NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF ENVIRONMENTAL SCIENCES AND ENGINEERING

LAURA TIÊ ZANCHI DA SILVA

Graduate in Environmental Engineering

EVALUATION OF THE IMPACT OF MICROPLASTICS ON MERCURY DETOXIFICATION STRATEGIES: A COMPARISON BETWEEN THE PERFORMANCE OF BACTERIA AND FUNGI

INTEGRATED MASTER'S IN ENVIRONMENTAL ENGINEERING, SANITARY ENGINEERING PROFILE

NOVA University of Lisbon
November, 2021

NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF ENVIRONMENTAL SCIENCES AND ENGINEERING

EVALUATION OF THE IMPACT OF MICROPLASTICS ON MERCURY DETOXIFICATION STRATEGIES: A COMPARISON BETWEEN THE PERFORMANCE OF BACTERIA AND FUNGI

LAURA TIÊ ZANCHI DA SILVA
Graduate in Environmental Engineering

Adviser: Marta Susana Silvestre Gouveia Martins, Assistant Professor, NOVA School of Science and Technology

Co-adviser: Neusa Figueiredo, Invited Assistant Professor, NOVA School of Science and Technology

Examination Comittee:

Chairperson: Prof.ª Doutora Maria da Graça Madeira Martinho
Professora Associada com Agregação da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

Rapporteurs: Prof. Doutor Vasco Rui Veloso Neves Branco,
Professor Auxiliar da Faculdade de Farmácia da Universidade de Lisboa

Members: Prof.ª Doutora Marta Susana Silvestre Gouveia Martins
Professora Auxiliar da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

INTEGRATED MASTER'S IN ENVIRONMENTAL ENGINEERING, SANITARY ENGINEERING PROFILE

NOVA University of Lisbon
November, 2021

Evaluation of the Impact of Microplastics on Mercury Detoxification Strategies: a Comparison between the Performance of Bacteria and Fungi.

Copyright © Laura Zanchi da Silva, School of Sciences and Technology, NOVA University Lisbon.
The School of Sciences and Technology and the NOVA University Lisbon have the right, perpetual and without geographical boundaries, to file and publish this dissertation through printed copies reproduced on paper or on digital form, or by any other means known or that may be invented, and to disseminate through scientific repositories and admit its copying and distribution for non-commercial, educational or research purposes, as long as credit is given to the author and editor.

Aos meus pais.

ACKNOWLEDGEMENTS

The conclusion of the present thesis and thus, of this academic chapter of my life, could not be done without my expression of gratitude towards all the people who helped, motivated, and inspired me along the way.

First, I would like to thank my advisers for the vote of confidence, sympathy, availability, and guidance provided in the laboratory and in the preparation of this work. Professor Marta Martins, I thank you for your help since the first email you quickly answered, and Professor Neusa Figueiredo, thank you for all the companionship in the lab and tranquility in times of stress. I also want to thank Professor Paula Ramos, from the School of Agriculture (ISA), who agreed to help introduce the mycotic component in the work.

My sincere gratitude towards the projects μScale and Plastisphere, the Marine and Environmental Sciences Centre (MARE) and the Marine Research and Environmental Risk Lab (MARlab) for providing me with the means and conditions for the elaboration of this work. A special thanks to the Faculty of Pharmacy of Lisbon for the utilization of the AMA, and the "Veríssimo de Almeida" Plant Pathology Laboratory of ISA.

To all MARlab members for their good disposition and help throughout all my work in the laboratory, and a special thanks to my laboratory colleague Sara Pereira, Biochemistry student, for her kindness and mutual help.

A special gratitude to those who made my academic journey at FCT-UNL Campus memorable. Sibele Parreira, thank you for being there, pushing and inspiring me. Thank you for making bad moments better. Beatriz Soares, Ricardo Alexandre, Laura Coelho and Andreia Silva, you were all amazing group partners, and life partners as well. Thank you for all the shared moments. Thank you all for the love and support.

Finally, the most heartfelt thanks to all my family in Portugal, Brazil, Spain, and USA. No matter how distant physically, your support was always felt. I love you.

"The task we face today is to understand the language of nature."
(Paul Stamets)

ABSTRACT

Estuaries are heavily impacted by anthropogenic contamination, serving as reservoirs of various pollutants such as heavy metals (e.g., mercury (Hg) and microplastics (MP, Ø <5mm). These pollutants can affect water quality and cause toxic effects in aquatic organisms. Mercury, a trace heavy metal, is characterized as neurotoxic, persistent, and bioaccumulative in the food chain. In systems contaminated by Hg, microorganisms are responsible for reducing Hg, contributing to its natural detoxification. However, the effects of the presence of MP in this process are still unknown. The growing concern of microplastics is the chemical additives in their constitution that can be released and their ability to act as vectors of other contaminants, namely Hg. Therefore, the following questions arise 1. The presence of microplastics affects bacterial and fungal-mediated mercury detoxification present in aquatic systems, and how this differs between the two microorganisms, and 2. What are the possible bioremediation strategies. Microbial communities (bacteria and fungi) were isolated from a Hg-contaminated area of the Tagus estuary, and the most resistant strains were selected to assess the effect of the presence of polystyrene (PS) MP (40 mg/L) on the detoxification of Hg. After 24h, 3, and 5 days of incubation for bacterial isolates and 24h, 5 days, and 10 days of incubation for fungal isolates, the following factors were analyzed: Hg detoxification capacity, acute toxicity (Microtox bioassay) of the leachate, and variation of pH. Isolates were identified through the amplification of 16SrRNA and ITS, respectively. The results showed that (i) strains isolated from the Tagus Estuary are resistant to Hg, (ii) fungal isolates are more resistant than bacteria and, consequently, have a greater capacity to detoxify Hg, (ii) the presence of PS MPs affect the availability of Hg and bacteria in the medium. It can be concluded that MPs interact with Hg detoxification processes and, therefore, should be considered in bioremediation strategies.

Keywords: Mercury, Microplastic, Microorganisms, Detoxification, Bioremediation.

RESUMO

Os estuários são fortemente impactados pela contaminação antropogénica, servindo de reservatórios de diversos poluentes tais como metais pesados (ex: mercúrio (Hg) e microplásticos (MP, Ø <5mm). Estes poluentes podem afetar a qualidade da água e causar efeitos tóxicos nos organismos aquáticos. O mercúrio, um metal pesado vestigial, é caracterizado como neurotóxico, persistente e bioacumulativo na cadeia alimentar. Nos sistemas contaminados pelo Hg, os microrganismos são responsáveis pela redução de Hg, contribuindo para a destoxificação natural. No entanto, não se conhecem os efeitos da presença MP neste processo. A crescente preocupação dos microplásticos diz respeito não apenas aos aditivos químicos em sua constituição que podem ser libertados, mas também à sua capacidade de atuar como vetores de outros contaminantes, nomeadamente o Hg. Portanto, surgem as seguintes questões: 1. A presença de microplásticos afeta a desintoxicação do mercúrio mediada por bactérias e fungos presentes nos sistemas aquáticos, e como isso difere entre os dois microrganismos, e 2. Quais são as possíveis estratégias de biorremediação. Comunidades microbianas (bactérias e fungos) foram isoladas de uma área contaminada por Hg do estuário do Tejo, e as estirpes mais resistentes foram selecionadas para avaliar o efeito da presença de poliestireno (PS) MP (40 mg/L) na destoxificação de Hg. Após 24h, 3 e 5 dias de incubação para isolados de bactérias e 24h, 5 dias e 10 dias de incubação para isolados de fungos. foram analisados os seguintes fatores: capacidade de destoxificação de Hg, toxicidade aguda (bioensaio Microtox) do lixiviado e variação de pH. Os isolados foram identificados através da amplificação do 16SrRNA e ITS., respetivamente. Os resultados demonstraram que (i) estirpes isoladas do Estuário do Tejo apresentam resistência ao Hg, (ii) isolados de fungos são mais resistentes do que bactérias e, consequentemente, apresentam maior capacidade de destoxificação de Hg, (ii) a presença de PS MPs afeta a disponibilidade de Hg e de bactérias no meio. Pode-se concluir que os MPs interagem nos processos de destoxificação de Hg e, portanto, devem ser considerados nas estratégias de biorremediação.

Palavras-chave: Mercúrio, Microplástico, Microrganismos, Destoxificação, Biorremediação.

CONTENTS

ACKNOWLEDGEMENTS **9**

ABSTRACT **13**

RESUMO **15**

CONTENTS **17**

LIST OF FIGURES **19**

LIST OF TABLES **21**

ACRONYMS **23**

1. INTRODUCTION **25**

1.1. PROBLEM DEFINITION 25
1.2. AIM 26
1.3. METHODOLOGY 27
1.4. STRUCTURE 27

2. AQUATIC SYSTEMS AND POLLUTION **29**

2.1. GLOBAL SCENARIO 29
2.2. URBAN CYCLE OF WATER 29
2.3. WATER POLLUTION 30
2.3.1. Heavy Metals 31
2.3.2. Mercury 31
2.3.3. Microplastics 33

3. MICROBIOLOGICAL COMMUNITIES **37**

3.1. IMPORTANCE IN ESTUARINE SYSTEMS 37
3.2. BACTERIA ROLE ON MERCURY DETOXIFICATION 38
3.3. FUNGI ROLE ON MERCURY DETOXIFICATION 39

4. BIOREMEDIATION STRATEGIES **41**

4.1. HEAVY METAL BIOREMEDIATION 41
4.1.1. Bacteria-based Heavy Metal bioremediation 42
4.1.2. Fungi-based Heavy Metal bioremediation 43

5. MATERIALS AND METHODS **45**

5.1. STUDY AREA AND SAMPLE COLLECTION 45
5.2. BACTERIAL ISOLATES 46
5.3. FUNGI ISOLATES 47
5.4. MERCURY SUSCEPTIBILITY TESTING 47
5.5. EXPERIMENT SETUP 47
5.6. TOTAL MERCURY ANALYSIS 49
5.7. PH ANALYSIS 49
5.8. ACUTE TOXICITY TEST 49
5.9. PCR ANALYSES 50
5.10. STATISTICAL ANALYSIS 51

6. RESULTS AND DISCUSSION ... **53**
6.1. Hg^{2+}-RESISTANT MICROORGANISMS, ISOLATED FROM THE TAGUS ESTUARY ... 53
6.1.1. Characterization of the Sampled Area ... *53*
6.1.2. Pure Isolated Colonies ... *54*
6.1.3. Mercury resistance levels ... *54*
6.1.4. Identification of Hg^{2+}-resistant strains ... *56*
6.2. EFFECTS OF MPS ON Hg^{2+} DETOXIFICATION MEDIATED BY BACTERIA ... 57
6.2.1. Bacterial-mediated detoxification of Hg-infused medium ... *57*
6.2.2. Effects of MP on the detoxification process ... *58*
6.2.3. Leachate Toxicity ... *59*
6.2.4. Identification of Hg-Resistant Bacteria ... *61*
6.2.5. Overview on the interaction of MPs on mercury's detoxification mediated by bacteria ... *61*
6.3. EFFECTS OF MPS ON Hg^{2+} DETOXIFICATION MEDIATED BY FUNGI ... 63
6.3.1. Fungi-mediated detoxification of Hg-infused medium ... *63*
6.3.2. Effects of MP on the detoxification process ... *64*
6.3.3. Leachate toxicity ... *65*
6.3.4. Overview on the interaction of MPs on mercury's detoxification mediated by fungi ... *68*

7. CONCLUSIONS AND FUTURE DEVELOPMENTS ... **71**

REFERENCES ... **73**

APPENDIX 1 ... **81**

APPENDIX 2 ... **82**

APPENDIX 3 ... **84**

APPENDIX 4 ... **85**

APPENDIX 5 ... **86**

LIST OF FIGURES

FIGURE 2-1. ILLUSTRATION OF MERCURY'S BIOGEOCHEMICAL CYCLE. 32
FIGURE 2-2. THE EFFECTS OF MICROPLASTICS IN THE AQUATIC SYSTEM; THE EXERTION OF TOXICITY ON ORGANISMS, BIOFILM FORMATION, AND ITS IMPLICATIONS (HE ET AL., 2022). 35
FIGURE 5-1. LOCATION OF SAMPLES AREA, BARREIRO ON TAGUS RIVER. 45
FIGURE 5-2. SAMPLING AREA, BARREIRO, SETUBAL. 46
FIGURE 5-3. SCHEME OF SUCCESSIVE DILUTIONS MADE (D1, D2 AND D3) FROM EACH REPLICATE OF WATER SAMPLE (A, B AND C) AND POREWATER SAMPLE (1,2 AND 3). 46
FIGURE 5-4. SCHEME OF THE ASSAY'S SETUP: FOR EACH ISOLATE, TWO CONDITIONS ARE IMPLEMENTED IN DUPLICATE, THAT OF HG^{2+}-INFUSED MEDIUM AND HG^{2+}+MP-INFUSED MEDIUM. FOR THE CONTROL NO INOCULUM IS ADDED. 48
FIGURE 6-1. CHOSEN RESISTANT-BACTERIA (A) ISOLATES AND RESISTANT-FUNGI (B) ISOLATES. 56
FIGURE 6-2. HG^{2+} RELATIVE VARIATION TO THE CONTROL (MEDIUM CONTAINING 1PPM OF HG), IN THE PRESENCE OF BACTERIA STRAINS AFTER 24H OF INCUBATION. (*) INDICATES DIFFERENCES IN RELATION TO HG (24H), ($P<0.05$, FISHER TEST). 57
FIGURE 6-3. MASS BALANCE FOR THE ISOLATES PW2.BL (A) AND PW2.LO (B). 57
FIGURE 6-4. HG^{2+} RELATIVE VARIATION TO THE CONTROL (MEDIUM CONTAINING 1PPM OF HG), IN THE PRESENCE OF BACTERIA ISOLATES AFTER 24 HOURS OF INCUBATION. (*) INDICATES DIFFERENCES IN RELATION TO HG (24H), ($P<0.05$, FISHER TEST). 58
FIGURE 6-5. HG^{2+} RELATIVE VARIATION TO HG:MP INFUSED MEDIUM, IN THE PRESENCE OF BACTERIA ISOLATES AFTER 5 DAYS OF INCUBATION. (*) INDICATE DIFFERENCES IN RELATION TO HG:MP (5 DAYS), ($P<0.05$, FISHER TEST). 58
FIGURE 6-6. THE VARIATION OF PH VALUES IN MEDIA CONTAINING HG, HG:MP, HG:BACTERIA AND HG:MP:BACTERIA, AFTER 3 AND 5 DAYS OF INCUBATION AT ROOM TEMPERATURE. 60
FIGURE 6-7. FUNGI CULTURE IN THE PRESENCE OF HG. THE ISOLATE PW01 WAS TAKEN AS AN EXAMPLE TO SHOW THE FORMATION OF THE SOLID FILM APPARENT ON THE FIFTH DAY OF INCUBATION. 63
FIGURE 6-8. HG2+ RELATIVE VARIATION TO THE CONTROL (MEDIUM CONTAINING 1PPM OF HG), IN THE PRESENCE OF FUNGI ISOLATES, AFTER 10 DAYS OF INCUBATION. (*) INDICATE DIFFERENCES IN RELATION TO THE CONTROL HG ($P<0.05$, FISHER TEST). 64
FIGURE 6-9. HG^{2+} RELATIVE VARIATION TO THE CONTROL,1 PPM OF HG, IN THE PRESENCE OF FUNGI ISOLATES, AFTER 10 DAYS OF INCUBATION. (*) AND (**) INDICATE DIFFERENCES IN RELATION TO THE INITIAL HG (CONTROL), AND IN RELATION TO HG:MP (10 DAYS), RESPECTIVELY ($P<0.05$, FISHER TEST AND *T*-TEST). 65
FIGURE 6-10. VALUES OF PH RELATIVE TO THE MEDIUM, FOR MEDIUM WITH HG AND HG:MP, ON THE FIFTH AND TENTH DAY OF INCUBATION. 67
FIGURE 6-11. PH VALUES RELATIVE TO THE FIFTH DAY OF INCUBATION FOR MEDIUM CONTAINING FUNGI (A) AND RELATIVE TO THE TENTH DAY OF INCUBATION FOR MEDIUM CONTAINING FUNGI (B), FOR HG:FUNGI AND HG:MP:FUNGI MEDIUMS. 67

LIST OF TABLES

TABLE 5-1. PRIMERS, THEIR RESPECTIVE SEQUENCES, AND ANNEALING TEMPERATURES, USED FOR BOTH BACTERIA AND FUNGI DNA AMPLIFICATION. 50
TABLE 6-1. CONCENTRATION OF HG^{2+} FOR THE TWO-SAMPLED MATRIX. THE DATA ARE EXPRESSED AS THE MEAN ± STANDARD DEVIATION OF THREE REPLICATES. 53
TABLE 6-2. NUMBER OF COLONIES PER DILUTION, IN MEDIA WITH AND WITHOUT HG FROM ENVIRONMENTAL SAMPLES (WATER AND POREWATER FROM THE TAGUS RIVER – BARREIRO) AFTER AN INCUBATION PERIOD OF 3 DAYS. THE DATA ARE EXPRESSED AS THE MEAN ± STANDARD DEVIATION OF THREE DILUTIONS. 54
TABLE 6-3. SELECTED COLONIES FOR EACH MICROORGANISM WITH THEIR RESPECTIVE SAMPLE ORIGIN AND DESIGNATION. 54
TABLE 6-4. INORGANIC HG MIC VALUES - FOR BACTERIAL ISOLATES FROM TAGUS ESTUARY. THE DATA ARE EXPRESSED AS THE MEAN ± SD. 55
TABLE 6-5. ACUTE TOXICITY OF MEDIUM LEACHATE CONTAINING HG:MP AND BACTERIA:HG:MP, AFTER 3-5 DAYS OF INCUBATION. PRESENCE OF TOXICITY (RED) AND ABSENCE OF TOXICITY (GREEN) OF LEACHATE. THE RESULTS ARE PRESENTED AS THE VALUE OF EC_{50} FOR THREE DIFFERENT READING TIMES (I5, I15 AND I25). 59
TABLE 6-6. ISOLATES DNA QUANTIFICATION (NG/ML). 61
TABLE 6-7. PERCENTAGE DECREASE OF EACH SAMPLE (HG, HG:MP, HG:ISOLATE AND HG:MP:ISOLATE) ALONG THE 5 DAYS OF INCUBATION. THE RESULTS ARE PRESENTED AS THE AVERAGE FOR THE TWO INDEPENDENT ASSAYS ± SD. 62
TABLE 6-8. HG^{2+} LOST (%), AND THEIR RESPECTIVE STANDARD DEVIATION, FROM 24H TO 10 DAYS OF INCUBATION, IN HG:FUNGI SAMPLES. 64
TABLE 6-9. HG^{2+} LOST (%), AND THEIR RESPECTIVE STANDARD DEVIATION, FROM 24H TO 10 DAYS OF INCUBATION, IN IN HG:MP:FUNGI SAMPLES. 65
TABLE 6-10. ACUTE TOXICITY OF MEDIUM LEACHATE CONTAINING FUNGI:MEDIUM, HG:FUNGI, HG:MP:FUNGI AND HG:MP, AFTER 5-10 DAYS OF INCUBATION. PRESENCE OF TOXICITY (RED), TOTAL OF INHIBITION (ORANGE) AND ABSENCE OF TOXICITY (GREEN) OF LEACHATE. 66
TABLE 6-11. VALUES OF EC_{50}, FOR HG:FUNGI AND HG:MP:FUNGI MEDIUMS, FOR THE LAST DAY OF INCUBATION, AS THE AVERAGE OF THREE DIFFERENT READING TIMES (I5, I15 AND I25) AND ITS RESPECTIVE STANDARD DEVIATION. 66

ACRONYMS

DNA – Deoxyribonucleic acid
ERM – Effects range-median
EPCRA – Emergency Planning and Community Right-to-Know Act
GEM - genetically engineered microorganisms
GPA - Global Programme of Action for the Protection of the Marine Environment from Land-based Activities
Hg – Mercury
Hg^0 – Elemental Mercury
Hg^{2+} – Mercuric Ion
IARC - International Agency for Research on Cancer
MeHg - Methylmercury
MH – Mueller-Hinton
MIC – Minimal Inhibitory Concentration
MP – Microplastic
MSFD - Marine Strategy Framework
PA - polyamide
PAHs - polycyclic aromatic hydrocarbons
PCBs - polychlorinated biphenyls
PCR - Polymerase chain reaction
PDA – Difco Potato Dextrose Agar
PE - polyethylene
PET - polyethylene terephthalate
POPs - persistent organic pollutants
PP - polypropylene
PS - polystyrene
PVC - polyvinyl chloride
rRNA – Ribonucleic acid of ribosomes
WFD - Water Framework Directive
WHO – World Health Organization
YEG – Yeast Extract Glucose

1. INTRODUCTION

1.1. Problem Definition

This dissertation is inserted in the research project "How microplastics shape microbial communities in aquatic systems: implications to environmental risk assessment and bioremediation strategies," part of the project μScale, coordinated by Professor Neusa Figueiredo and developed at Marine and Environmental Sciences Centre, FCT – NOVA (MARE NOVA).

Mercury is a naturally occurring element found in the environment. Although it exists naturally, human activity is the leading cause of mercury emissions, mainly due to artisanal and small-scale mining, stationary combustion of coal, and nonferrous metal production (EPA, n.d.). In the environment, it suffers chemical transformations through its biogeochemical cycle, impacting the toxicity effects upon organisms and ecosystems.

It is labeled a heavy metal due to its relatively high density (Tchounwou et al., 2012). It is classified as a persistent, bioaccumulative, and toxic compound by The Emergency Planning and Community Right-to-Know Act (EPCRA), section 313 (EPCRA, 2001). Furthermore, by the exact definition and under the EU Water Framework Directive (WFD) and its Marine Strategy Framework (MSFD), it is identified as a priority hazardous substance (Tornero & Hanke, 2018).

Being ubiquitous in nature, all organisms are exposed to mercury. Most predominantly, the exposure occurs at low levels, in what is referred to as chronic exposure (continuous or intermittent long-term contact), and a fewer portion is exposed to high levels of mercury, associated with acute exposure (over a short period) (Tchounwou et al., 2012). Regardless of the type of exposure, the health concerns are extensive. Its effects vary following the form of the metal, the kind of exposure, and entry routes in the organism. Mercury exists in the air, water, soil, and sediments and can be found in three forms: elemental (Hg^0), inorganic (mercurous – Hg_2^{2+} and mercuric – Hg^{2+}), and organic such as methylmercury (MeHg) (Teaf M, 2012).

Methylmercury is the most frequently encountered compound of the organic form found in the environment. It is the product of the methylation of inorganic (mercuric) forms of mercury by microorganisms found in soil and water (Tchounwou et al., 2012). It is bioaccumulative, biomagnifying, and neurotoxic to organisms (Selin, 2009).

Therefore, mercury is a matter of international concern as it is considered by WHO to be one of the top ten chemicals, or group of chemicals, of significant public health concern (WHO, 2021). In the European Union, mercury production stopped in 2003, and its exports and other mercury compounds have been banned since 2011. The Minamata Convention of 2013 laid the ground measures to reverse the increasing trend of mercury contamination in the environment and food chain. It was adapted in the EU, in 2018, as the Regulation on Mercury (European Union, 2017). Nevertheless, mercury-containing fuels still cause the unintentional release of emissions (European Commission, 2017).

In contrast to mercury, microplastic (MP) is categorized as an emerging contaminant which for this reason, the potential impacts aren't entirely understood yet. The continuous accumulation of plastic debris in the terrestrial and aquatic systems leads to progressive fragmentation into smaller pieces, forming microplastics. They are defined as fragments of less than five millimeters in diameter, harming the environment and animal health (NOAA, 2021).

Microplastics are primarily comprised of six types of petroleum-based polymers, those with lower density, such as polyethylene (PE), polypropylene (PP), and expanded polystyrene (PS), and those with higher density, including polyvinyl chloride (PVC), polyamide (PA), also known as nylon and polyethylene terephthalate (PET), affecting their deposition in the water column (Lusher et al., 2017). This contaminant results from commercial product development (primary microplastic) and larger plastics (secondary microplastic) breakdown. The extensive longevity of plastic materials contributes to these tiny fragments persisting in the environment (Lam et al., 2018).

Ultimately, plastic debris ends up in the ocean due to storms, wind-induced transport in the atmosphere, and runoffs. Terrestrial sources arise from a range of anthropogenic activities, including the primary microplastic production, terrestrial transportation, agricultural activities, wastewater plants, and maritime sources related to shipping, offshore industrial activities, and fisheries and aquaculture (GESAMP, 2016). Due to its small diameter, microplastics have already been detected in marine organisms from plankton to whales, in commercial seafood, in drinking water, and, more recently, in human lungs (Smith et al., 2018).

The effects of microplastic exposure on human and animal health are continuously studied. Yet, they can act as vectors of various chemical contaminants in the aquatic environment (Fred-Ahmadu et al., 2020). Once ingested, both the MP and the contaminant, acting as a host, can induce adverse effects on aquatic organisms - although evidence is still indefinite - from the subcellular level to the ecosystem level, such as genotoxicity and impaired fecundity (Sıkdokur et al., 2020).

Having the characteristics of abundance, non-degradability, and persistency - due to high stability and durability - plastics raise concerns regarding their adverse impacts on the environment and thus being subject to legal measures to reduce such effects (Lusher et al., 2017).

In general, legislation to prevent further mercury emissions and plastic littering exists and has been taken into action. Nevertheless, these contaminants are actively present in wastewaters and aquatic ecosystems, thus calling for the need of strategies to remediate the already contaminated environments.

1.2. Aim

The present work aims at: i) comparing the ability of bacteria and fungi to detoxify aqueous solution contaminated with mercury and ii) assessing the interaction effects of microplastics on detoxification processes. It is intended that the results obtained from this work contribute to the development of bioremediation strategies and the Environmental Risk Assessment of these environmental pollutants.

The present Masters' dissertation aims to address the following questions:

1. Which fungi and bacterial tolerant species are found in polluted mercury environments?
2. How does the presence of microplastic affect the processes of mercury detoxification?
3. Which bioremediation strategies could be proposed for mercury polluted areas?

1.3. Methodology

Bacteria and fungi will first be isolated from water and porewater samples collected in the Tagus estuary. An initial analysis of bacteria and fungi resistance to mercury through a broth microdilution test to determine the MIC (Minimum Inhibitory Concentration). After this, selected bacteria and fungi will be exposed to 1 mg/L Hg^{2+} and 40 mg/L of microplastics (powdered polystyrene polymer from a single-use disposable coffee cup) for five and ten days, respectively.

After exposure, several analyses will be carried out to i) analyze the variation in mercury concentration in water and porewater samples through an Atomic Absorption Spectrometer; ii) assess toxicity using the Microtox Bioassay (bioluminescence inhibition test of the bacterium *Allivibrio fischeri*), and iii) the most promising microorganisms for Hg detoxification will be identified by 16S rRNA amplification.

All results are presented as the mean ± standard deviation (n>2). Differences between experimental groups were determined by applying the Fisher Test and *t*-test for independent samples, considered significant at p-value < 0,5. Statistics were performed using the software Statistica (Statsoft), following (Zar, 1996). The EC_{50} determination was performed using the straight-line equation obtained by linear regression and its correction factor.

1.4. Structure

The present work is organized into seven chapters:

Chapter 1 addresses this study's framework, aim, and applied methodology.

Chapter 2 describes the state of aquatic systems, providing an overview of the urban water cycle and water pollution, focusing on mercury and microplastic pollution, its sources, pathways, and effects on the ecosystem.

Chapter 3 depicts the microbiological communities present in the aquatic systems, focusing on bacteria and fungi. It further explores these microorganisms' capacity at detoxifying mercury content in the environment.

Chapter 4 provides a synopsis on bioremediation strategies, what it consists of, and their applicability to the explored themes.

Chapter 5 describes the methodology used in this work to determine the interaction of microplastics on mercury detoxification mediated by bacteria and fungi.

Chapter 6 presents the results and discussion. The first sub-chapter outlines the mercury contamination in Tagus Estuary and the obtained Hg-resistant colonies from field samples, their respective characterization, and resistance. Additionally, an identification of the isolates is provided through the PCR method. In the following two sub-chapters, the Hg-detoxification capability of the Hg-resistant isolates is evaluated, followed by the analyses of the interaction of MPs on this through mercury concentration readings and evaluation of leachate's toxicity – acute toxicity and pH variance.

Finally, Chapter 7 presents the conclusions drawn and the future perspectives for mercury detoxification in the context of microplastic pollution and light for bioremediation strategies in this scope. This chapter also provides parameters for further tests to complement the obtained results.

2. AQUATIC SYSTEMS AND POLLUTION

2.1. Global Scenario

Aquatic ecosystems, including freshwater and marine ecosystems, cover the most significant portion of the biosphere (L. Wang & D'Odorico, 2013). Most of the world's water, about 96.3%, resides in the oceans. The fraction of water on which ecosystems and humans directly depend, freshwater in circulation, only accounts for 0.02% of the global water supply, distributed between rivers, lakes, wetlands, soils, and the biosphere (Marshall, 2013).

Aquatic ecosystems are mainly profited for food, transportation, and recreation. Specifically, freshwater ecosystems are exploited for drinking, sanitation, and agricultural and industrial purposes (Häder et al., 2020).

Urbanization represents one of the 21st century's most transformative trends. Currently, about 55% of the world's population resides in urban areas. By 2050, this figure is expected to increase 68%, where most of the predicted increase will be in the world's least developed regions. This trend contributes to both positive and negative impacts on the well-being of humans and the environment. One of the significant adverse impacts of urbanization is the demand for and access to water, sanitation, and rainwater drainage (Kookana et al., 2020).

A growing population and the economic patterns becoming ever more based on resource-intensive consumption leads to increased pressure on the aquatic systems, particularly on the global freshwater use for agricultural, industrial, and municipal purposes. According to the United Nations, water use has been growing globally at more than twice the rate of the population increases in the last century. Water scarcity - the lack of availability due to a physical shortage of appropriate infrastructures to provide a regular supply - already affects every continent (UN, 2021). Yet, this issue will continue to aggravate as anthropogenic activities, along with urban and tourism developments, remain unsustainable, furthermore clashing with the climate change.

2.2. Urban Cycle of Water

The hydrological cycle describes water's perpetual flux and exchange between different global reservoirs: biosphere, atmosphere, lithosphere, and hydrosphere (Marshall, 2013). This already complex yet natural cycle is enhanced by providing water services to the urban population, including water supply, drainage, wastewater collection, and management. Therefore, the concept of an urban water cycle is coined as material and energy fluxes are altered in these contexts of significant anthropogenic influences and interventions.

Generally, water is extracted from surface or groundwater sources and is later returned to a different source at another quality. Since the urban population demands high quantities of energy and raw materials, the consequential removal of waste will be proportionally prominent. Thus, pollution to the receiving water systems is a threat (Marsalek et al., 2014).

Additionally, impermeable surfaces are another variable that alters the hydrological cycle. Urban impermeable surfaces promote the accumulation of pollutants generated from various anthropogenic activities and natural processes in urban environments during dry weather, such as sediment, nutrients, bacteria, metals, and chemicals (Zhang et al., 2021). The kinetic energy of raindrops and water flow promotes the pollutant's wash-off as the accumulated particles are mobilized and transported. This characteristic of water-resistant surfaces represents a significant contributor to the deterioration of the quality of urban receiving waters (Gorgoglione et al., 2019).

2.3. Water Pollution

Wastes, solid and liquid, generated from urban centers and their respective management or lack of it is a leading contributor to the pollution of aquatic ecosystems (Marsalek et al., 2014). Hence, polluted water is when its quality and composition alter either naturally or due to anthropogenic activities, resulting in a resourceless suitable for consumption and uses for which it would have been otherwise in its natural state (Goel, 2006).

The main groups of water contaminants are pathogens, inorganic material - which includes heavy metals - organic material, including suspended solids, nutrients, agriculture pollutants, macroscopic pollutants, thermal and radioactive pollutants (Wasewar et al., 2020). Pollution of water bodies from agricultural, industrial, and municipal discharges, runoffs from urban areas and farmlands, as well as waste disposal and landfill leachate, are considered the primary sources of contamination (Mokarram et al., 2020). These, therefore, endanger the supply of clean drinking water and have negative impacts on marine and freshwater organisms (Häder et al., 2020).

Although developed countries treat their wastewaters, either for reutilization in irrigation or to ensure environmental protection (Kookana et al., 2020), an estimated 80% of municipal and industrial wastewater globally is released without any prior treatment (WWAP, 2021).

It is essential to highlight that both population growth and industrialization contribute, in a synergistic way, to increasing levels of pollution as more energy and resources are consumed as a result of higher economic levels (Goel, 2006).

Pollution sources can be divided into point sources and nonpoint sources. The first includes all identifiable localized sources of contaminants, and the second refers to sources distributed over a wide area and mobile sources (Schweitzer & Noblet, 2018). A typical example of a nonpoint source is urban runoff which represents the sum of many point sources over a wide geographic area. The effects of the various sources of pollution on human's and ecosystem's health vary in the quantity and composition of the pollution.

As a result, there are in place legislative instruments introduced on an international level that aims at creating integrated environmental management of water resources. The primary tool of the European Union's Water Policy is the Water Framework Directive, which establishes a framework for Community action to protect inland surface waters, transitional waters, coastal waters, and groundwater. From this directive, others are created with narrower concerns. The Marine Strategy Framework Directive (MSFD) defines the necessary measures to obtain or maintain good environmental status in the marine environment by 2020. Furthermore, the new EU Biodiversity Strategy for 2030 reinforces marine ecosystems' protection and restoration (European Commission, n.d.).

2.3.1. Heavy Metals

Heavy metals are defined as metallic elements with a relatively high density compared to water. They are considered trace elements, occurring in trace concentrations (ppb range to less than ten ppm) in various environmental matrices.

These exist naturally throughout the earth's crust and can enter ecosystems through weathering and volcanic eruptions. Nevertheless, most environmental contaminations and human exposure occur from anthropogenic activities. The sources of heavy metals in the environment include effluents from geogenic, industrial, agricultural, pharmaceutical, domestic activities, and atmospheric sources. The environmental pollution caused by these is very prominent in point source areas such as mining and other metal-based industrial operations (Tchounwou et al., 2012). Therefore, the primary source is industrial activities, and the dispersal mechanism is runoff, winds, and leaching (Häder et al., 2020).

The bioavailability of these elements in the environment is influenced by physical factors - temperature, phase association, adsorption, and sequestration - chemical factors - lipid solubility and octanol/water partition coefficients - and biological factors such as trophic interactions. (Tchounwou et al., 2012)

Several heavy metals are essential for life as they are critical elements in several vital enzymes and play crucial roles in various oxidation-reduction reactions. Some examples include cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), and manganese (Mn). Nevertheless, in more significant quantities than necessary, these metals can escape control mechanisms in plants and animals, such as homeostasis and binding to essential cell constituents due to their chemical coordination and oxidation-reduction properties. This means they displace original metals from their original binding sites, leading to cell malfunctioning and toxicity. An example of this is an oxidative deterioration of biological macromolecules due to the binding of heavy metals to the DNA and nuclear proteins (Jaishankar et al., 2014).

In contrast, other heavy metals aren't essential nutrients but toxic even in trace amounts. These include lead (Pb), cadmium (Cd), tin (Sn), and mercury (Hg). They produce reactive oxygen species (ROS), causing oxidative stress. Mercury in the inorganic binds to proteins containing thiols (e.g., cysteine) as well as thioethers (e.g., methionine), causing several dramatic changes to the SH group, the key to enzyme activity (Stratton et al., 2016). Furthermore, their metal ions interact with cell components, including the DNA, causing damage and conformational changes that may lead to cell cycle modulation, carcinogenesis, or apoptosis (Tchounwou et al., 2012). Hence, these non-essential heavy metals are a matter of great environmental concern and consequently a public health concern.

2.3.2. Mercury

Mercury, a non-essential heavy metal, is found in nature in three forms: elemental (Hg^0), inorganic mercuric (Hg^{2+}) and mercurous (Hg^{1+}), and organic, such as methylmercury (CH_3Hg^+), each with its degree of toxicity. It is mobilized from the earth's crust to the atmosphere through volcanic and geological activity. It undergoes a natural biogeochemical cycle through atmospheric transport, terrestrial and aquatic systems deposition, and revolatilization (Selin, 2009). Along this cycle, mercury undergoes complex chemical reactions, many of which are not entirely understood (Teaf M, 2012).

The Industrial Revolution and the succeeding rise of fossil fuel economies contributed to increased mercury emissions. Mercury levels are higher in the northern hemisphere due to higher emissions. In contrast, East and Southeast Asia contribute the most to the global mer-

cury release inventory due to its large population associated with industrial and other activities (Huang et al., 2018). Released to the atmosphere, these emissions travel globally, undergoing reactions within its biogeochemical cycle.

Nevertheless, emissions of mercury aren't the only concern. According to Liu et al., 2021, rivers are the primary source of mercury along the world's coasts, and more specifically, ten rivers are responsible for half of the riverine mercury. The Amazon River is at the top of the list, followed by the Ganges in India and Bangladesh and the Yangtze in China. The contributions include atmospheric deposition, anthropogenic sources like gold mining, and to a lesser extent, naturally occurring sources.

Once emitted to the atmosphere, elemental mercury vapor is solubilized and deposited by rain and water (WHO, 1990), entering terrestrial and aquatic systems through the inorganic form (Huang et al., 2018). Subsequently, microbes, such as sulfate-reducing bacteria, methylate the mercury entering the waterways, as seen in Figure 2-1.

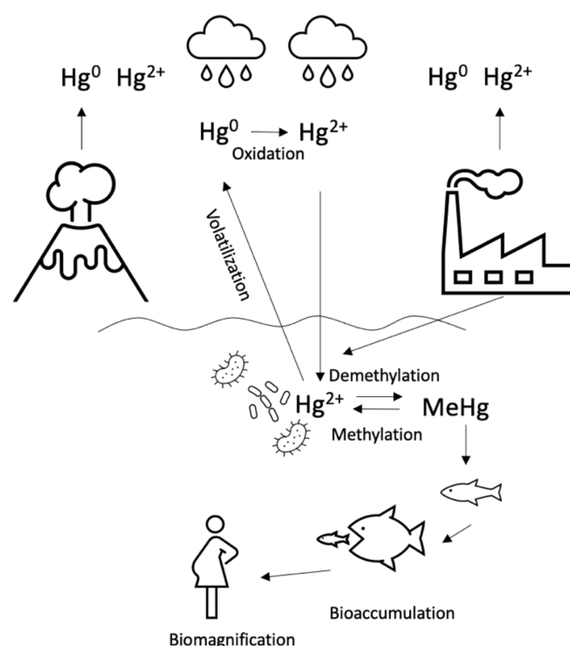

Figure 2-1. Illustration of mercury's biogeochemical cycle.

Estuarine and coastal environments, in particular, due to anthropogenic action, undergo accelerated rates of methylation due to the presence of inorganic mercury, favorable abiotic conditions, including anoxia, high levels of organic material and sulfates, as well as active microbial communities (de Almeida Rodrigues et al., 2019).

The most highly absorbed species are elemental mercury and methylmercury. Methylmercury is highly dangerous as it can cross placental and blood barriers (Teaf M, 2012). This organic form of mercury is toxic due to its bioaccumulation characteristics - accumulation of mercury in the animal tissue - and biomagnification – increase in mercury concentration throughout the trophic levels (Huang et al., 2018). Therefore, this form is the most common threat to human health as it is exposed through the consumption of contaminated fish and shellfish (Tchounwou et al., 2012).

Species that feed at high trophic levels are at an elevated risk for elevated exposure and potential toxicity. It has been determined that high exposure during embryonic stages can reduce foraging and reproductive success, alter sex ratios, cause morphological defects, and increase mortality (van Hees & Ebert, 2017). Fish, piscivorous birds, and mammals have

demonstrated significant behavioral, physiological, immunological, neurochemical, reproductive, and histological changes due to substantial MeHg exposure (Scheuhammer et al., 2007).

Methylmercury is a potent neurotoxin for humans, where the cerebellum and peripheral regions are most affected (WHO, 1990). Additionally, chromosome damage is associated with long-term exposure to methylmercury. IARC, the International Agency for Research on Cancer, has classified methylmercury as possibly carcinogenic to humans (Group 2B), though no quantitative potency is available (Teaf M, 2012).

As mercury pollution poses an environmental issue and a threat to human health, removing heavy metals from urban and industrial wastewaters is necessary. There is a range of techniques, such as chemical precipitation, ion exchange, adsorption - including the use of activated carbon and many waste materials, - membrane filtration, reverse osmosis, solvent extraction, and electrochemical treatment, which imply high operational costs and investment (Wołowiec et al., 2019).

The Minamata Convention of 2013 set the ground terms for reducing mercury pollution globally, which Portugal ratified. On a European level, the European Union's water policy in Portugal was adopted by The Water Framework Directive (Directive 2000/60/EC of the European Parliament and the Council, 23 October 2000). It establishes a framework for community action to protect inland surface waters, transitional waters, coastal waters, and groundwater. This Directive is complemented with "limit values" and "quality objectives" of mercury defined in two other directives, regarding mercury (84/156/CEE) and the discharge of mercury (82/176/CEE). Furthermore, the Decree-Law nº 306/2007, concerning the quality regime for water intended for human consumption, defines a parametric value for mercury of 1 $\mu g/L$ (Decreto-Lei n.º 306/2007 Do Ministério Do Ambiente, Do Ordenamento Do Território e Do Desenvolvimento Regional, 2007).

Legislation must be in place to prevent further release of mercury into the environment. However, the fraction of mercury that is already present and persistent in the environment due to anthropogenic actions also requires remediation methods, which will be addressed in Chapter 4.

2.3.3. Microplastics

In the last decade, the issue of microplastics gained enough attention from the research community. Initially, the matter caused concerns as a subset of the more general problem of marine plastic litter, but it has become mediatic as an environmental contaminant on its own.

Microplastics enter the aquatic environment from diverse sources and pathways. The sources can be divided between terrestrial and maritime. The first includes many activities such as primary microplastic production, agricultural activities, wastewater plants, degradation of plastic litter on beaches. Additionally, it can enter aquatic systems through the atmosphere, coastline, and runoff. Maritime sources are shipping, offshore industrial activities, and fisheries and aquaculture (GESAMP, 2016).

Rivers not only transport plastic from terrestrial to marine ecosystems, but they also efficiently retain part of the nano (< 0.1 μm) - and microplastic (< 5 mm) fraction in river systems. Smaller particles would probably be mobile in freshwater. In contrast, bigger particles at the higher end of this range would be retained efficiently in aquatic sediments, implying a potential risk to sediment-dwelling organisms (Besseling et al., 2017). Nevertheless, the ocean is the ultimate destination of most plastic particles. Microplastics are distributed between the five main ocean compartments - near the ocean surface, water column, seafloor, shoreline, and biota. Consequently, a wide range of marine organisms across all trophic levels

are exposed and contaminated with microplastics, where the main route of exposure is via feeding (GESAMP, 2016).

Microplastics have been reported on several aquatic organisms, as they can aggregate in the digestive system of plankton, crustaceans, fishes, and marine mammals. Furthermore, they can be caught on the surface of seagrass and macroalgal blades, which demonstrates an increase of bioavailability to marine herbivores (Seng et al., 2020), and as a consequence, the potential for microplastics to accumulate in the aquatic food web (Sıkdokur et al., 2020).

Alone, microplastics present toxicity. Their high surface area can lead to oxidative stress (W. Wang et al., 2019), produce neurotoxic, genotoxic, and chronic inflammatory effects in aquatic organisms (Prokić et al., 2019). They can alter reproductive performance (Anbumani & Kakkar, 1999) as energy reserves are disturbed, reducing growth and changing behavior, fecundity, and fertility (Galloway et al., 2017). Removing this pollutant becomes difficult because of its persistent characteristic, resulting in a potentially chronic inflammation that can increase cancer risk (Prata et al., 2020).

In humans, the primary entry route of microplastics is ingestion, followed by inhalation and dermal contact. Ingestion of contaminated food can lead to an estimated intake of 39,000–52,000 microplastic particles person^{-1} year^{-1} (Prata et al., 2020). Particles smaller than 20μm have a potential capacity to penetrate organs. Therefore those with sizes of about 10μm can access all organs, cross cell membranes, cross the blood-brain barrier, and enter the placenta (Campanale et al., 2020), promoting the enhancing plastic-derived contaminants bioavailability (W. Wang et al., 2019).

Recent studies have also found nano and microplastics in sugar, salt, alcohol, and bottles water (Prata et al., 2020). Therefore, it is fair to say that microplastics are ubiquitous in the environment and pose a severe threat to human and animal health.

Polystyrene, for example, in the form of microparticles and nanoparticles, has shown toxicity in animals, humans, and plants. Studies have found that the accumulation of PS in fish can lead to reproduction impairment over time, promoting DNA damage in erythrocytes and brain tissue. In humans, the accumulation of polystyrene in the red blood cells has led to hemolysis and local inflammation. As plants absorb nutrients through the roots, PS accumulation focuses on the root tissue, which can cause a biomass deficit, leading to a blockage in nutrient transportation and affecting seed germination and gene expression (Razi Othman et al., 2021).

The other aspect of microplastics in organisms is the capacity to act as vectors for other contaminants and microorganisms (Prata et al., 2020). Plastics contain several chemicals in their constitution, from a purposeful addition during their manufacture and from adsorbing or absorbing contaminants from the surrounding environment, due to the large surface-volume ratio and hydrophobicity (W. Wang et al., 2019). To provide specific properties to plastics, chemicals are added during their manufacture, including monomers, oligomers, plasticizers, and flame retardants. Then, once in the environment, these materials have the capability of adsorbing or absorbing contaminants, which include a wide range of substances, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs), trace metals, antibiotics and microrganisms. These all have their profile of toxicity and risks (Kedzierski et al., 2018).

Therefore, the toxic effects exerted by microplastics can be divided into two categories: physical effects, through the ingestion of particles, where the risk depends on the number, size, and type of particles, and chemical effects due to the contaminants present in the microplastics, where the risk depends on the duration of exposure, concentration and type of contaminants present and interaction with the organisms (Campanale et al., 2020).

Metals, for example, can adsorb to virgin and beached pellets, where the latter accumulate to a greater extent (Campanale et al., 2020). The toxic effects of simultaneous exposure to microplastics and metals through the water are still a growing scientific research area. A study by Barboza et al., 2018 with the European seabass (*Dicentrarchus labrax*), a common marine fish used for human consumption, demonstrated that microplastics, mercury, and their mixtures (ppb range concentrations) cause neurotoxicity, oxidative stress and damage, and changes in the activities of energy-related enzymes in juveniles of this species. Furthermore, it was noted that microplastics influence the bioaccumulation of mercury.

Hence, microplastics in the aquatic environment, together with the intrinsic (added during manufacture) and extrinsic (adsorbed or absorbed from the environment) contaminants, can be conveyed into the food web and later to humans, as bioaccumulation is likely to be an essential pathway for the introduction of microplastics (W. Wang et al., 2019).

Microplastics can also interact with microorganisms in the food web and the biogeochemical cycles on different environmental matrixes. Communities of microorganisms attach to the plastic particles, which act as a substrate, co-substrate, or carbon source, forming a biofilm and changing microplastic particles' physical characteristics (Rogers et al., 2020). This will be further explored in the upcoming chapters.

Figure 2-2 sums up the journey of microplastics in the ocean and their effects upon aquatic ecosystems through their intrinsic toxicity and extrinsic, where repercussions can travel through trophic levels; the formation of biofilm which promotes changes in the nature of the particle, adding new variables to the existence of these contaminants such as the addition of pathogenic microorganisms or contaminant-resistance microorganisms; and the transport and sedimentation of a microbe and contaminant-enriched particle throughout the ocean.

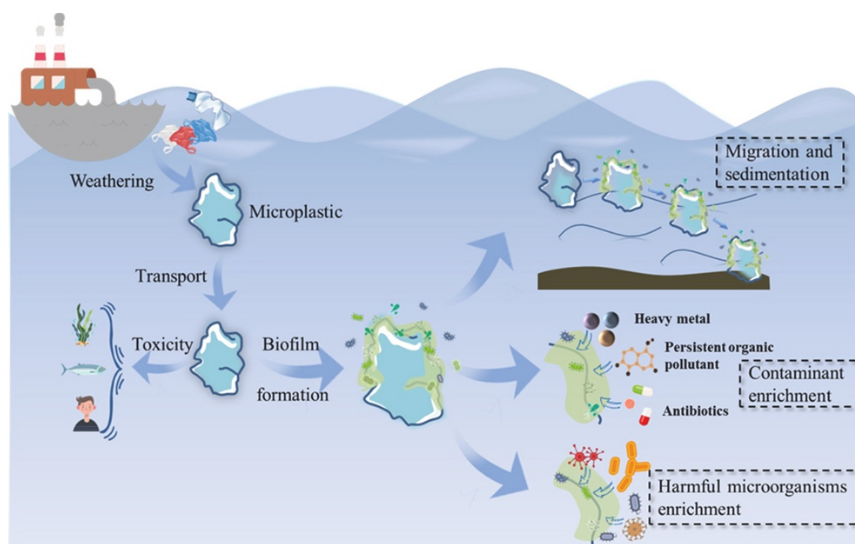

Figure 2-2. The effects of microplastics in the aquatic system; the exertion of toxicity on organisms, biofilm formation, and its implications (He et al., 2022).

Currently, there is no single European law that thoroughly covers microplastics. There are, however, various laws in place with partial objectives and others that target the production and release of these contaminants to the environment. The current legislation covering plastic and microplastic waste takes the form of levies, bans, and voluntary efforts through campaigns of reutilization and reduction (Lam et al., 2018).

On a global scale, the Global Programme of Action for the Protection of the Marine Environment from Land-based Activities (GPA), 1995, sets the base for the efforts on national

and local levels (Galgani & Pinto Da Costa, 2020). Based on the GPA, governments have created legislation that tackles marine pollution, such as legislation prohibiting the manufacture of microbeads in Canada, June 2017, and in the USA, through the Microbead-Free Water Act 2015 (Lam et al., 2018). At the local level, actions are directed towards larger plastics and plastic bags through taxation. In Portugal, a plastic bag tax was implemented in 2015 and consequently reduced 74% consumption (Martinho et al., 2017). However, for microplastics, there have been comparatively fewer initiatives developed at the local level (Galgani & Pinto Da Costa, 2020), and like mercury, remediation strategies for contaminated environments are still lacking.

3. MICROBIOLOGICAL COMMUNITIES

In aquatic environments, the activities of microorganisms range from the micro-level, through community dynamics, to large-scale environmental effects, as they play a pivotal role in the remineralization and restoration of nutrients, such as nitrogen, phosphorus, and sulfur, in aquatic systems through acting within the biogeochemical cycles. Additionally, they serve as decomposers, consumers, primary producers, and secondary producers, thus playing a vital role in the food web (Joshi, 2016). This shows how microorganisms are influenced by their surrounding factors and how they can exert their effects on their surroundings (Sigee, 2005).

Microbial communities aren´t constant. The succession of seasons leads to a difference in tides and precipitation and, subsequently, the content of dissolved oxygen on the surface of the sediment. These factors affect microbial adaptation, which contributes to their change of composition patterns. For industrialized and urbanized areas located near estuaries, the spatiotemporal behavior of microbial communities acts as indicators of water quality which can lead to ecological and human implications. Therefore, they serve as essential indicators of aquatic health. (Yi et al., 2020).

3.1. Importance in Estuarine Systems

Aquatic systems, primarily divided into freshwater and marine environments, provide rich habitats for microorganisms, even though they differ in many factors such as salinity, average temperature, depth, and nutrient content.

The estuarine environment - a transition zone between continent and ocean – faces constant changes due to different natural processes such as freshwater and marine inputs, terrestrial runoff, and anthropogenic impacts. Such variability contributes to incredible biodiversity in microbial communities in intertidal sediments compared to aquatic ecosystems (Yi et al., 2020).

In contrast, these environments are often polluted with toxic metals and organic chemicals as they are prone to anthropogenic activities. Estuaries act as buffers between the continent and ocean in urban and industrial areas by removing pollutants and filtering sediments through biochemical activities before reaching the ocean (Oyetibo et al., 2017).

The constant presence of pollutants, such as heavy metals, in estuaries, contributes to the advancement of microorganisms that can reduce and detoxify these pollutants, heavy metal-resistant microorganisms that perform their metabolic activity in the presence of the metals, and microorganisms, which are inactive in the presence of heavy-metals and only perform their metabolic activities once these have been reduced through extracellular processes (Oyetibo et al., 2017).

These traits are majorly developed in transposons and plasmids and are described as mechanisms of resistance or tolerance, which have significant interest for biotechnological strategies for polluted environments (Endo et al., 2002).

The mass introduction of plastic into aquatic environments also leads to interactions between microorganisms and microplastics through biofilm formation. To a further extent, the *plastisphere* - termed coined to the ecosystem that develops surrounding the surface of plastic in aquatic environments (Zettler et al., 2013).

This biofilm formation occurs through four stages: adsorption of dissolved organic molecules, attachment of bacterial cells, branch of unicellular eukaryotes, and attachment of larvae and spores (Lobelle & Cunliffe, 2011). When the cells attach, they can produce extracellular polymers to form structured and complex matrixes and alter microplastics' characteristics. The microbial composition of biofilm is diverse, including heterotrophs, autotrophs, predators, symbionts, and opportunistic predators. This shows that plastic, in this case, microplastics, can provide an ecological habitat in aquatic systems (Zettler et al., 2013).

Organisms such as diatoms, coccolithophores, bryozoans, barnacles, dinoflagellates, isopods, cyanobacteria, heterotrophic bacteria, and fungi have been cataloged on plastic debris. It is argued that symbiotic relations, rather than competitive prevail, form a cohesive structure (Rogers et al., 2020).

3.2. Bacteria role on mercury detoxification

Bacteria are prokaryotic unicellular organisms belonging to the Bacteria domain. Their genetic information is contained in a nucleoid in the cytoplasm, in a single strand of DNA. Some bacteria contain extra circular molecules and DNA, denominated plasmids. These include genes that might comprise beneficial information compared to other bacteria, such as resistance to a substance such as heavy metals (Parker, 2001).

Mercury polluted areas lead to organisms' constant exposure to mercurial compounds, which has enabled microbial communities, such as bacteria, to develop various types of tolerance and resistance mechanisms to defy the adverse effects of mercury-mediated toxicity.

These mechanisms include: blocking of the toxic ion from entering the cell, ejection of the metal ion from the cell, intracellular sequestration by binding proteins, extracellular sequestration by polysaccharides on the cell wall, and enzymatic conversion of the metal to less toxic or volatile forms (De et al., 2008). This last mechanism, which calls for the enzymatic reduction to less toxic forms involving Hg^{2+} reduction and MeHg demethylation, is also called detoxification due to the mer operon (Figueiredo, 2016).

The mer operon, the best well-known detoxification mechanism, confers mercury resistance to microorganisms, and it is found amongst mercury-resistant bacterial populations. It is a cluster of genes that vary in structure and constitute genes that encode functional proteins for the transport of mercury ions into the cell (*mer*T and *mer*P) and the transformation of mercuric forms to less toxic ones (*mer*A and *mer*B) (Mathema et al., 2011) which in turn the expression of these genes is controlled regulated by trans-acting activator-repressor protein encoded by *mer*R (Nucifora et al., 1989).

The *mer* operon can also be classified into two types of *mer* determinants - the narrow spectrum - confers resistance only to inorganic mercurial forms - and the broad spectrum - confers resistance to both inorganic and organic mercurial forms (Figueiredo, 2016). The narrow spectrum involves enzymatic reduction of Hg^{2+} to the volatile Hg^0 through mercuric reductase, encoded by *mer*A. In contrast, the broad spectrum involves additional enzymatic hydrolysis through the organomercurial lyse to cleave the $Hg\text{-}CH_3$, releasing Hg^{2+} (substrate for mercuric reductase) and reducing the organic components as methyl and phenyl to methane or benzene (Nucifora et al., 1989). The *mer* operon is usually located in transposons, inserted

in chromosomal DNA or in plasmids, which are bacterial mobile genetic elements (De et al., 2008) that occur in Gram-positive and Gram-negative bacteria.

Therefore, mercury-resistant bacteria possessing *mer* operon have considerable potential for bioremediation strategies (Figueiredo, 2016).

3.3. Fungi role on mercury detoxification

Belonging to the Eukarya domain, fungi are unicellular or multicellular and have a nucleus that stores genetic information. Fungi are spore-barring heterotrophs and contain a rigid cell wall made of chitin. These heterotrophic organisms release digestive enzymes by exocytosis to break down macro and organic molecules into more minor compounds to absorb them. Under aerobic conditions, they release CO_2 and H_2O, and under anaerobic conditions, the output is CH_4 if mineralization of the substrate occurs (Sánchez, 2020).

Unlike bacteria, information regarding the resistance mechanism in fungi is limited, impairing their application for bioremediation of Hg-contaminated environments. Nevertheless, fungi are considered essential bio-resources for bioremediation due to their large biomass, strong viability, and simple nutritional requirements (Chang et al., 2020). Furthermore, they have developed an extraordinary ability to adapt to changing environments, particularly those contaminated with pollutants. They can break down and use these pollutants to grow or make their chemical components available to other microorganisms. Their ability to resist and counteract pollutants includes intracellular and extracellular mechanisms. The enzymatic system - mediated by the cytochrome P450 family (CYP), in which fungi possess more diverse CYP families than animals, plants, and bacteria - is the principal intracellular mechanism. Regarding the extracellular process, the ability of adsorption and accumulation by the cell of substances can be regarded as a universal detoxification mechanism found in fungi (Sánchez, 2020).

Some scientific data has been reported on the isolation of resistant fungi to heavy metals and the subsequent use of their biomass to remove heavy metals from industrial wastewater and contaminated water. Regarding mercury, the fungi *Rhodotorula mucilaginosa* showed a capacity for Hg removal of 95.39 % after 48h, where metal sorption was directly proportional to the pH, as metal precipitation product of hydroxides formation, depends on the chemical interactions of metal ions with the biosorbent, which in turn depends on the pH and ion concentration (Grujić et al., 2017).

The fungus *Aspergillus niger* demonstrated a capacity of mercury removal of 83.2% (Acosta-Rodríguez et al., 2018), where both carboxyl and amine groups play a role in the heavy metal biosorption, as well as chitin and chitosan in chelating metal ions (Kapoor & Viraraghavan, 1997). Another study, conducted by Chang et al., 2020, verified that *Penicillium spp.* DC-F11 mechanism towards mercury detoxification, through volatilization, was mediated by the mer-mediated detoxification systems, thiol compound metabolism, and the oxidative stress defense and damage repair metabolism, which are the primary cellular adaptive responses of DC-F11 to Hg (II) stress. This demonstrates the variety and potential of application in the remediation of heavy metal-polluted environments of these fungi.

4. BIOREMEDIATION STRATEGIES

The rich and complex diversity of microorganisms leads to a world of possible applications in industry, as they produce valuable byproducts, agriculture, confer soil health maintenance, and environmental scope through bioremediation of soil and water from pollutants. Moreover, the enzymes produced by microbes also have a tremendous range and potential of applications in many areas, such as in textile, paper pulp, pharmaceuticals, and food industries (Joshi, 2016).

Bacteria and fungi possess genes for most elements that determine the transport systems for the uptake of needed nutrients. Furthermore, it allows for the equilibrium of intracellular concentrations and detoxification or even elimination of toxic elements such as Hg, Pb, As, Cr, Cd, and Ag (Umrania, 2006). This genetic information poses a great potential for environmental measurements, such as bioremediation.

Bioremediation can be viewed as a treatment for polluted areas. It is a process where harmful and dangerous substances are transformed into less or non-hazardous ones. This is done through microorganisms, like bacteria and fungi, capable of digesting and breaking down such matters (L. Kumar & Bharadvaja, 2019). They mineralize the contaminants to end-products such as carbon dioxide and water or metabolic intermediates, used as primary substrates for cell growth. Microorganisms are capable of two-way defense, producing degradative enzymes for the target pollutants and the resistance to relevant heavy metals (Dixit et al., 2015).

Bioremediation can be classified by the biological agents used and the location of treatment, whether *ex-situ* or *in-situ*, which poses different advantages and disadvantages (L. Kumar & Bharadvaja, 2019). Aerobic and anaerobic microorganisms are responsible for this process. Despite having different nutritional requirements, the basic needs are organic carbon and energy for both life forms. Under favorable conditions, successful microbial bioremediation is achieved when microbes interact within their niche (P. Kumar et al., 2019).

4.1. Heavy Metal Bioremediation

Bioremediation of heavy metals is more attractive than physicochemical methods as it can have lower costs and higher efficiency at low metal concentrations (De et al., 2008). However, it depends on the active metabolizing capabilities of microorganisms (Dixit et al., 2015).

Unlike organic pollutants, metals cannot be degraded into harmless forms like carbon dioxide and water but are degraded into more minor toxic forms. Microorganisms are already able to uptake heavy metals actively - through bioaccumulation - or passively - through adsorption (Dixit et al., 2015). Therefore, remediation processes depend on the presence of microbes with the appropriate enzymes and a range of other external factors. Those used in bioremediation processes should have acquired mercury-resistance mechanisms, such as the uptake and reflux of contaminating metals, their bioabsorption, intracellular assimilation, immobilization, complexion, precipitation, and release (Dash & Das, 2012).

There is a range of mechanisms capable of heavy metal bioremediation. All these mechanisms occur naturally, whereas their manipulation depends on bioremediation purposes.

Examples include biosorption, metal-microbe interactions, bioaccumulation, biomineralization, biotransformation, and bioleaching.

In bioleaching, for example, microorganisms solubilize metal oxides and sulfides. In biomineralization, the synthesis of minerals and their precipitation occurs through redox reactions, metabolic activities, and the action of enzymes (Rahman & Singh, 2020).

Another type of bioremediation is through genetically engineered microorganisms (GEM). Genetic information in microbes is altered, using recombinant DNA technology to generate a character-specific efficient strain for bioremediation of soil, water, and activated sludge. This can be applied *in* situ. These GEM also act as "microbial biosensors" to measure the degree of contamination in contaminated sites quickly and accurately (Dixit et al., 2015).

Biofilters, an example of *ex situ* application, involve a high-quality setup for detoxifying or removing heavy metals through columns with immobilized microbes (single or multiple populations, which may develop biofilms) attached to an inert surface, on porous carrier materials (Rahman & Singh, 2020).

Biosorption, another bioremediation method, arises due to the ability of certain microorganisms to bind and concentrate heavy metals from even the most diluted aqueous solutions through their metabolism or physio-chemical pathways of uptake. This presents a technically feasible and economically attractive alternative (Ismail & Moustafa, 2016). This process is influenced by many factors such as pH, temperature, and contact time. Yet, microorganisms as biosorbent materials provide a high surface-to-volume ratio and allow for selective removal of substances under various physicochemical properties, adsorption, and desorption (Oyewole et al., 2019). Furthermore, as these microbes will need constant nutrients for their active uptake of heavy metals, increasing the biological oxygen demand or chemical oxygen demand in the polluted environment, it seems more realistic for extensive scale application (Dixit et al., 2015).

4.1.1. Bacteria-based Heavy Metal bioremediation

Bacteria-based bioremediation requires the appropriate bacterial strain that contains the necessary genetic information. This can be obtained directly from contaminated areas as bacteria develop mechanisms to adapt to these contaminants; they are termed mercury-resistant bacteria. Their population is directly proportional to mercury contamination in the environment.

A wide range of Gram-positive and Gram-negative bacteria have a unique way of resisting and subsequently transforming the toxic forms of mercury into nontoxic forms. As mentioned before, the microbial pathway encoded by the *mer* operon is the most efficient and specific enzymatic reduction of mercury, from ions to a water-insoluble metallic form, meaning that Hg^{2+} is reduced to Hg^0, passively diffusing out of the cell without energy expenditure. Thus, the bacterial biomass can act as a catalyst without accumulating a large volume of contaminated biomass (Dash & Das, 2012).

Several studies have reported the removal of mercury by mercury-resistant bacteria and the mechanisms involved. Giovanella et al., 2017 showed that the isolate *Pseudomonas sp.* B50D presented reduction, biosorption, biofilm production, and siderophore production as its metal resistance mechanisms. The presence of the *mer*A gene indicated the possibility of mercury being removed by volatilization. It verified removal of 62% to 95%, which reflects the potential for implementation in bioremediation strategies.

Compared with the other group of microbes, bacteria have shown the most promising and efficient mechanism for the adsorption of metals. This can be a result of more extensive research directed to bacteria. Nevertheless, marine bacteria have successfully documented,

such as *Pseudomonas aeruginosa* and *Bacillus thuringiensis13*, *Lysinibacillussp*, *Bacillus cereus*, *Kocuriarosea Microbacteriumoxydans*, *Serratiamarcescens*, and *Achrobactriumsp* for the biosorption of heavy metals (Saranya et al., 2019).

Regarding genetically GEM, bacteria like *Escherichia coli* and *Moreaxella sp*. expressing phytochelatin 20 on the cell surface have been shown to accumulate 25 times more Hg than the wild-type strains (Dixit et al., 2015).

4.1.2. Fungi-based Heavy Metal bioremediation

The shift from using conventional methods to remove heavy metals from environment matrixes to biosorption includes fungi as great biosorbent candidates. Fungi have an excellent metal-binding capacity as their high percentage of cell-wall material confer a variety of functional groups, they are easy to cultivate at a large scale due to their short multiplication cycle, and they can be quickly grown using unsophisticated fermentation techniques and inexpensive growth media.

The mechanisms of biosorption through fungi involve a stoichiometric interaction between the metal and the reactive chemical groups in the cell wall, which offers several active sites capable of binding metal ions, followed by an inorganic deposition of increased amounts of heavy metals.

Based on the location where the metal removed from the solution is found, biosorption can be classified as 1. cell-surface sorption – due to a physicochemical interaction between the metal and the cell surface's functional groups, - 2. intracellular accumulation, as a result of the transport of metal across the cell membrane, and 3. precipitation/extracellular accumulation taking place both in the solution and on the cell surface.

Many species have shown the capacity to biosorb heavy metals, such as *Rhizopus arrhizus*, regarding Cr, Mn, Cu, Zn, Cd, Pb, Hg, U, and Ag; *A. oryzae* and *Penicillium spinulosum* regarding Cu, Cd and Zn and *Saccharomyces cerevisiae* regarding Cd, Cu, Th and U are some of the examples (Dhankhar & Hooda, 2011).

Fungi such as *Klebsiella oxytoca*, *Allescheriella sp*., *Stachybotrys sp*., *Phlebia sp*. *Pleurotus pulmonarius*, *Botryosphaeria rhodina* present metal-binding potential, which further indicates the potential for biosorption (Kelly et al., 2006).

Hg-resistant fungi (*Hymenoscyphus ericae*, *Neocosmospora vasinfecta*, and *Verticillum terrestre*) can biotransform a Hg (II) state to a non-toxic form. Furthermore, fungi of the genera *Penicillium*, *Aspergillus*, and *Rhizopus* have demonstrated potential acting as biocatalysts in transforming heavy metals into more minor toxic compounds (Dixit et al., 2015).

5. MATERIALS AND METHODS

5.1. Study Area and Sample Collection

Environmental samples were collected at Barreiro (Lat.: 38º70'45.40''N; Long.: -9 º1'40.51''W), Tagus Estuary, known to be highly contaminated with mercury (Figueiredo, 2016; LNEC, 2008) (Figure 5-1).

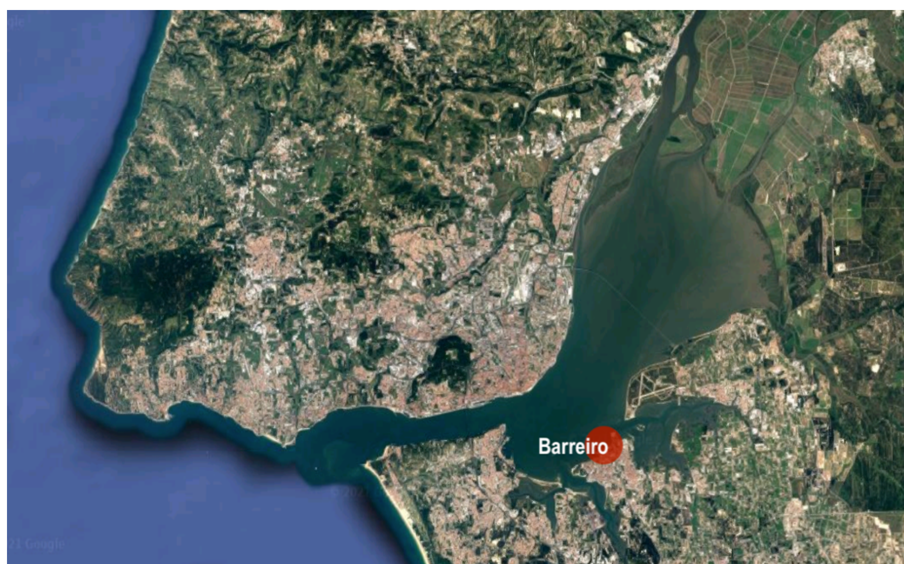

Figure 5-1. Location of samples area, Barreiro on Tagus River.

Barreiro, a city located in the district of Setubal, is characterized by having an industrial area by the Tagus Estuary. Since the sixties, this industrial park operated intensely in its chemical, metal-mechanical, and textile activities, contributing to high pollution by mercury, even after its deactivation. Values of Hg higher than 15 nmol/g are classified as contaminated or highly contaminated material, and the area of Barreiro accounted for a concentration of 246 nmol/g in 2005 (LNEC, 2008).

Three samples of water and sediments were taken at the shoreline (Figure 5-2) in May (late spring), during low tide. The sediment core was sampled in the intertidal zone.

Figure 5-2. Sampling area, Barreiro, Setubal.

Water and sediment samples were collected into sterile centrifuge tubes (50 mL). In the laboratory, sediments were centrifuged at 12 rpm for 2 minutes, and the porewater was collected.

These water and porewater samples were used to prepare 18 inoculums for the isolation of microbial communities. Triplicates of each sample were prepared by mixing 1 mL of samples with 9 mL of distilled sterile water, resulting in three successive dilutions of 1:10 (Figure 5-3).

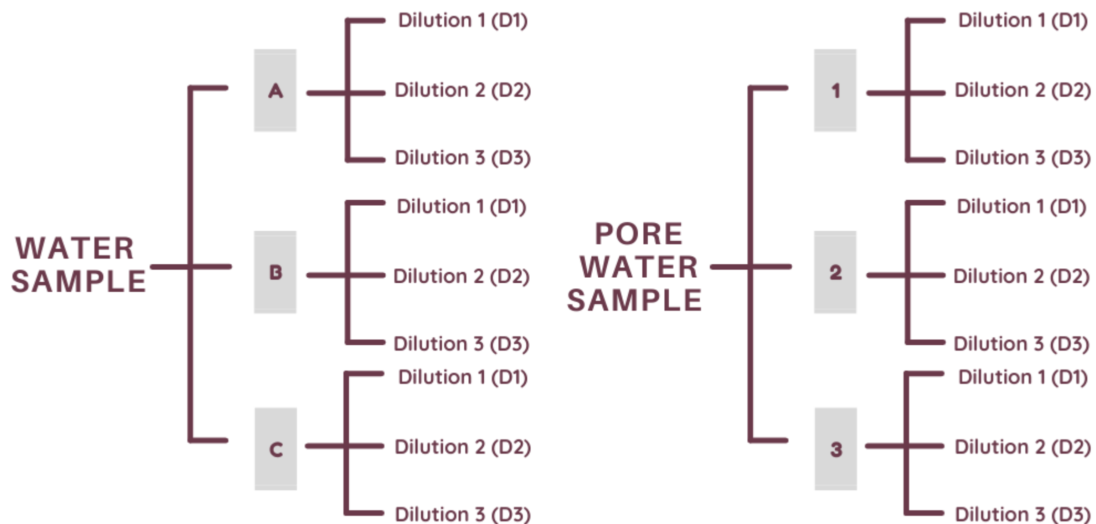

Figure 5-3. Scheme of successive dilutions made (D1, D2 and D3) from each replicate of water sample (A, B and C) and porewater sample (1,2 and 3).

5.2. Bacterial Isolates

For bacteria isolation, 500 µL of each diluted sample was taken and spread into 6 plates with a Mueller-Hinton (MH) agar media with and without Hg^{2+} selective pressure in a concentration of 1 ppm. This resulted in 12 plates left to incubate at room temperature.

After three days of growth, the colonies were characterized according to their qualitative characteristics, such as color, size, and texture. Afterward, they were isolated into a new plate with a selected pressure concentration of 1 ppm of Hg^{2+} to produce a pure culture. A total of 12 colonies were isolated and left to grow at room temperature.

5.3. Fungi Isolates

For fungi isolation, 500 μL of each diluted sample was taken and spread into a Difco Potato Dextrose Agar (PDA) media – previously prepared with 15 mg/L of Streptomycin Sulfate and 50 mg/L of Chloramphenicol – without and with Hg selective pressure in a concentration of 1 ppm. This resulted in 18 plates with selective pressure and 18 plates without selective pressure. These plates were placed in an incubator at 25 ^{o}C.

5.4. Mercury Susceptibility Testing

The Hg-resistance levels of the isolates to Hg were evaluated by determining MIC values, which represent the minimum concentration of mercury for which the isolates' growth is inhibited. This assay was done through the broth microdilution method in a 96-plate to provide a concrete range of limits of resistance to mercury. It is a microbiology standardized technique established by the Clinical and Laboratory Standards Institute.

Regarding the bacteria isolates, these were transferred to a liquid medium, of MH, with a selective pressure of Hg of 1 ppm, where they were incubated, at room temperature, for 24h. Following this, the absorbance at 630 nm of each was read to adjust the number of cells to $1x10^6$ CFU/mL.

Two mercury solutions were prepared, using different concentrations of 200 ppm and 100 ppm. To the 96-plate, 0.1 mL of the isolate solution in liquid medium was added to each well. Afterward, the same 0.1 mL of mercury solution, 100 and 200 ppm, was added to the first well of each row and a diluted, 1:2, along the line, leaving the last well as a positive control. This resulted in dilutions starting with 50 ppm and 100 ppm, with final dilutions of 0.0 ppm and 0.1 ppm, respectively. The plates were left to incubate, at room temperature, sheltered from light, for 24 hours.

The same procedure was used for the fungi isolates, except for the counting and adjustment of the number of spores. Each isolate was a spore suspension on Yeast Extract Glucose (YEG) media, 0.2% de YE + 1% glucose. The number of spores was counted following the hemacytometer method and adjusted to $1x10^6$ spores/mL.

The minimum inhibitory concentration was determined visually, - by the unaided eye - and corresponds to the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells, that is, that shows no turbidity.

5.5. Experiment Setup

As this study aims at understanding the correlation between microorganisms capable of detoxifying mercury and the effects of microplastics on this, it was set up an assay with two conditions, in duplicate, per Hg-resistant isolate (determined by the MIC assay):

i. Isolate + Hg (1 ppm)
ii. Isolate + Hg (1 ppm) + MP (40 mg/L)

Two controls were also set up, in duplicate:

i. Hg (1 ppm)
ii. Hg (1 ppm) + MP (40 mg/L)

It was defined as a concentration of 1 ppm of inorganic mercury to maintain the selective pressure in mediums containing Hg-resistant isolates. The aim is to assess the interaction of microplastics on the detoxification strategies upon the resistance mechanisms of these isolates. The 40 mg/L of microplastics concentration was determined based on previous literature (Figueiredo, 2016).

For fungi isolates, an additional third condition was set up, in duplicate, including only the isolate in the medium (YEG + sterilized river water), to act as control as a verification for the production of mycotoxins.

The MP used was from disposable polystyrene (PS) coffee cup, ground into fine particles (<1mm). Polystyrene is a synthetic aromatic hydrocarbon polymer, with the chemical formula C_8H_8, made from the monomer styrene, a liquid hydrocarbon, petroleum-based. This polymer is hard, rigid, solid at room temperature, and transparent, making it appropriate for food and packaging industries (Razi Othman et al., 2021). Furthermore, the hydrophobic characteristic confers resistance to hydrolysis, promoting forces of attraction between microorganisms and the substrate, thus enabling microorganisms to attach themselves to the polymer's surface (Yuan et al., 2017) and to move from water to organic, hydrocarbon phase, where biosurfactants, - extracellular surface-active compounds, with hydrophilic and hydrophobic groups, able to interact at interfaces and reduce the surface tension (Thavasi, 2011) - and enzymes are released, allowing for degradation of the polymer (Krasowska & Sigler, 2014).

This assay was set up in erlenmeyers of 100 mL, each containing one-part media and one-part sterilized river water to recreate the environmental conditions, along with the necessary nutrients to experiment. Figure 5-4 demonstrates the assay's setup.

Bacteria isolates were inoculated from a liquid suspension in MH medium with 1 ppm of Hg selective pressure after 24h of incubation. The number of cells for inoculation was adjusted to $1x10^6$ CFU/mL through the absorbance reading.

The fungi isolates were also inoculated from a spore suspension in a liquid YEG medium with 1 ppm of Hg selective pressure after 48h of incubation. Using a hemacytometer, the number of spores was adjusted to $1x10^6$ spores/mL.

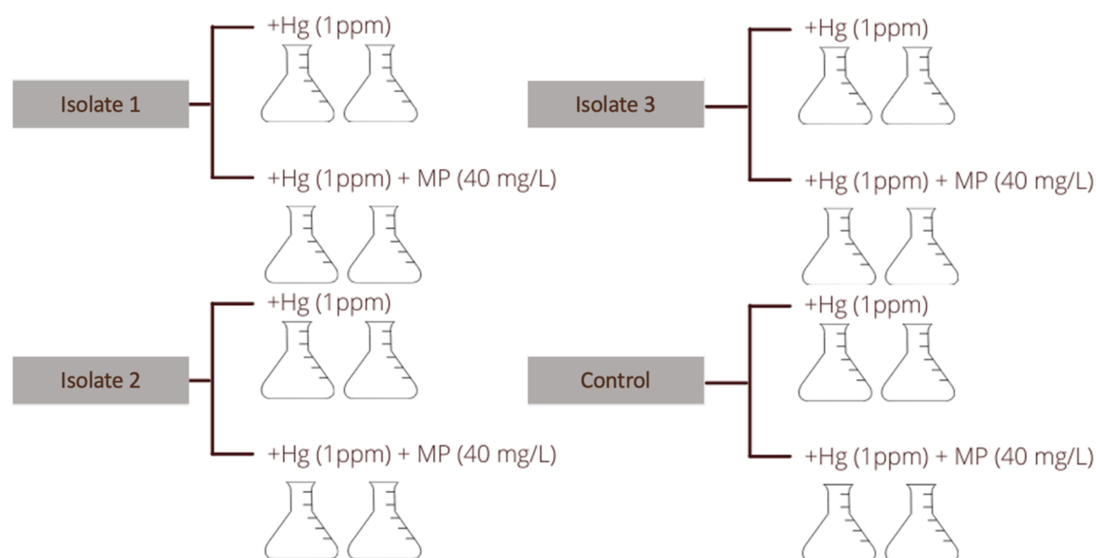

Figure 5-4. Scheme of the assay's setup: for each isolate, two conditions are implemented in duplicate, that of Hg^{2+}-infused medium and Hg^{2+}+MP-infused medium. For the control no inoculum is added.

Three-time points were defined, for sampling, considering the four phases of microbial growth – the lag phase, the log phase (first sampling point), the stationary phase (second sampling point), and the death phase (third sampling point).

Due to the difference in time growth for each test microorganism, the three-time points for bacteria (24h, 3 days, and 5 days) and fungi (24h, 5 days, and 10 days) differed. Therefore, for each time point, the following was sampled:

1. At the log phase, 1 mL of each treatment was sampled and centrifugated (12 rpm for 2 minutes). The collected supernatant and pellet were stored at -80 ^{o}C.
    a. For fungi isolates in Hg:MP:Fungi medium, 5 units of MP were collected and stored into an appropriate vessel from the DNeasy Power Water Kit, at -80 ^{o}C.
2. At the stationary phase, 5 mL of each treatment were sampled for pH analysis, and from this:
    a. 1 mL sampled, centrifugated (12 rpm for 2 minutes), and both supernatant and pellet stored at -80 ^{o}C.
    b. 3 mL sampled, centrifugated (12 rpm for 2 minutes), and supernatant stored at -4 ^{o}C.
3. At the death phase, 5 mL of each treatment were sampled for pH analysis, and from this:
    a. 1 mL sampled, centrifugated (12 rpm for 2 minutes), and both supernatant and pellet stored at -80 ^{o}C.
    b. 3 mL sampled, centrifugated (12 rpm for 2 minutes), and supernatant stored at -4 ^{o}C. From the pellet, for bacteria isolates in Hg:MP:Bacteria medium, 25 units of MP were collected and stored into an appropriate vessel from the DNeasy Power Water Kit, at -80 ^{o}C.

Medium samples collected from each treatment after the three-time points described previously were stored for total mercury, sample toxicity, pH analysis, and species identification.

5.6. Total Mercury Analysis

Total mercury was analyzed using an Atomic Absorption Spectrometer, model AMA 254 Mercury Analyzer (without sample pre-treatment or sample preconcentration). Mineral water was used for blank readings, and all readings were performed in duplicate. Data was collected in ppm and expressed in relative Hg^{2+} to the control of 1 ppm and % of Hg^{2+}.

5.7. pH Analysis

The pH of each sample was read by a benchtop pH/mV/ºC meter, pHenomenal ® pH 1100L, calibrated using two buffers of 4 and 7 units of pH. Readings were performed in duplicate. Data is expressed in relative units of pH to the control (culture medium and medium:isolates).

5.8. Acute Toxicity Test

The toxicity of each sample collected during the assay was evaluated by an in vitro acute toxicity bioassay (Microtox Bioassay), which uses the reduction of bioluminescence of the bacterium *Allivibrio fischeri* as a measure of samples toxicity. The analyses were carried out using

a Microtox Model 500 Toxicity Analyzer System (Azur Environmental Carlsbad, CA), and the procedure was in accordance with the model's software.

The toxicity of the leachate on *Allivibrio fischeri* was evaluated by reading the bioluminescence after incubation for 5, 15, and 25 min. Negative control was used. EC50 value was obtained using the straight-line equation obtained by linear regression and its correction factor. The toxicity was determined by considering the proportion of leachate (0, 0.13, 0.25, 0.5, and 1) and the effect percentage. The presence of toxicity in the leachate is considered when at least one reading results in the EC_{50} below 1.

5.9. PCR Analyses

Bacterial and fungi DNA was extracted through pure colonies and from units of MPs collected from each replicate of each isolate, using the DNeasy Power Water Kit. The procedure performed was in accordance with the protocol provided by the kit. The samples were then read by a NanoDrop spectrophotometer which quantified, in ng/ul and assessed the purity of the DNA present.

For the identification of the isolates, the 16SrRNA gene was amplified. This gene encodes for a small subunit of the ribosomal RNA molecules of ribosomes, which are responsible for converting genetic messages to functional cell components through the translation of mRNA to proteins (Byrne et al., 2018). The sequencing of the 16SrRNA gene is frequently used to provide genus and species identification for isolates. Several studies have demonstrated the success of the 16SrRNA gene sequencing in providing genus identification in most cases, and to a lesser extent, regarding species identification (Janda & Abbott, 2007).

The amplification was done through the Polymerase Chain Reaction method, using the NZY Supreme qPCR Green Master Mix, a premixed solution containing the recombinant modified form of Taq DNA polymerase with a hot start like PCR capacity. The PCR was executed following NZYTech's standard protocol. The primer used is presented in Table 5-1.

Table 5-1. Primers, their respective sequences, and annealing temperatures, used for both bacteria and fungi DNA amplification.

Isolate	Primer	Sequence	Annealing Temperature (^{0}C)
Bacteria	16SV1V3	F: 5- GAG TTT GAT CNT GGC TCA G -3 R: 5- GTN TTA CNG CGG CKG CTG -3	58
Fungi	ITS1ITS4	F: 5- TCC GTA GGT GAA CCT GCG G -3 R: 5- TCC TCC GCT TAT TGA TAT GC -3	61

The verification of the amplification after the PCR was made through an agarose gel electrophoresis – 2% gel (2g of agarose to 200 mL of TAE) – running for 60 min at 110 V. Afterwards, the gel was placed in a solution of ethidium bromide (EtBr) with a final concentration of approximately 0.2-0.5 μg/mL, for 20 min, as the EtBr binds to the DNA and allows to visualize the DNA under ultraviolet (UV) light.

Once verified the presence of bands, confirming the amplification, the DNA purification was done following the NZY Gelpure Kit's procedure, a protocol for PCR clean-up. After this procedure, once more a verification of DNA presence was made through electrophoresis. The amplified DNA than was sequenced by STAB-Vida (Lisbon, Portugal), using the specific primers for amplification. Finally, the obtained sequences were crossed with The National Center for Biotechnology Information database, through the programme FinchTV, to obtain a match

for the genus (≥ 70%) and a match for the species (≥ 95%) which was additionally complemented with existing literature for a further verification of the isolate's identity.

5.10. Statistical Analysis

Results in figures are presented as average ± standard deviation (n >2). Differences between experimental groups were determined applying the Fisher Test and t-test for independent samples, considered significant at p-value < 0,5. Statistics were performed using the software Statistica (Statsoft), following (Zar, 1996).

6. RESULTS AND DISCUSSION

6.1. Hg^{2+}-Resistant Microorganisms, Isolated from the Tagus Estuary

6.1.1. Characterization of the Sampled Area

The Tagus Estuary has been documented to be highly affected by toxic metals as a consequence of industrial and urban effluents (Figueiredo, 2016). A study by Chainho et al., (2008), classified 30% of sampled areas from the Tagus estuary, near industrial areas, as poor/bad status. Furthermore, concentrations of Hg exceeded ERM (effects range-median) value, a measure of toxicity in marine sediment, which therefore indicates occurrence of harmful effects (Long et al., 1995).

The concentration of Hg^{2+} in the initial samples taken from the water and porewater matrix was measured by the AMA, resulting in the values (ppm) presented on Table 6-1.

Table 6-1. Concentration of Hg^{2+} for the two-sampled matrix. The data are expressed as the mean ± standard deviation of three replicates.

Environmental Matrix Sample	Hg^{2+}(ppm) ± SD
H_2O Sample	0.155 ± 0.143
PW Sample	0.130 ± 0.019

Both water and porewater samples, taken during late spring, resulted in similar values. Levels of total mercury concentrations have been previously determined (Canário et al., 2007; Figueiredo, 2006). For sediment samples, a study carried out by Canário et al., (2007) determined the total mercury concentrations in July, - between 0.02 ppm and 49.1 ppm, - and in December, - between 0.04 ppm and 66.7 ppm, in areas with high concentrations, in the vicinity of industrial complexes. Figueiredo, (2016) subsequently quantified total mercury concentrations in sediment from Barreiro to be up to 126 ppm, in the summer. For water samples, Figueiredo et al., (2014) quantified levels of total mercury contamination ranging between $9.00x10^{-7} – 6.80x10^{-6}$ ppm.

Hence, the results obtained, of Hg^{2+} concentration from the two-sample matrix, are higher than those determined by Figueiredo, (2014) and fall within the range determined by Canário et al., (2007), demonstrating to be highly contaminated which concern water samples.

6.1.2. Pure Isolated Colonies

Inoculums of three successive dilutions from the initial water and porewater samples, were spread into plates containing growth media with and without Hg^{2+} selective pressure of 1 ppm. After an incubation period of three days (room temperature for bacteria and at 25°C for fungi), the numbers of colonies were counted, per dilution plate (Table 6-2).

Table 6-2. Number of colonies per dilution, in media with and without Hg from environmental samples (water and porewater from the Tagus River – Barreiro) after an incubation period of 3 days. The data are expressed as the mean ± standard deviation of three dilutions.

Microorganisms	Environmental Matrix	Medium	Medium + Hg
Bacteria	Water	10.0 ± 3.46	16.7 ± 2.08
	Porewater	29.3 ± 10.38	37.7 ± 21.17
Fungi	Water	3.0 ± 2.28	7.0 ± 5.56
	Porewater	4.0 ± 1.15	2.0 ± 1.15

Regarding colonies obtained from field samples, for bacteria, a greater number was quantified for a medium with selective mercury pressure and more colonies from pore water samples. The same was not true in fungi: more colonies were found in a medium with selective pressure from water samples. Results are not statistically significant ($p>0.05$, Fisher test).

A total of 12 different colonies of bacteria, in qualitative terms, was found to grow in media containing 1 ppm of Hg^{2+} after 3 days of incubation. For fungi, 22 different colonies grew in media with selective pressure. Bacteria showed a higher number of colonies, but fungi shower more diversity. Additionally, colonies from porewater samples presented less variety for both microorganisms, which can be attributed to being a more polluted matrix, as mercury can be adsorbed on sediment particles (Gworek et al., 2016).

Besides counting, these colonies were grouped according to their qualitative characteristics. For bacteria, colonies were categorized in terms of color, texture, and/or size (Appendix 1). Fungal colonies were characterized across three universes (water with selective pressure, water without selective pressure, and porewater) using a code name based on the qualitative characteristics of each fungus (Appendix 2). From this characterization, 3 colonies of bacteria and 3 colonies of fungi were selected, based on their predominance across samples and/or dilutions (Table 6-3).

Table 6-3. Selected colonies for each microorganism with their respective sample origin and designation.

Sample	Bacterial Strains	Fungi Strains
Porewater	PW2.BL PW2.LO	PW01
Water	B.RL	AGH01 AGH03

6.1.3. Mercury resistance levels

The MIC values for the 4 selected bacterial strains and 3 selected fungi strains ranged from 0.8 to 25 ppm and 37.5 to 50 ppm, respectively (Table 6-4). The highest values - 25ppm and 50ppm - were exhibited by PW2.BL and PW01, both bacterial and fungi strains isolated from pore water samples. In general, the pore water isolates showed higher MIC values when compared with those from water samples (Table 6-3).

Table 6-4. Inorganic Hg MIC values - for bacterial isolates from Tagus estuary. The data are expressed as the mean ± SD.

Microorganism	Isolate	MIC values (ppm) ± SD
Bacteria	PW2.BL	25.0 ± 0.0
	PW2.LO	17.2 ± 6.5
	B.RL	6.3 ± 0.0
	A.AG	0.8 ± 0.0
Fungi	PW01	50.0 ± 0.0
	AGH01	37.5 ± 13.4
	AGH03	37.5 ± 13.4

The isolate A.AG was excluded for the subsequent assays due to its very low resistance value and kept the remaining six due to a higher resistance capability and compared different sources of samples (water and porewater).

Overall, these results show that bacteria and fungi with high Hg-resistance exist in the pore water of Barreiro (Tagus estuary). This can result from historical Hg contamination in this location (Figueiredo et al., 2014, 2016). Previous studies conducted by Figueiredo (2016) reported the existence of highly resistant bacteria, which were isolated from sediment samples of Barreiro (Tagus estuary) (MIC values ranging from 0.16 to 140 ppm). To the best of our knowledge, no study reported the isolation of fungi displaying from Tagus estuary. However, samples from a historic Hg-contaminated area, a former chloralkaline plant in Romania, originated fungi isolate that exhibited very high MIC values for Hg, ranging from 140 to 200 mg/L. These included *Cladosporium* sp., *Didymella glomerata*, *Fusarium oxysporum*, *Phoma costaricensis*, and *Sarocladium kiliense* (Văcar et al., 2021). Another study by Sanjaya et al., 2021 aimed at isolating and characterizing mercury-resistant microbes from a gold mine area in Indonesia. It identified two microbial strains: fungus strain *Cladosporium halotolerans* Hg32 and the bacterial strain *Mycolicibacterium peregrinum* Hg37, with a mercury resistance level of up to 3000 ppm. Therefore, the magnitude of contamination on a determined area influences the response of microbes, translated into high MIC values.

Therefore, due to its greater resistance to Hg, the bacterial and fungal community isolated (Figure 6-1) was used in the following assays to assess their mercury reduction potential and the influence of MP in this process.

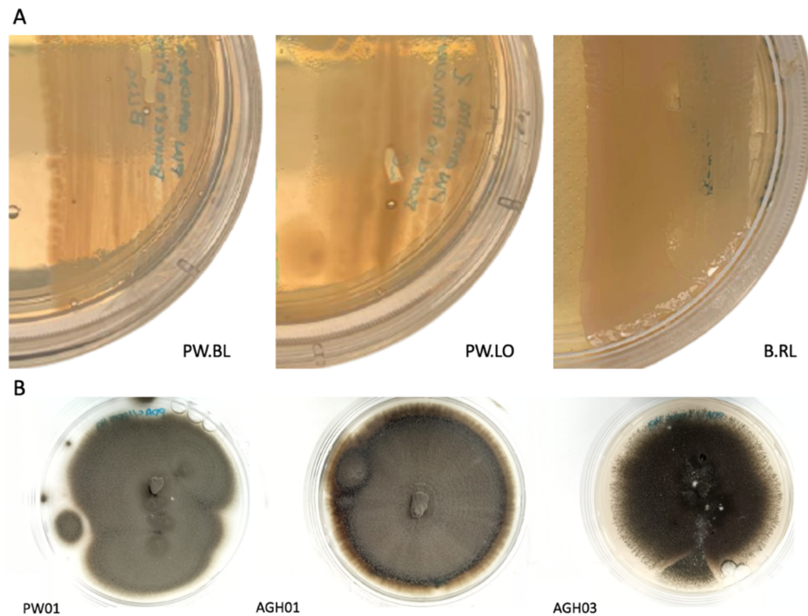

Figure 6-1. Chosen resistant-bacteria (A) isolates and resistant-fungi (B) isolates.

Regarding the bacteria isolates, B.RL lost its ability to grow in media containing Hg after a while. This can be explained through the plasmid's loss. In bacteria, mercury reduction with subsequent volatilization is mediated by mercuric reductase, whose genes are often located in a plasmid (Olson et al., 1979). Therefore, the loss of plasmids leads to the loss of genetic resistance information.

6.1.4. Identification of Hg^{2+}-resistant strains

The selected bacterial and fungi isolates selected for this study were identified through 16S rRNA and ITS amplification. The obtained sequences (Appendix 5) were crossed with the National Center for Biotechnology Information's (NCBI) databases. It was regarded matches superior to 95% for identifying species, and the chosen genus was supported by literature review, mainly regarding the environmental source and use.

All bacterial three strains, B.RL, PW2.BL and PW2.LO corresponds to the genus *Pseudomonas* with a 100% and 99% identity. Regarding the species, the possible matches include *Pseudomonas chengduensis* (Tao et al., 2014) (MK583559.1) and *Pseudomonas pseudoalcaligenes* (Manso Cobos et al., 2015; Estepa, 2016) (DQ071558.1) with 100% match and *Pseudomonas alcaliphila* (Yumoto et al., 2001) (EU082832.1) *Pseudomonas oleovorans* (Manickam et al., 2008) (MF612156.1) with 99% match.

Regarding fungi strains: PW01 is from the genus *Cladosporium* (Sanjaya et al., 2021; Văcar et al., 2021), and the possible species' match, both with 99%, include *Cladosporium pseudocladosporioides* (MT582794.1) and *Cladosporium cladosporioides* - (MT466517.1).

To confirm the species' match further, it would be necessary to draw its primer and proceed with its amplification to obtain a match. Furthermore, biochemical tests would complement the verification.

6.2. Effects of MPs on Hg^{2+} Detoxification mediated by Bacteria

To evaluate the bacterial-mediated detoxification of medium containing Hg and the subsequent effect of MP on this process, a combination of Hg, MP, and each bacteria isolates (Hg:MP, Hg:PW2.BL, Hg:MP:PW2.BL, Hg:PW2.LO, Hg:MP:PW2.LO) was incubated up to 5 days. Samples were taken after 1, 3, and 5 days to evaluate bacterial detoxification potential and the effect of MP on these processes.

6.2.1. Bacterial-mediated detoxification of Hg-infused medium

Total Hg concentration was determined in the supernatant and pellet fraction to evaluate bacterial-mediated detoxification of medium containing Hg. Figure 6-2 shows the results of Hg concentration in the supernatant, which represents the culture medium, and Figure 6-3 shows the mass balance with the pellet fraction.

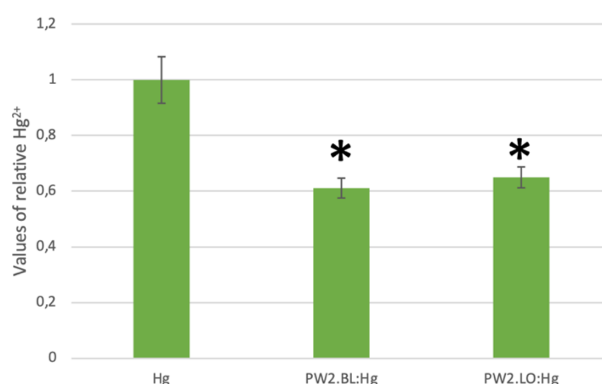

Figure 6-2. Hg^{2+} relative variation to the control (medium containing 1ppm of Hg), in the presence of bacteria strains after 24h of incubation. (*) indicates differences in relation to Hg (24h), ($p<0.05$, Fisher test).

On the first 24h of incubation, the medium containing Hg:Bacteria (PW2.BL and PW2.LO) showed a clear decrease in Hg concentration. Concerning the control, the results from PW2.BL and PW2.LO are statistically significant (p-value < 0.05). This Hg concentration decrease can be related to the detoxification potential displayed by such highly Hg-resistant bacteria. These results are in good agreement with the aerobic bacteria-mediate reduction and subsequent volatilization of Hg reported by Figueiredo et al. (2016).

Considering the mass balance, it can be observed that 57 and 38 % of Hg are missing on the first 24h from PW2.BL and PW2.LO strains, respectively. This reduction is a consequence of the resistance capability of the isolates, which in turn is validated by the MIC values. Therefore, the reduction of Hg^{2+} justifies the resistance (Figueiredo, 2016).

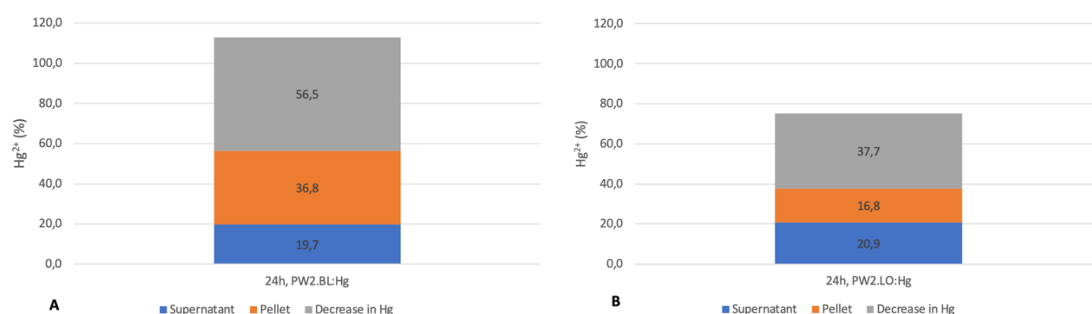

Figure 6-3. Mass balance for the isolates PW2.BL (A) and PW2.LO (B).

Mercury concentration measured in the supernatant represents soluble mercury in the media, while mercury content in the pellet illustrates accumulated mercury in the cells. Greater mercury content was analyzed in PW2.BL's pellet fraction, rather than in the supernatant, reflects bacteria's heavy metal detoxification. In contrast, the presence of mercury in PW2.LO's pellet was similar to that of the supernatant, reflecting a greater percentage of mercury left to be detoxified. Therefore, PW2.BL reduced more Hg^{2+} than PW2.LO.

6.2.2. Effects of MP on the detoxification process

The concentration of Hg^{2+} in the supernatant medium was determined after 24h and 5 days of incubation from samples containing Hg:MP, Hg:MP:Bacteria. Figures 6-4 and 6-5 show the 24h and 5 days reading results, respectively.

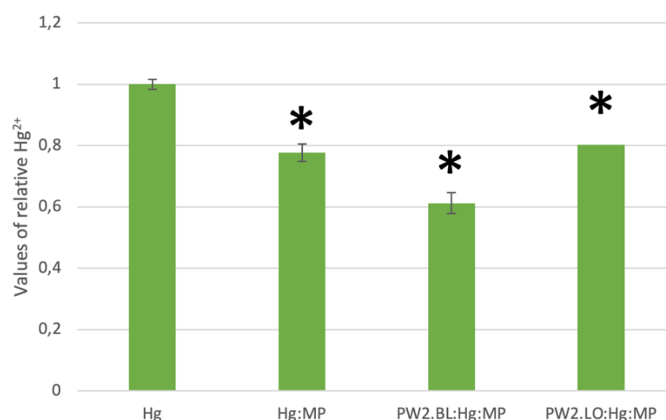

Figure 6-4. Hg^{2+} relative variation to the control (medium containing 1ppm of Hg), in the presence of bacteria isolates after 24 hours of incubation. (*) indicates differences in relation to Hg (24h), ($p<0.05$, Fisher test).

After 24h of incubation time, the medium containing the isolate PW2.LO showed Hg concentration slightly higher than the Hg:MP sample, which can be attributed to mercury's association with the MPs, leading to less available mercury in the medium. Adsorption of Hg to MPs has already been described by Barboza et al., (2018) and Oliveira et al., (2018).

In relation to the control, the results are all statistically significant (p-value < 0.05). Between samples (Hg:MP with PW2.BL:Hg:MP, and PW2.BL:Hg:MP with PW2.LO:Hg:MP), results are all statistically significant (p-value < 0.05).

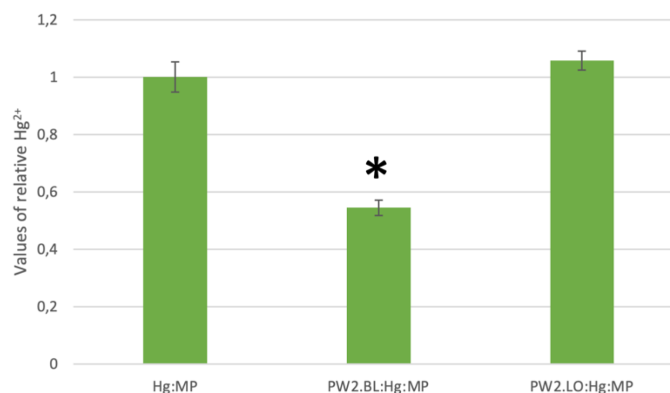

Figure 6-5. Hg^{2+} relative variation to Hg:MP infused medium, in the presence of bacteria isolates after 5 days of incubation. (*) indicate differences in relation to Hg:MP (5 days), ($p<0.05$, Fisher test).

On the fifth day of incubation, compared to the sample containing Hg:MP, Hg:MP:PW2.BL showed a decrease and Hg:MP:PW2.LO, no significant difference. Therefore, the difference between isolates in Hg:MP medium is significant.

In relation to Hg's initial concentration, after 5 days, on this last day of incubation, the decrease in Hg from the medium is less significant from PW2.BL and PW2.LO strains. The isolate PW2.LO saw a bigger decrease than PW2.BL on the 5th day, but along the time PW2.BL reduced more mercury than PW2.LO.

This dynamic change can be explained by bacteria death, releasing mercury to the medium (Figueiredo et al., 2016), or Hg dissociation from MP (Oliveira et al., 2018). The second isolate's poorer reduction can be attributed to mercury's association with the MPs or biofilm formation. Several authors have already documented this biofilm formation on the plastic surface (Yang et al., 2020).

On the fifth day of incubation, the Hg concentration of the medium decreases, as previously mentioned. This supports the decrease in mercury detoxification by bacteria. Therefore, the presence of PS MPs influences the availability of Hg in the medium, and consequently, the detoxification process, as it can act both as a vector for mercury and bacteria through the formation of biofilm.

6.2.3. Leachate Toxicity

To evaluate the toxicity associated with the resulting leachate, samples were taken from the supernatant to assess pH variation and perform the Microtox acute toxicity test. The toxicity was determined by considering the proportion of leachate (0, 0.13, 0.25, 0.5, and 1) and the EC_{50} value.

6.2.3.1. Acute toxicity

The leachate proportion was considered toxic when at least one reading time (5min, 15min and 25min) resulted in the EC_{50} below 1. The results are shown in the Table 6-5.

Table 6-5. Acute toxicity of medium leachate containing Hg:MP and Bacteria:Hg:MP, after 3-5 days of incubation. Presence of toxicity (red) and absence of toxicity (green) of leachate. The results are presented as the value of EC_{50} for three different reading times (I5, I15 and I25).

Isolate	Incubation Time	Acute Toxicity	EC_{50} (leachate proportion)
Hg:MP	3 days	Toxicity	I5= 0.427; I15= 0.551; I25= 0.537
	5 days	No toxicity	
PW2.BL:Hg:MP	3 days	Toxicity	I5= 0.433; I15= 0.142
	5 days	No toxicity	
PW2.LO:Hg:MP	3 days	Toxicity	I5= 0.728; I15= 0.627
	5 days	Toxicity	I25= 0.771

Hg^{2+} expresses a toxic effect between the proportions of 0.25 and 0.13 with an EC_{50} value on the first 5min reading of 0.223. This indicates the great and immediate toxicity of the heavy metal.

The toxicity of Hg:MP decreased within the samples of three days of incubation, as EC_{50} values throughout reading times increased and decreased on the fifth day of incubation. This can result from the mercury's association with the available MPs (Oliveira et al., 2018).

The same is seen for the samples containing the strain PW2.BL in which the EC_{50} value was given only for the third day of incubation. Isolate PW2. However, LO resulted in toxicity for both incubation times with lower toxicity as the EC_{50} value was closer to 1. The result given to this latter can also be corroborated by the Hg readings (Figure 6.2-4), as PW2.LO:Hg:MP demonstrated higher values than the sample Hg:MP, suggesting that MPs act as vectors of bacteria, and by doing so, more mercury is left to detoxify in the medium, contributing to the toxicity presented in Table 6.2-1.

Therefore, in general, the toxicity was PW2.BL (average EC_{50} of 0.287 ± 0.206 leachate proportion) > Hg:MP (EC_{50} of 0.505 ± 0.068) > PW2.LO (EC_{50} of 0.709 ± 0.074), with the control of Hg^{2+} leachate showing greater toxicity. Furthermore, the presence of MP affects the environment's toxicity by acting as a vector for mercury and bacteria, thus contributing to either mercury being unavailable from the medium or bacteria being unavailable in the medium for mercury's detoxification.

The difference in Hg concentration values between the samples Hg:MP (5th day of incubation) and PW2.LO:Hg:MP (5th day) isn't significant, as shown in Figure 6.2-4. Consequently, the toxicity present on the second sample can also be due to the release of other contaminants or additives from the MPs, which must be verified.

6.2.3.2. Variation of pH

The pH plays a crucial role in the microbial population, enzyme activity, and rate of degradation, thus being a key factor for the activity and survival of microorganisms (Auta et al., 2018).

The presence of Hg^{2+} on the medium (MH+H_2O) doesn't significantly alter the pH; between the third and fifth day of incubation, the pH increases about 0,03 units of pH. With the introduction of MPs, in contrast, occurs a slight decrease between both incubation periods, that of 0.02 units of pH. Results in relation to the control are not statistically significant.

Regarding the isolates (Figures 6-6), both in the presence of only Hg^{2+} and Hg^{2+}+MPs, the pH suffers a slight increase from the third to the fifth day of incubation compared to that of the medium. Overall, in the presence of the bacteria strains, the pH is basic, ranging around 8 units of pH, about one pH unit higher than that of the medium. Differences between mediums are not statistically significant.

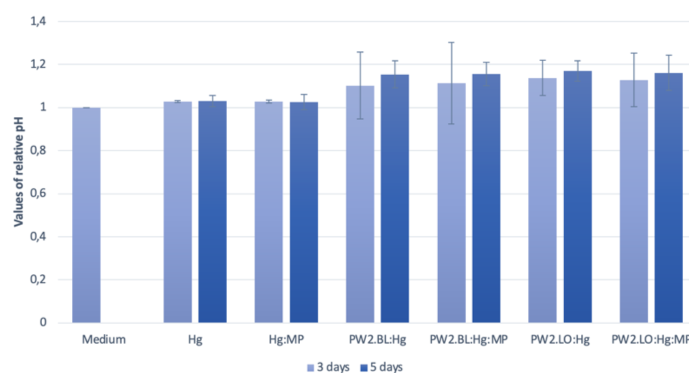

Figure 6-6. The variation of pH values in media containing Hg, Hg:MP, Hg:Bacteria and Hg:MP:Bacteria, after 3 and 5 days of incubation at room temperature.

In a study carried out by Auta et al., (2018), for an incubation period of 40 days, the pH showed a continuous increase, with the maximum pH values of 9.51 and 9.53 for *Bacillus sp.* strain 27 and *Rhodococcus sp.* strain 36, respectively, in a Bushnell Haas medium with 0.5 g PP

polymer. Another study by Habib et al., (2020), with the same incubation period and conditions (BH media infused with PP microplastic), also showed an increase in the pH value; from 7.12 to 8.17 for the strain ADL15 and 7.17 to 7.94 for the strain ADL36. The authors suggested that this increase of pH towards a more alkaline state is related to bacterial exoenzymes' production and accumulation of oligomers and intermediate products (Habib et al., 2020).

Overall, the data obtained here suggest that the presence in the medium of PS MPs (40 mg/L) for samples with Hg^{2+} does not induce changes in pH values. The same happens with samples containing the bacteria isolates, where the addition of MP has no impact on pH values after 5 days of incubation.

A change in the pH value would indicate that the chemical additives present in MPs would be dissociating from it once in suspension. An eventual release of compounds with basic properties would translate into an increase in pH (equivalent to a relative pH value > 1). If compounds with acid content were to be dissociated, the opposite effect would be verified.

6.2.4. Identification of Hg-Resistant Bacteria

The DNA obtained from microplastic units on the fifth day of incubation was quantified by a Nanodrop (Table 6-6) and subsequent sequenced by StabVida (Lisbon, Portugal) resulting in the sequences present in Appendix 5.

Table 6-6. Isolates DNA quantification (ng/µL).

Isolates	DNA (ng/ µL)
PW2.BL	0,70
PW2.LO	2,50
B.RL	1,00

All three isolates, B.RL, PW2.BL and PW2.LO corresponds to the genus *Pseudomonas* with 99% of identity. The possible species' matches, all with 99%, includes: *Pseudomonas alcaliphila*, *Pseudomonas chengduensis*, *Pseudomonas pseudoalcaligenes* and *Pseudomonas indoloxydans* (Manickam et al., 2008) (KP462871.1). These results are in accordance with the ones demonstrated in Chapter 6.4, which suggests the isolates are forming a biofilm on the MPs.

Furthermore, the response is shown in Figure 6.2-4, in relation to the sample Hg:MP:PW2.LO suggests the possibility of MPs acting as vectors of microorganisms. This is backed up by the DNA quantities extracted from units of MPs (Table 6.2-2)—the isolate PW2.LO has more DNA than PW2.BL indicates the presence of more cells attached to the MPs, therefore suggesting biofilm formation. This implies the capacity of MPs acting as vectors for bacteria through the formation of a biofilm, thus making bacteria unavailable to perform Hg-detoxification in the medium.

6.2.5. Overview on the interaction of MPs on mercury's detoxification mediated by bacteria

The isolated Hg-resistant bacteria strains proved to have Hg-detoxification potential. In the first 24h of incubation, an apparent decrease in Hg concentration in respect to the control (medium containing 1ppm of Hg) is verified and statistically significant. PW2.BL displayed the higher drop of mercury in the medium, and the lower was by PW2.LO (Figure 6-2). This is further sustained by the mass balance, which demonstrated less Hg in the medium, left to be detoxified, in the presence of the strain PW2.BL (Figure 6-3).

Furthermore, PW2.BL's pellet fraction contained a higher percentage of Hg, which indicates the bacteria's detoxification as it shows accumulated mercury in the cells. The strain

PW2.LO also demonstrated Hg reduction, however less than PW2.BL, which consequently indicates that PW2.BL reduced more Hg than PW2.LO.

The introduction of MPs in the medium containing Hg (Figure 6-4) indicates the possibility of MPs' interaction in mercury detoxification processes mediated by bacteria. Although Hg:MP:PW2.BL sample still demonstrates a decrease in Hg both for 24h and 5 days of incubation (figures 6-4 and 6-5), the sample Hg:MP:PW2.LO shows a Hg concentration higher than the Hg:MP sample for both incubation periods. This can be attributed to the MPs capacity to act as a vector of (i) mercury, as it is adsorbed into the MPs, and (ii) microorganisms, through the formation of biofilm, which is supported by the quantity of DNA extracted from units of MP (Table 6-5). Thus, MPs contribute to mercury accumulation in the environment by acting as vectors for contaminants and bacteria.

Table 6-7 resumes the overall percentage decrease of the samples after 5 days of incubation. The presence of bacteria isolates in medium contaminated with mercury (Hg:Isolate), compared to the samples Hg and Hg:MP, shows a higher decrease of mercury from the medium, corroborating the argument of Hg-resistant bacteria's detoxification potential of mercury. The same is verified for the samples containing both contaminants and the isolates (Hg:MP:Isolate) except for the strain PW2.LO, which is in agreement with the previous discussion. Overall, it is verified a detoxification potential from the bacteria strains and an effect of the presence of MPs on this potential.

Table 6-7. Percentage decrease of each sample (Hg, Hg:MP, Hg:Isolate and Hg:MP:Isolate) along the 5 days of incubation. The results are presented as the average for the two independent assays ± SD.

Medium	% Decrease of Hg ± SD
Hg	58.5 ± 0.09
Hg:MP	54.2 ± 0.05
PW2.BL:Hg	67.0 ± 0.03
PW2.LO:Hg	59.6 ± 0.03
PW2.BL:Hg:MP	69.0 ± 0.03
PW2.LO:Hg:MP	52.2 ± 0.03

Furthermore, a higher capacity of Hg-detoxification by the isolate PW2.BL produces leachate that loses toxicity on the 10th day of incubation, like the sample Hg:MP (Table 6-5). On the contrary, the isolate PW2.LO's poor reduction of Hg contributed to toxic leachate, both on the third and fifth day of incubation, as the quantity of mercury in the medium was higher than for the sample Hg:MP (figures 6-4 and 6-5). Therefore, the presence of toxicity is hypothesized to be a result of the mercury present in the medium instead of dissociating chemical additives from the MPs since no significant changes were verified in the pH's medium (Figure 6-6).

6.3. Effects of MPs on Hg^{2+} Detoxification mediated by Fungi

To evaluate the fungi-mediated detoxification of medium containing Hg and the subsequent effect of MP on this process, combinations of Hg, MP and each fungi isolates (PW01, AGH01, AGH03, Hg:MP, Hg:PW01, Hg:MP:PW01, Hg:AGH01, Hg:MP:AGH01, Hg:AGH03 and Hg:MP:AGH03) was incubated up to 10 days. Samples was taken after 1, 5 and 10 days to evaluate fungi detoxification potential and the effect of MP on these processes.

6.3.1. Fungi-mediated detoxification of Hg-infused medium

6.3.1.1. Fungi growth in the presence of Hg

For the first 24h of incubation, fungi showed little growth, as expected. Nevertheless, on the fifth, it was verified a formation of a solid film was. The spores also showed a quick attachment to the erlenmeyer's wall, demonstrating the fungi's affinity to substrates (Figure 6-7 and Appendix 3). The same was not verified for the control, where, without the presence of Hg, fungi strains didn't form any type of biofilm (Appendix 4).

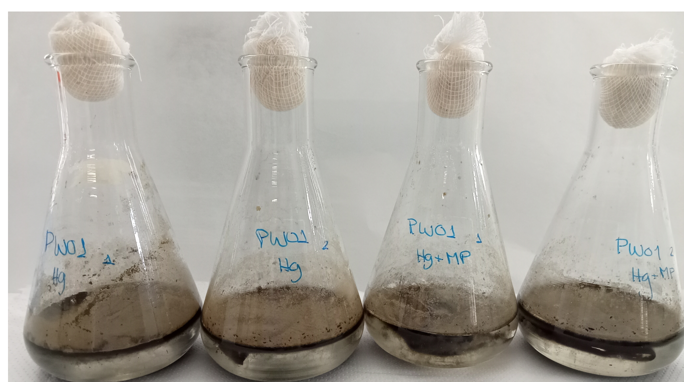

Figure 6-7. Fungi culture in the presence of Hg. The isolate PW01 was taken as an example to show the formation of the solid film apparent on the fifth day of incubation.

The formation of biofilm in the presence of Hg^{2+}, a cell-cell or solid-surface-attached population encased in a self-produced matrix of extracellular polymers (Harrison et al., 2007), can respond to stress raised by the presence of this toxic HM. This can be supported by the study by (Grujić et al., 2017), which concluded that biofilm tolerance is higher than with planktonic cells. Metal exposure also affects the normal process of cellular differentiation during the biofilm development in yeast strains of *Candida*. This process is part of the ecological cycle for many yeasts (Chandra et al., 2001). Furthermore, the location of biofilm formation in the microtiter plate wells changed with Hg^{2+} concentration; lower concentrations (≤0.08 mM) resulted in biofilm growth being surface adherent, and higher concentrations (≥2.5 mM) led to the formation of pellicles at the air-liquid interface.

Furthermore, the isolate AGH03 showed some qualitative difference on the tenth day from the first independent isolate to the second, on the erlenmeyers containing MP (40 mg/L). The supernatant formed an orange coloration, as seen in Appendix 3.

6.3.1.2. Fungi Hg-detoxification potential

Total Hg concentration was determined in the supernatant fraction of medium containing Hg to evaluate fungi-mediated detoxification of the heavy metal. Figure 6-8 shows the results of Hg concentration in the supernatant after 10 days of incubation.

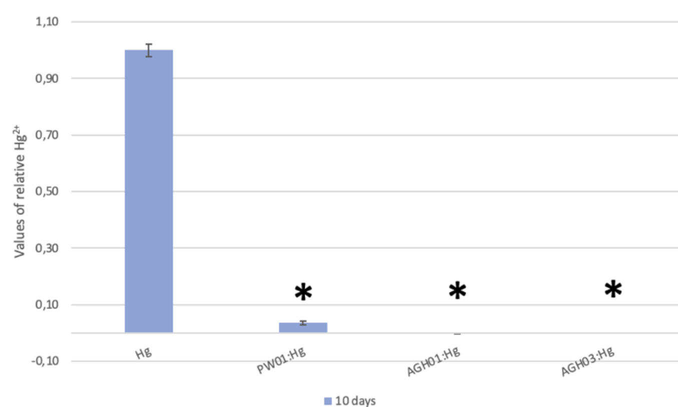

Figure 6-8. Hg2+ relative variation to the control (medium containing 1ppm of Hg), in the presence of fungi isolates, after 10 days of incubation. (*) indicate differences in relation to the control Hg (p<0.05, Fisher test).

On the tenth day of incubation, a significant decrease of Hg concentration occurred for all three Hg:Fungi mediums, where AGH01 showed a higher decrease, followed by AGH03 and PW01. Results, in relation to the control (Hg medium after 10 days of incubation), are all statistically significant (p-values < 0.05).

From the first 24h to the last 10 days of incubation, the overall Hg lost in the supernatant is presented in Table 6-8, where the highest decrease was verified with strains AGH03 and AGH01 (100%) followed by PW01 (99%). Therefore, AGH03 reduced more mercury than the other two strains.

Table 6-8. Hg^{2+} lost (%), and their respective standard deviation, from 24h to 10 days of incubation, in Hg:Fungi samples.

Strain	Hg^{2+} Lost (%) ± SD
PW01+Hg	98,68 ± 0,016
AGH01+Hg	100,00 ± 0,012
AGH03+Hg	100,00 ± 0,013

Generally, fungi have a higher resistant-mercury ability than bacteria. It has been documented that some fungi are able to survive with a MIC of more than 1000 mg/L, where mercury biovolatilization is the major filamentous fungal detoxification mechanism (Sanjaya et al., 2021). These results support the fungi-mediate reduction and subsequent volatilization of Hg reported by Sanjaya et al., (2021) and biosorption reported by Martínez-Juárez et al., (2012). Therefore, this Hg concentration decrease can be related to the detoxification potential displayed by such highly Hg-resistant fungi.

It was not possible to analyze the mercury in the pellet as (i) the presence of MP went against safety procedures of the AMA equipment, and (ii) fungi pellet wasn't collected as they formed a slimy and low-density structure after 24h and a solid structure on the latest day, unable to acquire pellet sample. Therefore, a mass balance wasn’t made.

6.3.2. Effects of MP on the detoxification process

The concentration of Hg^{2+} in the supernatant medium was determined after 24h and 10 days of incubation from samples containing Hg:MP, Hg:MP:Fungi. Figure 6-9 shows the results for 10 days reading.

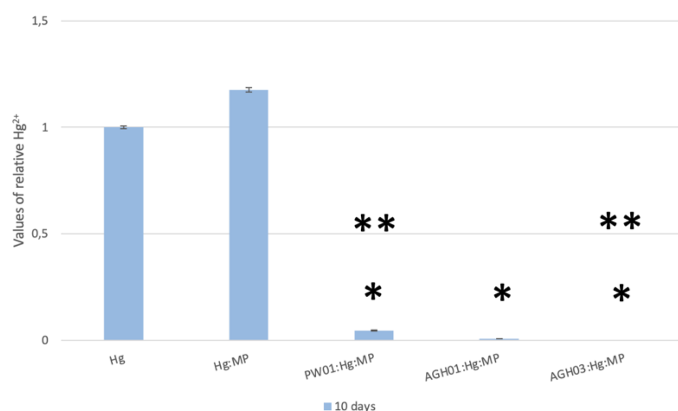

Figure 6-9. Hg^{2+} relative variation to the control,1 ppm of Hg, in the presence of fungi isolates, after 10 days of incubation. (*) and (**) indicate differences in relation to the initial Hg (control), and in relation to Hg:MP (10 days), respectively (p<0.05, Fisher test and *t*-test).

A significant decrease was observed after 10 days of incubation in the following order AGH03 > AGH01 > PW01. The results, in relation to both the control (1 ppm of Hg) and Hg:MP (10 days), are statistically significant (p-values < 0.05). The results are also statistically significant between Fungi:Hg:MP samples for 10 days of incubation (p-value < 0.05). Between Hg and Hg:MP, results are not statistically different.

The overall Hg lost in the supernatant, from the first to the last day of incubation is presented in Table 6-9, where the highest decrease was once again verified with strain AGH03 (100%) followed by AGH01 (99%) and PW01 (89%), which supports the decrease in mercury detoxification by fungi.

Table 6-9. Hg^{2+} lost (%), and their respective standard deviation, from 24h to 10 days of incubation, in in Hg:MP:Fungi samples.

Sample	Hg^{2+} lost (%) ± SD
PW01:Hg:MP	89.12 ± 0.02
AGH01:Hg:MP	99.42 ± 0.02
AGH03:Hg:MP	100.00 ± 0.01

Therefore, AGH03 reduced more mercury than the other two strains. These changes are as significative as in medium without the presence of MPs. The only difference is regarding AGH01 and PW01 response to Hg decrease, as in the presence of MP, they inverted places. Hg's association with the MPs could have influenced this. In general, the reduction of the Hg^{2+} trend was AGH03 > AGH01 > PW01.

6.3.3. Leachate toxicity

The toxicity associated with the resulting leachate was evaluated through samples taken from the supernatant to determine pH variation and perform the Microtox acute toxicity test. The toxicity was determined by considering the proportion of leachate (0, 0.13, 0.25, 0.5, and 1) and the EC_{50} value. The control was the medium containing fungi strains.

6.3.3.1. Acute toxicity

The leachate proportion was considered toxic when at least one reading time (5min, 15min and 25min) resulted in the EC_{50} below 1. The results are shown in the Table 6-10.

Table 6-10. Acute toxicity of medium leachate containing Fungi:Medium, Hg:Fungi, Hg:MP:Fungi and Hg:MP, after 5-10 days of incubation. Presence of toxicity (red), total of inhibition (orange) and absence of toxicity (green) of leachate.

Strain	Incubation Time	Acute Toxicity		
		Medium	Medium:Hg	Medium:Hg:MP
No strain	5 days	-	-	Toxicity
	10 days	-	-	No toxicity
PW01	24h	No toxicity	-	-
	5 days	Total inhibition	-	Toxicity
	10 days	No toxicity	No toxicity	Toxicity
AGH01	24h	No toxicity	-	-
	5 days	Total inhibition	-	Toxicity
	10 days	No toxicity	Toxicity	Toxicity
AGH03	24h	No toxicity	-	-
	5 days	Total inhibition	-	Toxicity
	10 days	No toxicity	Toxicity	Toxicity

The samples containing only fungi isolates in the medium were read for three periods of incubation: 24h, 5 days, and 10 days. Both 24h and 10 days didn't produce toxicity on the leachate; nevertheless, the 5th day occurred a total inhibition, meaning that the decrease in the bioluminescence of *Aliivibrio fischeri* to 100% was immediate.

The samples containing Hg:Fungi medium, for the last incubation period of 10 days, resulted in toxic effects from two of the three isolates, AGH01, and AGH03. Those containing Hg:MP:Fungi medium, for 5 and 10 days of incubation, produced toxic leachates. The values of EC_{50} for the acute toxicity response is given in Table 6-11.

Table 6-11. Values of EC_{50}, for Hg:Fungi and Hg:MP:Fungi mediums, for the last day of incubation, as the average of three different reading times (I5, I15 and I25) and its respective standard deviation.

Strain	EC_{50} (leachate proportion) ± SD	
	Hg:Fungi	Hg:MP:Fungi
PW01	-	0.361 ± 0.151
AGH01	0.487 ± 0.005	0.337 ± 0.342
AGH03	0.602 ± 0.280	0.525 ± 0.205

The toxicity for Hg:Fungi medium was AGH01 > AGH03, with the Hg^{2+} leachate presenting higher toxicity (EC_{50} value of 0.223 leachate proportion), and the toxicity for Hg:MP:Fungi medium was AGH01 > PW01 > AGH03, where the leachate of Hg:MP medium demonstrates the same toxicity as AGH01 (EC_{50} value of 0.337 ± 0.080 leachate proportion).

Information regarding fungi's response to stress induced by mercury and/or MPs is still lacking, nevertheless studies by Kettner et al., 2017 and Gkoutselis et al., 2021 both concluded that MP-associated communities differed from fungal ones in the surrounding aquatic and

terrestrial environment, respectively as MPs serve as a habitat for certain opportunistic pathogens, such as parasitic fungi, and therefore it should be regarded as a persistent reservoir and potential vector for fungal pathogens. Furthermore, fungi are able to detoxify mercury and form cell-to-cell biofilms to increase extracellular response (Grujić et al., 2017). It has been documented, for example, that some fungi can secrete organic acid to increase heavy metal mobilization (Sanjaya et al., 2021). Therefore, the response depends on the nature of the isolate.

6.3.3.2. Variation of pH

The introduction of Hg in the acidic medium (6.85 units of pH) promotes a small increase of about 0,6 units of pH. The medium containing Hg:MP, in contrast, occurs a slight decrease, that of 0,03 units of pH, compared to that with only mercury (Figure 6-10). Differences are not statistically significant.

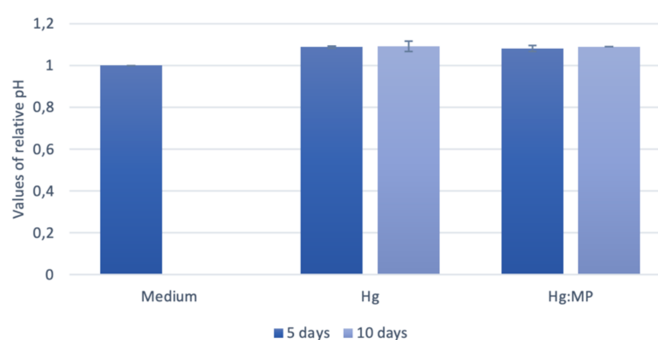

Figure 6-10. Values of pH relative to the medium, for medium with Hg and Hg:MP, on the fifth and tenth day of incubation.

On the fifth day of incubation, the medium containing the fungi strains generates an acidic medium, from 2.5 to 3 units of pH, which sees a rise on the tenth day of incubation to 5 - 6 units of pH. In the presence of both Hg:Fungi and Hg:MP:Fungi, the same occurs; a rise in pH values from the 5th day to the 10th day; from 5 - 6.5 to 7 - 7.5 units of pH. Concerning the control's first reading (Fungi medium, 5 days of incubation), the pH values for the control's last reading and for Hg:Fungi and Hg:MP:Fungi medium readings are all superior (Figure 6-11). Therefore, in the presence of mercury, the pH response increases in pH values rather than without the heavy metal in the medium.

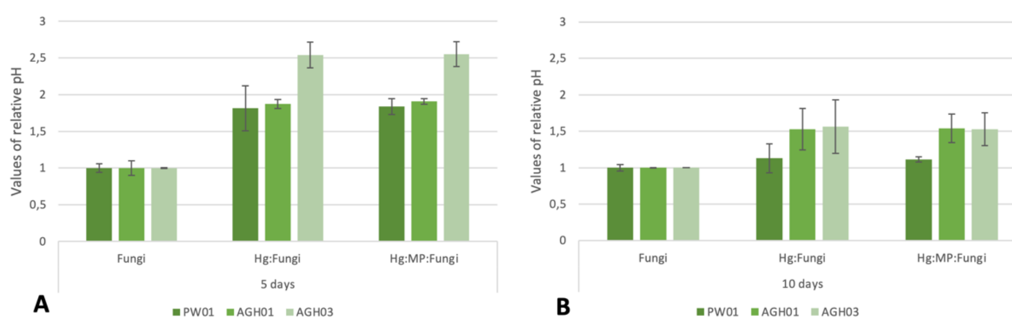

Figure 6-11. pH values relative to the fifth day of incubation for medium containing fungi (A) and relative to the tenth day of incubation for medium containing fungi (B), for Hg:Fungi and Hg:MP:Fungi mediums.

The isolate PW01 has the most acidic response, followed by the isolate AGH01 and AGH03. The respective samples are not statistically significant concerning the controls (Fungi 5 days and Fungi 10 days). The difference isn't also significant between Hg:Fungi and Hg:MP:Fungi samples.

Changes in the pH from the mercury-infused medium indicate both a metabolic diversity and individual fungal response to mercury contamination; in other words, it's dependent on strain origin and sensitivity (Urík et al., 2014). A study by Kurniati et al., 2014 also saw the pH of the culture media tends toward acidic during incubation. The presence of fungi naturally lowered the pH of the media, consistent with its role as fermenter and decomposer in the natural life cycle.

Another explanation for low pH values in fungi strains is the production of mycotoxins, secondary low-molecular-weight metabolites produced by filamentous fungi. They constitute a toxigenically and chemically heterogeneous assemblage and display overlapping toxicities to invertebrates, plants, and microorganisms (Bennett et al., 2003). These metabolites' production results from the interaction among the fungus, the host, and the environment, such as the substrate and other growth conditions (Pitt et al., 2000). Studies have demonstrated that for different mycotoxins, the pH of the medium has an important influence on the expression of the respective biosynthesis genes. A study by Brzonkalik et al., (2012) demonstrated that the pH increases with the biomass increasing, as mycotoxins cannot be produced unless fungal growth occurs throughout the days of cultivation. It was hypothesized that the initial pH fungal growth was possible after a pH change by the fungus. It also demonstrated that the highest mycotoxin concentrations were produced at acidic pH values. Therefore, the possibility of mycotoxin production is in agreement with the acute toxicity results discussed above.

Overall, the data obtained suggests that the presence in the medium of PS MPs (40 mg/L), for samples with Hg^{2+}, does not induce changes in pH values. The same happens with samples containing fungi isolates, where the addition of MP has no impact on pH values. The common variable which induces changes in the pH values is the presence of Mg. Therefore, it can be concluded that, for the incubation period of 10 days, there is no evidence that chemical additives are being released into the medium with the potential to vary the pH value.

6.3.4. Overview on the interaction of MPs on mercury's detoxification mediated by fungi

The current study shows that the fungi strains' growth induces changes in the medium. On the 5th day of incubation, the pH decreases in relation to the medium, about 4 units of pH (not statistically significant). This is aligned with the results obtained for the acute toxicity test (Table 6-7), where the presence of toxicity through a hormesis effect was observed. On the 10th day of incubation, pH values were raised to the original value with no toxicity evidence. This suggests that the metabolism of the fungi strains naturally promotes changes in the environment. These changes can be explained as a result of metabolic characteristics of fungi as fermenters and decomposers (Kurniati et al., 2014).

Nevertheless, the toxic response aligned with the decrease in pH value could be attributed to the production of mycotoxins. Brzonkalik et al., (2012) hypothesized that fungi growth occurred after a pH change by the fungus. It was verified that the pH of the medium increased with an increase in the fungal biomass and that mycotoxin production occurred at acidic pH values. As this aligns with the obtained results, natural mycotoxin production from the fungi strains is a possibility.

In the presence of $Hg^{2+,}$ the pH variance also demonstrates the same behavior: decreasing after 5 days to values smaller than control (not statistically significant). After 10 days, pH

values are slightly higher than the medium (not statistically significant). Yet, on this last incubation period of ten days, toxicity was verified for the strains AGH01 and AGH03, which showed higher results in mercury reduction than 100%. Isolate PW01 also showed a high decrease in Hg contents, 98,7%, and no toxicity. Furthermore, in the presence of this contaminant, all three strains produced a biofilm, evident on the 5th day of incubation (Figure 6-7 and Appendix 3) in comparison to the samples without the contaminant (Appendix 4). This can be explained as a stress response to the presence of such contaminant, which is in agreement with Grujić et al., (2017), which concluded that biofilm tolerance is higher than with planktonic cells. Hence, biofilm formation allows for higher tolerance of mercury and, consequently, its detoxification. Nevertheless, fungi can be producing toxic metabolites as a stress response or from Hg's detoxification process.

Finally, in the presence of both Hg and MPs, the overall response is the same as in Hg:Fungi medium. The pH decreases on the fifth day and increases again on the tenth day (Figure 6-11). Hg reduction is still significant, although the performance between strains changes as AGH03 demonstrated the highest reduction (100%), followed by AGH01 (99%) and PW01 (89%). Toxicity is verified for all three isolates on the last day of incubation. The biofilm formation occurs, and it is also evident on the 5th day of incubation. The main difference between the two mediums (Hg and Hg:MP) is the response from PWO1 and AGH01 (figures 6-7 and 6-8). In the Hg:MP:Isolate medium, both isolates verified a lesser Hg reduction than in the Hg:Isolate medium, but not statistically significant. However, the difference in toxicity produced on the isolate's PW01 sample was significative. This might suggest an interaction from the MPs on the Hg-detoxification processes, which can be attributed to Hg's association with the polymer or a response to stress induced by Hg. Indeed, it has been documented that some fungi can secrete organic acid to increase heavy metal mobilization (Sanjaya et al., 2021). Further analysis of the leachate would clarify this hypothesis.

7.
CONCLUSIONS AND FUTURE DEVELOPMENTS

The Tagus Estuary has been documented as a highly contaminated aquatic system by toxic metals due to industrial and urban effluents. Samples of water and porewater from the historic industrial area of Barreiro analyses in the present work proved that such pollution persists in these environmental matrixes (0,134 ppm and 0,155 ppm, respectively). The obtained results reveal to be greater than those obtained by other studies carried out in the same sample area ($9.00x10^{-7}$ – $6.80x10^{-6}$ ppm).

From this, the first proposed question was, "Which fungi and bacterial tolerant species are found in polluted mercury environments?" This study shows that bacteria *Pseudomonas sp.* and fungi *Cladosporium sp.,* present in the water and porewater of Tagus estuary, exhibited high resistance to Hg (25 ppm, isolate the bacteria PW2.BL, and 50 ppm, attributed to the fungi isolate PW01).

Addressing the second question of this study, "How does the presence of microplastic affect the processes of mercury detoxification?" This study showed that (i) fungi isolates are more resistant to mercury than bacteria and consequently have higher mercury-detoxification results (up to 100% on the first 24h compared to 57% for the same incubation time), (ii) the presence of PS MPs interacts with Hg detoxification mediated by bacteria, as it interacts with the availability of mercury and microorganisms on the medium, (iii) fungi isolates interact with the medium's composition as it alters the pH and induces toxicity, which is enhanced in the presence of Hg, a stressor, for which they respond by forming a biofilm and, (iv) the presence of PS MPs interacts with Hg detoxification mediated by fungi, however not significantly as the primary response to stress induced by Hg.

Concerning the last proposed question, "Which bioremediation strategies could be proposed for polluted mercury areas?" This study demonstrates that bacteria and fungi isolated from contaminated areas have potential bioremediation strategies as they liberate mercury from the medium through their detoxification processes. Bacteria reduced Hg from the medium, whereas fungi seem to reduce and bioaccumulate Hg, both good strategies for bioremediation. Nevertheless, it was verified that the presence of microplastics affects the natural detoxification processes as these contaminants act as vectors of mercury and bacteria through the formation of biofilm on its surface, thus making microorganisms unavailable to detoxify the environment's mercury. Therefore, to draw a bioremediation strategy, the interaction of microplastics needs to be considered.

Thus, the results obtained from this study hold great scientific value. It was the first time demonstrated that there are mercury-resistant bacteria and fungi in the water and porewater matrix of the Tagus Estuary. This contributes to the efforts towards bioremediation and the development of such strategies. Nonetheless, more exhaustive studies on this theme are still needed, namely assessing the biodegradability of MP by microorganisms through a Fourier transform infrared (FTIR). Additionally, further analysis of the leachate to determine what

metabolites bacteria and fungi produce and any toxic relevance. A study of protein expression upon exposure to contaminants would allow for understanding the resistance mechanisms in bacteria and fungi. Finally, a study regarding the effects of MPs on the processes of methylation and demethylation as it is part of the biogeochemical cycle of mercury in which microorganisms play a vital role.

REFERENCES

Acosta-Rodríguez, I., Cárdenas-González, J. F., Rodríguez Pérez, A. S., Oviedo, J. T., & Martínez-Juárez, V. M. (2018). Bioremoval of Different Heavy Metals by the Resistant Fungal Strain Aspergillus niger. *Bioinorganic Chemistry and Applications*, *2018*, 1–7. https://doi.org/10.1155/2018/3457196

Anbumani, S., & Kakkar, P. (1999). *Ecotoxicological effects of microplastics on biota: a review*. https://doi.org/10.1007/s11356-018-1999-x

Auta, H. S., Emenike, C. U., Jayanthi, B., & Fauziah, S. H. (2018). Growth kinetics and biodeterioration of polypropylene microplastics by Bacillus sp. and Rhodococcus sp. isolated from mangrove sediment. *Marine Pollution Bulletin*, *127*(April 2017), 15–21. https://doi.org/10.1016/j.marpolbul.2017.11.036

Barboza, L. G. A., Vieira, L. R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., & Guilhermino, L. (2018). Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, Dicentrarchus labrax (Linnaeus, 1758). *Aquatic Toxicology*, *195*(July 2017), 49–57. https://doi.org/10.1016/j.aquatox.2017.12.008

Bennett, J. W., Klich, M., & Mycotoxins, M. (2003). *Mycotoxins*. *16*(3), 497–516. https://doi.org/10.1128/CMR.16.3.497

Besseling, E., Quik, J. T. K., Sun, M., & Koelmans, A. A. (2017). Fate of nano- and microplastic in freshwater systems: A modeling study. *Environmental Pollution*, *220*, 540–548. https://doi.org/10.1016/j.envpol.2016.10.001

Brzonkalik, K., Hümmer, D., Syldatk, C., & Neumann, A. (2012). Influence of pH and carbon to nitrogen ratio on mycotoxin production by Alternaria alternata in submerged cultivation. *AMB Express*, *2*(1), 1–8. https://doi.org/10.1186/2191-0855-2-28

Byrne, S. J., Butler, C. A., Reynolds, E. C., & Dashper, S. G. (2018). *Taxonomy of Oral Bacteria* (Vol. 45, pp. 171–201). Academic Press. https://doi.org/10.1016/bs.mim.2018.07.001

Campanale, Massarelli, Savino, Locaputo, & Uricchio. (2020). A Detailed Review Study on Potential Effects of Microplastics and Additives of Concern on Human Health. *International Journal of Environmental Research and Public Health*, *17*(4), 1212. https://doi.org/10.3390/ijerph17041212

Canário, J., Branco, V., & Vale, C. (2007). Seasonal variation of monomethylmercury concentrations in surface sediments of the Tagus Estuary (Portugal). *Environmental Pollution*, *148*(1), 380–383. https://doi.org/10.1016/j.envpol.2006.11.023

Chainho, P., Chaves, M. L., Costa, J. L., Costa, M. J., & Dauer, D. M. (2008). Use of multimetric indices to classify estuaries with different hydromorphological characteristics and different levels of human pressure. *Marine Pollution Bulletin*, *56*(6), 1128–1137. https://doi.org/10.1016/j.marpolbul.2008.03.018

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., & Ghannoum, M. A. (2001). Biofilm Formation by the Fungal Pathogen Candida albicans : Development, Architecture, and Drug Resistance. *Journal of Bacteriology*, *183*(18), 5385–5394. https://doi.org/10.1128/JB.183.18.5385-5394.2001

Chang, J., Shi, Y., Si, G., Yang, Q., Dong, J., & Chen, J. (2020). The bioremediation potentials and mercury(II)-resistant mechanisms of a novel fungus Penicillium spp. DC-F11 isolated from contaminated soil. *Journal of Hazardous Materials*, *396*(April), 122638. https://doi.org/10.1016/j.jhazmat.2020.122638

Dash, H. R., & Das, S. (2012). Bioremediation of mercury and the importance of bacterial mer

genes. *International Biodeterioration & Biodegradation*, *75*, 207–213. https://doi.org/10.1016/j.ibiod.2012.07.023
de Almeida Rodrigues, P., Ferrari, R. G., dos Santos, L. N., & Conte Junior, C. A. (2019). Mercury in aquatic fauna contamination: A systematic review on its dynamics and potential health risks. *Journal of Environmental Sciences (China)*, *84*, 205–218. https://doi.org/10.1016/j.jes.2019.02.018
De, J., Ramaiah, N., & Vardanyan, L. (2008). Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. *Marine Biotechnology*, *10*(4), 471–477. https://doi.org/10.1007/s10126-008-9083-z
Decreto-Lei n.º 306/2007 do Ministério do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional, Pub. L. No. Diário da República: I série, n.º 164, 5747 (2007). https://dre.pt/pesquisa/-/search/640931/details/maximized
Dhankhar, R., & Hooda, A. (2011). Fungal biosorption – an alternative to meet the challenges of heavy metal pollution in aqueous solutions. *Environmental Technology*, *32*(5), 467–491. https://doi.org/10.1080/09593330.2011.572922
Dixit, R., Wasiullah, Malaviya, D., Pandiyan, K., Singh, U., Sahu, A., Shukla, R., Singh, B., Rai, J., Sharma, P., Lade, H., & Paul, D. (2015). Bioremediation of Heavy Metals from Soil and Aquatic Environment: An Overview of Principles and Criteria of Fundamental Processes. *Sustainability*, *7*(2), 2189–2212. https://doi.org/10.3390/su7022189
Endo, G., Narita, M., Huang, C.-C., & Silver, S. (2002). Microbial Heavy Metal Resistance Transposons and Plasmids.Pdf. *Journal of Environmental Biotechnology*, *2*(No. 2), 71–82.
EPA. (n.d.). *Mercury Emissions: The Global Context*. https://www.epa.gov/international-cooperation/mercury-emissions-global-context
EPCRA. (2001). Emergency Planning and Community Right-to-Know Act of 1986 (EPCRA) Section 313. *United States Environmental Protection Agency*. http://www2.epa.gov/sites/production/files/documents/2001hg.pdf
Estepa, J. (2016). *Degradación bacteriana de cianuro y compuestos nitrogenados tóxicos*. Universidad de Córdoba.
European Commission. (n.d.). *The Marine Strategy Framework Directive*. Directorate-General for Environment. https://ec.europa.eu/environment/marine/eu-coast-and-marine-policy/marine-strategy-framework-directive/index_en.htm
European Commission. (2017). *Science for Environment Policy (2017) Tackling mercury pollution in the EU and worldwide. In-depth Report 15 produced for the European Commission, DG Environment by the Science Communication Unit, UWE, Bristol.* (Issue November). https://doi.org/10.2779/44615
European Union. (2017). Regulation (EU) 2017/852 of the European Parliament and of the Council on mercury, and repealing Regulation (EC) No 1102/2008. *Official Journal of the European Union*, *137*(2), 21. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R0852&from=EN
Figueiredo, N. L. L. (2016). *Microbial Community Composition and Mercury Cycling in Sediments of Tagus Estuary*.
Figueiredo, N. L. L., Areias, A., Mendes, R., Canário, J., Duarte, A., & Carvalho, C. (2014). Mercury-Resistant Bacteria From Salt Marsh of Tagus Estuary: The Influence of Plants Presence and Mercury Contamination Levels. *Journal of Toxicology and Environmental Health, Part A*, *77*(14–16), 959–971. https://doi.org/10.1080/15287394.2014.911136
Fred-Ahmadu, O. H., Bhagwat, G., Oluyoye, I., Benson, N. U., Ayejuyo, O. O., & Palanisami, T. (2020). Interaction of chemical contaminants with microplastics: Principles and perspectives. *Science of the Total Environment*, *706*(December). https://doi.org/10.1016/j.scitotenv.2019.135978
Galgani, F., & Pinto Da Costa, J. (2020). *The Role of Legislation, Regulatory Initiatives and Guidelines on the Control of Plastic Pollution*. https://doi.org/10.3389/fenvs.2020.00104
Galloway, T. S., Cole, M., & Lewis, C. (2017). Interactions of microplastic debris throughout the marine ecosystem. *Nature Ecology and Evolution*, *1*(5), 1–8. https://doi.org/10.1038/s41559-017-0116
GESAMP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection. (2016). Sources, fate and effects of microplastics in the marine environment: part 2 of a global assessment. (IMO, FAO/UNESCO-

IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP). In: Kershaw, P.J. (Ed.), Rep. Stud. GESAMP No. 90 (96 pp). *Reports and Studies GESAMP, No. 93, 96 P., 93*.

Giovanella, P., Cabral, L., Costa, A. P., de Oliveira Camargo, F. A., Gianello, C., & Bento, F. M. (2017). Metal resistance mechanisms in Gram-negative bacteria and their potential to remove Hg in the presence of other metals. *Ecotoxicology and Environmental Safety*, *140*(October 2016), 162–169. https://doi.org/10.1016/j.ecoenv.2017.02.010

Gkoutselis, G., Rohrbach, S., Harjes, J., Obst, M., Brachmann, A., Horn, M. A., & Rambold, G. (2021). Microplastics accumulate fungal pathogens in terrestrial ecosystems. *Scientific Reports*, *11*(1), 13214. https://doi.org/10.1038/s41598-021-92405-7

Goel, P. K. (2006). General. In *Water Pollution: Causes, Effects and Control* (p. 418). New Age International.

Gorgoglione, A., Bombardelli, F. A., Pitton, B. J. L., & Oki, L. R. (2019). Uncertainty in the parameterization of sediment build-up and wash-off processes in the simulation of sediment transport in urban areas Environmental Modelling & Software Uncertainty in the parameterization of sediment build-up and wash-off processes in th. *Environmental Modelling and Software*, *111*(December 2018), 170–181. https://doi.org/10.1016/j.envsoft.2018.09.022

Grujić, S., Vasić, S., Radojević, I., Čomić, L., & Ostojić, A. (2017). Comparison of the Rhodotorula mucilaginosa Biofilm and Planktonic Culture on Heavy Metal Susceptibility and Removal Potential. *Water, Air, & Soil Pollution*, *228*(2), 73. https://doi.org/10.1007/s11270-017-3259-y

Gworek, B., Bemowska-Kałabun, O., Kijeńska, M., & Wrzosek-Jakubowska, J. (2016). Mercury in Marine and Oceanic Waters—a Review. *Water, Air, & Soil Pollution*, *227*(10), 371. https://doi.org/10.1007/s11270-016-3060-3

Habib, S., Iruthayam, A., Abd Shukor, M. Y., Alias, S. A., Smykla, J., & Yasid, N. A. (2020). Biodeterioration of Untreated Polypropylene Microplastic Particles by Antarctic Bacteria. *Polymers*, *12*(11), 2616. https://doi.org/10.3390/polym12112616

Häder, D. P., Banaszak, A. T., Villafañe, V. E., Narvarte, M. A., González, R. A., & Helbling, E. W. (2020). Anthropogenic pollution of aquatic ecosystems: Emerging problems with global implications. *Science of The Total Environment*, *713*, 136586. https://doi.org/10.1016/J.SCITOTENV.2020.136586

Harrison, J. J., Ceri, H., Yerly, J., Rabiei, M., Hu, Y., Martinuzzi, R., & Turner, R. J. (2007). Metal Ions May Suppress or Enhance Cellular Differentiation in Candida albicans and Candida tropicalis Biofilms. *Applied and Environmental Microbiology*, *73*(15), 4940–4949. https://doi.org/10.1128/AEM.02711-06

He, S., Jia, M., Xiang, Y., Song, B., Xiong, W., Cao, J., Peng, H., Yang, Y., Wang, W., Yang, Z., & Zeng, G. (2022). Biofilm on microplastics in aqueous environment: Physicochemical properties and environmental implications. *Journal of Hazardous Materials*, *424*, 127286. https://doi.org/10.1016/j.jhazmat.2021.127286

Huang, C. R., Hsu, S. P., Chang, W. N., Lui, C. C., Wu, H. S., Chen, H. J., & Chen, D. (2018). Global Mercury Assessment. In *United Nations Environmental Programme* (Vol. 7, Issue 3).

Ismail, I., & Moustafa, T. (2016). Biosorption of heavy metals. *Heavy Metals: Sources, Toxicity and Remediation Techniques*, *October*, 131–174.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology*, *7*(2), 60–72. https://doi.org/10.2478/intox-2014-0009

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Joshi, P. (2016). Microbial Diversity of Aquatic Ecosystem and its Industrial Potential. *Journal of Bacteriology & Mycology: Open Access*, *3*(1), 177–179. https://doi.org/10.15406/jbmoa.2016.03.00048

Kapoor, A., & Viraraghavan, T. (1997). Heavy metal biosorption sites in Aspergillus niger. *Bioresource Technology*, *61*(3), 221–227. https://doi.org/10.1016/S0960-8524(97)00055-2

Kedzierski, M., D'Almeida, M., Magueresse, A., Le Grand, A., Duval, H., César, G., Sire, O., Bruzaud, S., & Le Tilly, V. (2018). Threat of plastic ageing in marine environment.

Adsorption/desorption of micropollutants. *Marine Pollution Bulletin*, *127*(July 2017), 684–694. https://doi.org/10.1016/j.marpolbul.2017.12.059
Kelly, D. J. A., Budd, K., & Lefebvre, D. D. (2006). The biotransformation of mercury in pH-stat cultures of microfungi. *Canadian Journal of Botany*, *84*(2), 254–260. https://doi.org/10.1139/B05-156
Kookana, R. S., Drechsel, P., Jamwal, P., & Vanderzalm, J. (2020). Urbanisation and emerging economies: Issues and potential solutions for water and food security. *Science of The Total Environment*, *732*, 139057. https://doi.org/10.1016/J.SCITOTENV.2020.139057
Krasowska, A., & Sigler, K. (2014). How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in Cellular and Infection Microbiology*, *4*(AUG), 1–7. https://doi.org/10.3389/fcimb.2014.00112
Kumar, L., & Bharadvaja, N. (2019). Enzymatic bioremediation: a smart tool to fight environmental pollutants. *Smart Bioremediation Technologies: Microbial Enzymes*, 99–118. https://doi.org/10.1016/B978-0-12-818307-6.00006-8
Kumar, P., Jyoti, B., Kumar, A., & Paliwal, A. (2019). Biotechnological and microbial standpoint cahoot in bioremediation. *Smart Bioremediation Technologies: Microbial Enzymes*, 137–158. https://doi.org/10.1016/B978-0-12-818307-6.00008-1
Kurniati, E., Arfarita, N., Imai, T., Higuchi, T., Kanno, A., Yamamoto, K., & Sekine, M. (2014). Potential bioremediation of mercury-contaminated substrate using filamentous fungi isolated from forest soil. *Journal of Environmental Sciences (China)*, *26*(6), 1223–1231. https://doi.org/10.1016/S1001-0742(13)60592-6
Lam, C. S., Ramanathan, S., Carbery, M., Gray, K., Vanka, K. S., Maurin, C., Bush, R., & Palanisami, T. (2018). A Comprehensive Analysis of Plastics and Microplastic Legislation Worldwide. *Water, Air, and Soil Pollution*, *229*(11). https://doi.org/10.1007/s11270-018-4002-z
Liu, M., Zhang, Q., Maavara, T., Liu, S., Wang, X., & Raymond, P. A. (2021). Rivers as the largest source of mercury to coastal oceans worldwide. *Nature Geoscience*. https://doi.org/10.1038/s41561-021-00793-2
LNEC. (2008). Avaliação comparativa das alternativas existentes para a terceira travessia do tejo na área metropolitana de lisboa. *LNEC*, *62/2008*, 325.
Lobelle, D., & Cunliffe, M. (2011). Early microbial biofilm formation on marine plastic debris. *Marine Pollution Bulletin*, *62*(1), 197–200. https://doi.org/10.1016/j.marpolbul.2010.10.013
Long, E. R., Macdonald, D. D., Smith, S. L., & Calder, F. D. (1995). Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environmental Management*, *19*(1), 81–97. https://doi.org/10.1007/BF02472006
Lusher, A., Hollman, P., & Mandoza-Hill, J. (2017). Microplastics in fisheries and aquaculture. In *FAO Fisheries and Aquaculture Technical Paper* (Vol. 615, Issue July). http://www.fao.org/3/a-i7677e.pdf
Manickam, N., Ghosh, A., Jain, R. K., & Mayilraj, S. (2008). Description of a novel indole-oxidizing bacterium Pseudomonas indoloxydans sp. nov., isolated from a pesticide-contaminated site. *Systematic and Applied Microbiology*, *31*(2), 101–107. https://doi.org/10.1016/j.syapm.2008.02.002
Manso Cobos, I., Ibáñez García, M. I., de la Peña Moreno, F., Sáez Melero, L. P., Luque-Almagro, V. M., Castillo Rodríguez, F., Roldán Ruiz, M. D., Prieto Jiménez, M. A., & Moreno Vivián, C. (2015). Pseudomonas pseudoalcaligenes CECT5344, a cyanide-degrading bacterium with by-product (polyhydroxyalkanoates) formation capacity. *Microbial Cell Factories*, *14*(1), 77. https://doi.org/10.1186/s12934-015-0267-8
Marsalek, J., Jiménez-Cisneros, B., Karamouz, M., Malmquist, P. A., Goldenfum, J., & Chocat, B. (2014). Urban water cycle processes and interactions: Urban water series - UNESCO-IHP. *Urban Water Cycle Processes and Interactions: Urban Water Series - UNESCO-IHP*, *78*, 1–131.
Marshall, S. J. (2013). Hydrology. *Reference Module in Earth Systems and Environmental Sciences*. https://doi.org/10.1016/B978-0-12-409548-9.05356-2
Martínez-Juárez, V. M., Cárdenas-González, J. F., Torre-Bouscoulet, M. E., & Acosta-Rodríguez, I. (2012). Biosorption of Mercury (II) from Aqueous Solutions onto Fungal

Biomass. *Bioinorganic Chemistry and Applications*, *2012*(Ii), 1–5. https://doi.org/10.1155/2012/156190
Martinho, G., Balaia, N., & Pires, A. (2017). The Portuguese plastic carrier bag tax: The effects on consumers' behavior. *Waste Management*, *61*, 3–12. https://doi.org/10.1016/J.WASMAN.2017.01.023
Mathema, V. B., Thakuri, B. C., & Sillanpää, M. (2011). Bacterial mer operon-mediated detoxification of mercurial compounds: a short review. *Archives of Microbiology*, *193*(12), 837–844. https://doi.org/10.1007/s00203-011-0751-4
Mokarram, M., Saber, A., & Sheykhi, V. (2020). Effects of heavy metal contamination on river water quality due to release of industrial effluents. *Journal of Cleaner Production*, *277*, 123380. https://doi.org/10.1016/J.JCLEPRO.2020.123380
NOAA. (2021). *What are Microplastics?* https://oceanservice.noaa.gov/facts/microplastics.html
Nucifora, G., Chu, L., Silver, S., & Misra, T. K. (1989). Mercury operon regulation by the merR gene of the organomercurial resistance system of plasmid pDU1358. *Journal of Bacteriology*, *171*(8), 4241–4247. https://doi.org/10.1128/jb.171.8.4241-4247.1989
Oliveira, P., Barboza, L. G. A., Branco, V., Figueiredo, N., Carvalho, C., & Guilhermino, L. (2018). Effects of microplastics and mercury in the freshwater bivalve Corbicula fluminea (Müller, 1774): Filtration rate, biochemical biomarkers and mercury bioconcentration. *Ecotoxicology and Environmental Safety*, *164*, 155–163. https://doi.org/10.1016/j.ecoenv.2018.07.062
Olson, B. H., Barkay, T., & Colwell, R. R. (1979). Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. *Applied and Environmental Microbiology*, *38*(3), 478–485. https://doi.org/10.1128/aem.38.3.478-485.1979
Oyetibo, G. O., Miyauchi, K., Huang, Y., Chien, M. F., Ilori, M. O., Amund, O. O., & Endo, G. (2017). Biotechnological remedies for the estuarine environment polluted with heavy metals and persistent organic pollutants. *International Biodeterioration and Biodegradation*, *119*, 614–625. https://doi.org/10.1016/j.ibiod.2016.10.005
Oyewole, O. A., Zobeashia, S. S. L.-T., Oladoja, E. O., Raji, R. O., Odiniya, E. E., & Musa, A. M. (2019). Biosorption of heavy metal polluted soil using bacteria and fungi isolated from soil. *SN Applied Sciences*, *1*(8), 857. https://doi.org/10.1007/s42452-019-0879-4
Parker, J. (2001). Bacteria. In *Encyclopedia of Genetics* (pp. 146–151). Elsevier. https://doi.org/10.1006/rwgn.2001.0102
Pitt, J. I., Basílico, J. C., Abarca, M. L., & López, C. (2000). Mycotoxins and toxigenic fungi. *Medical Mycology*, *38*(s1), 41–46. https://doi.org/10.1080/mmy.38.s1.41.46
Prata, J. C., da Costa, J. P., Lopes, I., Duarte, A. C., & Rocha-Santos, T. (2020). Environmental exposure to microplastics: An overview on possible human health effects. *Science of The Total Environment*, *702*, 134455. https://doi.org/10.1016/J.SCITOTENV.2019.134455
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. https://doi.org/10.1016/J.TRAC.2018.12.001
Rahman, Z., & Singh, V. P. (2020). Bioremediation of toxic heavy metals (THMs) contaminated sites: concepts, applications and challenges. *Environmental Science and Pollution Research*, *27*(22), 27563–27581. https://doi.org/10.1007/s11356-020-08903-0
Razi Othman, A., Abu Hasan, H., Hafizuddin Muhamad, M., Ismail, I., & Rozaimah Sheikh Abdullah, S. (2021). Microbial degradation of microplastics by enzymatic processes: a review. *Environmental Chemistry Letters*, *19*, 3057–3073. https://doi.org/10.1007/s10311-021-01197-9
Rogers, K. L., Carreres-Calabuig, J. A., Gorokhova, E., & Posth, N. R. (2020). Micro-by-micro interactions: How microorganisms influence the fate of marine microplastics. *Limnology and Oceanography Letters*, *5*(1), 18–36. https://doi.org/10.1002/lol2.10136
Sánchez, C. (2020). Fungal potential for the degradation of petroleum-based polymers: An overview of macro- and microplastics biodegradation. *Biotechnology Advances*, *40*, 107501. https://doi.org/10.1016/J.BIOTECHADV.2019.107501
Sanjaya, W. T. A., Khoirunhisa, N. S., Ismiani, S., Hazra, F., & Santosa, D. A. (2021). Isolation and characterization of mercury-resistant microbes from gold mine area in Mount

Pongkor, Bogor District, Indonesia. *Biodiversitas Journal of Biological Diversity*, *22*(7), 2656–2666. https://doi.org/10.13057/biodiv/d220714
Saranya, K., Sundaramanickam, A., Shekhar, S., & Swaminathan, S. (2019). Biosorption of mercury by Bacillus thuringiensis (CASKS3) isolated from mangrove sediments of southeast coast india. *Indian Journal of Geo-Marine Sciences*, *48*(1), 143–150.
Scheuhammer, A. M., Meyer, M. W., Sandheinrich, M. B., & Murray, M. W. (2007). Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *Ambio*, *36*(1), 12–18. https://doi.org/10.1579/0044-7447(2007)36[12:EOEMOT]2.0.CO;2
Schweitzer, L., & Noblet, J. (2018). Water Contamination and Pollution. *Green Chemistry: An Inclusive Approach*, 261–290. https://doi.org/10.1016/B978-0-12-809270-5.00011-X
Selin, N. E. (2009). Global biogeochemical cycling of mercury: A review. *Annual Review of Environment and Resources*, *34*, 43–63. https://doi.org/10.1146/annurev.environ.051308.084314
Seng, N., Lai, S., Fong, J., Saleh, M. F., Cheng, C., Cheok, Z. Y., & Todd, P. A. (2020). Early evidence of microplastics on seagrass and macroalgae. *Marine and Freshwater Research*, *71*(8), 922. https://doi.org/10.1071/MF19177
Sigee, D. C. (2005). Freshwater Microbiology: Biodiversity and Dynamic Interactions of Microorganisms in the Aquatic Environment. In J. W. & Sons (Ed.), *Freshwater Microbiology: Biodiversity and Dynamic Interactions of Microorganisms in the Aquatic Environment* (p. 516).
Sıkdokur, E., Belivermiş, M., Sezer, N., Pekmez, M., Bulan, Ö. K., & Kılıç, Ö. (2020). Effects of microplastics and mercury on manila clam Ruditapes philippinarum: Feeding rate, immunomodulation, histopathology and oxidative stress. *Environmental Pollution*, *262*, 114247. https://doi.org/10.1016/J.ENVPOL.2020.114247
Smith, M., Love, D. C., Rochman, C. M., & Neff, R. A. (2018). Microplastics in Seafood and the Implications for Human Health. *Current Environmental Health Reports*, *5*(3), 375–386. https://doi.org/10.1007/s40572-018-0206-z
Stratton, A., Ericksen, M., Harris, T. V, Symmonds, N., & Silverstein, T. P. (2016). *Mercury(II) binds to both of chymotrypsin's histidines, causing inhibition followed by irreversible denaturation/aggregation*. https://doi.org/10.1002/pro.3082
Tao, Y., Zhou, Y., He, X., Hu, X., & Li, D. (2014). Pseudomonas chengduensis sp. nov., isolated from landfill leachate. *International Journal of Systematic and Evolutionary Microbiology*, *64*(Pt_1), 95–100. https://doi.org/10.1099/ijs.0.050294-0
Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Molecular, clinical and environmental toxicicology Volume 3: Environmental Toxicology. *Molecular, Clinical and Environmental Toxicology*, *101*, 133–164. https://doi.org/10.1007/978-3-7643-8340-4
Teaf M, C. (2012). Mercury Exposure Considerations: Evaluating the Chemical Form and Activities of the Individual. *Sediments, Water and Energy*, *17*.
Thavasi, R. (2011). Microbial Biosurfactants: From an Environmental Application Point of View. *Journal of Bioremediation and Biodegradation*, *02*(05), 1000104. https://doi.org/10.4172/2155-6199.1000104e
Tornero, V., & Hanke, G. (2018). Marine chemical contaminants -support to harmonized MSFD reporting: Substances considered for MSFD descriptor 8, EUR 29281. In *Publications Office of the European Union*. European Unio. https://doi.org/10.2760/804610
Umrania, V. V. (2006). Bioremediation of toxic heavy metals using acidothermophilic autotrophes. *Bioresource Technology*, *97*(10), 1237–1242. https://doi.org/10.1016/j.biortech.2005.04.048
UN. (n.d.). *Water Scarcity*. https://www.unwater.org/water-facts/scarcity/
Urík, M., Hlodák, M., Mikušová, P., & Matúš, P. (2014). Potential of Microscopic Fungi Isolated from Mercury Contaminated Soils to Accumulate and Volatilize Mercury(II). *Water, Air, & Soil Pollution*, *225*(12), 2219. https://doi.org/10.1007/s11270-014-2219-z
Văcar, C. L., Covaci, E., Chakraborty, S., Li, B., Weindorf, D. C., Frențiu, T., Pârvu, M., & Podar, D. (2021). Heavy Metal-Resistant Filamentous Fungi as Potential Mercury Bioremediators. *Journal of Fungi*, *7*(5), 386. https://doi.org/10.3390/jof7050386
van Hees, K. E., & Ebert, D. A. (2017). An evaluation of mercury offloading in two Central

California elasmobranchs. *Science of the Total Environment*, *590–591*, 154–162. https://doi.org/10.1016/j.scitotenv.2017.02.191
Wang, L., & D'Odorico, P. (2013). Decomposition and Mineralization. *Encyclopedia of Ecology*, 280–285. https://doi.org/10.1016/B978-0-12-409548-9.00688-6
Wang, W., Gao, H., Jin, S., Li, R., & Na, G. (2019). The ecotoxicological effects of microplastics on aquatic food web, from primary producer to human: A review. *Ecotoxicology and Environmental Safety*, *173*, 110–117. https://doi.org/10.1016/J.ECOENV.2019.01.113
Wasewar, K. L., Singh, S., & Kansal, S. K. (2020). Process intensification of treatment of inorganic water pollutants. In *Inorganic Pollutants in Water* (pp. 245–271). Elsevier. https://doi.org/10.1016/B978-0-12-818965-8.00013-5
WHO. (1990). Environmental Health Criteria 101: Methylmercury. *Environmental Health Criteria*, *101*, 1–144.
WHO. (2021). *Mercury and health*. https://www.who.int/news-room/fact-sheets/detail/mercury-and-health
Wołowiec, M., Komorowska-Kaufman, M., Pruss, A., Rzepa, G., & Bajda, T. (2019). Removal of Heavy Metals and Metalloids from Water Using Drinking Water Treatment Residuals as Adsorbents: A Review. *Minerals*, *9*(8), 487. https://doi.org/10.3390/min9080487
WWAP. (2021). *The United Nations World Water Development Report 2021: Valuing Water*.
Yang, Y., Liu, W., Zhang, Z., Grossart, H.-P., & Gadd, G. M. (2020). Microplastics provide new microbial niches in aquatic environments. *Applied Microbiology and Biotechnology*, *104*(15), 6501–6511. https://doi.org/10.1007/s00253-020-10704-x
Yi, J., Lo, L. S. H., & Cheng, J. (2020). Dynamics of Microbial Community Structure and Ecological Functions in Estuarine Intertidal Sediments. *Frontiers in Marine Science*, *7*(October), 1–15. https://doi.org/10.3389/fmars.2020.585970
Yuan, Y., Hays, M. P., Hardwidge, P. R., & Kim, J. (2017). Surface characteristics influencing bacterial adhesion to polymeric substrates. *RSC Advances*, *7*(23), 14254–14261. https://doi.org/10.1039/C7RA01571B
Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Suemori, A., Nakajima, K., Inoue, N., & Kawasaki, K. (2001). Pseudomonas alcaliphila sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2), 349–355. https://doi.org/10.1099/00207713-51-2-349
Zettler, E. R., Mincer, T. J., & Amaral-Zettler, L. A. (2013). Life in the "plastisphere": Microbial communities on plastic marine debris. *Environmental Science and Technology*, *47*(13), 7137–7146. https://doi.org/10.1021/es401288x
Zhang, T., Xiao, Y., Liang, D., Tang, H., Xu, J., Yuan, S., Wang, N., & Luan, B. (2021). A two-layer model for studying 2D dissolved pollutant runoff over impermeable surfaces. *Hydrological Processes*, *35*(5). https://doi.org/10.1002/hyp.14152

APPENDIX 1

Bacterial colony growth with and without Hg^{2+} (1 ppm) from environmental samples (water and pore water from the Tagus River – Barreiro) after 3 days of incubation.

	Number of colonies – color, texture/shape	
Environmental Matrix	MH	MH + Hg^{2+}
Water	Sample A 3- Yellow, milky 1- Pink, opaque 2- Yellow, dot 1- Pink, milky 1- White, opaque	Sample A 2- Pink, milky 3- White, milky 4- Yellow, dot 1- White, branches 3- Orange, milky 2- Orange, opaque 1- Yellow, big 3- White, opaque
	Sample B 1- Yellow, dot 1- Yellow, milky 6- Orange, milky 5- White, milky 1- Red, milky	Sample B 3- Yellow, dot 7- Orange, milky 4- Pink, milky 2- White, milky
	Sample C 1- Yellow, dot 1- Yellow, milky 6- Orange, milky 5- White, milky 1- Red, milky	Sample C 3- Yellow, dot 7- Orange, milky 4- Pink, milky 2- White, milky
Total number of colonies	30	50
Porewater	Sample 1 0	Sample 1 0
	Sample 2 9- White, milky 9- Pink, opaque 1- Yellow, dot 43- Orange, lumpy	Sample 2 9- White, smooth 10- White, milky 92- Orange, opaque
	Sample 3 24 – Yellow, dot 2- Pink, milky	Sample 3 2- Pink, opaque
Total number of colonies	88	113

APPENDIX 2

Fungi colony growth with and without Hg^{2+} (1 ppm) from environmental samples (water and porewater from the Tagus – Barreiro estuary) after an incubation of 3 days.

	Number of colonies – designation	
Environmental Matrix	PDA	PDA + Hg^{2+}
Water	Sample A 3- AG01 1- AG02 1- AG03 2- AG04 1- AG05 2- AG06 1- AG07 1- AG08	Sample A 16- AGH01 9- AGH02 1- AGH03 1- AGH04 1- AGH05 1- AGH06 1- AGH07 1- AGH08 1- AGH09 1- AGH10
	Sample B 6- AG01 1- AG09 3- AG10 1- AG11 1- AG12 1- AG13	Sample B 1- AGH01 10- AGH10 2- AGH11 1- AGH12 1- AGH13 1- AGH14 2- AGH15
	Sample C 3- AG01 1- AG13	Sample C 9- AGH01 1- AGH17 3- AGH18 1- AGH19
Total number of colonies	29	65
Porewater	Sample 1 2- PW01 1- PW02	Sample 1 1- PW01
	Sample 2 2- PW01 2- PW03 1- PW04	Sample 2 2- PW03 1- PW05
	Sample 3	Sample 3

	1- PW01 1- PW03 1- PW06	1- PW01
Total number of colonies	11	5

APPENDIX 3

Qualitative results of both independent assays for fungi in mediums infused with Hg^{2+} and Hg^{2+}+MP.

From top to bottom, the three fungi isolates (PW01, AGH01 and AGH03), in two different mediums (Hg^{2+} and Hg^{2+}+MP), in replicate, on the 10th day of incubation for the first independent assay (left) and second independent assay (right).

APPENDIX 4

Qualitative results of the independent assays for the three fungi isolates (PW01, AGH01 and AGH03) in YEG + distilled water mediums, in replicate, on the 10th day of incubation.

APPENDIX 5

Amplified sequences from bacteria DNA.

Isolate	Sequence
B.RL Isolate's sequence, obtained from microplastics	ACCGGGGCGGCTAAACATGCAAGTCGAGCGGATGAG AGGAGCTTGCTCCTTGATTTAGCGGCGGACGGGTGAG TAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACG TTCCGAAAGGAACGCTAATACCGCATACGTCCTACG GGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCA GATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAA TGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG AGGATGATCAGTCACACTGGAACTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGT GTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTT GGGAGGAAGGGCATTAACCTAATACGTTAGTGTTTTG ACGTTACCGACAGAATAAGCACCGGCTAACTTCGTG CCAGCCGCCGCAGTAAAACA
PW2.BL Isolate's sequence, obtained from microplastics	ATTGGCTGGCGGAGGCCTACACATGCAAGTCGAGCG GATGAGAGGAGCTTGCTCCTTGATTTAGCGGCGGACG GGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG ATAACGTTCCGAAAGGAACGCTAATACCGCATACGT CCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCG CTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGG TCTGAGAGGATGATCAGTCACACTGGAACTGAGACA CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT ATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCC GCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT TAAGTTGGGAGGAAGGGCATTAACCTAATACGTTAGT GTTTTGACGTTACCGACAGAATAAGCACCGGCTAACT TTGTGCCAGCCGCCGCCGTAAAACAA
PW2.BL Isolate's sequence, obtained from liquid suspension	CCAGGGCGGCTACCATGCAAGTCGAGCGGATGAGAG GAGCTTGCTCCTTGATTTAGCGGCGGACGGGTGAGTA ATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTT CCGAAAGGAACGCTAATACCGCATACGTCCTACGGG AGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGA TGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATG GCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG GATGATCAGTCACACTGGAACTGAGACACGGTCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT

	GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGG GAGGAAGGGCATTAACCTAATACGTTAGTGTTTTGAC GTTACCGACAGAATAAGCACCGGCTAACTTCGTGCC AGCAGCCGCACGTAACACAA
PW2.LO Isolate's sequence, obtained from microplastics	CCCGGGGGGCTACCATGCAAGTCGAGCGGATGAGAG GAGCTTGCTCCTTGATTTAGCGGCGGACGGGTGAGTA ATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTT CCGAAAGGAACGCTAATACCGCATACGTCCTACGGG AGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGA TGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATG GCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG GATGATCAGTCACACTGGAACTGAGACACGGTCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGG GAGGAAGGGCATTAACCTAATACGTTAGTGTTTTGAC GTTACCGACAGAATAAGCACCGGCTAACTTCGTGCC AGCCGCCGCCGGTAACACAACAA
PW2.LO Isolate's sequence, obtained from liquid suspension	CCCGGCGGAGCCTACACATGCAAGTCGAGCGGATGA GAGGAGCTTGCTCCTTGATTTAGCGGCGGACGGGTGA GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAAC GTTCCGAAAGGAACGCTAATACCGCATACGTCCTAC GGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATC AGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTA ATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGA GAGGATGATCAGTCACACTGGAACTGAGACACGGTC CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGG ACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGT TGGGAGGAAGGGCATTAACCTAATACGTTAGTGTTTT GACGTTACCGACAGAATAAGCACCGGCTAACTTCGT GCCAGCCGCCGCCGGTAACACAAAA
PWO1 Isolated from solid PDA culture	NNNNNNNNNNCAGNTGNNCCCGGTCTAACNNCCG GGATGTTNNTNNNNNNNNNNNTGTTGTCCNNNTCT GTGCCTCCGGGGCGACCCTGNNTTCGGGCGGGGGCT CCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAG TCTGAGTAAACTTAATTAATAAATTAAAACTTTTAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA GTGAATCATCGAATCTTTGAACGCACATTGCGCCCCC TGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTT CACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGT CCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTG TCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTG TTCGGGAGGCTACGCCGTAAAACAACCCCATTTCTAA GGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACT TAAGCATATCAATAAGCGGAGGA

⟨2021⟩

LAURA TIÊ ZANCHI DA SILVA

EVALUATION OF THE IMPACTS OF MICROPLASTICS ON MERCURY DETOXIFICATION STRATEGIES: A COMPARISON BETWEEN THE PERFORMANCE OF BACTERIA AND FUNGI

NOVA SCHOOL OF SCIENCE & TECHNOLOGY