-1). Measurements were performed on 10 normal-developed *plutei* per each replicate and expressed as % of body-size reduction compared to controls.

Quality Assurance and Quality Control

Bioscience Research Center is a certified laboratory (ISO 9001:2015) and applies a severe control procedure under guidelines of the UNI EN ISO 17025:2018 to ensure the quality of produced data (ACCREDIA 1715L). QA/QC tests were performed as described by reference methods. Specific variable of interest defined by the applied method were standardized and monitored during tests (**Table S1**). Positive and negative controls were tested during the experiments and results are reported in supplementary materials (**Table S2)**.

Data analyses

Statistics (mean, standard deviation, t-Test Student and F-test), if appropriate, were calculated by Excel on experimental raw data and the applied formulae were those detailed reported in each of the reference method. Results of positive controls performed on opportune dilution of reference substances during tests on *P. tricornutum* and *P. lividus* were utilized to calculate the EC_{50} values and their related confidential limits. This calculation was performed using US EPA Toxicity Relationship Analysis Program (TRAP) version 1.30 and setting Gaussian distribution and logarithmic transformation of exposure variable. The positive control carried out on *A. fischeri* did not need the calculation of EC_{50} value according to the reference method.

Recorded effects were used in Sediqualsoft® software to perform integrated evaluations and weighted risk assessment derived by cigarette butts' leachates/elutriates. Eco-toxicological classification and the related Class of Hazard were elaborated by weigh integration of the results gathered by all components of the biological battery. Weigh integration criterions applied by the software consider important aspect and specific characteristic of each applied biological assay (i.e., the statistical significance of the difference between sample and control, the strictness of the biological effect consider by the assay, the type of exposition acute or chronic, etc.). The limit, that represents the minimum variation biological significant for each experimental condition, is reported in **Table 2** (extracted by the Tab. A1 of the Italian Minister Decree n. 173/2016). Furthermore, each assay was weighted respect to *i)* biological endpoint measured, *ii)* exposition time, and *iii)* matrix (represented in **Table 3** and extracted by the Tab. A2 of the Italian Minister Decree n. 173/2016). The criteria applied from Sediqualsoft® to weight ecotoxicological effects and to perform integrated classifications are detailed in Piva et al., 2011 (Piva et al., 2011).

***Table 2** Limits of biological assays required by the Italian D.M. n. 173/2016 (translation of the Table A1).*

Species	Endpoint (E)	Limit (%)	Exposition (T)	Matrix (M)
Paracentrotus lividus	Fertilization Development	15 15	Acute Chronic	Interstitial water-Leachate Interstitial water-Leachate
Phaeodactylum tricornutum	Algal growth	10	Chronic	Interstitial water-Leachate
Aliivibrio fischeri	Bioluminescence	15 25	Acute	Interstitial water-Leachate Sediment-Moist sediment

***Table 3** Weights assigned according to the relevance of the biological endpoint, the matrix, the time of exposure. Translation of the Table A2, Italian D.M. n. 173/2016. * Variation (%) measured and then corrected using Abbott's correction in relation to negative control.*

Biological Endpoint (En)		Matrix (M)	
Fertilization	1.5	Sediment	1
Development	1.9	Interstitial water	0.8
Algal growth	2.1	Leachate	0.7
Bioluminescence Mortality	2.4 3	Moist sediment	0.6
Exposition (T)		**Algal biostimulation (Ei) ***	
Acute	1	$E \leq 40\%$ $40\% < E \leq 100\%$	0 1.25
Chronic	0.7	$E > 100\%$	1.5

Results

Effect of "combustion" on classic and electronic-CBs toxicity

A synthetic view of results obtained is reported in **Figure 2** in which Smoked classic CB (S-CCB) showed the highest negative score in terms of Battery Bioassay Hazard Quotient (HQ = 1.99) and the corresponding Class of Hazard (i.e., moderate).

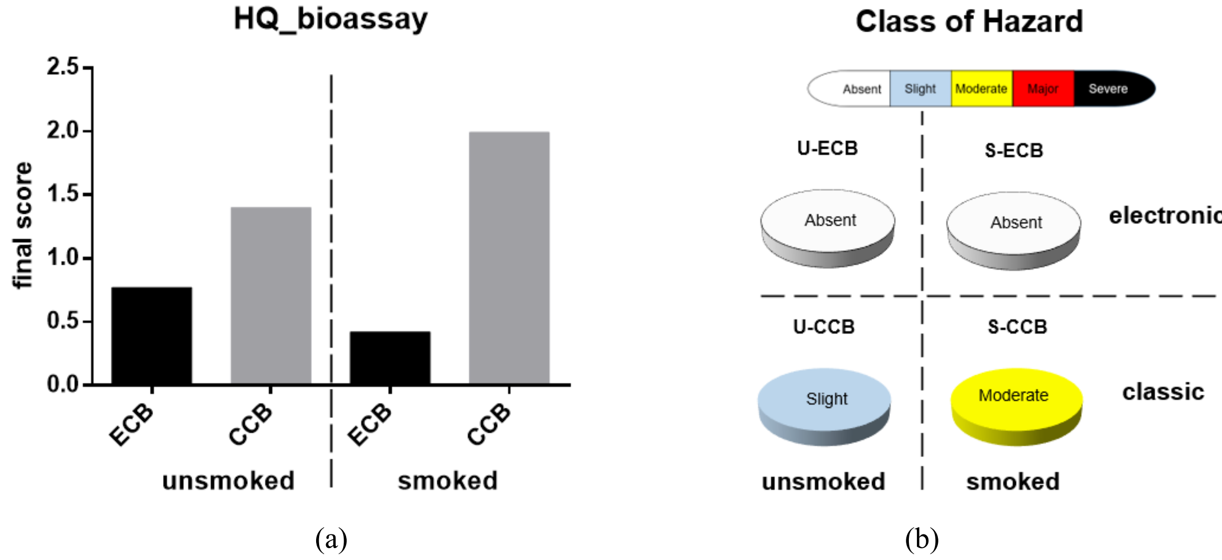


Figure 2 *Evaluation of eco-toxicological bioassay. Marine species were exposed to leachates of smoked (S-) and unsmoked (U-) cigarette butts from classic (CCB) and electronic cigarettes (ECB). a) Hazard Quotient of the battery of bioassays; b) Class of hazard. Calculations and categorization are those reported by Sediqualsoft®.*

A more detailed report of the eco-toxicological responses for each tested species is described in **Table 4.** Exposure to leachates caused inhibition of natural bioluminescence of bacteria (*A. fischeri*, values between 8.46 and 35.03 %,), inhibition of growth in phytoplanktonic primary producers (*P. tricornutum*, between 7.01 and 32.43 %) and reduction in fertilization success of grazers (*P. lividus*, between 16.65 and 52.54 %).

Table 4 *Eco-toxicological results recorded on tested species on the basis of which HQ_battery and Class of Hazard were calculated. "Negative controls" and "samples" are expressed as mean ± standard deviation (SD). S-ECB = smoked electronic cigarette butt; U-ECB= unsmoked electronic cigarette butt; S-CCB = smoked classic cigarette butt; U-CCB = unsmoked classic cigarette butt. Z effect = the effect (biological response) multiplied for the z factor (t-test considering the replicates of the samples and the negative controls). HQ = Hazard Quotient, elaborated by the software, considering the biological endpoint, the tested matrix, the time of exposure, and the significant differences from the negative control (using specific thresholds and the z factor). * = corrected with Abbott's correction: (X-Y)/(100-Y)*100, where X is the effect caused by the sample (%) and Y is the effect caused by the control (%).*

Litter	Species	Endpoint	Negative Control (100%-Effect)	Sample (100%-Effect)	Effect % *	Weighted Effect	z Effect	HQ	Lower HQ	Max HQ
S-ECB	*A. fischeri*	Inhibition of bi-oluminescence	100.00 ± 0.00	91.54 ± 0.82	8.46	0.56	8.46	0.94	1.68	11.20
	P. lividus	Fertilization	100.00 ± 0.00	83.35 ± 15.93	16.65	0.22	3.28	0.23	1.05	7.00
	P. tricornutum	Growth inhibition	100.00 ± 1.61	92.99 ± 0.43	7.01	0.70	7.01	0.72	1.03	10.29
	P. lividus	Larval development	94.33 ± 1.15	90.48 ± 21.29	4.08	0.07	0.99	0.07	0.93	6.21
U-ECB	*P. lividus*	Fertilization	100.00 ± 0.00	49.98 ± 26.01	50.02	0.51	7.60	0.54	1.05	7.00
	A. fischeri	Inhibition of bi-oluminescence	100.00 ± 0.00	74.05 ± 2.59	25.95	1.73	25.95	2.91	1.68	11.20
	P. tricornutum	Growth inhibition	100.00 ± 1.61	106.17 ± 0.23	26.82	0.00	26.82	0.00	1.03	10.29
	P. lividus	Larval development	94.33 ± 1.15	82.52 ± 13.47	12.52	0.17	2.61	0.16	0.93	6.21
S-CCB	*P. tricornutum*	Growth inhibition	100.00 ± 1.61	67.57 ± 3.10	32.43	3.24	32.43	3.33	1.03	10.29
	A. fischeri	Inhibition of bi-oluminescence	100.00 ± 0.00	64.97 ± 0.17	35.03	2.34	35.03	3.93	1.68	11.20
	P. lividus	Fertilization	100.00 ± 0.00	47.46 ± 32.54	52.54	0.53	7.98	0.56	1.05	7.00
	P. lividus	Larval development	94.33 ± 1.15	81.04 ± 10.17	14.09	0.17	2.62	0.16	0.93	6.21
U-CCB	*P. lividus*	Fertilization	100.00 ± 0.00	82.40 ± 15.74	17.60	0.23	3.38	0.24	1.05	7.00
	P. tricornutum	Growth inhibition	100.00 ± 1.61	73.18 ± 3.64	26.82	2.68	26.82	2.76	1.03	10.29
	A. fischeri	Inhibition of bi-oluminescence	100.00 ± 0.00	73.89 ± 3.20	26.11	1.74	26.11	2.92	1.68	11.20
	P. lividus	Larval development	94.33 ± 1.15	85.96 ± 16.83	8.87	0.13	2.02	0.12	0.93	6.21

P. lividus larvae after 72 h from fertilization, also reported abnormal development in up to 14.09 % of cases. The more sensitive organism (namely, echinoderms) also reported a statistically significant reduction (*p-value* < 0.05) in mean arm length of normo-formed *plutei* exposed to classic CCB, with meaningless differences within the factor "combustion" (*p-value <0.01*) (**Figure 3)**. Specifically, larvae exposed to smoked-CCB reported a mean reduction of 21 %, whereas unsmoked-CCB of 18.5 %. No significant differences were induced by electronic cigarettes elutriates.

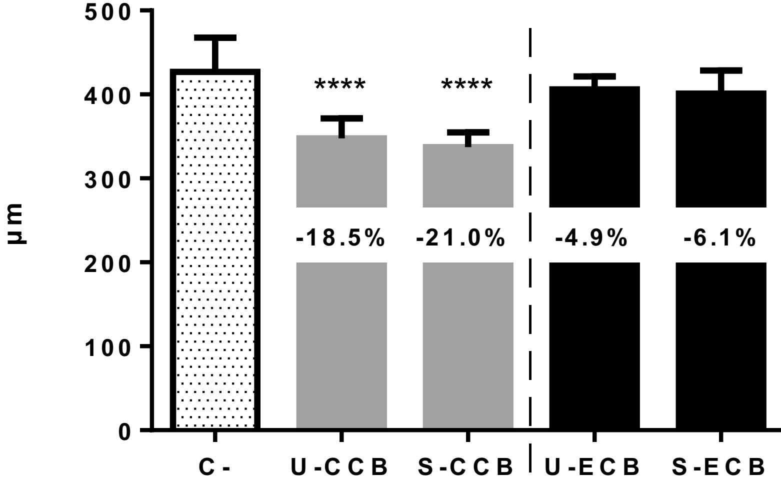


*Figure 3 Mean length (± SD) of arms in normo-formed 72h-old plutei of P. lividus exposed to leachates of smoked (S-) and unsmoked (U-) cigarette butts from classic (CCB) and electronic cigarettes (ECB). C- = negative controls. Statically significant differences, compared to controls, were calculated in specimens exposed to CCBs (**** = p-value < 0.0001), and corresponded to an arm length reduction of up to 21.0 % and meaningless differences within the factor "combustion".*

Effects of "combustion"- "time" and "atmospheric condition" on classic-CBs toxicity

A synthetic view of results obtained after "1 week" of weathering is reported in **Figure 4.** Specifically, smoked classic CB (S-CCB) under "dry" conditions, showed the highest score in terms of Battery Bioassay Hazard Quotient (HQ = 3.86) and Class of Hazard (namely, major). Such elutriates induced inhibition of bioluminescence in bacteria (*A. fischeri*, 68.87 %), inhibition of growth in phytoplanktonic primary producers (*P. tricornutum*, 47.7 %) and reduction in fertilization success of grazers (*P. lividus*, 62.12 %). *P. lividus* larvae after 72 h from fertilization, also reported abnormal development in up to 58.59 % of cases. On the contrary, Unsmoked CCB under "dry" conditions corresponded to an "absent" Class of Hazard. CCBs pre-treated under "rain" conditions produced elutriates characterized by "moderate" (smoked-CCB) and "slight" (unsmoked-CCB) Class of Hazard. A more detailed report of the eco-toxicological responses for each tested species is described in **Table 5.**

Table 5 *Eco-toxicological results recorded on tested species, after 1 week of CCB conditioning under different atmospheric conditions (rain vs dry). Such results were used to calculate the HQ_battery and Class of Hazard. "Negative controls" and "sample" are expressed as mean ± standard deviation (SD). * = corrected with Abbott's correction: (X-Y)/(100-Y)*100, where X is the effect cause by the sample (%) and Y is the effect cause by the control (%).*

Treatment		Species	Endpoint	Negative Control (100%-Effect)	Sample (100%-Effect)	Effect % *	Weighted Effect	z Effect	HQ	Lower HQ	Max HQ
Dry	Smoked	*P. tricornutum*	Growth inhibition	100.00 ± 1.61	52.26 ± 0.48	47.74	4.77	47.74	4.91	1.03	10.29
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	31.13 ± 0.14	68.87	4.59	68.87	7.71	1.68	11.20
		P. lividus	Fertilization	100.00 ± 0.00	37.88 ± 57.74	62.12	0.82	12.26	0.86	1.05	7.00
		P. lividus	Larval development	94.33 ± 1.15	39.06 ± 57.74	58.59	0.79	11.81	0.74	0.93	6.21
	Unsmoked	*P. lividus*	Fertilization	100.00 ± 0.00	79.59 ± 12.38	20.41	0.21	3.10	0.22	1.05	7.00
		P. tricornutum	Growth inhibition	100.00 ± 1.61	72.39 ± 2.80	27.61	2.76	27.61	2.84	1.03	10.29
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	90.3 ± 0.53	9.67	0.64	9.67	1.08	1.68	11.20
		P. lividus	Larval development	94.33 ± 1.15	87.91 ± 18.16	6.80	0.11	1.59	0.10	0.93	6.21

Treatment		Species	Endpoint	Negative Control (100%-Effect)	Sample (100%-Effect)	Effect % *	Weighted Effect	z Effect	HQ	Lower HQ	Max HQ
Rain	Smoked	*A. fischeri*	Inhibition of bioluminescence	100.00 ± 0.00	94.41 ± 0.74	5.59	0.37	5.59	0.62	1.68	11.20
		P. lividus	Fertilization	100.00 ± 0.00	72.92 ± 8.95	27.08	1.81	27.08	1.90	1.05	7.00
		P. tricornutum	Growth inhibition	100.00 ± 1.61	60.96 ± 0.21	39.94	3.90	39.04	4.01	1.03	10.29
		P. lividus	Larval development	94.33 ± 1.15	80.52 ± 10.03	14.64	0.18	2.73	0.17	0.93	6.21
	Unsmoked	*P. lividus*	Fertilization	100.00 ± 0.00	72.38 ± 2.44	27.62	1.84	27.62	1.93	1.05	7.00
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	91.53 ± 1.63	8.47	0.07	1.00	0.12	1.68	11.20
		P. tricornutum	Growth inhibition	100.00 ± 1.61	60.64 ± 6.35	39.36	3.94	39.36	4.05	1.03	10.29
		P. lividus	Larval development	94.33 ± 1.15	80.39 ± 18.06	14.78	0.21	3.17	0.20	0.93	6.21

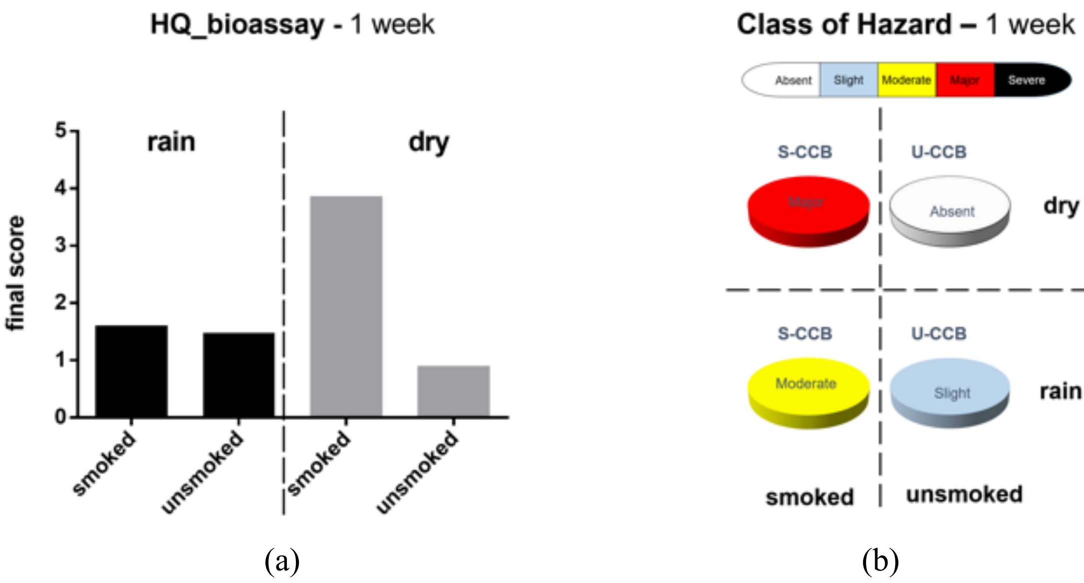


Figure 4 *Evaluation of eco-toxicological bioassay. Marine species were exposed to elutriates prepared from both smoked and unsmoked CCBs subjected to different atmospheric conditions (rain vs dry) for 1 week. a) Hazard Quotient of the battery of bioassays; b) Cl ass of hazard. Calculations and categorization are those reported by Sediqualsoft®.*

After 2 weeks, the HQ and ecotoxicological risk (Class of Hazard) changed for all treatments: under "dry" condition, S-CCB passed from "major" to "slight"; and U-CCB from "absent" to "slight". Under "rain" condition, S-CCB passed from "moderate" to "slight" and U-CCB from "slight" to "absent". Results are graphically reported in **Figure 5** and detailed in **Table 6.**

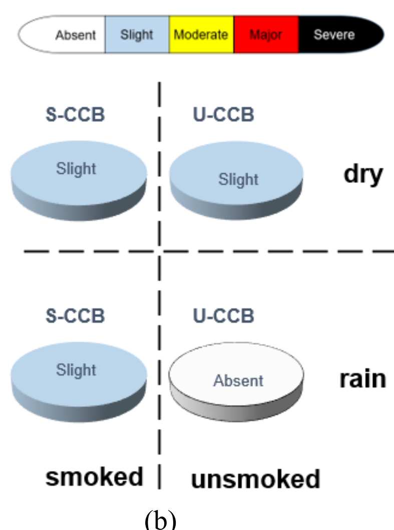
HQ_bioassay - 2 weeks

final score

rain | dry

smoked | unsmoked | smoked | unsmoked

(a)



(b)

Figure 5 *Evaluation of eco-toxicological bioassay. Marine species were exposed to elutriates prepared from both smoked and unsmoked CCBs subjected to different atmospheric conditions (rain vs dry) for 2 weeks. a) Hazard Quotient of the battery of bioassays; b) Class of hazard. Calculations and categorization are those reported by Sediqualsoft®.*

Table 6 *Eco-toxicological results recorded on tested species, after 2 weeks of CCB conditioning under different atmospheric conditions (rain vs dry). Such results were used to calculate the HQ_battery and Class of Hazard. "Negative controls" and "sample" are expressed as mean ± standard deviation (SD). * = corrected with Abbott's correction: (X-Y)/(100-Y)*100, where X is the effect cause by the sample (%) and Y is the effect cause by the control (%).*

Treatment		Species	Endpoint	Negative Control (100%-Effect)	Sample (100%-Effect)	Effect % *	Weighted Effect	z Effect	HQ	Lower HQ	Max HQ
Rain	Smoked	*A. fischeri*	Inhibition of bioluminescence	100.00 ± 0.00	102.04 ± 0.49	2.07	0.00	0.43	0.00	1.68	11.20
		P. lividus	Fertilization	100.00 ± 0.00	37.88 ± 57.74	62.12	0.82	12.26	0.86	1.05	7.00
		P. tricornutum	Growth inhibition	100.00 ± 1.61	60.25 ± 5.15	39.75	3.98	39.75	4.10	1.03	10.29
		P. lividus	Larval development	94.33 ± 1.15	90.48 ± 21.29	4.08	0.07	0.99	0.07	0.93	6.21
	Unsmoked	*P. lividus*	Fertilization	100.00 ± 0.00	79.51 ± 12.35	20.49	0.21	3.11	0.22	1.05	7.00
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	97.52 ± 0.69	2.48	0.02	0.29	0.03	1.68	11.20
		P. tricornutum	Growth inhibition	100.00 ± 1.61	74.73 ± 8.51	25.27	2.53	25.27	2.60	1.03	10.29
		P. lividus	Larval development	94.33 ± 1.15	90.48 ± 21.29	4.08	0.07	0.99	0.07	0.93	6.21
Dry	Smoked	*P. tricornutum*	Growth inhibition	100.00 ± 1.61	65.54 ± 6.26	34.46	3.45	34.46	3.55	1.03	10.29
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	100.60 ± 1.15	2.07	0.00	0.43	0.00	1.68	11.20
		P. lividus	Fertilization	100.00 ± 0.00	74.51 ± 6.34	25.49	1.70	25.49	1.78	1.05	7.00
		P. lividus	Larval development	94.33 ± 1.15	81.04 ± 10.17	14.09	0.17	2.62	0.16	0.93	6.21
	Unsmoked	*P. lividus*	Fertilization	100.00 ± 0.00	69.52 ± 5.36	30.48	2.03	30.48	2.13	1.05	7.00
		P. tricornutum	Growth inhibition	100.00 ± 1.61	61.20 ± 3.10	38.80	3.88	38.80	3.99	1.03	10.29
		A. fischeri	Inhibition of bioluminescence	100.00 ± 0.00	105.60 ± 5.03	2.07	0.00	0.43	0.00	1.68	11.20
		P. lividus	Larval development	94.33 ± 1.15	85.96 ± 16.83	8.87	0.13	2.02	0.12	0.93	6.21

Discussion and Conclusions

Cigarette butts' leachates, prepared in ASW at high concentration (10 CB/L of ASW), have been aimed at identifying evident ecotoxicological responses in tested species and therefore facilitate a comparison between the toxicity of the traditional and electronic cigarettes. Our results highlighted that classic cigarette butts, particularly the smoked ones, produced higher hazard and higher ecotoxicological risks compared to electronic cigarette butts. Electronic cigarettes were assigned an "absent" Class of Hazard, on the contrary, classic CBs represented a "slight" and "moderate" ecotoxicological risk, for unsmoked and smoked CB respectively. In particular, smoked CCB leachates induced inhibition of bioluminescence in *A. fischeri*, inhibition of algal growth and reduction in fertilization success of *P. lividus*. Smoked CCB affected the development of *plutei* increasing the percentage of abnormal larvae respect to controls, but also induced impairment in biometrics of normal-formed larvae. The body size reduction in normo-formed (72h) *plutei*, showed ecotoxicological efficacy for both smoked and unsmoked cigarette butts, thus confirming as sensitive endpoint, as reported in other studies (Piccardo et al., 2020). These results are consistent with the literature showing that classic smoked cigarettes are worse than the unsmoked ones. As example, Slaughter et al. (2011) recorded in marine fish LC_{50} values of 1.8 CB/L of water and 5.1 CB/L of water respectively in smoked and unsmoked cigarette butts. Caridi and collegues (2020), showed that 4.0 CB/L of water are sufficient to determine shall decalcification and death in benthic foraminifera (Protista). The low ecotoxicological risk for electronic cigarettes is in accordance with results collected by Parker and Rayburn (2017) who tested the potential developmental toxicities of three different cigarette butt leachates (regular cigarette butt, menthol and electronic) in the frog embryo teratogenesis assay–*Xenopus* (FETAX). *Xenopus laevis* embryos were exposed to concentrations ranging from 0 to10 ECB/L and the ECB leachate resulted to be much less toxic than all the other treatments, with an overall 96-h LC_{50} of 14.6 CBs/L. ECB leachate was at least 10-fold less toxic than the regular cigarette butts. If on one hand, EC do not release second-hand smoke, and thus they are thought as being a safe alternative to traditional cigarette (Parker and Rayburn, 2017), on the other side, researchers found impurities in both e-liquid and composition of the emitted vapour (e.g., lead, nickel, silver, silicate beads, and nanoparticles) [39] potentially toxic for aquatic organisms. Considered the paucity of data at disposal and the great variety in EC configurations (e.g., e-liquid composition in terms of % of nicotine, excipient and flavouring agents), further studies are needed to better elucidate the toxicity of this new form of personal litter. Undoubtedly, butts derived from electronic cigarettes cannot be considered a prominent hazard today. However, future efforts should be put starting from the monitoring of their relative abundance in the environment. For example, a specific category of litter may be created in beach litter protocol in order to facilitate their analysis and trend tracking.

Considering the entirety of the study, the major environmental concern was associated to elutriates derived from low level of pollution (0.11 CB/L of sediment), in presence of combusted cigarettes exposed, for a relatively short frame of time (1 week), to dry conditions. Increased toxicity of CB subjected to combustion can be explained by the production of new compounds such as polycyclic aromatic hydrocarbons (Savino et al., 1989) already proven, in other studies, to be toxic for aquatic organisms (Honda and Suzuki, 2020; Wang et al., 2021). The highest score in terms of Battery Bioassay Hazard Quotient (HQ = 3.86) and Class of Hazard (namely, major) for the treatment "1 week" + "dry" conditions, showed that the mere exposure of butts to natural air and solar radiation, is sufficient to cause the desorbing and leaching of chemical compounds able to affect the ecotoxicological responses of marine species. On the contrary, CCBs pre-treated under "rain" conditions, produced elutriates characterized by "moderate" (smoked-CCB) and "slight" (unsmoked-CCB) Class of Hazard representing an alternative source of variability in elutriates toxicity. Finally, the results of this study show that longer exposure times (2 weeks) reduced the ecotoxicological effectiveness of Classic CB, probably due to a relevant dilution and/or inhibition of the toxicant substances by the rainwater.

The use of a battery of bioassays, involving species belonging to different trophic levels, makes our results of particular interest from an ecological point of view. Recorded impacts on tested species owning to bacteria, phytoplanktonic communities and ecological groups of grazers could produce significant effects on their relative trophic webs. The decrease of Bacteroidates and Cyanobacteria Phyla in marine ecosystems, to the advantage of other bacterial groups, such as the Gammaproteobacteria, the Firmicutes and the Thermotogae, has been already reported as a consequence of the exposure to smoked and unsmoked Classic CB (Quéméneur et al., 2020). A research performed on *Aliivibrio fischeri* showed that about 0.03 CB/L are able to induce chronic toxicity causing, also, the inhibition of the population growth (Warne et al., 2002). Impacts on grazers, in marine ecosystems, are reported by the literature to affect algal communities, reduce grazer effects, and significantly impair ecosystem dynamics and species associations (Bulleri et al., 1999). Based on the results recorded in the study, it emerges that classic cigarette butts can also represent indirect and long-term impacts for marine species: reducing reproductive success and body-size of the larval stages of grazers, reducing population growth of primary producers (algae), and unbalancing the transfer of energy into the marine trophic web.

This study wanted to test the integration and synthesis capacity of the Sediqualsoft®, and its underlying theoretical approach, in an experimental context unusual compared to the traditional use of the computer tool. This context was represented by the artificially pollution, under laboratory conditions, of environmental matrices. Traditionally, the software is suggested for the

implementation of the Italian Minister Decree n. 173/2016 (the Regulation laying down detailed rules and technical criteria for the re-use of marine, brackish and coastal waste sediments). According to the law, before their re-use, sediments must be subject to a physical, chemical and ecotoxicological characterization process; different destinations of the sediments, according to their quality, are then provided. Ecotoxicological analyses are evaluated as a whole at the level of "battery" (not of individual test), weighing the biological relevance of the measured effects, the statistical significance of the result, the ecological relevance of the tested matrix, as well as the type of exposure (Piva et al., 2011). The final output of the software is a classification of sediment samples in a 5-level ecotoxicological risk scale: absent, slight, moderate, major and severe. From our point of view, the advantages of using the Sediqualsoft® and its underlying theoretical approach are: i) it is a free software, obtainable on request; ii) it allows to produce integrated, synthetic and "in accordance with the law" data; iii) thanks to the extended version of the software, it is possible to integrate data collected from the Line of Evidence (LOE) of Bioassay with the other LOEs, following a weight of evidence approach (WOE, Piva et al., 2011). Other LOEs may derived from future studies on chemical composition of the elutriates, assessment of bioavailability, and sub-lethal effects on battery of biomarkers. In this regard, the use of a weight of evidence approach (WOE, Piva et al., 2011) appears to be a powerful tool to support more complex processes of environmental risk assessment and furnish a comprehensive assessment of hazard associated to sediments polluted with contaminants of various kinds. A wide literature is available on this aspect (ISPRA, 2019; Regoli et al., 2014; Maradonna et al., 2020; Pittura et al., 2018). However, the Sediqualsoft® could be improved by making WOE-useful extensions easily available.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/ 10.3390/jmse9070734/s1, S.1. Materials and Methods: Cigarette composition, Artificial Sea water (ASW) composition, Composition of the nutrients used for *P. tricornutum* test, Preparation of Leachates and Elutriates. Table S1: Experimental conditions required by the applied methods; Table S2: Endpoint values obtained from the analyses of negative and positive controls during experiments.

S.1. Materials and Methods

Cigarette Composition

Below is the composition of the cigarettes used for the experiment:

ECB:

Tobacco = 203.3 mg/stick (nicotine = 0.5 mg/stick)

Glycerol = 47.1 mg/stick

Water = 36.1 mg/stick

Cellulose = 10.5 mg/stick

Guar gum = 6.1 mg/stick

Propylene glycol = 2.4 mg/stick

Natural and artificial flavourings = 0.638 mg/stick

Filtration material = 357.5 mg/stick

Paper and wrappers = 100.9 mg/stick

Tipping paper and tipping paper inks = 20.2 mg/stick

Adhesives = 10.8 mg/stick

CCB (NA = Not-Available. About the CCB the producer doesn’t explain the single concentration of the stick components):

Tobacco = NA (nicotine = 0.7 mg/stick)

Water = NA

Propylene glycol = NA

Sugars (sucrose and/or invert sugar) = NA

Glycerol = NA

Natural and artificial flavouring = NA

Filtration material = NA

Paper and wrappers = NA

Tipping paper and tipping paper inks = NA

Adhesives = NA

Artificial Sea Water (ASW) Composition

1- NaCl = 22.0 g/L

2- $MgCl_2 * 6H_2O$ = 9.7 g/L

3- Na_2SO_4 = 3.7 g/L

4- $CaCl_2 * 2H_2O$ = 1.32 g/L

5- KCl = 0.65 g/L

6- $NaHCO_3$ = 0.2 g/L

7- H_3BO_3 = 0.023 g/L

Composition of the Nutrients used for *P. tricornutum* Test

Solution 1 (S1)

$FeCl_3*6H_2O$ = 48 mg/L

$MnCl_2*4H_2O$ = 144 mg/L

$ZnSO_4*7H_2O$ = 45 mg/L

$CuSO_4*5H_2O$ = 0.157 mg/L

$CoCl_2*6H_2O$ = 0.404 mg/L

H_3BO_3 = 1140 mg/L

Na_2EDTA = 1000 mg/L

Solution 2 (S2)

Thiamin hydrochloride = 50 mg/L

Biotin = 0.01 mg/L

Vitamin B_{12} (cyanocobalamin) = 0.10 mg/L

Solution 3 (S3)

K_3PO_4 = 3.0 g/L

$NaNO_3$ = 50.0 g/L

Na_2SiO_3*5H_2O = 14.9 g/L

Preparation of Leachates and Elutriates

Before exposure to target species, in some case parameters were corrected to comply with the standardized requirements of the eco-toxicological tests. In detail, pH was corrected by the addition of few microliters of NaOH and HCl 1M. Corrections are specified in **Table 1** by means of *.

Table S1 *Experimental conditions required by the applied methods.*

Species	Endpoint	Type	Method	Salinity PSU	Temperature °C	Test duration	Illumination
A. fischeri	Inhibition of bioluminescence	Acute	UNI EN ISO 11348-3:2019	> 20	15 ± 1	15 minutes 30 minutes	-
P. lividus	Larval development	Chronic	EPA/600/R-95-136/Sezione 15 + ISPRA Quaderni Ricerca Marina 11/2017	35 ± 1	18 ± 1	72 hours	Darkness
P. lividus	Fertilization	Acute	EPA/600/R-95-136/Sezione 16 + ISPRA Quaderni Ricerca Marina 11/2017	35 ± 1	18 ± 1	40 minutes (20 min exposition + 20 min fertilization)	-
P. tricornutum	Growth inhibition	Chronic	UNI EN ISO 10253:2017	30 ± 1	20 ± 2	72 hours	24/24 h light 6000-10,000 lux from both side

Table S2 *Endpoint values obtained from the analyses of negative and positive controls during experiments.*

Species	Negative Control			Positive Control		
	Medium	Mean	Standard Deviation	Substance	Value	Standard Deviation Confidential limit
A. fischeri	ASW	0.0 %	0.0	3,5-dichlorophenol 3.4 mg/L	15′: 37.46% 30′: 36.11%	15′: 1.88 30′: 2.27
P. lividus (Larval development)	ASW	5.67 %	1.15	Copper (II) nitrate	EC_{50}: 28.26 μg/L	26.90–29.69 μg/L
P. lividus (Fertilization)	ASW	0.0 %	0.0	Copper (II) nitrate	EC_{50}: 57.43 μg/L	33.58–98.24 μg/L
P. tricornutum	algal culture medium	0.0 %	1.61	Potassium dichromate	EC_{50}: 21.61 mg/L	16.78–27.83 mg/L

References

Abraham, A., Park, H., Choi, O., & Sang, B. I. (2021). Anaerobic co-digestion of bioplastics as a sustainable mode of waste management with improved energy production–a review. Bioresource Technology, 322, 124537.

Ach, A. (1993). Biodegradable plastics based on cellulose acetate. Journal of Macromolecular Science, Part A: Pure and Applied Chemistry, 30(9-10), 733-740.

Araújo, M. C. B., & Costa, M. F. (2019). A critical review of the issue of cigarette butt pollution in coastal environments. Environmental research, 172, 137-149.

Ariza, E., & Leatherman, S. P. (2012). No-smoking policies and their outcomes on US beaches. Journal of Coastal Research, 28(1A), 143-147.

Becherucci, M. E., & Pon, J. P. S. (2014). What is left behind when the lights go off? Comparing the abundance and composition of litter in urban areas with different intensity of nightlife use in Mar del Plata, Argentina. Waste management, 34(8), 1351-1355.

Bergmann, M., Gutow, L., & Klages, M. (2015). Marine anthropogenic litter (p. 447). Springer Nature.

Blickley, L. C., Currie, J. J., & Kaufman, G. D. (2016). Trends and drivers of debris accumulation on Maui shorelines: Implications for local mitigation strategies. Marine pollution bulletin, 105(1), 292-298.

Booth, D. J., Gribben, P., & Parkinson, K. (2015). Impact of cigarette butt leachate on tidepool snails. Marine pollution bulletin, 95(1), 362-364.

Bulleri, F., Benedetti-Cecchi, L., & Cinelli, F. (1999). Grazing by the sea urchins *Arbacia lixula* L. and *Paracentrotus lividus* Lam. in the Northwest Mediterranean. Journal of Experimental Marine Biology and Ecology, 241(1), 81-95.

Caridi, F., Sabbatini, A., Birarda, G., Costanzi, E., De Giudici, G., Galeazzi, R., ... & Negri, A. (2020). Cigarette butts, a threat for marine environments: Lessons from benthic foraminifera (Protista). Marine Environmental Research, 162, 105150.

D. M. 173/2016. Regolamento Recante Modalità e Criteri Tecnici per l’autorizzazione All’immersione in Mare Dei Materiali Di Escavo Di Fondali Marini.; 2016; pp 23–35.

Dutra, L. M., Grana, R., & Glantz, S. A. (2017). Philip Morris research on precursors to the modern e-cigarette since 1990. Tobacco control, 26(e2), e97-e105.

Etter, J. F., Bullen, C., Flouris, A. D., Laugesen, M., & Eissenberg, T. (2011). Electronic nicotine delivery systems: a research agenda. Tobacco control, 20(3), 243-248.

Fortibuoni, T., Amadesi, B., & Vlachogianni, T. (2021). Composition and abundance of macrolitter along the Italian coastline: The first baseline assessment within the european Marine Strategy Framework Directive. Environmental Pollution, 268, 115886.

Glantz, S. A.; Slade, J.; Bero, L. a; Hanauer, P.; Barnes, B. The Cigarette Papers; 1998.

Hidalgo-Ruz, V., Honorato-Zimmer, D., Gatta-Rosemary, M., Nuñez, P., Hinojosa, I. A., & Thiel, M. (2018). Spatio-temporal variation of anthropogenic marine debris on Chilean beaches. Marine pollution bulletin, 126, 516-524.

Honda, M., & Suzuki, N. (2020). Toxicities of polycyclic aromatic hydrocarbons for aquatic animals. International Journal of Environmental Research and Public Health, 17(4), 1363.

ISPRA. L'ecotossicologia Come Strumento Di Gestione Degli Ambienti Acquatici e Terrestri; 2019; Vol. 8th BECOME. https://doi.org/10.1017/CBO9781107415324.004.

ISPRA. Saggio di fecondazione e saggio di sviluppo embrionale con il riccio di mare *Paracentrotus lividus* (Lamarck) (Echinodermata: Echinoidea). (2017). https://doi.org/10.1017/CBO9781107415324.004.

Kenyon, K. W., & Kridler, E. (1969). Laysan albatrosses swallow indigestible matter. The Auk, 86(2), 339-343.

Kunst, 2020. Tobacco product usage in Italy 2020. Stastista. https://www.statista.com/forecasts/1000709/tobacco-product-usage-in-italy

Lee, W., & Lee, C. C. (2015). Developmental toxicity of cigarette butts–An underdeveloped issue. Ecotoxicology and environmental safety, 113, 362-368.

Loizidou, X. I., Loizides, M. I., & Orthodoxou, D. L. (2018). Persistent marine litter: small plastics and cigarette butts remain on beaches after organized beach cleanups. Environmental monitoring and assessment, 190(7), 414.

Maradonna, F., Ancillai, D., Notarstefano, V., Valenti, A., Leoni, T., & Carnevali, O. (2020). An integrated approach to evaluate port sediment quality: From chemical characterization to multispecies bioassays. Science of The Total Environment, 746, 141204.

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., & Pennati, R. (2018). Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environmental Pollution, 237, 1080-1087.

Micevska, T., Warne, M. S. J., Pablo, F., & Patra, R. (2006). Variation in, and causes of, toxicity of cigarette butts to a cladoceran and microtox. Archives of Environmental Contamination and Toxicology, 50, 205-212.

Moriwaki, H., Kitajima, S., & Katahira, K. (2009). Waste on the roadside,'poi-sute'waste: its distribution and elution potential of pollutants into environment. Waste management, 29(3), 1192-1197.

Nelms, S. E., Coombes, C., Foster, L. C., Galloway, T. S., Godley, B. J., Lindeque, P. K., & Witt, M. J. (2017). Marine anthropogenic litter on British beaches: a 10-year nationwide assessment using citizen science data. Science of the Total Environment, 579, 1399-1409.

Ocean Conservancy. The Beach and Beyond; 2019. https://doi.org/10.1215/9780822391265-013.

Ocean Conservancy. Together, We Are Team Ocean 2020; 2020.

Oigman-Pszczol, S. S., & Creed, J. C. (2007). Quantification and classification of marine litter on beaches along Armação dos Búzios, Rio de Janeiro, Brazil. Journal of Coastal Research, 23(2), 421-428.

Parker, T. T., & Rayburn, J. (2017). A comparison of electronic and traditional cigarette butt leachate on the development of Xenopus laevis embryos. Toxicology reports, 4, 77-82.

Piccardo, M., Provenza, F., Grazioli, E., Cavallo, A., Terlizzi, A., & Renzi, M. (2020). PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations. Science of the Total Environment, 715, 136947.

Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., ... & Regoli, F. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. Frontiers in marine science, 5, 103.

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., & Regoli, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. Chemosphere, 83(4), 475-485.

Quéméneur, M., Chifflet, S., Akrout, F., Bellaaj-Zouari, A., & Belhassen, M. (2020). Impact of cigarette butts on microbial diversity and dissolved trace metals in coastal marine sediment. Estuarine, Coastal and Shelf Science, 240, 106785.

Regoli, F., Pellegrini, D., Cicero, A. M., Nigro, M., Benedetti, M., Gorbi, S., ... & Frenzilli, G. (2014). A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: integrative indices from Mussel Watch. Marine environmental research, 96, 92-104.

Santos, I. R., Friedrich, A. C., Wallner-Kersanach, M., & Fillmann, G. (2005). Influence of socio-economic characteristics of beach users on litter generation. Ocean & Coastal Management, 48(9-10), 742-752.

Savino, J. F., & Tanabe, L. L. (1989). Sublethal effects of phenanthrene, nicotine, and pinane on Daphnia pulex. Bulletin of Environmental Contamination and Toxicology;(USA), 42(5).

Savino, J. F., & Tanabe, L. L. (1989). Sublethal effects of phenanthrene, nicotine, and pinane on Daphnia pulex. Bulletin of Environmental Contamination and Toxicology;(USA), 42(5).

Slaughter, E., Gersberg, R. M., Watanabe, K., Rudolph, J., Stransky, C., & Novotny, T. E. (2011). Toxicity of cigarette butts, and their chemical components, to marine and freshwater fish. Tobacco control, 20(Suppl 1), i25-i29.

Spahn, J. E., Stavchansky, S. A., & Cui, Z. (2021). Critical research gaps in electronic cigarette devices and nicotine aerosols. International Journal of Pharmaceutics, 593, 120144.

UNEP. Marine Litter: A Global Challenge; 2009.

Wang, Y., Liu, M., Dai, Y., Luo, Y., & Zhang, S. (2021). Health and ecotoxicological risk assessment for human and aquatic organism exposure to polycyclic aromatic hydrocarbons in the Baiyangdian Lake. Environmental Science and Pollution Research, 28(1), 574-586.

Warne, M., Patra, R. W., Cole, B., & Lunau, B. (2002). Toxicity and a hazard assessment of cigarette butts to aquatic organisms. Interact: Sydney, Australia, 1.

Williams, M., Villarreal, A., Bozhilov, K., Lin, S., & Talbot, P. (2013). Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. PloS one, 8(3), e57987.

Wright, S. L., Rowe, D., Reid, M. J., Thomas, K. V., & Galloway, T. S. (2015). Bioaccumulation and biological effects of cigarette litter in marine worms. Scientific reports, 5(1), 14119.

Chapter 5

Ecotoxicity of basil (*Ocimum basilicum*) extract in aquaculture feeds: is it really eco-safe for the aquatic environment?

Paolo Pastorino[1], Marino Prearo[1], Serena Anselmi[2], Andrea Broccoli[2], Francesca Provenza[2], Damià Barcelò[3,4], Monia Renzi[5]

[1] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, 10154 Torino, Italy

[2] Bioscience Research Center (BsRC), 58015 Orbetello, Italy

[3] Institute of Environmental Assessment and Water Research (IDAEA-CSIC), 08034 Barcelona, Spain

[4] Catalan Institute for Water Research (ICRA-CERCA), 17003 Girona, Spain

[5] Dipartimento di Scienze della Vita, Università degli Studi di Trieste, 34127 Trieste, Italy

Ecological Indicators

Volume 142, September 2022, 109173

https://doi.org/10.1016/j.ecolind.2022.109173

Abstract

Plant extract and essential oils are gaining application in aquaculture, but data about their environmental impact are limited and their potential effects on aquatic organisms are largely unknown. For this study, ecotoxicity tests were performed under standardized conditions on fish feed supplemented with 3% w/w of a basil supercritical extract (F1-BEO; substance A), F1-BEO extract (substance B), and fish feed without F1-BEO extract (substance C) on three model species of different trophic levels (bacteria, primary producer, primary consumer) considered representative for freshwater (*Aliivibrio fischeri*, *Raphidocelis subcapitata, Daphnia magna*) and marine (*A. fischeri*, *Phaeodactylum tricornutum*, *Paracentrotus lividus*) ecosystems. Ecotoxicological response was largely comparable within the same trophic level (whichever the ecosystem). EC_{50} was not calculable in the concentration range here tested (3.9-500 mg/L) for freshwater and marine microalgae, suggesting that none of the substances was toxic for primary producers. Reduction of *A. fischeri* bioluminescence at the tested concentration (0.5-10 mg/L) was observed only for substance A (EC_{50} 9.53 mg/L and 9 mg/L for freshwater and marine ecosystems, respectively). Notably, in *P. lividus* embryotoxicity was higher for substances A (EC_{50} 1.80 mg/L) and C (EC_{50} 4.6 mg/L) than for substance B (EC_{50} 7.10 mg/L), suggesting a toxic effect due to feed dissolution. In contrast, substance B was more toxic (EC_{50} 0.34 mg/L) in *D. magna* than substances A (EC_{50} 3.98 mg/L) and C (EC_{50} 5.50 mg/L). Based on the Globally Harmonized System of Classification and Labelling of Chemicals, all substances were categorized Acute 2, except for substance A which was categorized Acute 1 for *D. magna*. Overall, the substances were found to be potentially toxic for an aquatic ecosystem, especially for primary consumer. Further study of plant extract and essential oils is needed to better understand their effects and fate on the aquatic environment.

Keywords

Acute toxicity; aquatic ecosystems; *Daphnia magna*; *Paracentrotus lividus*; plant extract; trophic level

Introduction

The ever wider use of antibiotics in human and veterinary medicine has led to an increase in the circulation of antibiotic-resistant bacteria (Amarasiri et al., 2020; Serwecińska, 2020). In veterinary medicine, antibiotics have long been used in therapy and animal production (Sicuro et al., 2020; Palma et al., 2020). The World Health Organization (WHO) has stated that antimicrobial

resistance is a global concern for the 21st century (Talebi Bezmin Abadi et al., 2019). In addition to measures to improve surveillance and diagnosis of infection and promote rational antibiotic use (Ben et al., 2019; Tse Sum Bui et al., 2022), strengthened and coordinated actions are needed to achieve successful results.

In this context, plant extracts (PEs) and essential oils (EOs) could play a key role as natural antimicrobials (Yu et al., 2020; El-Tarabily et al., 2021). Such compounds are a liquid, volatile, rarely coloured and lipid-soluble mixture of terpenes and terpenoid that are biosynthesized by aromatic plants for attractive or defensive purposes (Mohammedi et al., 2020). EOs for example typically have 20-60 constituents, of which two or three are major compounds (20-70 % of total amount) and the other components occur in traces (Ferraz et al., 2022a).

PEs and EOs have traditionally been used for their antimicrobial activity in folk medicine (Stefanello et al., 2011). Scientific studies demonstrating this property are relatively recent, however, and in most cases concern pathogens that affect humans (Ahmad et al., 2021). The use of PEs and EOs in veterinary medicine for pet animals and livestock holds interest since they could offer an alternative to synthetic antimicrobials (Ebani and Mancianti, 20220; Sicuro et al., 2020) against infection and improve production quality (i.e., meat, eggs, milk, honey, seafood), without the residues of conventional drugs in food (Evangelista et al., 2021).

Essential oils are volatile liquids obtained by distillation of any part of a plant or mechanical extraction from the epicarp of a citrus fruit at room temperature (van Beek and Joulain, 2018). Hydrolates can be obtained as a by-product of distillation to extract EOs (ISO, 2013a). The International Organization for Standardization (ISO) defines a hydrolate as the distilled water that remains after distillation and is typically rich in water-soluble essential oil components (ISO, 2013a; Bicchi and Joulain, 2018), whereas an extract is “a product obtained by treating a natural raw material with a solvent and then, after filtration, removing the solvent by distillation, unless a non-volatile solvent is used” (ISO, 1997).

Supercritical fluid extraction (SFE) of plant materials with solvents such as carbon dioxide (CO_2) is gaining popularity. SFE allows plant material to be processed at low temperatures, limiting thermal degradation, without the use of toxic solvents (Khajeh et al., 2004). Currently, SFE is used primarily for large-scale decaffeination of coffee and tea and the production of hop extracts. It is attracting growing interest for other industrial applications at various scales of operation (Babova et al., 2016). SFE with CO_2 can extract natural compounds, especially those unstable at high temperature. It is the most widely used method in the food and pharmaceutical industry as the extracts contain no organic residues (Wang et al., 2021). Furthermore, extraction can be carried out at low temperature and moderate pressure (Yang et al., 2020).

Despite the growing interest in EO and PE in livestock, the scientific community has paid far less attention to their potential environmental impact (Ferraz et al., 2022a). One explanation for this lack of research is the general belief that plants, and their components are generally natural and safe. Some plants, however, produce highly toxic metabolites (Falkowski et al., 2020; Zárybnick et al., 2018), necessitating assessment of their potential toxic effects on non-target organisms.

The number of drugs authorized in aquaculture is limited and the spread of antibiotic resistance has significantly reduced current options for treating fish diseases (Santos and Ramos, 2018). Several EOs and PEs from aromatic plants are known to have biological activity (Radünz et al., 2019). Basil (*Ocimum basilicum*), for instance, one of the world's most popular aromatic herbs, has been shown to be an effective antioxidant, antimicrobial, insecticidal, nematocidal, and fungistatic agent in aquaculture (Brum et al., 2018; El-Ekiaby, 2019; Amor et al., 2021; Magara et al., 2022).

Two recent studies assessed the long-term changes in serum blood biochemical parameters and biomarkers of antioxidant stress in rainbow trout (*Oncorhynchus mykiss*) fed with a commercial fish diet supplemented with a basil supercritical extract (F1-BEO) up to 3% w/w (Magara et al. 2022; Pastorino et al., 2022). Its ecotoxicity profile in aquatic ecosystems has not been assessed to date, however. For the present study, ecotoxicity tests with F1-BEO extract (fish feed supplemented with 3% w/w F1-BEO) and fish feed without F1-BEO extract (comparison condition) were performed on three species of different trophic levels and considered representative for freshwater (*Aliivibrio fischeri*, bacterium; *Raphidocelis subcapitata* primary producer, *Daphnia magna*, primary consumer) and marine ecosystems (*Aliivibrio fischeri*, bacterium; *Phaeodactylum tricornutum*, primary producer; *Paracentrotus lividus*, primary consumer) (Parvez et al., 2006; Baudo et al., 2011). Ecotoxicological bioassays on three species at different levels of biological complexity and ecological niches can yield information about the effects of contaminants on community and ecosystems (Baudo et al., 2011). The use of assay batteries for evaluation of the ecotoxicity of chemical substances was introduced in European legislation by the REACH Regulation, which refers to the OECD protocols for the choice of the most suitable methods and indicators for acute and chronic toxicity tests (Parvez et al., 2006; Oliva et al., 2021).

Material and methods

Chemical profile of basil supercritical fluid extract (F1-BEO)

The basil supercritical fluid extract (F1-BEO) was the same as that used in previous studies (Magara et al., 2022; Pastorino et al., 2022). F1-BEO was extracted by Exenia Group s.r.l. (Pinerolo, Italy) from dried, clean sweet basil leaves (size 0.3 to 0.5 cm; residual humidity 10%) using a supercritical fluid extractor (SCF-100; Separeco s.r.l, Pinerolo, Italy). Spectrophotometric analysis showed that the F1-BEO contained bioactive compounds, total polyphenol content and total flavan-3-ol content of 32.97±1.63 mmol gallic acid equivalent (GAE) per 100 g of fresh weight and 21.21±1.04 mmol A2-type proanthocyanidin content equivalent (A2-PACE) per 100 g of fresh weight, respectively. Several polyphenolic compounds were identified in the F1-BEO extract by HPLC-ESI-MS/MS (Fig. 1). The F1-BEO also contained several volatile organic compounds, mainly linalool (25.29%), α-bergamotene (19.34%) and estragole (18.79%) (Table 1). The F1-BEO fraction was composed of about 10% fats: palmitic acid, linoleic acid, and oleic acid accounted for 77% of the total fatty acid content (GC-MS and GC-FID analysis).

***Figure 1** Polyphenols in F1-BEO identified via HPLC-ESI-MS/MS (Magara et al., 2022).*

Table 1 *Formulae, identification, and chemical abstracts service identification number (CAS ID) of volatile organic compounds with quantification (in percentage; %) in the F1-BEO extract.*

Formula	**Compound**	**CAS ID**	**Percentage (%)**
$C_{10}H_{18}O$	1,8-Cineole	470-82-6	9.33 ± 0.45
$C_{10}H_{18}O$	Linalool	78-70-6	25.29 ± 0.81
$C_{10}H_{12}O$	Estragol	140-67-0	18.79 ± 0.78
$C_{10}H_{12}O_2$	Eugenol	97-53-0	4.49 ± 0.12
$C_{10}H_{10}O_2$	Methylcinnamylate	103-26-4	8.71 ± 0.15
$C_{11}H_{14}O_2$	Methyleugenol	93-15-2	6.58 ± 0.08
$C_{15}H_{24}$	b-Caryophyllene	87-44-5	7.47 ± 0.29
$C_{15}H_{24}$	α-Bergamotene	17699-05-7	19.34 ± 1.09

Ecotoxicity bioassay of substances

Ecotoxicity bioassays were performed on: commercial feed (Alterna Eel, Skretting; ingredients: fish meal, fish oil, wheat red dog, wheat gluten, blood meal from poultry, a soya bean protein concentrate, swine haemoglobin, whey powder; proximate composition: protein 48%, lipid 11%, ash 8%, fibre 1%) supplemented with 3% w/w F1-BEO (substance A) which is the feed with the higher basil extract inclusion used by Magara et al. (2022) and Pastorino et al. (2022); the F1-BEO extract (substance B); a commercial feed (Alterna Eel, Skretting) without F1-BEO supplement (substance C).

Exposure concentrations were determined by pilot testing to define the correct range of dilutions and based on relevant literature (Ferraz et al., 2022b): 0.5-10 mg/L (*A. fisheri*), 3.9-500 mg/L (*R. subcapitata*), 0.01-100 mg/L (*D. magna*), 3.9-500 mg/L (*P. tricornutum*), 0.5-10 mg/L (*P. lividus*). Fish feed samples (3% w/w F1-BEO and feed without F1-BEO) were prepared as previously reported (Magara et al., 2002). Fish feed samples (pellets) were powdered with a pestle and stock solutions of 500 mg/L were prepared with 0.5% DMSO as solvent (Ferraz et al., 2022b). The solutions were treated by sonication at 40 Hz for 20 min to disaggregate the clusters. Toxicity of the 0.5% DMSO solution was also tested as negative control to minimize any possible effects of the solvent on the results (OECD, 2019).

Toxicity bioassay: freshwater organisms

Tests were performed on three species of different trophic levels and considered representative for freshwater ecosystems: *Aliivibrio fischeri* (Gram-negative bacteria; ISO, 2019); *Raphidocelis subcapitata* (algae; ISO, 2012), *Daphnia magna* (Cladocera; ISO, 2013b).

Briefly, the endpoint for *Aliivibrio fischeri* was inhibition of bacteria when exposed to the sample. Bioluminescence was measured using a luminometer set at 430 nm. The test was performed in

triplicate at 15±1 °C for 30 min. The initial bacterial concentration was 10^6 cells, and the maximum testable concentration of the sample was 90%.

Growth inhibition was the endpoint for the freshwater algae *Raphidocelis subcapitata*. The alga, which was in the exponential growth phase, was exposed to the samples and placed under continuous light for 72 h to stimulate rapid growth. The samples were shaken every 24 h and measured using a spectrophotometer at a wavelength of 670 nm. The test was performed in triplicate at 20±2 °C. The initial concentration of the samples was 10^4 cells, and the sample concentration was 100%. Nutrients (four stock solutions) were added to each sample according to UNI EN ISO 8692:2012 (ISO, 2012). The test was considered valid if the algal concentration in the negative controls was 16 times the initial concentration after 72 h and if the EC_{50} of the positive controls was 1.19±0.27 mg/L.

Five juvenile *Daphnia magna* aged less than 24 h were exposed to different concentrations of the substances at 20±2 °C and a photoperiod of 16 h of light and 8 h of dark for 48 h. At the end of the test, the number of immobilized specimens was counted. Four replicates were performed for each sample. The criteria in the UNI EN ISO 6341:2013 guideline (ISO, 2013b) were followed to meet the test validity criteria. All tests were performed under standardized conditions using negative and positive controls for each batch of analyses (Table 1); results were within the range of acceptability reported by the specific testing method (Table 1).

Table 2 *Positive and negative controls performed on the species of different trophic levels and considered representative of freshwater and marine ecosystems. Negative controls are not available for Aliivibrio fischeri. CV denotes coefficient of variation.*

Species	Negative control	Value	Positive control	Value
Aliivibrio fischeri	Artificial seawater	-	3,5- dichlorophenol (3.4 mg/L)	20-80% of inhibition
Raphidocelis subcapitata	culture medium	CV% (μ): max 5%	$K_2Cr_2O_7$	EC_{50}: 1.19±0.27 mg/L
Daphnia Magna	standard ISO freshwater	percentage of immobility: max 10%	$K_2Cr_2O_7$	EC_{50}: 0.6–2.1 mg/L (24 h)
Phaeodactylum tricornutum	culture medium	CV% (μ): max 5%	$K_2Cr_2O_7$	EC_{50}: 20.1±5.3 mg/L
Paracentrotus lividus	Artificial seawater	-	$Cu(NO_3)_2$	22.60–68.34 μg/L Cu^{2+}.

Toxicity bioassay: marine organisms

The tests were performed on three species of different trophic levels and considered representative for marine ecosystems: *Aliivibrio fischeri* (ISO, 2019), *Phaeodactylum tricornutum* (ISO, 2017), *Paracentrotus lividus* (Echinoderm, EPA/600/R-95-136/Section 15). Growth inhibition was the endpoint for *P. tricornutum* (ISO, 2017). Algae in the exponential growth phase were placed under continuous light for 72 h to stimulate rapid growth. The samples were shaken and measured with a spectrophotometer at a wavelength of 670 nm every 24 h. The tests were carried out in triplicate at 20 ±2 °C. The initial concentration of the samples was 10^4 cells. Except for the negative controls

(the algal culture medium was already nutrient rich), specific nutrients (S1 + S2 + S3; Piccardo et al., 2021) were added to each sample in accordance with ISO (2017). Positive controls (n=3) with potassium dichromate were set up. The test was considered valid if the algal concentration in the negative controls after 72 h was 16 times the initial concentration and the EC_{50} of the positive controls was 20.1 ± 5.3 mg/L.

The larval development of the sea urchin *P. lividus* after 72 h of exposure was the endpoint of this study (Piccardo et al., 2021). Eggs were fertilized with sperm, and the fertilized eggs were exposed to the solutions for 20 min. Each sample was added with 2–3 drops of Lugol's fixative to stop cell division at 72 h, and the results were examined under microscopy. Fertilized eggs reached the pluteus larval stage at 72 h of normal development: 100 *plutei* were counted per replicate to determine the percentage of abnormal larvae. Larvae were considered abnormal if their development was halted, arms were absent or of different length, there were extra arms or cross-lateral rods, body width was asymmetrical or displayed other abnormalities according to the literature (ISPRA, 2017). Tests were performed in triplicate. Artificial sea water was used as a negative control and copper (II) nitrate as a positive control (Table 2). Tests were considered valid when the negative control had more than 80% normally developed larvae and the EC_{50} of the positive control was between 22.60 and 68.34 g/L Cu^{2+} (Table 2).

Statistical analysis and toxicological categorization

Concentrations that induced the endpoint in 50% (EC_{50}), 20% (EC_{20}), and 10% (EC_{10}) of the exposed samples were calculated by statistical interpolation from experimental data using the US EPA Toxicity Relationship Analysis Program (TRAP version 1.30), with a Gaussian distribution and logarithmic transformation of exposure variables sized for ecotoxicological tests. Principal component analysis (PCA) was performed to check for trends in ecotoxicological response (EC_{50}, EC_{20}, percentage of effects, maximum concentration, percentage of effects at minimum concentration) between the freshwater (*Aliivibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna*) and the marine (*Aliivibrio fischeri*, *Phaeodactylum tricornutum*, *Paracentrotus lividus*) model organisms of different trophic roles (bacteria, primary producers, primary consumers) after exposure to the three substances (A, B, C). The PCA results were plotted using open-source data analysis software RStudio® (RStudio, Inc.).

Solution toxicity was classified according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) adopted by the United Nations (UN, 2019). The system consists of three short-term (acute) categories based on acute toxicity data (mean EC_{50}): Acute 3 in the range of 10–100 mg/L; Acute 2 in the range of 1–10 mg/L; Acute 1 when $EC_{50} \leq 1$ mg/L (UN, 2019).

Results

Freshwater

Ecotoxicological response to the substances is reported in Table 3. Effects at the maximum concentration were observed only for *Daphnia magna* in all substances tested, with higher effects (100%) noted for substance B (F1-BEO extract), whereas effects at the minimum concentration were observed for substance A (feed with 3% F1-BEO). EC_{50} was calculated only in *A. fisheri* (9.53 mg/L) exposed to substance A and in *D. magna* in all substances tested, with EC_{50} from 0.34 mg/L (substance B) to 5.50 mg/L (substance C; feed without F1-BEO). Finally, EC_{20} was calculated only for substance B in *A. fisheri* (5.40 mg/L) and *D. magna* (0.05 mg/L). EC_{10} was never calculable at the concentrations in any of the freshwater organisms.

Table 3 *Ecotoxicological response to substances (A - feed with 3% F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) in freshwater organisms. LCL and UCL are 95% confidence lower-level concentration and upper-level concentration intervals associated with EC_{20} and EC_{50}, respectively. NC denotes not calculable; SD standard deviation; %E (max conc) percentage of effect at the maximum concentration; %E (min conc) percentage of effect at the minimum concentration. Data are expressed as mg of substance per litre of solution (mg/L).*

Species	Sample	% E (max conc)	SD	% E (min conc)	SD	EC_{50}	95% LCL	95% UCL	EC_{20}	95% LCL	95% UCL
A. fischeri (10-0.5 mg/L)	A	-0.5	0.0	-10.5	0.1	NC	NC	NC	NC	NC	NC
	B	50.5	0.7	-21.0	0.2	9.53	8.41	10.80	5.40	4.55	6.41
	C	-7.4	0.7	-27.8	0.4	NC	NC	NC	NC	NC	NC
R. subcapitata (500-3.9 mg/L)	A	-11.2	0.3	-9.4	1.2	NC	NC	NC	NC	NC	NC
	B	-17.8	6.7	-10.2	0.8	NC	NC	NC	NC	NC	NC
	C	-10.8	2.1	-10.3	0.8	NC	NC	NC	NC	NC	NC
D. magna (100-0.01 mg/L)	A	80.0	0.0	50.0	34.6	3.98	NC	NC	NC	NC	NC
	B	100.0	0.0	35.0	10.0	0.34	0.12	1.01	0.05	0.01	0.37
	C	65.0	10.0	10.0	11.5	5.50	NC	NC	NC	NC	NC

Marine organisms

Ecotoxicological response to the substances is reported in Table 3. Effects at the maximum concentration were observed only in *P. lividus* for all substances tested, with higher effects (100%) noted for substance A, followed by substances B (95.7%) and C (90%). Effects at the minimum concentrations were observed for all substances in *P. lividus* (12%, 18.3%, and 17% effect for substances B, C, and A, respectively). EC_{50} was calculated only in *A. fisheri* (9 mg/L) exposed to substance B and in *P. lividus* for all tested substances, with EC_{50} from 1.8 mg/L (substance A) to 7.1 mg/L (substance B). Finally, EC_{20} was calculated only in *P. lividus* for substance A (1.3 mg/L), substance B (5.3 mg/L), and substance C (1.5 mg/L). EC_{10} was never calculable at the tested concentrations in any of the marine organisms.

Table 4 *Ecotoxicological response to substances (A - feed with 3% F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) in marine organisms. LCL and UCL were 95% confidence lower-level concentration and upper-level concentration intervals associated with EC_{20} and EC_{50}, respectively. NC denotes not calculable; SD standard deviation; %E (max conc) percentage of effect at the maximum concentration; %E (min conc) percentage of effect at the minimum concentration. Data are expressed as mg of substance per litre of solution (mg/L).*

Species	Sample	%E (max conc)	SD	% E (min conc)	SD	EC_{50}	95% LCL	95% UCL	EC_{20}	95% LCL	95% UCL
A. fischeri (10-0.5 mg/L)	A	-6.5	0.1	-13.5	0.8	NC	NC	NC	NC	NC	NC
	B	53.6	0.7	-16.0	1.5	9.0	8.0	10.2	NC	NC	NC
	C	-5.4	0.7	-20.6	1.8	NC	NC	NC	NC	NC	NC
P. tricornutum (500-3.9 mg/L)	A	-12.1	0.6	-13.3	0.3	NC	NC	NC	NC	NC	NC
	B	-13.5	0.6	-13.0	0.6	NC	NC	NC	NC	NC	NC
	C	-12.9	0.2	-12.9	0.2	NC	NC	NC	NC	NC	NC
P. lividus (10-0.5 mg/L)	A	100.0	0.0	18.3	0.6	1.8	1.6	1.9	1.3	1.0	1.5
	B	95.7	2.5	12.0	2.6	7.1	6.8	7.5	5.3	4.8	5.8
	C	90.0	1.0	17.0	5.2	4.6	2.8	4.5	1.5	0.0	4.5

Principal component analysis

Eigenvalues revealed that the first two principal components (PC1 and PC2) accounted for a significant portion of total variance (94.6%), while the two other components (PC3 and PC4) accounted for a much smaller portion of variance (5.4%). Interpretation of the principal components (PCs) was evaluated using eigenvalues (only PCs with eigenvalues greater than one were retained). The biplot of loadings (variables) and scores (observations) shows which trophic roles are closest to them (Fig. 2). The scores of each trophic role are distinguished by a different symbol and colour (largest symbol denotes average value). Almost all variables moved to PC1 because they were more related to it. The bacteria (*A. fisheri*) and the primary producers (*R. subcapitata* and *P. tricornutum*) are located in the right quadrant of the plot in relation to higher EC_{20} and EC_{50} (fewer sensitive species), whereas primary consumers (*D. magna* and *P. lividus*) are located in the upper left quadrant in relation to higher percentage effects due to exposure to tested substances (more sensitive species).

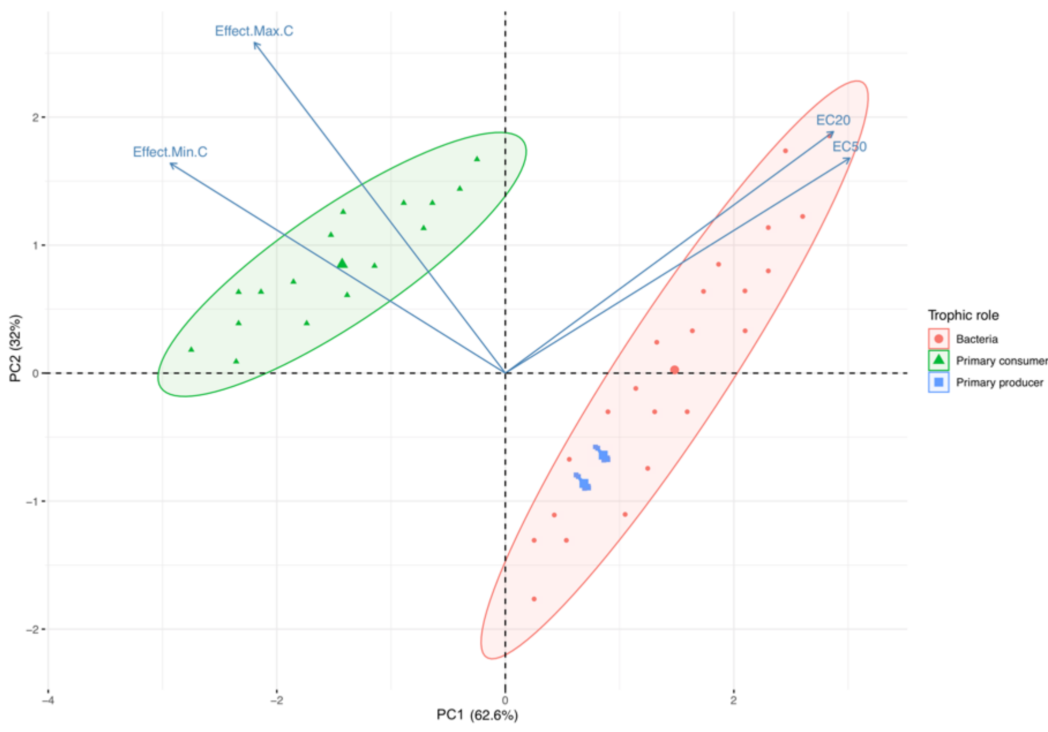


Figure 2 *Biplot of loadings (variables) and scores (observations) in the principal component analysis. The scores of each trophic role are denoted by a symbol (largest symbol denotes average value); each trophic role has a different colour (red for bacteria, blu e for primary producer, green for primary consumer).*

Toxicological categorization

Table 5 presents the toxicity category according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). All substances resulted Acute 2 in the freshwater and the marine ecosystem for bacteria (*A. fischeri*) and primary consumers (*D. magna* and *P. lividus*), except for substance A (F1-BEO extract) categorized as Acute 1 for *D. magna*. The GHS classification is not reported for algae (*R. subcapitata* and *P. tricornutum*) since the EC_{50} was not calculated in the concentration range tested (3.9-500 mg/L). It can be concluded that none of the three substances were toxic for primary producers.

Table 5 *Substances (A - feed with 3% F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) tested in freshwater and marine model organisms and their toxicological categorization (EC_{50}) according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Acute 3: 10–100 mg/L; Acute 2: 1–10 mg/L; Acute 1: $EC_{50} \leq 1$ mg/L.*

Environment	Species	Substance	EC_{50} (mg/L)	GHS classification
Freshwater	*A. fischeri*	B	9.53	Acute 2
	D. magna	A	3.98	Acute 2
		B	0.34	Acute 1
		C	5.50	Acute 2
Marine	*A. fischeri*	B	9.0	Acute 2
	P. lividus	A	1.8	Acute 2
		B	7.1	Acute 2
		C	4.6	Acute 2

Discussion

The available data on the toxicity of EOs and other PEs in model aquatic organisms are very scarce (Ferraz et al. 2022a). For this study, we report the ecotoxicological response of freshwater and marine model organisms exposed to F1-BEO bail extract and fish feed supplemented with 3% w/w of F1-BEO. The ecotoxicity of fish feed without F1-BEO is reported for comparison. Despite the difference in ecosystem (freshwater or marine), the ecotoxicological response was generally comparable for the same trophic level, with higher sensitivity noted in primary consumers.

Aliivibrio fischeri lives as a free-living (planktonic) organism or a mutualistic symbiont that colonizes the light-producing organ (photophore) of squids and fish, imparting luminescence for camouflage or prey attraction by the host (Kaeding et al., 2007). Through the electron transport chain, bioluminescence is directly linked to respiration and thus reflects cellular metabolic status as a determinant of xenobiotic-mediated toxicity (Girotti et al., 2008; Abbas et al., 2018). Exposure to toxic substances reduces the production of luminescence: bacterial metabolism is inhibited by the decrease in light emittance that corresponds to the toxicity level of the substance (Abbas et al., 2018). In the present study, a reduction in *A. fischeri* bioluminescence at the tested concentrations (0.5-10 mg/L) was observed only for the F1-BEO extract (mean EC_{50} 9.53 mg/L and 9 mg/L for freshwater and marine ecosystems, respectively). Shukla et al. (2020) investigated the volatile effect of linalool and eugenol (major components of the F1-BEO extract) and found that both compounds could reduce the bioluminescence of *A. fischeri* even at very low concentrations. In contrast, no inhibition of bioluminescence was observed for the other two substances (A and C) tested here.

Microalgae inhabit the world's oceans and seas where they occupy a key trophic level in aquatic ecosystems as primary producers at the base of the marine food chain (Mucha et al., 2003). Because phytoplankton are at the bottom of the aquatic food chain, they are vital to the entire ecosystem; however, very few studies to date have examined the toxic effects of PEs and EOs (papaveraceae, pinaceae, fabaceae, malvaceae, cupressaceae) on microalgae (mainly *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Chlamydomonas reinhardtii*) (Jančula et al., 2007; Duringer et al., 2010; Oliveira et al., 2016; Pino-Otín et al., 2019; Ferraz et al., 2022). In the present study, the EC_{50} for freshwater and marine microalgae was not calculated at the tested concentrations, suggesting no acute toxic effects on microalgae growth (endpoint). For the F1-BEO extract (substance B), we may note that linalool was highest in composition percentage, followed by α-bergamotene and estragol. In a previous study, linalool toxicity was tested in *Scendedesmus subspicatus* (growth inhibition test; 96-h period), with an EC_{50} of 141.4 mg/L (Api et al., 2015), confirming the low toxicity of the compound on microalgae.

Unfortunately, the literature offers no data on basil extract toxicity for comparison. With regard to other PEs and EOs, however, Duringer et al. (2010) assessed the ecotoxicity of steam-extracted oils derived from western juniper foliage (*Juniperus occidentalis*) and Port Orford cedar heartwood (*Chamaecyparis lawsoniana*) on *R. subcapitata*; the EC_{50} for *J. occidentalis* EO was 1.7 mg/L at 96 h and was considered moderately toxic to *R. subcapitata*. After exposure to EO from *C. lawsoniana*, the EC_{50} for algal cell growth was reported to be higher than 5 mg/L, leading to the conclusion that the release of *C. lawsoniana* EO into the aquatic environment had no expected acute toxic effects on microalgae. The aqueous extracts from the roots of five papaveraceae plants were tested for their effects on *R. subcapitata* (Jancula et al., 2007); the extracts from *Dicranostigma lactucoides* and *Sanguinaria canadensis* were found to be the most toxic to microalgae after 96 h of exposure (EC_{50} of 21.27 and 23.90 mg/L, respectively) (Jancula et al., 2007).

As regards the toxicity of substances A and C on microalgae, fish feed is a known primary source of waste with the greatest environmental impact in aquaculture. The quantity and quality of waste excreted by fish are determined by dietary intake, digestion, and metabolism (Bureau and Hua, 2010). There is also a link between feed quality, feeding strategy, and waste production (Schneider et al., 2004). Aquacultural waste can be divided into solid and dissolved waste; unused and/or spilled feed by the fish, as well as excreted faeces are the main sources of solid waste, while dissolved waste is nutrient (mainly phosphorus and nitrogen) disintegration/suspension from the solid waste fraction. The increase in organic loading from fish feed into waters may influence the structure, composition, dominance, and biomass of phytoplankton communities (San Diego-Mcglone et al., 2008).

In the present study, dissolution of fish feed in the algal media enhanced the growth of the microalgal species tested with no toxic effects. Differently, the primary consumers in the freshwater and the marine ecosystem displayed acute toxicity in response to all three substances. *Daphnia magna* exposed to F1-BEO (substance B) showed the lowest EC_{50} (0.34 mg/L) compared to the EC_{50} in response to exposure to feed supplemented with F1-BEO (3.98 mg/L) and feed without basil extract (5.50 mg/L). Such findings are corroborated by the chemical composition of the F1-BEO extract as previously stated. A *D. magna* immobilization test (48 h) performed using linalool reported an EC_{50} of 20 mg/L. Eugenol (4.49% in F1-BEO) was found to be highly toxic at low concentrations for *D. magna* (EC_{50} 0.70 mg/L) (Gueretz et al., 2017). Estragol (18.79% in F1-BEO) is reported to be toxic for houseflies (Palacios et al., 2009), fruit flies (Cheng et al., 2009), and house dust mites (Lee, 2004), but no data on aquatic organisms are available. Although the toxic effects of PEs and EOs on crustaceans such as *Daphnia magna*, *Daphnia pulex*,

Scapholeberis kingi, and *Artemia salina* have been studied (Andreu et al., 2018; Seremet et al., 2018; Ishimota et al., 2019; Pavela et al., 2020), only one study (Ferraz et al., 2022b) reported toxicity data for basil extract. *O. basilicum* hydrolate (Ferraz et al. 2022b), composed mainly of 52.5 eugenol and 38.3% linalool, showed no acute toxic effects on *D. magna* up to very high concentrations (8000 mg/L). The study did not mention whether the solution was sonicated and had solubilization issues, however.

Daphnia magna was sensitive to fish feed dissolution, with higher toxicity in response to fish feed supplemented with F1-BEO, most likely due to the synergic effect of feed powder and basil extract. The reason for the toxicity was probably due to the solid waste (also known as particulate organic matter) that causes oxygen depletion and ammonia toxicity when it decomposes. Furthermore, suspended solids (feed powder) floating in the water column can cause gill irritation to *D. magna*, also filling the intestinal tract (Capper, 2006).

Likewise, *Paracentrotus lividus* showed acute toxicity to all substances tested here: a higher percentage of abnormal larvae compared to controls and greater sensitivity to feed supplemented with F1-BEO, followed by feed without basil extract. *Paracentrotus lividus* is a key marine species with larval and adult populations inhabiting planktonic and benthic marine ecosystems, respectively. *P. lividus* has been demonstrated to be highly sensitive to various compounds, and it is widely used to assess the toxicological effects and the environmental impact of a variety of pollutants. The embryos are a highly duplicative cell system; exposure to chemicals has an adverse effect on delicate embryo development (Piccardo et al., 2021; Gharred et al., 2022). As reported for *D. magna*, the dissolution of fish feed probably caused the release of particulate organic matter that altered the water's physiochemical characteristics, making it toxic to *P. lividus* embryos. Changes in the physicochemical parameters of seawater (e.g., total organic content, nitrate, turbidity) could be indicators of eutrophication and have a negative impact on sea urchin embryo-larvae development and animal growth (Ternengo et al., 2018). Zúñiga et al. (1995) found that exposure to organic-waste discharges caused embryotoxicity in the sea urchin *Arbacia spatuligira*.

Finally, no data are available for comparison of acute toxicity of F1-BEO to *P. lividus* embryos. Novaes Simões et al. (2017) evaluated the use of *Lippia alba* EO (composed mainly of linalool [48.69%] and eucalyptol [10.51%]) as a sedative in the sea urchin *Echinometra lucunter* and found that a concentration of 150 ppm is sufficient to induce anaesthesia in adult specimens, with possible adverse effects on sea urchin embryos.

Conclusions

For this study, the effects of a basil extract (F1-BEO) as-is and supplemented in a commercial fish feed (based on the effectiveness observed in fish [Magara et al., 2022; Pastorino et al., 2022]) were assessed using model organisms for marine and freshwater ecosystems. Our findings suggest that the ecotoxicological responses were comparable in the freshwater and the marine ecosystem for organisms at the same trophic level. The substances appear to be safe for microalgae, whereas they caused toxic effects on primary consumers (*D. magna* and *P. lividus*), particularly the F1-BEO as-is in *D. magna*. The widespread belief that plant-based products are green and safer alternatives to their chemical counterparts lacks empirical data to support this claim. Hence there is an urgent need to assess the safety of other PEs and EOs to better understand their effects on ecosystems.

References

Abbas, M., Adil, M., Ehtisham-ul-Haque, S., Munir, B., Yameen, M., Ghaffar, A., ... & Iqbal, M. (2018). *Vibrio fischeri* bioluminescence inhibition assay for ecotoxicity assessment: a review. Science of the Total Environment, 626, 1295-1309.

Ahmad, A., Elisha, I. L., van Vuuren, S., & Viljoen, A. (2021). Volatile phenolics: A comprehensive review of the anti-infective properties of an important class of essential oil constituents. Phytochemistry, 190, 112864.

Amarasiri, M., Sano, D., & Suzuki, S. (2020). Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. Critical Reviews in Environmental Science and Technology, 50(19), 2016-2059.

Amor, G., Sabbah, M., Caputo, L., Idbella, M., De Feo, V., Porta, R., ... & Mauriello, G. (2021). Basil essential oil: Composition, antimicrobial properties, and microencapsulation to produce active chitosan films for food packaging. Foods, 10(1), 121.

Andreu, V., Levert, A., Amiot, A., Cousin, A., Aveline, N., & Bertrand, C. (2018). Chemical composition and antifungal activity of plant extracts traditionally used in organic and biodynamic farming. Environmental Science and Pollution Research, 25(30), 29971-29982.

Api, A. M., Belsito, D., Bhatia, S., Bruze, M., Calow, P., Dagli, M. L., ... & Wilcox, D. K. (2015). RIFM fragrance ingredient safety assessment, Linalool, CAS registry number 78-70-6. Food Chem Toxicol, 82(Suppl), S29-S38.

Babova, O., Occhipinti, A., Capuzzo, A., & Maffei, M. E. (2016). Extraction of bilberry (Vaccinium myrtillus) antioxidants using supercritical/subcritical CO2 and ethanol as co-solvent. The Journal of Supercritical Fluids, 107, 358-363.

Baudo, R., Faimali, M., Onorati, F., & Pellegrini, D. (2011). Batterie di saggi ecotossicologici per sedimenti di acque salate e salmastre. I manuali di Ecotossicologia, Manuali e linee guida, ISPRA Istituto Superiore per la Protezione e la Ricerca Ambientale, 67, 2011.

Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M. H., & Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. Environmental research, 169, 483-493.

Bicchi, C., & Joulain, D. (2018). Techniques for preparing essential oils and aromatic extracts. Flavour and Fragrance Journal, 33(2), 133-134.

Brum, A., Cardoso, L., Chagas, E. C., Chaves, F. C. M., Mouriño, J. L. P., & Martins, M. L. (2018). Histological changes in Nile tilapia fed essential oils of clove basil and ginger after challenge with Streptococcus agalactiae. Aquaculture, 490, 98-107.

Bureau, D. P., & Hua, K. (2010). Towards effective nutritional management of waste outputs in aquaculture, with particular reference to salmonid aquaculture operations. Aquaculture Research, 41(5), 777-792.

Capper, N. A. (2006). The Effects of suspended sediment on the aquatic organisms *Daphnia magna* and *Pimephales promelas* (Doctoral dissertation, Clemson University).

Cheng, S. S., Chua, M. T., Chang, E. H., Huang, C. G., Chen, W. J., & Chang, S. T. (2009). Variations in insecticidal activity and chemical compositions of leaf essential oils from Cryptomeria japonica at different ages. Bioresource Technology, 100(1), 465-470.

Duringer, J. M., Swan, L. R., Walker, D. B., & Craig, A. M. (2010). Acute aquatic toxicity of western juniper (*Juniperus occidentalis*) foliage and Port Orford cedar (C*hamaecyparis lawsoniana*) heartwood oils. Environmental monitoring and assessment, 170(1), 585-598.

Ebani, V. V., & Mancianti, F. (2020). Use of essential oils in veterinary medicine to combat bacterial and fungal infections. Veterinary Sciences, 7(4), 193.

El-Ekiaby, W. T. (2019). Basil oil nanoemulsion formulation and its antimicrobial activity against fish pathogen and enhance disease resistance against *Aeromonas hydrophila* in cultured Nile tilapia. Egyptian Journal for Aquaculture, 9(4), 13-33.

El-Tarabily, K. A., El-Saadony, M. T., Alagawany, M., Arif, M., Batiha, G. E., Khafaga, A. F., ... & Abd El-Hack, M. E. (2021). Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. Saudi Journal of Biological Sciences, 28(9), 5145-5156.

Evangelista, A. G., Corrêa, J. A. F., Pinto, A. C. S. M., & Luciano, F. B. (2022). The impact of essential oils on antibiotic use in animal production regarding antimicrobial resistance–a review. Critical Reviews in Food Science and Nutrition, 62(19), 5267-5283.

Ferraz, C. A., Sousa, A. C. A., Caramelo, D., Delgado, F., de Oliveira, A. P., & Pastorinho, M. R. (2022b). Chemical profile and eco-safety evaluation of essential oils and hydrolates from Cistus ladanifer, Helichrysum italicum, Ocimum basilicum and *Thymbra capitata.* Industrial Crops and Products, 175, 114232.

Ferraz, C. A., Pastorinho, M. R., Palmeira-de-Oliveira, A., & Sousa, A. C. (2022a). Ecotoxicity of plant extracts and essential oils: A review. Environmental Pollution, 292, 118319.

Gharred, C., Jenzri, M., Bouraoui, Z., Guerbej, H., Jebali, J., & Gharred, T. (2022). Application of the *Paracentrotus lividus* sea-urchin embryo-larval bioassay to the marine pollution biomonitoring program in the Tunisian coast. Environmental Science and Pollution Research, 29(4), 5787-5797.

Girotti, S., Ferri, E. N., Fumo, M. G., & Maiolini, E. (2008). Monitoring of environmental pollutants by bioluminescent bacteria. Analytica chimica acta, 608(1), 2-29.

Gueretz, J. S., Somensi, C. A., Martins, M. L., & Souza, A. P. D. (2017). Evaluation of eugenol toxicity in bioassays with test-organisms. Ciência Rural, 47.

Ishimota, M., Nakajima, D., Sakamoto, M., & Miyabara, Y. (2019). Water-soluble bioactive natural compounds in *Trapa japonica* leaves: Temporal changes in chemical composition and effects on cladocerans. Ecological Research, 34(2), 328-335.

ISO (1997). Aromatic raw materials – vocabulary, international standard ISO 9235 Standardization.

ISO (2012). UNI EN ISO 8692:2012 Qualità Dell'acqua - Prova Di Inibizione Della Crescita Di Alghe d'acqua Dolce per Mezzo Di Alghe Verdi.

ISO (2013a). ISO 9235: 2013: aromatic natural raw materials: vocabulary.

ISO (2013b). UNI EN ISO 6341:2013 Qualità Dell'acqua - Determinazione Dell'inibizione Della Mobilità Di *Daphnia Magna* Straus (Cladocera, Crustacea) - Prova Di Tossicità Acuta.

ISO (2017). UNI EN ISO 10253:2017. Water quality, marine algal growth inhibition test with *Skeletonema* sp. and *Phaeodactylum tricornutum*.

ISO (2019). UNI EN ISO 11348-1:2019 Qualità Dell'acqua - Determinazione Dell'effetto Inibitorio Di Campioni Acquosi Sull'emissione Di Luce Di *Vibrio fischeri* (Prova Su Batteri Luminescenti) - Parte 1: Metodo Con Batteri Preparati Di Fresco.

ISPRA (2017). Saggio di Fecondazione e Saggio Di Sviluppo Embrionale Con Il Riccio di Mare *Paracentrotus lividus* (Lamarck) (Echinodermata: Echinoidea); ISPRA: Livorno, Italy, 2017.

Jančula, D., Suchomelová, J., Gregor, J., Smutná, M., Maršálek, B., & Táborská, E. (2007). Effects of aqueous extracts from five species of the family Papaveraceae on selected aquatic organisms. Environmental Toxicology: An International Journal, 22(5), 480-486.

Kaeding, A. J., Ast, J. C., Pearce, M. M., Urbanczyk, H., Kimura, S., Endo, H., ... & Dunlap, P. V. (2007). Phylogenetic diversity and cosymbiosis in the bioluminescent symbioses of "Photobacterium mandapamensis". Applied and Environmental Microbiology, 73(10), 3173-3182.

Khajeh, M., Yamini, Y., Sefidkon, F., & Bahramifar, N. (2004). Comparison of essential oil composition of *Carum copticum* obtained by supercritical carbon dioxide extraction and hydrodistillation methods. Food chemistry, 86(4), 587-591.

Lee, H. S. (2004). p-Anisaldehyde: acaricidal component of Pimpinella anisum seed oil against the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Planta medica, 70(03), 279-281.

Magara, G., Prearo, M., Vercelli, C., Barbero, R., Micera, M., Botto, A., ... & Pastorino, P. (2022). Modulation of antioxidant defense in farmed rainbow trout (*Oncorhynchus mykiss*) fed with a diet supplemented by the waste derived from the supercritical fluid extraction of basil (*Ocimum basilicum*). Antioxidants, 11(2), 415.

Mohammedi, H., Mecherara-Idjeri, S., Hassani, A. (2020). Variability in essential oil composition, antioxidant and antimicrobial activities of *Ruta montana* L. collected from different geographical regions in Algeria. Journal of essential oil research, 32(1), 88-101.

Mucha, A. P., Leal, M. F. C., Bordalo, A. A., & Vasconcelos, M. T. S. (2003). Comparison of the response of three microalgae species exposed to elutriates of estuarine sediments based on growth and chemical speciation. Environmental Toxicology and Chemistry: An International Journal, 22(3), 576-585.

Novaes Simões, L., Carine Campos Medeiros, L., Maria Heinzmann, B., Lucia Loro, V., de Carvalho Gomes, L., Thomas da Silva, D., ... & Baldisserotto, B. (2017). Essential oil of *Lippia alba* as a sedative and anesthetic for the sea urchin *Echinometra lucunter* (Linnaeus, 1758). Marine and Freshwater Behaviour and Physiology, 50(3), 205-217.

OECD (2019). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixture

Oliva, M., De Marchi, L., Cuccaro, A., Pretti, C. (2021). Bioassay-based ecotoxicological investigation on marine and freshwater impact of cigarette butt littering. Environmental Pollution, 288, 117787.

Oliveira, A. E., Duarte, J. L., Amado, J. R., Cruz, R. A., Rocha, C. F., Souto, R. N., ... & Carvalho, J. C. (2016). Development of a larvicidal nanoemulsion with *Pterodon emarginatus* Vogel oil. PLoS One, 11(1), e0145835.

Palacios, S. M., Bertoni, A., Rossi, Y., Santander, R., & Urzúa, A. (2009). Insecticidal activity of essential oils from native medicinal plants of Central Argentina against the house fly, Musca domestica (L.). Parasitology Research, 106(1), 207-212.

Palma, E., Tilocca, B., & Roncada, P. (2020). Antimicrobial resistance in veterinary medicine: An overview. International Journal of Molecular Sciences, 21(6), 1914.

Parvez, S., Venkataraman, C., & Mukherji, S. (2006). A review on advantages of implementing luminescence inhibition test *(Vibrio fischeri)* for acute toxicity prediction of chemicals. Environment international, 32(2), 265-268.

Pastorino, P., Bergagna, S., Vercelli, C., Pagliasso, G., Dellepiane, L., Renzi, M., ... & Prearo, M. (2022). Changes in Serum Blood Parameters in Farmed Rainbow Trout (*Oncorhynchus mykiss*) Fed with Diets Supplemented with Waste Derived from Supercritical Fluid Extraction of Sweet Basil (*Ocimum basilicum*). Fishes, 7(2), 89.

Pavela, R., Morshedloo, M. R., Lupidi, G., Carolla, G., Barboni, L., Quassinti, L., ... & Benelli, G. (2020). The volatile oils from the oleo-gum-resins of *Ferula assa-foetida* and *Ferula gummosa*: a comprehensive investigation of their insecticidal activity and eco-toxicological effects. Food and Chemical Toxicology, 140, 111312.

Piccardo, M., Provenza, F., Anselmi, S., Broccoli, A., Terlizzi, A., & Renzi, M. (2021). Use of Sediqualsoft® to Determine the Toxicity of Cigarette Butts to Marine Species: A Weather Simulation Test. Journal of Marine Science and Engineering, 9(7), 734.

Pino-Otín, M. R., Ballestero, D., Navarro, E., González-Coloma, A., Val, J., & Mainar, A. M. (2019). Ecotoxicity of a novel biopesticide from Artemisia absinthium on non-target aquatic organisms. Chemosphere, 216, 131-146.

Radünz, M., da Trindade, M. L. M., Camargo, T. M., Radünz, A. L., Borges, C. D., Gandra, E. A., & Helbig, E. (2019). Antimicrobial and antioxidant activity of unencapsulated and encapsulated clove (*Syzygium aromaticum*, L.) essential oil. Food chemistry, 276, 180-186.

San Diego-McGlone, M. L., Azanza, R. V., Villanoy, C. L., & Jacinto, G. S. (2008). Eutrophic waters, algal bloom and fish kill in fish farming areas in Bolinao, Pangasinan, Philippines. Marine Pollution Bulletin, 57(6-12), 295-301.

Santos, L., & Ramos, F. (2018). Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. International Journal of Antimicrobial Agents, 52(2), 135-143.

Schneider, O., Sereti, V., Eding, E. H., & Verreth, J. A. J. (2005). Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacultural engineering, 32(3-4), 379-401.

Seremet, O. C., Olaru, O. T., Gutu, C. M., Nitulescu, G. M., Ilie, M., Negres, S., ... & Margina, D. M. (2018). Toxicity of plant extracts containing pyrrolizidine alkaloids using alternative invertebrate models. Molecular Medicine Reports, 17(6), 7757-7763.

Serwecińska, L. (2020). Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water, 12(12), 3313.

Shukla, D. M., Bajwa, V., Gajic, D., & Saxena, P. K. Quorum sensing inhibition in *Vibrio fischeri*: An efficient system to assess antibacterial properties of medicinal plants and their volatile compounds.

Sicuro, B., Pastorino, P., Barbero, R., Barisone, S., Dellerba, D., Menconi, V., ... & Prearo, M. (2020). Prevalence and antibiotic sensitivity of bacteria isolated from imported ornamental fish in Italy: A translocation of resistant strains?. Preventive veterinary medicine, 175, 104880.

Stefanello, M. É. A., Pascoal, A. C., & Salvador, M. J. (2011). Essential oils from neotropical Myrtaceae: chemical diversity and biological properties. Chemistry & biodiversity, 8(1), 73-94.

Talebi Bezmin Abadi, A., Rizvanov, A. A., Haertlé, T., & Blatt, N. L. (2019). World Health Organization report: current crisis of antibiotic resistance. BioNanoScience, 9, 778-788.

Ternengo, S., Marengo, M., El Idrissi, O., Yepka, J., Pasqualini, V., & Gobert, S. (2018). Spatial variations in trace element concentrations of the sea urchin, *Paracentrotus lividus*, a first reference study in the Mediterranean Sea. Marine pollution bulletin, 129(1), 293-298.

Tse Sum Bui, B., Auroy, T., & Haupt, K. (2022). Fighting Antibiotic-Resistant Bacteria: Promising Strategies Orchestrated by Molecularly Imprinted Polymers. Angewandte Chemie International Edition, 61(8), e202106493.

UN (2019). Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (2019), 10.18356/f8fbb7cb-en

van Beek, T. A., & Joulain, D. (2018). The essential oil of patchouli, *Pogostemon cablin*: A review. Flavour and Fragrance Journal, 33(1), 6-51.

Wang, W., Rao, L., Wu, X., Wang, Y., Zhao, L., & Liao, X. (2021). Supercritical carbon dioxide applications in food processing. Food Engineering Reviews, 1-22.

Yang, Y. C., Wang, C. S., & Wei, M. C. (2020). Development and validation of an ultrasound-assisted supercritical carbon-dioxide procedure for the production of essential oils from *Perilla frutescens*. LWT, 128, 109503.

Yu, Z., Tang, J., Khare, T., & Kumar, V. (2020). The alarming antimicrobial resistance in ESKAPEE pathogens: Can essential oils come to the rescue?. Fitoterapia, 140, 104433.

Zúñiga M, Roa R, Larrain A (1995) Sperm cell bioassays with the sea urchin *Arbacia spatuligera* on samples from two polluted Chilean coastal sites. Mar Pollut Bull 30(5):313–319.

Conclusions and Future perspective

Conclusions

Delineating the impact of emerging contaminants and materials of ecotoxicological concern in a complex and changing environmental context is a complex goal that will require the efforts of researchers in the coming decades. Much has been done in recent years, both in the implementation of the scientific basis and in the legal regulation. Among European countries, Italy is the most sensitive to ecotoxicological problems in the environment.

The context, however, is objectively dynamic and complex and requires a high degree of expertise. Regulatory standards that are rigid and slow to implement are unlikely to achieve the goal of environmental protection in this context, even considering the impact of global changes that may lead to important variations in ecotoxicological responses.

The results of the research carried out have made it possible to implement the overall framework of scientific knowledge on the subject, on the effects of emerging pollutants, complex mixtures such as soaps, materials of recent commercial interest such as glitter, common litter such as cigarette butts and newly introduced biomolecules such as basil extract. In summary, the results are reported below.

Concerning materials of emerging concern (i.e., microplastic, packaging and glitter) and marine litter of ecotoxicological concern (i.e., cigarette butts):

- different types of plastics cause different toxicity on tested species owning to different trophic levels; PET has the higher ecotoxic effect.
- the shape and length of plastics, especially in the case of glitter, cause different rates of uptake by organisms and different effects, so smaller plastics are worse.
- ingestion and expulsion of fragments can lead to a change in the length of microplastics and consequently change the rate of uptake by other organisms.
- some types of emerging pollutants can generate new toxic components, such as polycyclic aromatic hydrocarbons from combustion.

Concerning newly introduced biomolecules (i.e., basil extract for aquaculture disinfection):

- plant-based products used as antimicrobials, such as *O. basilicum* in aquaculture, appear to be an environmentally friendly and safer alternative, but there is no supporting data yet.
- the ecotoxicological responses of some analyzed newly introduced biomolecules were comparable in the freshwater and the marine ecosystem for organisms at the same trophic level.
- the substances of basil appear to be safe for primary producer (microalgae), but they can cause toxic effects on primary consumers (for example, *D. magna* in freshwater and *P. lividus* in marine ecosystem), particularly where the substance is evaluated as-is.
- the widespread belief that plant-based products are green and safer alternatives to their chemical counterparts lacks empirical data to support this claim. So, it is urgent to estimate the safety of other newly introduced molecules and, on consequence, their impacts on ecosystems.
- newly introduced molecules can have ecotoxicological impact on ecosystem and can have different effects when combined each other.

Concerning the effect of global change on ecotoxicology of tested emerging pollutants and materials:

- global changes increase ecotoxicological impacts and can change the behavior and toxicity of molecules that are already known and considered safe; in fact, molecules can show different toxicity when used outside standard conditions.
- the use of different and integrated approaches (ecotoxicological and biochemical analyses, application of different markers in standardized analyses such as biometrics, use of data integration) gives more answers to complex problems.
- according to some report, previously cited, can be possible some interactions between the availability and effects of toxicants and natural environmental reactions related to symptoms of global change.
- there is not enough data at this moment to understand how toxins could interact with one another, how to assess toxicity, and how global changes affect this interaction.
- there is a lacking in long-term experiments than can be investigate possible effects of newly emerging problematic issues; this lack in literature regards in particular marine ecotoxicological studies.

Future Perspective

The results obtained allow a small step forward in the knowledge of the subject but open the scenario for new research needed to implement the general knowledge and allow a more environmentally friendly use of chemical substances and materials of interest and informed regulation.

The future perspectives for the research presented can be summarised as follows:

- evaluate the toxicity of well-known chemicals and compare the results with data on standard conditions and expand the knowledge of emerging pollutants and materials in the context of global change and analyse personal care products and fragrances in a changing scenario.
- analyse contaminant-derived by-products and their behaviour in environmental complex matrices.
- biometric variations, more innovative endpoint in same analyses, could be considered a precocious marker of stress in echinoderm larvae.
- there is a significant lack of knowledge regarding the effects of micro- and nanoplastics on aquatic biota, particularly polymers such as poly-methyl-methacrylate (PMMA).
- further studies are needed to test the toxicity of phthalates and metals (alone or combined) on model aquatic organisms. A future area of focus is also to experiment with different types of glitter and different exposure times in other aquatic organisms.
- more LOEs (Line of Evidence) of bioassays may derived from future studies on chemical composition of the elutriates, assessment of bioavailability, and sub-lethal effects on battery of biomarkers. The use of a weight of evidence approach appears to be a powerful tool to support more complex processes of environmental risk assessment and furnish a comprehensive assessment of hazard associated to sediments polluted with contaminants of various kinds.
- explore ecotoxicity of plant-based chemicals and new commercial products.
- the widespread belief that plant-based products are green and safer alternatives to their chemical counterparts lacks empirical data to support this claim. Hence there is an urgent need to assess the safety of other plant extracts (PEs) and essential oils (EOs) to better understand their effects on ecosystems.
- improve the use of an integrated ecotoxicological approach and innovative endpoints to better weight impacts for the environment and to detect early occurring effects on target species.