UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

MICROBIAL COMMUNITY COMPOSITION AND MERCURY CYCLING IN SEDIMENTS OF TAGUS ESTUARY

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Orientadores: Prof. Doutora Cristina Maria Leitão de Carvalho

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Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia (Especialidade de Toxicologia)

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This work is dedicated to God

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ABSTRACT

Mercury is a pervasive pollutant well known to cause several disorders in humans and wildlife. The major concern related with mercury pollution is the neurotoxicity associated to methylmercury and its presence in aquatic systems, as it undergoes bioaccumulation and biomagnification in the food chain. In aquatic ecosystems, mercury-resistant microorganisms are the main responsible for methylation of Hg^{2+} and also for processes of detoxification (reduction of Hg^{2+} and demethylation of methylmercury). High levels of mercury, including methylmercury, have been shown to exist in the Tagus Estuary.

This study aims to give an insight about the involvement of microorganisms in the cycle of mercury in the Tagus Estuary, based on their phenotypic and genetic characterization. To achieve this, mercury-resistant microorganisms were isolated from sediments of four mercury-polluted areas of the Tagus Estuary (Barreiro, Cala do Norte, Rosário and Alcochete) and, after their characterization their potential to transform mercury compounds was evaluated.

The isolates encompassed aerobic microorganisms, such as *Bacillus* sp., *Vibrio* sp., *Aeromonas* sp. and *Enterobacteriacea* sp., and anaerobic microorganisms, such as *Clostridium* sp., *Enterobacteriaceae* sp. and the *Archaea* sulfate-reducing bacteria (e.g. *Desulfovibrio desulfuricans*). Their resistance to mercury compounds ranged from 0.41-140 µg/mL for Hg^{2+} and 0.04-50.1 µg/mL for CH_3Hg. The genetic system conferring detoxification ability (*mer* operon genes) was found only in 7% of the isolates, being all aerobes. This set of data indicated the involvement of these microorganisms in the processes of methylation and detoxification of mercury in the Tagus Estuary.

To evaluate this hypothesis, isolated microorganisms and microbial communities were incubated with $HgCl_2$. The results showed that these microorganisms are able to reduce Hg^{2+} into Hg^0, resulting in the removal of around 50% of the total added mercury. The highest removal rates were observed among isolates of high contaminated areas (Barreiro and Cala do Norte). It was also observed the formation of organomercurials, including methylmercury. The rate of methylation among the isolates ranged between 1-8%. Moreover, it was found that bacteria isolated from salt marsh are influenced by plants species such as *Sacocornia fruticosa* and *Spartina maritima* since the kinetics of mercury

mobility between plant's roots and the surrounding environment affects mercury-resistant microorganisms' selection. Thus, these results are the first evidence of the relevance of interaction between bacteria and plants in Hg cycling in the Tagus Estuary.

To understand better the conditions promoting methylation and demethylation, three microbial communities (aerobic, anaerobic and sulphate-reducing bacteria communities) were incubated with isotope enriched mercury species ($^{199}HgCl$ and $CH_3{}^{201}HgCl$). The results showed that microbial communities are actively involved in methylation and demethylation processes, being the methylation directly related with sulphate-reducing bacteria communities with rates up to 0.07% (after 48h), while the demethylation process is strongly promoted (rates up to 100%) by aerobic community.

To obtain optimal conditions for mercury reduction, the effects of ambient factors, such as organic matter (glucose), sulphate, iron and chloride, on microbial reduction were evaluated by factorial design methodology. The results revealed that sulphate enhances microbial reduction, while chloride inhibits it.

Overall, the results showed that microorganisms of Tagus Estuary are involved in processes that change mercury speciation through reduction and demethylation and formation of methylmercury. The removal is a pathway for detoxification and can be used on the bioremediation strategies. Meanwhile, the formation of methylmercury represents a risk for human health. Thus, this study's set of data is useful for both risk assessment and bioremediation purposes.

KEYWORDS: Mercury; Methylmercury; Tagus Estuary; Microorganisms; Mercury cycle

RESUMO

O mercúrio é um poluente de ocorrência natural, podendo ser encontrado no ambiente em diversas formas: mercúrio elementar (Hg^0), espécies inorgânicas tais como o mercúrio mercuroso e mercúrico (Hg_2^{2+} e Hg^{2+}) e organomercuriais (ex. metilmercúrio (MeHg)). No entanto, a contaminação de sistemas aquáticos com o mercúrio proveniente de fontes antropogénicas tem sido considerado um problema de elevada magnitude e de impacto mundial. Em particular, a presença do MeHg nestes sistemas ganhou maior ênfase depois da descoberta de elevados níveis do mesmo em peixes na Baia de Minamata (Japão), o que levou à morte de 46 pessoas e à morbilidade de um elevado número. Desde então, a presença do MeHg nos sistemas aquáticos passou a ser considerado um problema de saúde pública, o que levou a que várias agências reguladoras estabelecessem limites aos níveis de mercúrio aceitáveis no peixe. A maior preocupação inerente à presença do MeHg nos sistemas aquáticos prende-se principalmente com a neurotoxicidade associada ao mesmo e às suas capacidades de bioacumulação e biomagnificação na cadeia alimentar.

Nos sedimentos dos sistemas aquáticos, o mercúrio existente sofre diversas transformações. Os microrganismos são os principais responsáveis pelas três principais transformações:

1) Metilação de Hg^{2+}, formando o MeHg;
2) Redução de formas inorgânicas, libertando o Hg^0;
3) Desmetilação de MeHg, degradando-o a formas inorgânicas.

A redução e a desmetilação são processos de destoxificação que normalmente estão associados à presença de mecanismos de resistência conferidos pelo operão *mer*. A metilação constitui igualmente um mecanismo de resistência e é normalmente associado às bactérias redutoras de sulfato (SRB) existentes em ambiente anóxicos.

O estuário do Tejo é um dos mais importantes estuários da Europa e tem grande relevância para o desenvolvimento económico e para a riqueza ecológica de Portugal. No entanto, as diversas atividades industriais que tiveram lugar nas margens deste estuário, deram origem a um historial de deterioração resultante da poluição. Entre os poluentes

encontrados neste estuário, o mercúrio é um dos mais problemáticos, sendo desde 1985 relatados níveis elevados do mesmo. Em particular, duas áreas localizadas nas margens Norte e Sul – Cala do Norte e Barreiro – continuam até aos dias de hoje a apresentar elevados níveis de contaminação nos sedimentos. Os estudos subsequentes estimaram a presença de 21 toneladas de mercúrio total e 23 kg de MeHg em sedimentos (até 5 cm), o que levanta diversas preocupações ao nível de segurança alimentar e da proteção da saúde humana. No entanto, até agora nenhum estudo foi realizado para esclarecer concretamente os processos de transformação de mercúrio mediado pelos microrganismos neste estuário. Assim, neste trabalho de Doutoramento, investigou-se o papel dos microrganismos nos processos de transformação do mercúrio no Estuário do Tejo, através da caraterização efetiva da sua atividade sobre o mercúrio existente, partindo do pressuposto de que este estudo poderá ser uma ferramenta para a melhor compreensão dos riscos associados a esta contaminação e estímulo para que sejam traçadas futuras estratégias de remediação. Assim, os objetivos específicos deste trabalho foram:

1) Isolamento e caracterização fenotípica e genética de microrganismos existentes no Estuário do Tejo que apresentam resistência ao mercúrio;
2) Avaliação da influência dos isolados para modificar a especiação do mercúrio em meios de cultura: metilação *vs*. desmetilação; redução e volatilização;
3) Avaliação do papel das comunidades microbianas nos processos de transformação dos compostos mercuriais;
4) Estudo das condições ótimas que mitigam a formação e/ou acumulação do MeHg (proposta de biorremediação).

Para dar cumprimento a estes objetivos, o trabalho foi subdivido em diversas etapas, como apresentado na Figura 1:

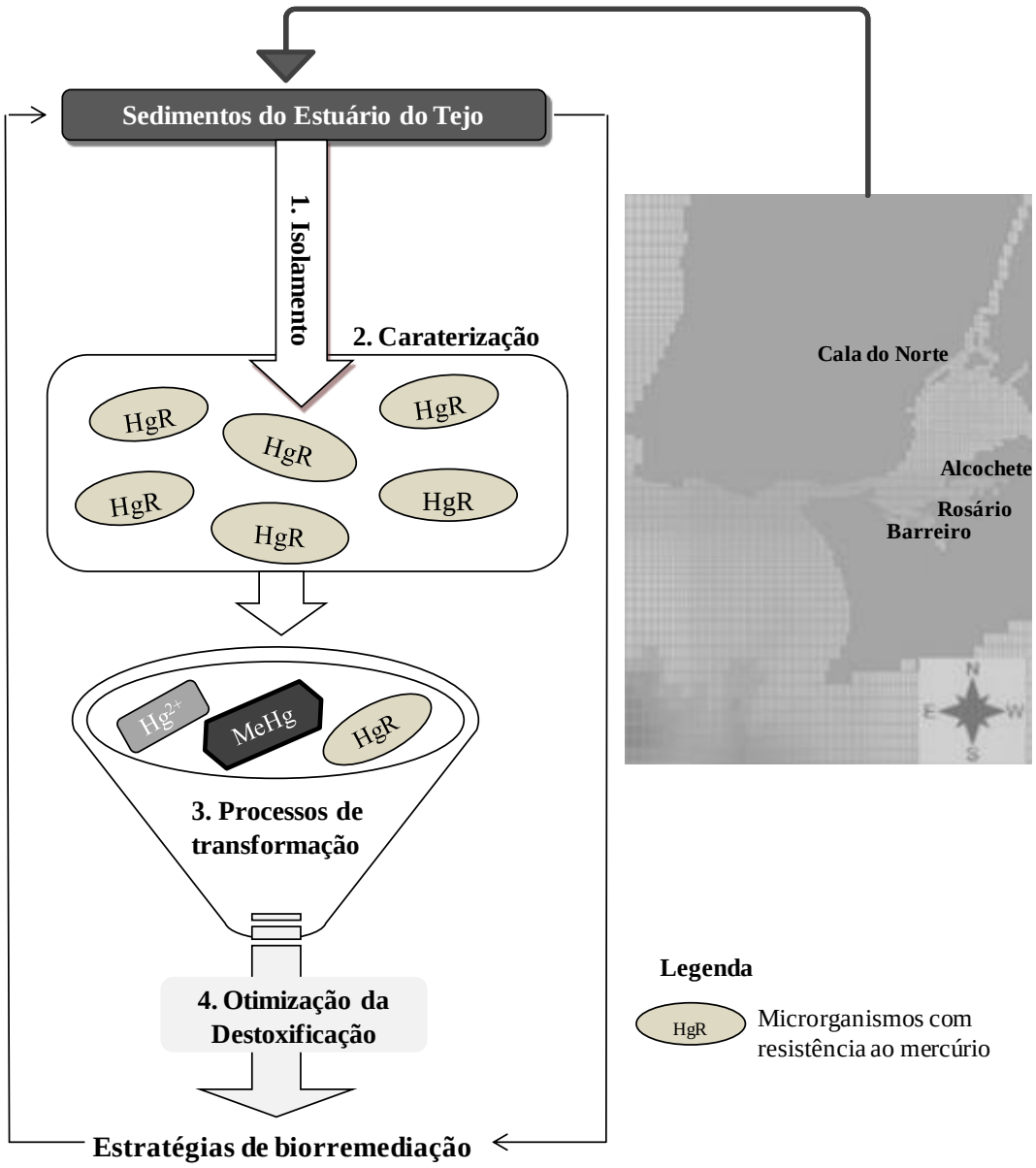

Figure 1: Resumo gráfico do trabalho experimental desenvolvido.

Os microrganismos incluídos neste estudo foram isolados a partir de sedimentos de quatro áreas do Estuário do Tejo - Barreiro, Cala do Norte, Alcochete e Rosário. Estas áreas foram selecionadas de acordo com os seus níveis de contaminação (Barreiro> Cala do Norte> Rosário> Alcochete), de forma a obter-se uma amostragem representativa da situação do estuário. De entre os isolados, selecionaram-se os microrganismos que têm resistência ao mercúrio por serem os que terão capacidade de promover as transformações das espécies de mercúrio. Para isso, utilizou-se uma pressão seletiva de MeHg (0,22 µg/mL) no meio de cultura, durante todo o processo de isolamento. Seguidamente, os microrganismos foram caraterizados, recorrendo a métodos

bioquímicos, para a diferenciação de microrganismos, e genéticos, para a identificação pelo 16SrRNA e pesquisa do operão *mer*.

De entre os isolados, foram identificados microrganismos aeróbios, pertencentes as espécies de *Bacillus*, *Vibrio*, *Aeromonas* e *Enterobacteriaceae*, e microrganismos anaeróbios, pertencentes aos géneros *Enterobacteriaceae* e *Clostridium* e espécies pertencentes ao domínio *Archaea*, nomeadamente a espécie *Desulfovibrio desulfuricans* que pertence ao grupo das SRB. As resistências destes isolados aos compostos mercuriais variaram entre 0,41-140 µg/mL para o Hg^{2+} e 0,04-50,1 µg/mL para o MeHg. A ocorrência de genes *mer* foi observada em apenas 7% dos isolados, dentre os quais se encontram os aeróbios dos géneros *Bacillus*, *Citrobacter* e *Aeromonas*. Estes primeiros resultados revelaram que:

1) A maior diversidade de espécies e a maior resistência ao mercúrio estão associadas às áreas de maior contaminação;
2) Os microrganismos do estuário do Tejo estão envolvidos nos processos de transformação do mercúrio, nomeadamente processos de redução e volatilização i.e., destoxificação;
3) A baixa ocorrência de genes *mer* sugere a existência de outros mecanismos de resistência.

Para dar cumprimento ao segundo objetivo, procedeu-se a investigação da capacidade destes microrganismos em transformar os compostos de mercúrio. Destas investigações, comprovou-se a ocorrência de processos de destoxificação e metilação levados a cabo pelos isolados. Os processos de destoxificação incluem a redução de Hg^{2+} a Hg^{0} e foi verificada através da incubação dos microrganismos isolados ou da comunidade microbiana com $HgCl_2$, resultando na remoção em média de 50% de mercúrio total existente inicialmente. As taxas de remoção mais elevadas foram observadas entre os isolados das áreas de maior contaminação (Barreiro e Cala do Norte). Aquando da incubação dos isolados com 1 µg/mL de Hg^{2+} (na forma de $HgCl_2$), foi também possível observar a formação de espécies orgânicas de mercúrio, que posteriormente se verificou que eram em parte constituídas pelo MeHg. A taxa de metilação variou entre 1-8%.

Face a estes resultados, tornou-se mandatório compreender em que condições estão a ocorrer a maior taxa de metilação e desmetilação mediada pelos microrganismos no Estuário do Tejo. Assim, para dar cumprimento ao terceiro objetivo, os processos de metilação e desmetilação foram avaliados através da incubação de três comunidades microbianas – aeróbias, anaeróbias e SRB - com isótopos enriquecidos de mercúrio ($^{199}HgCl$ e $CH_3{}^{201}HgCl$), numa proporção semelhante à existente no estuário do Tejo (0,106 µg/mL de ^{199}Hg:0,002 µg/mL de $CH_3{}^{201}Hg$). A produção de $CH_3{}^{199}Hg$ foi utilizada para calcular a percentagem de metilação e o desaparecimento de $CH_3{}^{201}HgCl$ foi utilizado para calcular a percentagem de MeHg degradado (desmetilação). A maior taxa de metilação (0,07% após 48h de incubação) foi observada entre a comunidade microbiana composta por SRB e a maior percentagem de desmetilação (100%) foi observada pela comunidade microbiana constituída por microrganismos aeróbios. A partir destes resultados, pode-se concluir que:

1) Os processos de destoxificação estão principalmente associados a atividade dos microrganismos aeróbios;
2) Os microrganismos anaeróbios são os principais responsáveis pela metilação, nomeadamente as SRBs;
3) Os processos de metilação e destoxificação mediados pelas comunidades microbianas ocorrem em simultâneo no Estuário do Tejo.

Na sequência destes resultados, optou-se pelo estudo da otimização do processo de redução como proposta de estratégia de biorremediação, tendo em conta que a redução de Hg^{2+} à forma de Hg^0 mitiga a formação de MeHg através da redução da concentração de Hg^{2+} disponível no meio para o processo de metilação. Para isso, estudou-se o efeito de alguns fatores relevantes nos processos de transformação do mercúrio, nomeadamente a matéria orgânica (glucose), o sulfato, o ferro e o cloreto, usando uma gama de concentrações próximas das existentes no Estuário do Tejo. Os resultados revelaram que o sulfato e o cloreto são os fatores que mais afetam o processo de redução, favorecendo e inibindo-o, respetivamente.

Em suma, os resultados obtidos neste trabalho dão cumprimento aos objetivos propostos, demonstrando o papel crítico dos microrganismos nos processos de redução e remoção do mercúrio e na formação de MeHg no Estuário do Tejo. No entanto, a formação de MeHg

também existe e representa risco para a saúde humana assim como risco ambiental. Os resultados evidenciam que a redução e a remoção constituem uma via de destoxificação promissora que poderá ser utilizada no desenvolvimento de estratégias de remediação. Neste contexto, considera-se que este estudo apresenta um conjunto de dados relevantes para a avaliação de riscos associados à contaminação do Estuário do Tejo pelo mercúrio e no desenvolvimento de futuras estratégias de remediação.

PALAVRAS-CHAVE: Mercúrio; Metilmercúrio; Estuário do Tejo; Microrganismos; Ciclo biogeoquímico do mercúrio

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INITIAL STATEMENT

- OUTPUTS

This thesis represents my original work and involved the collaboration between the Toxicology and the Microbiology laboratories at Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia and Centro de Química Estrutural in Instituto Superior Técnico, as well as in Centro de Química e Bioquímica from Faculdade de Ciências, all of them affiliated to Universidade de Lisboa.

Part of this work was also performed at Chemistry Department, Trent University (Canada).

Parts of this thesis are already published in international peer-reviewed journals:

1. Figueiredo, N.L.L., Canário, J., O'Driscoll, Duarte, A. and Carvalho, C. 2016. **Aerobic mercury-resistant bacteria alter Mercury speciation and retention in the Tagus estuary (Portugal)**. Ecotoxicology and Environmental Safety, 124:60-67. doi: 10.1016/j.ecoenv.2015.10.001. Epub 2015 Oct 19.

2. Figueiredo, N.L.L., Areias, A., Mendes, R., Canário, J., Duarte, A. and 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: Current Issues, 77:14-16, 959-971. doi: 10.1080/15287394.2014.911136

3. Figueiredo, N.L.L., Canário, J., Duarte, A., Serralheiro, M. L. and Carvalho, C., 2014. **Isolation and characterization of mercury-resistant bacteria from sediments of Tagus Estuary (Portugal): Implications for environmental and human health risk assessment.** Journal of Toxicology and Environmental Health, Part A: Current Issues, 77:155-168. doi: 10.1080/15287394.2014.867204

Some papers are <u>in preparation</u>:

1. Figueiredo, N.L.L., Hintelmann, H., Canário, J., Duarte, A., Serralheiro, M.L. and Carvalho, C., 2016. Evidence of mercury methylation and demethylation by the estuarine microbial communities obtained in stable Hg isotope studies. *In prep to be submitted*

2. Figueiredo, N.L.L., Canário, J., Serralheiro, M.L. and Carvalho, C., 2016. Optimization of microbial detoxification for a mercury-contaminated environment. *In prep to be submitted*

3. Figueiredo, N.L.L. and Carvalho, C., 2016. The Impact of Microbial Mercury Transformations in Aquatic Systems: Environmental and Human Health (review). *In prep to be submitted*

4. Figueiredo, N.L.L. and Carvalho, C., 2016. Environmental Importance of Estuaries: Tagus Estuary (Portugal) as a case-study (review). *In prep to be submitted.*

The results also have been presented as oral (3) and poster (10) communications in international and national meetings.

International scientific meetings

Oral communications:

1. Canário, J., Carvalho, C., Caetano, M., Figueiredo, N., Cesário, R., Hintelmann, H., O'Driscoll, N.J., 2015. Mercury and methylmercury cycle in the Tagus Estuary, Portugal: Major findings of the PROFLUX Project. 12th International Conference on Mercury as a Global Pollutant, June 14-19, Jeju, South Korea.

2. Figueiredo, N., Canário, J., Duarte, A. and Carvalho, C. Relationship between Bacteria Susceptibility to Mercury and Mercury Contamination on Tagus Estuary (Portugal). 2nd International Conference on Occupational and Environmental Toxicology (ICOETox), 16th – 17th September 2013, Porto, Portugal.

Posters:

1. Figueiredo, NL, Hintelmann, H., Canário, J., Duarte, A., Serralheiro, M.L. and Carvalho, C. Evaluation of mercury methylation/demethylation by the microbial community of Tagus estuary using stable Hg isotopes. 51st Congress of the European Societies of Toxicology (Eurotox), 13st - 16th September 2015, Porto, Portugal. Toxicology Letters. 10/2015; 238(2):S135. DOI: 10.1016/j.toxlet.2015.08.424

2. Figueiredo, NL, Duarte, A. and Carvalho, C. *Mer* operon genes occurrence among mercury-resistant bacteria of Tagus Estuary (Portugal). Trends in Environmental Microbiology for Public Health (TEMPH), 18 - 21 September 2014, Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL) Lisboa, Portugal.

3. Figueiredo, NL, Canário, J., Duarte, A. and Carvalho, C. Bacterial Transformation of Mercury in a Polluted Environment: Impact on Human Health. 50th Congress of the European Societies of Toxicology 7th-10th September 2014 Edinburgh, Scotland. Toxicology Letters, Volume 229, Supplement, 10 September 2014, Page S1-S252. P-2.36. http://dx.doi.org/10.1016/j.toxlet.2014.06.382

4. Figueiredo, NL, Areias, A., Canário, J., Duarte, A. and Carvalho, C. Isolation and characterization of mercury-resistant bacteria from salt marsh of Tagus estuary. 2nd International Conference on Occupational and Environmental Toxicology (ICOETox), 16th – 17th September 2013, Porto, Portugal.

5. Figueiredo, NL, Canário, J, Duarte, A and Carvalho, C. Mercury-Resistant *Citrobacter freundii* strain 1.1SvA isolated from Tagus Estuary – Characterization and Mercury Detoxification Potential. 11th International Conference on Mercury as a Global Pollutant (ICMGP) 28th July –2nd August 2013, Edinburgh, Scotland.

6. Figueiredo, NL, Canário, J, Duarte, A and Carvalho, C. Mercury-Resistant Bacteria from Tagus Estuary -Characterization and Mercury Reduction Potential. 49th Congress of the European Societies of Toxicology (Eurotox), 1st - 4th September 2013, Interlaken, Switzerland. Toxicology Letters, Volume 221, Supplement, 28 August 2013, Page S110, ISSN 0378-4274, http://dx.doi.org/10.1016/j.toxlet.2013.05.176

7. Figueiredo, N.L., Canário, J., Duarte, A. and Carvalho, C. Bacteria from Tagus Estuary: Characterization and Mercury Transformation Potential. 48th Congress of the European Societies of Toxicology (Eurotox), 17th-20th June 2012, Stockholm, Sweden. Toxicology Letters, Volume 211, Supplement, doi: 10.1016/j.toxlet.2012.03.351.

National scientific meetings

Oral communication:

1. Figueiredo, N., Canário, J. and Carvalho, C. PROFLUX – Bacteria Role in Mercury Cycle in Tagus Estuary. Seminário de grupo de investigação do iMed.UL - Chemical, Biology and Toxicology (CBT) - da Faculdade de Farmácia da Universidade de Lisboa. 2012.

Posters:

1. Figueiredo, NL, Canário, J, O'Driscoll, N, Duarte, A and Carvalho, C. Bacterial Transformation of Mercury in Tagus Estuary: Implication in Environment and Human Neurotoxicity. 5th iMed Postgraduate Students Meeting, 18th July 2013 Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal.

2. Figueiredo, NL, Canário, J, Duarte, A and Carvalho, C. Mercury and Antibiotic Resistance in Bacteria Isolated from Tagus Estuary. Congresso Nacional de Farmacêuticos, Novembro 2012, Lisbon, Portugal.

3. Figueiredo, NL, Canário, J, Duarte, A and Carvalho, C. Hg-resistant bacteria community in Tagus Estuary and their antibiotic resistance. 4th iMed Postgraduate Students Meeting, December 2012 Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal.

AIMS OF THIS THESIS

The main objective of this study is to give insight about the involvement of microorganisms in the cycle of mercury in the Tagus Estuary based on their complete characterization (phenotypic and genetic) and activity. In this sense, some specific goals were drawn:

1. To isolate and characterize phenotypically and genetically the microorganisms from the Tagus Estuary that have more resistance to mercurials compounds;

2. To evaluate the capacity of the isolated microorganisms to alter mercury speciation in growth media: methylation vs. demethylation; reduction and volatilization;

3. To evaluate the role of microbial communities in the processes of mercury transformations;

4. To study the optimal conditions to impair MeHg formation and/or accumulation (bioremediation proposal).

OUTLINE OF THE THESIS

Chapter 1 corresponds to the Introduction and is divided in two parts: A and B.

Part A gives an overview about mercury toxicology, namely human exposure to the three main forms of mercury (elemental, inorganic and methylmercury). The main health effects, especially the neurotoxicity are discussed. Furthermore, the state-of-the-art of mercury transformations in aquatic systems, with emphasis to the formation of methylmercury by microorganisms and the microbial transformations affecting MeHg net and fate is analyzed. The impact of methylmercury formation is also revised.

Part B gives an overview on the importance of estuaries and the impacts of anthropogenic pressure on these systems, including the strategies to recover impacted estuaries. The special case of Tagus estuary is detailed by discussing its historical pollution, namely mercury contamination.

Chapter 2 reports the results of the isolation and characterization of mercury-resistant microorganisms from sediments of the two hotspots of mercury pollution and one area of low mercury contamination in the Tagus Estuary.

Chapter 3 provides the data about the isolation and genetic characterization of mercury-resistant bacteria from a salt marsh area of Tagus Estuary. The results showed their prevalence in the rhizosphere and the consequences for mercury cycling are presented in this chapter.

Chapter 4 details the results about the mercury conversions performed by aerobic bacteria isolated from the Tagus Estuary, showing the occurrence of methylation and reduction of Hg^{2+}. The impacts of these transformations for human health are discussed here.

Chapter 5 relates the research carried out with isotope enriched mercury species that were analysed by ICP-MS to evaluate the capacity of different microbial communities of Tagus Estuary to methylate mercury and demethylate methylmercury.

Chapter 6 reports the study of the optimization of the reduction process in the Tagus Estuary, using the factorial design methodology. The results obtained for the influence of four factors (glucose, sulphate, iron and chloride) and their interactions are discussed.

Chapter 7 summarizes the main conclusions of this work and analyses the perspectives for future work.

ABBREVIATIONS

AAS - Atomic absorption spectroscopy

Acetyl-CoA - Acetylcoenzyme A

AM - Ampicillin

AMC - Aerobic microbial community

AnMC - Anaerobic microbial community

As L Arsenic

CCD - Central Composite Design

Cd - Cadmium

CH_4 - Methane

CH_3 - Methyl

CFU - Colony-forming units

CLSI - Clinical and Laboratory Standards Institute

CNS - Central nervous systems

Co - Cobalt

CO_2 - Carbon dioxide

Cr - Chromium

Cu - Copper

CV-AFS - Cold vapour atomic fluorescence spectroscopy

Cys - Cysteine

DDT - Dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide

DOC - Dissolved organic carbon

DOM - Dissolved organic matter

ERL - Effects Range Low

ERM - Effects Range Medium

FAD - Flavin Adenine Dinucleotide

GC-AFS - Gas chromatography with atomic fluorescence spectroscopy

GC/ICP-MS - Gas Chromatographic/Inductively Coupled Plasma - Mass Spectrometry

GPxs - Glutathione peroxidase

GR - Glutathione reductase

GSH - Glutathione

HCHs - Hexachlorocyclohexanes

HCB - Hexachlorobenzene

Hg - Mercury

hgcA - putative corrinoid protein gene

hgcB - Ferredoxin-like protein gene

$HgCl_2$ - Mercury chloride

HNO_3 - Nitric acid

HgR - Mercury-resistant (referring to microorganisms such as bacteria)

HgS - Cinnabar

HgT - Total mercury

Hg^0 - Elemetal mercury

Hg^{2+} - mercuric ion

H_2O_2 - Hydrogen peroxide

K - Kanamycin

LAS - Linear alkylbenzene Sulfonates

Mer - mercury resistance operon

MeHg - Methylmercury

MeHgCl - Methylmercury chloride

MeSn - Methyl tin

MIC - Minimal Inhibitory Concentrations

MH - Mueller-Hinton broth

Mn - Manganese

MTr - Methyltransferase

NA - Nalidixic acid

NADPH - Nicotinamide adenine dinucleotide phosphate

NCCLS - National Committee for Clinical Laboratory Standards

Ni - nickel

NO - Nitric oxide

NRC - National Research Council

O_2^- - Superoxide anion

PAHs - Polycyclic aromatic hydrocarbons

Pb - lead

PCBs - Polychlorinated Biphenyls

PCR - Polymerase chain reaction

PHs - Petroleum hydrocarbons

PTWI - Provisional Tolerable Weekly Intake

RA - Rifampicin

RfC - Reference Concentration

RfD - Reference Dose

ROS - Reactive oxygen species

rRNA - Ribonucleic acid of ribosomes

S - Streptomycin

SeH - Selenol group

SH - Thiol group

SO_4 - Sulphate

SOD - Superoxide dismutase

SO_4-RMC - Sulphate-reducing microbial community

SRB - Sulphate-reducing bacteria

STD - Standard deviation

TBT - Tributyltin

TE- Tetracycline

THF - Tetrahydrofolate

Trx - Thioredoxin

TrxR - Thioredoxin reductase

TWI - Tolerable Weekly Intake

UNDP - United Nations Development Programme

UNEP - United Nations Environment Program

US EPA - United States Environmental Protection Agency

Unesco - United Nations Educational, Scientific and Cultural Organization

UV - Ultraviolet

WFD - Water Framework Directive

WHO - World Health Organization

Zn - Zinc

CHAPTER I - GENERAL INTRODUCTION

PART A - THE IMPACT OF MICROBIAL MERCURY TRANSFORMATIONS IN AQUATIC SYSTEMS: ENVIRONMENTAL AND HUMAN HEALTH †

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ABSTRACT CHAPTER IA

In natural systems, mercury can be found in different chemical forms such as elemental, inorganic and organic compounds. Although all forms of mercury are toxic, methylation of inorganic mercury with the formation of methylmercury is of great risk, as it undergoes bioaccumulation and biomagnification in food chain, which represents a risk of poisoning for a population who routinely consumes fish. Methylmercury is known to be highly neurotoxic in humans, especially to foetuses and children. Methylmercury also represents a risk for wildlife due to its adverse effects, namely toxic effects on reproduction.

It is in the aquatic ecosystems - oceans, lakes, and rivers – where methylation of mercury is promoted by microorganisms including both 1) *Archeae*, such as sulphate-reducing bacteria (e.g. *Desulfovibrio desulfurican*) and methanogens, and 2) *Bacteria*, such as *Clostridium* sp., iron-reducing bacteria (e.g. *Geobacter sulfurreducens*), *Enterobacteriaceae* sp. and *Bacillus* sp. (*Bacillus megaterium*). However, microorganisms are also involved in processes that mitigate methylmercury formation and accumulation, namely Hg^{2+}-reduction and demethylation; these processes are mainly associated to aerobic bacteria which possess *mer* operon in their genes. This work reviews the abiotic and biotic, i.e. microbial, transformations which affect formation, net and fate of methylmercury in aquatic systems. The impact of these transformations for both human health and environmental risk assessment is further discussed.

IA.1. INTRODUCTION

Mercury is one of 188 hazardous air pollutants (EPA, 2007) and it is among the most prominent toxic heavy metals, such as cadmium and lead (Syversen and Kaur, 2012). The Emergency Planning and Community Right-to-Know Act (EPCRA) classified mercury as a persistent, bioaccumulative and toxic compound (Section 313) (EPA, 2007). Mercury exists naturally in our environment, being present in air, aquatic systems and soil (Syversen and Kaur, 2012) and, as chemical element, it cannot be created nor destroyed, so the same amount exists since the formation of the Earth, in different chemical forms. Mercury species undergo chemical transformations in a complex biogeochemical cycle and each of them exhibit different toxic effects upon interaction with biological structures and processes (Syversen and Kaur, 2012).

Mercury appears normally in three species: elemental (Hg^0), inorganic (mercurous - Hg_2^{2+} and mercuric - Hg^{2+}) and organomercurial compounds (including alkyl mercury compounds, such as methylmercury (MeHg), ethylmercury and phenylmercury) (Tchounwou et al., 2003). All of these three species are found in the environment and each one has specific properties, such as solubility and chemical reactivity (Clarkson, 2002; Tchounwou et al, 2003), which determine their environmental persistence and toxicity.

Since ancient times, mercury adverse effects have been recognized as a concern, however, in the last 30 years a flurry interest in mercury as an environmental pollutant arose. In particular, the presence of mercurial compounds in aquatic systems is a concern nowadays, as it was realized that Hg^{2+}, the most common form discharged into the environment by human activities, suffers abiotic and biotic conversions into organic mercurial compounds, mainly MeHg (Morel et al., 1998; Yu, 2000). Besides the formation of MeHg, there are other transformations interfering with the aquatic concentration of mercury such as Hg^{2+}-reduction and demethylation.

Here, it is reviewed the toxicological effects of mercury, highlighting MeHg effects, and abiotic and biotic transformations which affect formation, net and fate of MeHg in

aquatic systems. The impact of these transformations for both human health and environmental risk assessment is also discussed.

IA.2. MERCURY TOXICOLOGY

Mercury exists in various chemical forms. All forms of mercury are known to cause adverse health effects in a number of tissues and organs, depending of its chemical form, as well as, the levels, duration and route of exposure (Ung et al., 2010). In general, its toxic effects include neurotoxicity, teratogenicity, nephrotoxicity, immunotoxicity and cardiotoxicity (Clarkson and Magos, 2006; Clarkson, 2002).

IA.2.1. Exposure routes

Exposure of living organisms to mercury species comes from inhalation of air, drinking of contaminated water and eating of mercury contaminated fish. Humans have been also exposed through the use of a range of products containing mercury compounds, such as dental amalgams and thermometers (Hg^0) (Rooney, 2007), antiseptic agents (Hg^{2+}) (Tchounwou et al., 2003), biocides, pesticides and preservatives in vaccines (organic forms) (Clarkson, 2002). Nevertheless, the usage of ethylmercury and MeHg as a fungicide or on food grain was banned by U.S. Environmental Protection Agency (USEPA) and World Health Organization (WHO) and the addition of phenylmercuric acetate in paints was ceased in 1991 (EPA, 2007). Thimerosal (ethylmercury thiosalicylate) has been incorporated as a preservative in some vaccines and other pharmaceuticals, however, is being phased out or significantly reduced in many countries (WHO/UNEP, 2008).

IA.2.2. Health effects

From the toxicological viewpoint, elemental mercury, inorganic forms and MeHg are the most important mercurial forms, since most of the people are exposed to these three forms as a result of their normal activities (WHO and UNEP, 2008). Because of this, regulatory agencies worldwide established safety guidelines for these compounds

(Table IA.1). In this section, a brief review of the toxicological effects of mercury compounds is made.

Table IA.1: Toxicological effects of elemental mercury, inorganic mercury compounds and MeHg and their reference levels.

	Elemental mercury	**Inorganic Hg forms**	**MeHg**
Exposure routes	Inhalation	Ingestion	Ingestion
Target organs	Central nervous system, Peripheral nervous system, Kidney	Kidney	Central nervous system
Health effects	Neurotoxicity Nephrotoxicity Teratogenicity	Nephrotoxicity Cardiotoxicity Immunotoxicity [3]Dermatitis	Neurotoxicity Immunotoxicity Teratogenicity Death
Symptoms	[1]Tremors, erethism, insomnia, neuromuscular changes, headaches, [2]paresthesia, hyperactive tendon reflexes, slowed sensory and motor nerve conduction velocities and memory loss.	Inflammation of kidney (formation of autoimmune glomerulonephritis), increased heart rate and blood pressure and damage in the digestive tract.	[2]Paraesthesia, [4]ataxia, sensorial disturbances, [1]tremors, impairment of hearing, constriction of visual field and loss of balance.
Reference levels	[a]RfC = 0.3 µg/m^3 of air (US EPA)	[b]RfD = 0.3 µg/kg day^{-1} (US EPA)	[c]PTWI = 1.6 µg/kg [d]bw /week (WHO) [e]TWI = 1.3 µg/kg bw/week (EFSA) [b]RfD = 0.1 µg/kg day^{-1} (US EPA)
References	WHO and UNEP, 2008	WHO and UNEP, 2008	Ahlmark, 1948, Clarkson and Magos, 2006; EFSA, 2015; Magos, 1997; US EPA, 2001WHO, 2004; WHO and UNEP, 2008;

[1]Tremors: shaking or quivering, especially in the hands. [2]Paraesthesia: Numbness and tingling. [3]Dermatitis: Inflammation of the skin. [4]Ataxia: Failure of muscular coordination. (WHO, 2016).
[a]RfC – Reference Concentration, [b]RfD – Reference Doses, [c]PTWI - Provisional Tolerable Weekly Intake, and [d]bw – body weight, and [e]TWI - Tolerable Weekly Intake.

IA.2.2.1. Elemental mercury

Elemental mercury is liquid at room temperature and has a high vapor pressure. People may be exposed to elemental through the inhalation of ambient air during occupational activities or at home (e.g. mercury-containing thermometers) and from dental amalgams (WHO and UNEP, 2008; Clarkson, 2002; Rooney, 2007). Dental amalgams contain 50% of Hg^0 in their composition (Dye et al., 2005) and its release in the form of Hg^{2+} and/or Hg^0 is inhaled, being 3 to 17 µg of mercury absorbed from the mercury vapours (Clarkson and Magos, 2006; UNEP, 2002).

When ingested or absorbed, Hg^0 is almost completely excreted in the faeces with little to mild toxic damage to the organism (WHO and UNEP, 2008). About 80% of the inhaled Hg^0 are absorbed by the lung tissues and, once absorbed, it is readily distributed throughout the body and eventually can cross both placental and blood-brain barriers (WHO and UNEP, 2008). Once it crosses placental and blood-brain barriers, is oxidized into inorganic mercury ions, which can be retained for several weeks or months in the brain. Effects on the central nervous systems (CNS) are thus the most sensitive toxicological end-point of Hg^0 exposure. In humans, neurological and behavioural disorders have been observed, through various symptoms (Table IA.1). At higher concentrations, adverse effects can be observed also in kidney, thyroid, lung and visual system (WHO and UNEP, 2008).

IA.2.2.2. Inorganic mercury

People are exposed to inorganic mercury mainly at working places, namely where mercury compounds are produced, used in processes or incorporated in products (WHO and UNEP, 2008). Absorption of inorganic mercury may occur after inhalation of aerosols of mercuric chloride ($HgCl_2$) (Clarkson, 1989).

In the gastrointestinal tract, as much as 20% of Hg^{2+} may be absorbed, being the majority of the ingested Hg^{2+} excreted through the faeces and in the urine (WHO and UNEP, 2008). The portion that is absorbed remains in the body for a considerable length of time (e.g. half-life in blood is 20-66 days). Conjugation with glutathione (GSH) forming a complex similar to oxidized glutathione, can also occur (Clarkson and

Magos, 2006), being a fraction of this complex secreted into the bile followed by Hg^{2+} elimination in the faeces (Ballatori and Clarkson, 1985). However, the same complex can also enter systemic circulation and eventually reach the kidney where, due to its small size, is not retained by glomerular filtration (Zalups, 2000).

Damage to the kidney associated to inflammation is the key end-point in inorganic mercury exposure (Table IA.1) (WHO and UNEP, 2008). Cardiac effects, damage in digestive tract and adverse effects to the skin after dermal exposure have been also reported (WHO and UNEP, 2008) (Table IA.1).

IA.2.2.3. MeHg

MeHg is 100 times more toxic than the inorganic forms (Robinson and Tuovinen, 1984). People are exposed to MeHg mainly through their diet, especially through the consumption of fish and other marine species (WHO and UNEP, 2008). After chronic MeHg exposure, paresthesia and ataxia can be observed (Ahlmark, 1948, Clarkson and Magos, 2006), followed by other neurological symptoms (Table IA.1); dementia and death will follow then (Ahlmark, 1948).

The first case of MeHg poisoning was registered in 1860, when two laboratory technicians died after its synthesis (Clarkson, 2002). However, the potent antifungal properties of the short-chain alkyl mercury compounds lead to the usage of such compound in agriculture. Although, few cases of poisoning were reported, in the first half of 20^{th} century there was already a concern about the usage of MeHg in industry and agriculture. In the late 1950s and early 1960s serious outbreaks of alkyl mercury poisoning erupted in several developing countries (Clarkson, 2002). The largest outbreak resulted from agriculture use and occurred in Iraq (1971–1972). It was caused by the preparation of homemade bread directly from the MeHg-treated seeds grain. About 6,000 cases were admitted to hospitals and an epidemiologic follow-up estimated that around 40,000 individuals may have been poisoned (Clarkson, 2002).

Another serious outbreak occurred in Minamata (Japan) and that was the first well-documented acute MeHg poisoning by consumption of contaminated fish. The values of MeHg in the seafood were exceptionally high (> 20 µg/g), due to the direct industrial

discharge of effluents loaded with MeHg and other mercury compounds to the Minamata bay water (Clarkson et al., 2003; Clarkson and Magos, 2006). This caused chronic mercury poisoning from 1956 up to 1998 (Ekino et al., 2007), with more than 2,200 cases of clinical manifestations that became known as the Minamata disease (Ekino et al., 2007). Infants exposed in uterus to MeHg were born suffering from severe brain damage, such as mental retardation, seizure disorders, cerebral palsy, blindness and deafness (NRC, 2000; Clarkson et al., 1992; 2003). Since the Minamata outbreak, both the scientific community and the general public realized that fish consumption is the major route by which humans are exposed to MeHg, which forced regulatory agencies worldwide to established food safety guidelines for mercury intake by human populations (Table IA.1).

Followed, several observational epidemiological studies were performed in human populations that consumed significant quantities of fish or seafood. For instance, in populations from Seychelles Islands, Faroe Islands and New Zealand (Table IA.2) known for their diet habits that included frequent fish-meals, occasional whale meat (mercury levels around 1.6 ppm) and shark meat (mercury levels > 4 ppm), which contained high mercury content (Clarkson and Magos, 2006). Indigenous population of Amazon were exposed to MeHg as a result of gold mining in the Brazilian Amazon Basin, which leads to the contamination of freshwater fish in downstream areas with concentrations of MeHg often exceeding 0.5 ppm (Castoldi et al., 2001). The main observations and conclusions resulting from these four studies are shown in Table IA.2; with the exception of the Seychelles study, all other studies found an association between neuropsychological deficit and prenatal exposure to MeHg.

Table IA.2: Large-scale epidemiological studies reporting prenatal MeHg exposure and consequent adverse effects (Adapted from Castoldi et al., 2001).

Epidemiological study	Levels of exposure	Observations and conclusions
Seychelles (Davidson et al., 1995; Myers et al., 1997)	Mean: 6.1 ppm [0.6-36 ppm] (Hair levels of mothers during pregnancy)	No adverse influence of prenatal or postnatal MeHg exposure on neurodevelopment was demonstrated indicating that the high nutritional value of a fish-based diet may help to prevent some of the chronic effects of mercury exposure in human populations.
Faroe Islands (Grandjean et al., 1997).	Mean: 4.3 ppm [2.6-7.7 ppm] (Hair levels of mothers during pregnancy)	Children showed a pronounced mercury-related neuropsychological deficit in the domains of language, attention, memory, and to a lesser extent in visuospatial and motor functions.
New Zealand (Castoldi et al., 2001; Kjellsrom, 1989)	>6 ppm (Hair levels of mothers during pregnancy)	Poorer performances of children submitted to neurodevelopmental tests, such as Denver Developmental Screening Test, Wechsler Intelligence Scale for Children-Revised and Test of Language Development; this poor performance was then related with maternal hair mercury level (13–15 ppm).
Indigenous population of Amazon (Cordier et al., 2002; Grandjean et al., 1999; Lebel et al., 1998; Yokoo et al., 2003)	Mean: 11.0 pg/g (Hair levels of children) Mean: 11.6 pg/g (Hair levels of mothers)	Neuropsychological examination (motor function, attention, and visuospatial performance) of 351 children between 7 and 12 years of age from four comparable Amazonian communities showed decreased performance in neurobehavioral outcomes associated with hair mercury concentration.

Despite the unique susceptibility of the foetus, cases of MeHg poisoning in adults exposed acutely have also been described and symptoms included blurred vision, hearing impairment, olfactory and gustatory disturbances, ataxic gait, clumsiness of the hands, dysarthria, and somatosensory and psychiatric disorders (Ekino et al., 2007).

IA.2.3. Mechanisms of action

Mercury compounds enter the body's circulation via different exposure routes mentioned above. Inhaled mercury accumulates in red blood cells and is carried out to all tissues in the body in less than 24 hours, whereas the ingested mercury is absorbed in

the gastrointestinal tract and is distributed to all tissues in about 30 hours (Clarkson, 2002). The high mobility of mercury in the body is attributed to the formation of water-soluble mercury complexes, being that 99% of the mercury in blood circulation exists as mercury-SH-groups complexes (Clarkson, 1972; Lurscheider et al., 1995). This affinity to SH-groups facilitates its permeabilization through cell membranes and promotes its cytotoxic effects (Figure IA.1) (Clarkson, 1972; Lurscheider et al., 1995). Additionally, mercury crosses the blood-brain and placental barriers in the form of L-cysteine complex.

Once inside the cell, mercury disrupts vital cellular functions by interfering with the integrity and function of enzymes and proteins (Ung et al., 2010) (Figure IA.1). For instance, the modification or damage of proteins induces cell structure disruption, interfering with cell morphology and motility, which is responsible for cell transportation, division, mobility and signalling (Alberts et al., 2002). On the other hand, mercury compounds induce oxidative stress, by increasing reactive oxygen species (ROS) (Ung et al., 2010) and consequently lead to the depletion of antioxidants or enzymes possessing SH-groups that are involved in the reduction of ROS, such as GSH, GSH-peroxidase and GSH-reductase and superoxide dismutase (SOD) (Livardjani et al., 1991; Oh and Lee, 1981; Ung et al., 2010; Franco et al., 2009). Furthermore, oxidative stress induces DNA damage and dysfunction of cell's organelles, such as mitochondria that increase oxidative phosphorylation and the disruption of the electron transport chain may occur (Ung et al., 2010) leading to cell apoptosis (Figure IA.1).

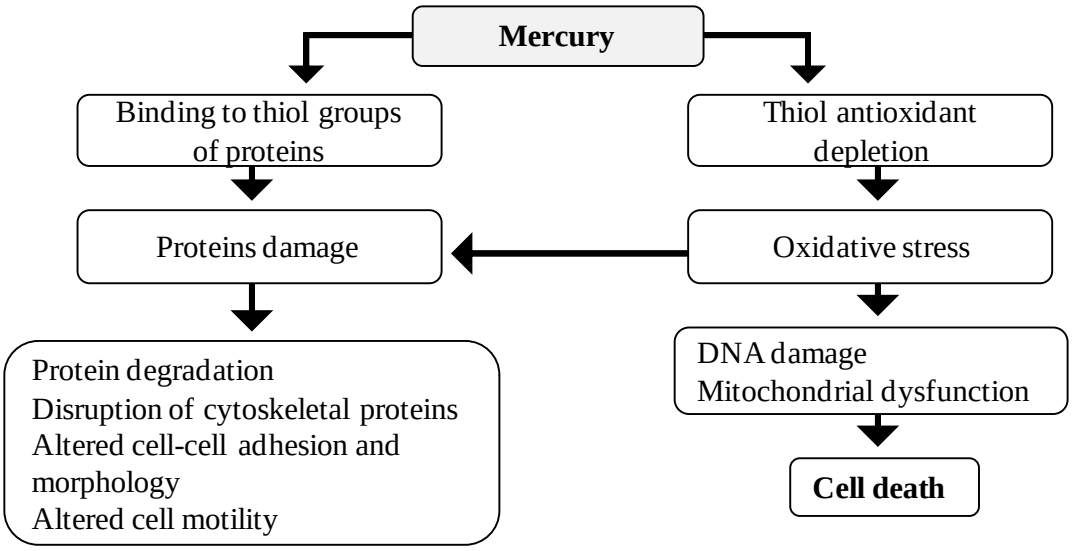

Figure IA.1: Primary mechanisms behind mercury toxicological effects, which can lead to cell death (Adapted from Ung et al., 2010).

Besides the affinity to SH-groups, mercury is also known to interact with selenols (-SeH) (Carvalho et al., 2008). Thus, selonoproteins such as glutaredoxin peroxidase (GPxs) and the thioredoxin system are good targets for mercury (Branco et al., 2012a,b; Carvalho et al., 2008). In particular, the thioredoxin system inhibition has been proposed as the key mechanism in mercury toxic effects due to its particular higher sensitivity to mercury compounds (Branco et al., 2011, 2012a,b, 2014; Carvalho et al., 2008). The thioredoxin system includes NADPH (Nicotinamide Adenine Dinucleotide Phosphate), the flavoprotein thioredoxin reductase (TrxR) and thioredoxin (Trx), which are essential for several cellular functions, such as protein repair and regulation of the cellular cycle (Lillig and Holmgren, 2007). Trx is responsible for the reduction of SH groups in several proteins, while TrxR is responsible for the reduction of Trx after its oxidation (Holmgren, 1989). As an example, Trx is known as an hydrogen donor for ribonucleotide reductase, an essential enzyme providing deoxyribonucleotides for DNA replication (Holmgren, 1989) thus, the loss of Trx and TrxR activity has been implicated in the development of several pathologies, such as cardiac disease, embriogenic disorders, neurodegenerative diseases, and carcinogenesis (Conrad, 2009; Matés et al., 2010; Venardos et al., 2004; Wu et al., 2003).

Regarding mercury effect over the thioredoxin system enzymes, in the last decade, studies have been shown the inhibitory effect of Hg^{2+} and MeHg over both TrxR and Trx *in vitro* (Carvalho et al., 2008) and *in vivo* (Branco et al., 2011, 2012a,b). For instance, a decrease of about 50% in the TrxR activity was observed in brain, liver and kidney of zebra-seabreams (Branco et al., 2012a,b). Ethylmercury was also found to inhibit this system *in vitro* (Rodrigues et al., 2015). The mechanism behind this inhibition is related to the binding of mercurial compounds to the selenocysteine and cysteine residues in the active site of Trx and TrxR (Carvalho et al., 2008, 2011).

Mercuric mercury can be conjugated with selenium forming mercuric selenide (HgSe) precipitates, which have been observed in the liver of several species and are thought to be a detoxification mechanism for mercury (Groth et al., 1976; Carvalho et al., 2011; Branco et al., 2012b). In fact, Branco et al. (2012a,b) already showed that co-exposure of Hg^{2+} with selenium prevent its inhibitory effect over TrxR activity by removing it from the active site of the enzyme.

Both Hg^0 and MeHg are able to cross the brain blood barrier and cause neurotoxic effects (Clarkson and Magos, 2006); however the toxicokinetics of the compounds is different. For instance, toxic symptoms related with MeHg neurotoxicity is delayed relatively to the peak of exposure to mercury (Clarkson and Magos, 2006).

One of the proposed mechanisms for mercury neurotoxicity is related with the disruption of glutamate transport (Figure IA.2), the main excitatory neurotransmitter in central nervous system (Aschner et al., 1993; Fonfria et al., 2005). MeHg and Hg^{2+} enhance glutamate release from the pre-synaptic terminal hindering their uptake by astrocytes. High levels of glutamate in the synaptic cleft promote an increased calcium influx to the post synaptic terminal, which affect the mitochondria through the increase of nitric oxide synthase activity and consequently nitric oxide production. Moreover, the increase of ROS production (Figure IA.2) has been considered the major mechanism behind MeHg-induced toxicity (Syversen and Kaur, 2012). For instance, MeHg affects the mitochondrial electron transfer chain, mainly at the level of complex II-III leading to the increased formation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which in turn can produce the highly toxic hydroxyl radical anion via Fenton's reaction.

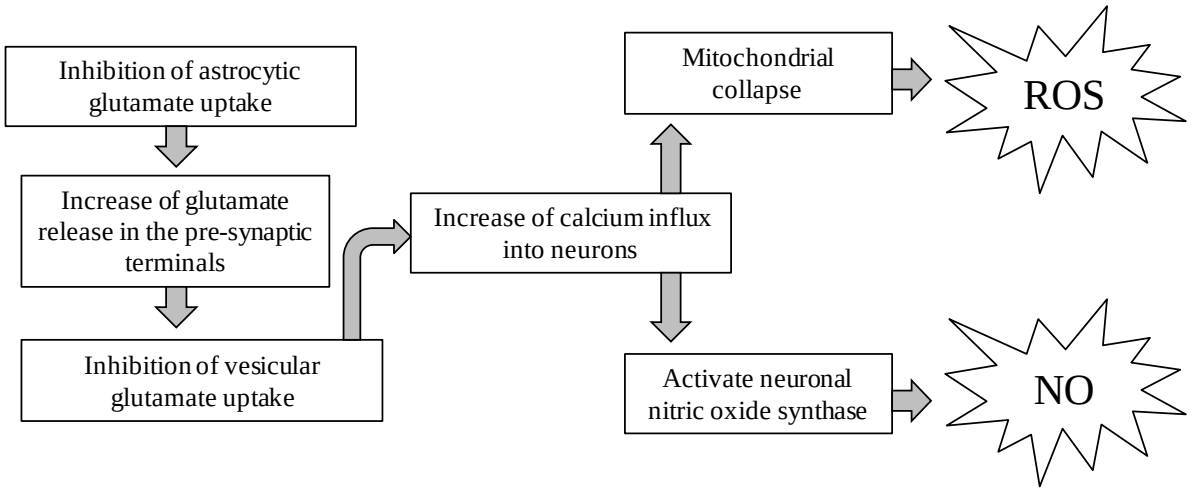

Figure IA.2: Disruption of neuronal glutamate transport and reactive species as mediators of mercury induced neurotoxicity (adapted from Farina et al., 2011). ROS - reactive oxygen species - and NO – nitric oxide.

Moreover, there are also evidences of MeHg adverse effects in other systems such as the cardiovascular (Salonen et al., 1995) and the immune systems (IIback, 1991). This effects may be related with the ability of mercury to increase the lipid peroxidation through the Fenton reaction in the cardiovascular system (Salonen et al., 1995) and cause alterations in thymus and the killer cell activity of the immune system (IIback, 1991).

IA.3. MERCURY TRANSFORMATIONS IN AQUATIC ENVIRONMENT

IA.3.1. Mercury cycle

Mercury cycle involves volatilization from soil and water surfaces, atmospheric transport, deposition and adsorption of mercurial compounds in soil or aquatic sediments, and can be divided into a global cycle (air movement of Hg^0) and a local cycle (abiotic and biotic transformations) (Boening, 2000). In the global cycle, the Hg^0 released by degassing of the earth's crust and oceans (10,000 tonnes) and anthropogenic sources (approximately 20,000 tonnes/year) (Hansen and Dasher, 1997) is transported as gas to the atmosphere where it can eventually be oxidized (Figure IA.3) (Morel et al., 1998).

In aquatic systems, mercury undergoes successive transformations: Hg^0-oxidation, Hg^{2+}-reduction, Hg^{2+}-methylation and MeHg-demethylation (Figure IA.3). Inorganic mercury is the predominant species found in aquatic systems and appears usually bounded to chlorides, sulphides and organic acids (Morel et al, 1998; Yu, 2000). Its accumulation on aquatic systems results from Hg^0-oxidation or industrial waste release (Figure IA.3). Hg^0 can be oxidized to Hg^{2+} by both abiotic and biotic processes (Colombo et al, 2013; Siciliano et al., 2002). The abiotic processes involved photochemical reactions mediated by sunlight (UV-B radiation) in the water surface whereas in the dark, oxygen or SH compounds also originate Hg^0-oxidation (Amyot et al., 2005; Yamamoto, 1996). Oxidation can also be promoted by geochemical reactions with humic substances (Ravichandran, 2004) and mineral-associated ferrous iron (Charlet et al., 2002).

Biotic oxidation of Hg^0 into Hg^{2+} is mediated by enzymatic reduction performed by microorganisms possessing for instance, catalase enzymatic activity (Barkay and Wagner-Dobler, 2005; Siciliano et al., 2002; Smith et al., 1998).

The inorganic species can later undergo methylation by the transfer of a methyl group to the Hg^{2+} ion and originate MeHg that bioaccumulates and biomagnifies in the aquatic and terrestrial food chain (Figure IA.3).

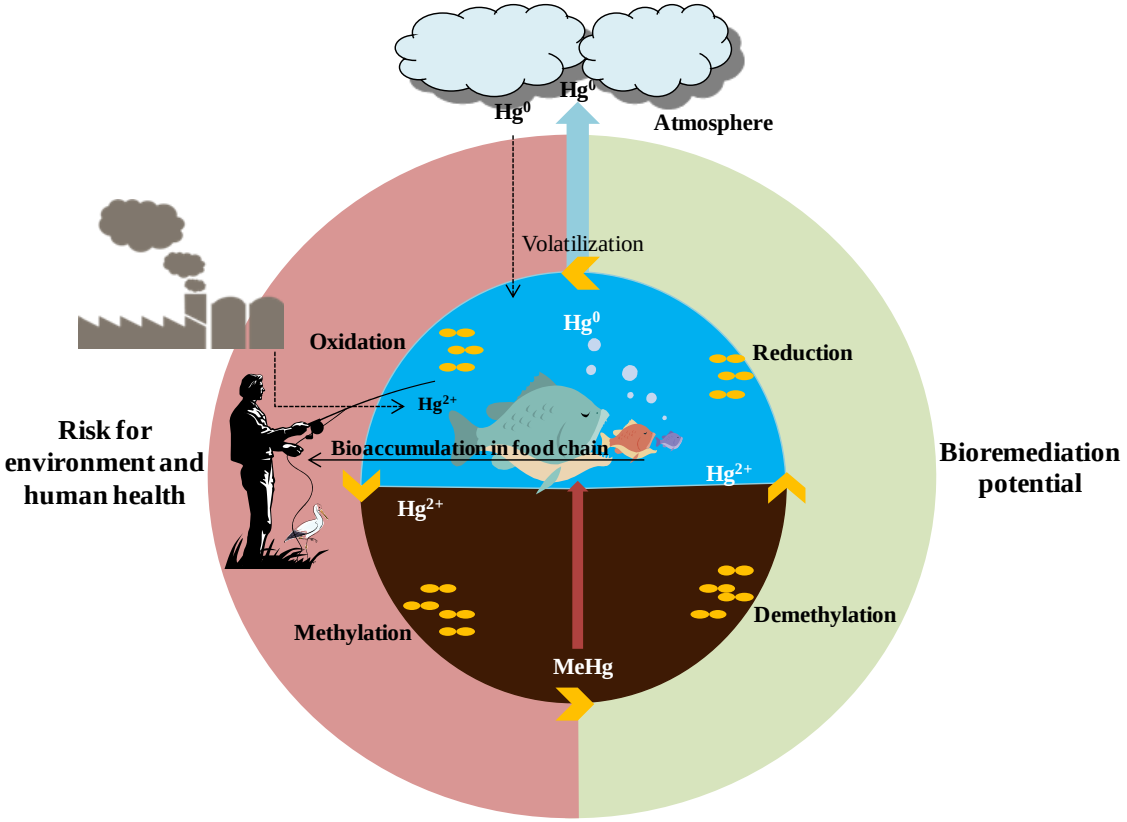

Figure IA.3: Mercury cycle in aquatic systems, highlighting the transformations performed by microbial community (oxidation, methylation, demethylation and reduction) and the fate of mercury species, as well as their impact in environment and human health.

However, the formation of MeHg can be mitigated by the inverse process – demethylation - which consists in the break of the bound between the methyl group and mercury ion, releasing of CH_4, CO_2 and Hg^{2+} (Barkay-Dobler and Wagner, 2005) (Figure IA.3). These transformations determine the prevalence, formation and degradation of mercury species as well as the local mercury cycling.

IA.3.2. The formation of MeHg

Mercury methylation can be biologically (microbial processes) or chemically (abiotic processes) mediated under aerobic and anaerobic conditions (Barkay Wagner-Dobler,

2005; Celo et al., 2006). For long time, it was assumed that mercury methylation was exclusively a biotic process since high MeHg concentrations were found in sediments with high microbial activity and the microbial activity inhibition by chemicals or sterilization resulted in a decrease of MeHg production (Celo et al., 2006). In fact, in sediments of aquatic environments, biological processes are the main responsible for the formation of MeHg (Berman and Bartha, 1986; Celo et al., 2006; Coelho-Souza et al., 2006). However the environmental formation of MeHg is not exclusively biotic as demonstrated by the presence of MeHg in environments with little or any biological activity, such as Polar Regions like Arctic wetlands (Celo et al., 2006; Loseto et al., 2004; Raposo et al., 2008).

IA.3.2.1. Microbial MeHg-formation

Microbial or biotic methylation of mercury was first observed by Wood (1968) in the methylcobalamin-utilizing methanogenic bacteria. Nevertheless, Jensen and Jernelov (1969) were the first providing evidences of microbial-mediated MeHg production from Hg^{2+} in sediments, by showing that this activity was inhibited by sterilization (Barkay and Wagner -Dobler, 2005). Since then, microorganisms from several taxonomic groups have been shown to methylate inorganic mercury in laboratory conditions (Coelho-Souza et al., 2006; Macalady et al., 2000; Furutani and Rudd, 1980; Fischer et al., 1995; Vaithiyanathan et al., 1996; Farrell et al., 1998; Pak and Bartha, 1998a; Siciliano et al., 2002) although, the mechanism behind this process remained obscure for a long time.

IA.3.2.1.1. Mechanisms of microbial MeHg-formation

The first mechanism proposed to explain microbial mercury methylation assumed that the only compounds capable of transferring a methyl group to Hg^{2+} in environment were methylcorrinoids. The studies in *Desulfovibrio desulfuricans* and *Geobacter sulfurreducens* showed that MeHg production involves cellular uptake of Hg^{2+} by active transport, followed by its methylation in the cytosol, and then exportation of MeHg from the cell (Figure IA.4) (Parks et al., 2013). The study of *Desulfovibrio desulfuricans* LS using isotopically labeled carbon (^{14}C), allowed the identification of an enzymatic pathway involving acetylcoenzyme A (acetyl-CoA) (Figure IA.4) (Barkay and Wagner-Dobler, 2005; Choi and Bartha, 1993; Choi et al., 1994a,b). However, the

connection between the acetyl-CoA pathway and the ability of microorganisms to methylate mercury could not be established for all methylators. For instance, Ekstrom et al. (2003) showed that SRB strains lacking acetyl-CoA pathway could perform mercury methylation and also that after inhibition of acetyl–CoA pathway some SRB still kept methylation activity.

Therefore, other pathways have been proposed such as a non-enzymatic transfer of the methyl group from methyl B12 (Yamada and Tonomura, 1972) and the "incorrect" synthesis of methionine (Landner, 1971; Siciliano and Lean, 2002).

Recent studies on the genetic and biochemical basis of microbial mercury methylation showed that two genes - *hgcA* and *hgcB* - are key components of the bacterial mercury methylation pathway (Parks et al., 2013). The *hgcA* codifies for a putative corrinoid protein, which facilitates methyl transfer from CH_3-cob(III)alamin-HgcA to a mercury substrate (mercury in a complex involving either free cellular SH or cysteine residues from a protein). On the other hand, *hgcB* codifies for a ferredoxin-like protein HgcB, that carries out the thermodynamically difficult reduction of Co(II) to Co(I) necessary for corrinoid reduction and required for turnover (Parks et al., 2013) (Figure IA.4).

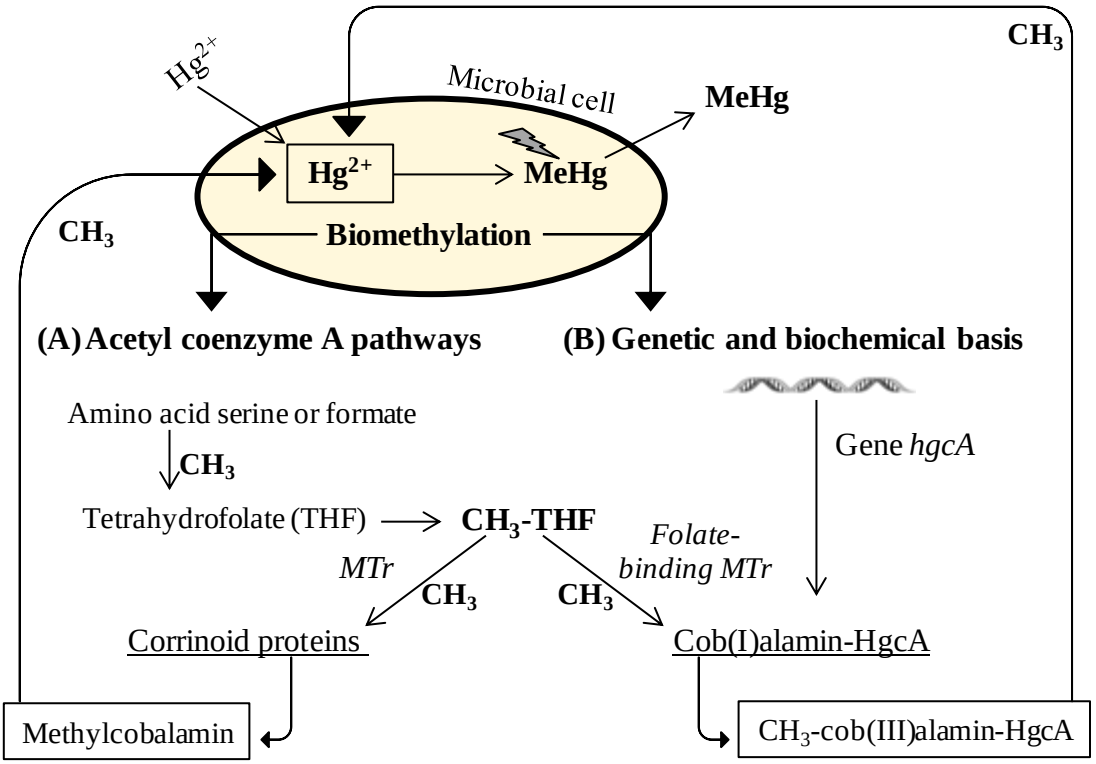

Figure IA.4: Diagram integrating all the mechanisms proposed until now for microbial mercury methylation. (A) Enzyme-catalyzed pathway involving acetyl coenzyme A: a methyl group (CH_3) is transferred from methyl tetrahydrofolate (CH_3-THF) to a corrinoid protein by methyltransferase (MTr). Intracellular cobalamin has been proposed to be the one receiving the methyl group from CH_3-THF to form methyl cobalamin (methyl B_{12}) and then transfers it to Hg^{2+} to form MeHg. (B) Genetic and biochemical basis involving genes *hgcA* and *hgcB*: a methyl group originated from CH_3-THF is likely first transferred (as CH_3^+) to cob(I)alamin-HgcA to form CH_3-cob(III)alamin-HgcA by a folate-binding MTr, and then to Hg^{2+} to form MeHg.

IA.3.2.1.2. Microorganisms involved in MeHg-formation

Environmental production of MeHg has been mainly pointed out as an anaerobic microbial process generally driven by anaerobic *Archaea* that use sulphate (SO_4) as late electron acceptor, reducing it to sulphide (S^{2-}) (Barton and Fauque, 2009), the so-called SRB (Benoit et al., 2003). It is consensual that SRB are the primary mercury methylators in freshwater and estuarine anoxic sediments (Compeau and Bartha, 1985; Gilmour et al., 1992; King et al., 2000, 2001, 2002). Among SRB, the family *Desulfobacteriaceae* was suggested to be the most likely involved in mercury methylation process and the general trend for mercury methylation rate among the genera is *Desulfobacterium* > *Desulfobacter* > *Desulfococcus* > *Desulfovibrio* ≈ *Desulfobulbus* (King et al., 2000).

However, many other studies have identified non-SRB microorganisms, belonging to *Archaea* as well as to *Bacteria* domains, performing mercury methylation processes (Table IA.3). For instance, *Clostridium cochlearium* (Yamada and Tonomura, 1972), methanogens such as *Methanococcus maripaludis* (Hamelin et al., 2011; Pak and Bartha, 1998) and iron-reducing bacteria (Fleming et al., 2006; Kerin et al., 2006; Lin et al., 2014; Warner et al., 2003) were also identified as mercury methylators.

Mercury methylation was also observed in oxic conditions mediated by aerobes microorganisms (Montperrus et al., 2007) in bacteria that possess methyl transfer enzymes like coenzymes N5-methyltetrahydrofolate, S-adenosylmethionine and methyl B_{12} (Wood et al., 1968; Robinson and Touvinen, 1984) (Table IA.3).

Table IA.3: Microorganisms identified to be involved in mercury methylation processes.

Group	Microorganisms	References
Sulphate-reducing bacteria	*Desulfovibrio desulfuricans*	Choi and Bartha (1993) King et al. (2000)
	Desulfovibrio africanus	Ekstrom et al. (2003) Ranchou-Peyruse et al. (2009)
	Desulfobacter sp	King et al. (2000)
	Desulfobacterium sp.	King et al. (2000)
	Desulfococcus multivorans	King et al. (2000) Ekstrom et al. (2003)
	Desulfobulbus propionicus	King et al. (2000) Ekstrom et al. (2003) Ranchou-Peyruse et al. (2009)
Methanogens	*Methanococcus maripaludis*	Pak and Bartha (1998)
Iron-reducing bacteria	*Geobacter*	Fleming et al. (2006) Kerin et al. (2006)
	Desulfuromonas	Kerin et al. (2006)
Anaerobic bacteria	*Clostridium cochlearium*	Pan-Hou and Imura (1982) Yamada and Tonomura (1972)
Aerobic bacteria	*Enteroboacter aerogenes*	Hamdy and Noyes (1975)
	Klebsiella sp.	Achá et al. (2012)
	Bacillus megaterium	Ramamoorthy et al. (1982)
	Proteobacteria	Achá et al. (2012)
	Pleomorphomonas sp.	Achá et al. (2012)

The recent discovery of *hgcA* and *hgcB* genes involvement in mercury methylation process can facilitate the identification of methylators. Indeed, Parks and co-works (2013) already suggested that the presence of the *hgcAB* cluster or the two genes cluster in the genomes of some *Bacteria* and *Archaea*, such as *Proteobacteria* and *Firmicutes*, *Euryarchaeota*, *Deltaproteobacteria*, *Clostridia*, *Negativicutes*, *Methanomicrobia*, may signify that these organisms are able to perform mercury methylation.

IA.3.2.2. Abiotic MeHg-formation

The abiotic mercury methylation processes do not require biological activity, being chemically mediated through transmethylation reactions (transfer of a methyl group) involving organometallic complexes (Celo et al., 2006; Morel et al., 1998; Weber, 1993). These reactions occur when CH_3^+, CH_3^- or even $CH_3^\cdot$ are transferred to mercury

(Celo et al., 2006). However, the transfer only occurs if there are suitable methyl donors such as methyl cobalamin, methyl tin compounds and humic matter in aquatic environment (Celo et al., 2006; Weber, 1998).

Methyl cobalamin is normally associated with microbial methylation (Pak and Bartha, 1998), however, it was also demonstrated that methyl cobalamin is capable of transferring a methyl carbanion to the mercuric ion in aqueous abiotic systems (Bertilsson and Neujahr, 1971; Celo et al., 2006; DeSimone et al., 1973; Hintelmann and Evans, 1997; Imura et al., 1971) being MeHg production from Hg^{2+} verified after 30 minutes of incubation (Celo et al., 2006). Compeau and Bartha (1985) also observed that MoO_4^{2-}methyl cobalamin methylated Hg^{2+}.

Methyl tin (MeSn) compounds are common in natural waters, sediments and aquatic organisms, as a result of pollution and natural methylation processes (Celo et al., 2006; Weber, 1993) and they have been considered since 1986 by Howell and co-workers for their potential involvement in mercury methylation (Weber, 1993). MeHg production was demonstrated by Celo et al. (2006) through the incubation of solutions containing Me_2Sn^{2+}, $MeSn^{3+}$ and Me_3Sn^{+} with Hg^{2+}. This study also gave an insight for optimal abiotic mercury methylation in the aquatic environments mediated by methyl tin compounds.

Although the compounds mentioned above are methyl donors, humic matter consisting in a mixture of metal-complexing organic compounds that exists in sediments and water of rivers, oceans, estuaries, etc. is the most promising methyl donor to Hg^{2+} (Weber, 1993). The formation of MeHg by humic matter has been demonstrated, namely the methylation mediated by fluvic acid (Siciliano et al., 2005; Weber et al., 1993) through the electrophilic attack of Hg^{2+} on fluvic acid (Weber, 1993).

Moreover, chemical reagents such as methyl iodide and dimethyl sulfide are also thought to cause abiotic MeHg formation (Celo et al., 2006; Weber, 1993). For instance, methyl iodide methylates Hg^0 in a rate similar to the one reported for SRB (Celo et al., 2006).

Notwithstanding, studies are needed to clarify the contribution of abiotic mercury methylation in aquatic environments to the mercury cycle.

IA.3.2.3. Factors affecting MeHg-formation

In the case of biological methylation, its efficiency depends on both: 1) factors that influence the activity, distribution and composition of the microbial community and 2) factors affecting the availability of the substrate (Hg^{2+}) (Marvin-DiPasquale and Agee, 2003). On the other hand, the abiotic methylation is conditioned only by factors affecting mercury speciation (Celo et al., 2006).

The factors affecting microbial activity include temperature, pH, salinity, redox potential and the availability of electron donors (e.g. acetate, lactate, methanol, etc.) and acceptor of electrons (e.g. O_2, Fe^{3+}, Mn^{4+}, SO_4^{2-} and CO_2) (Marvin-DiPasquale and Agee, 2003). The mercury availability is usually affected by the mercury species existing in ecosystem, and the presence of organic or inorganic agents that form complexes with Hg^{2+}, such as dissolved organic matter (DOM) (humic or fluvic acids), chlorides or sulphides (Marvin-DiPasquale and Agee , 2003). Some of these factors can affect both microbial activity and mercury availability. For instance, DOM can affect mercury availability by forming stable complexes with Hg^{2+} (Barkay et al., 1997), but can also affect microbial growth. Likewise, pH and salinity also affect both; low pH values increase the availability of Hg^{2+} for methylation by decreasing the formation of complexes with DOM (Barkay et al., 1997) and liming Hg^0 volatilization (Roy et al., 2009), whereas pH fluctuations in the surrounding environment can affect microbial community composition (Macalady et al., 2000). Another example is the salinity (NaCl) since it is directly related with the concentration of chloride which forms complexes with Hg^{2+} (Barkay et al., 1997) and also affects microbial growth.

Others factors that affect mercury methylation are contaminants such as antibiotics (Lima-Bittencourt et al., 2007) and chemical compounds, including different heavy metals (Nascimento and Chartone-Souza, 2003). Furthermore, elements such as Al, Mn and Fe when present together with Hg^{2+} limit its transport and prevent the methylation process (Fleming et al., 2006).

IA.3.3. Microbial transformations affecting MeHg net and fate

Besides mercury methylation, other microbial mercury transformations can be observed in aquatic systems, such as: 1) precipitation of insoluble inorganic complexes - HgS complexes - and 2) enzymatic reduction to a less toxic compound encompassing Hg^{2+}-reduction and MeHg-demethylation (detoxification) (Essa et al., 2002; Nascimento and Chartone-Souza, 2003).

The best studied detoxification mechanism is promoted by a system encoded by genes of the mercury resistance operon *mer* (see 3.3.1) (Lloyd and Lovley, 2001) that is responsible for enzymatic reduction of Hg^{2+} and MeHg. However, Hg^{2+} reduction has also been reported as an Fe^{2+}-dependent mechanism involving cytochrome c oxidase, in the thermophilic *Streptomyces* and *Thiobacillus ferrooxidans* (Lloyd and Lovley, 2001). Likewise, demethylation of MeHg by a pathway independent of *mer* operon (oxidative demethylation) was also observed among anaerobic bacteria (Barkay and Poulain, 2007).

All these processes affect the net MeHg and its fate by decreasing Hg^{2+} available for methylation and degrading the existing MeHg. In this context, microorganisms possessing *mer* operon have deserved considerable attention for bioremediation strategies (Born et al., 2003).

IA.3.3.1. Genetic basis for mercury reduction and demethylation

Mer operon is a cluster of genes which confers mercury-resistance to the microorganisms that carry it in their genes (Nascimento and Chartone-Souza, 2003). This genetic machinery occurs both, in Gram-positive and in Gram-negative bacteria such as the genus *Pseudomonas*, *Staphylococcus*, *Bacillus*, *E. coli*, etc. (Silver and Phung, 2005) as well as in *Archaea*, such as *Sulfolobus solfataricus* (Schelert et al., 2004), living in environmental, clinical and industrial environments (Liebert et al., 1997; Silver and Phung, 2005). *Mer* operon is usually located in transposons (e.g. Tn 21) inserted in chromosomal DNA or in plasmids (Mindlin et al., 2001; Silver and Phung, 2005).

According to its constitution, the *mer* operon can be classified into two types of *mer* determinants - the narrow spectrum and the broad spectrum, conferring resistance only to inorganic mercurial forms or to both inorganic and organic mercurial forms, respectively (Nascimento and Chartone-Souza, 2003). The biochemical basis for narrow spectrum determinants involves enzymatic reduction of Hg^{2+} to Hg^{0}, while broad spectrum involves additional enzymatic hydrolyses of the bond carbon-mercury (Hg-C) (Figure IA.5) (Nascimento and Chartone-Souza, 2003). From the structural arrangement viewpoint, *mer* operon encompasses four to five genes that encode for proteins of mercury transport (*merP*, *T*, *C*, and *F*), transformation (*merA* and *B*) and regulatory genes (*merR* and *merD*) (Mathema et al., 2011; Silver and Phung, 2005) (Figure IA.5).

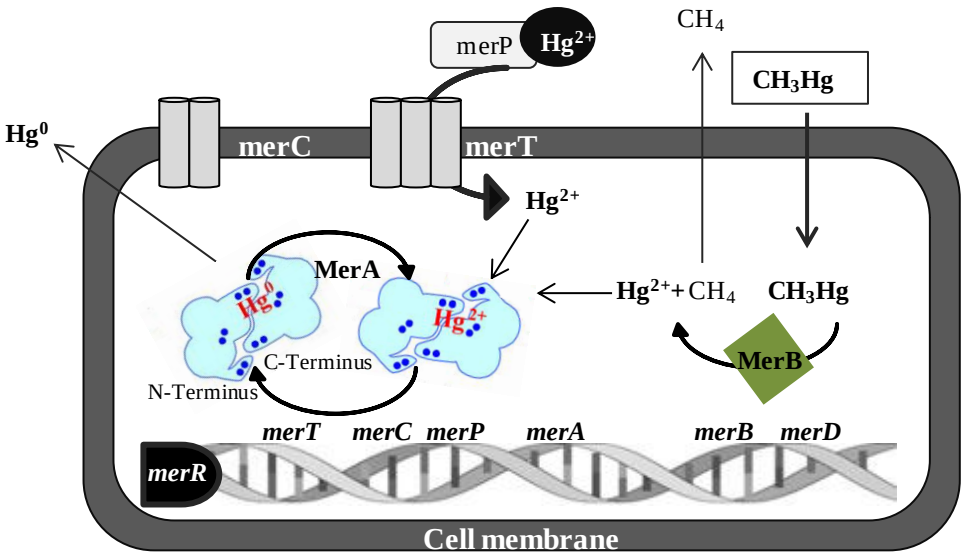

Figure IA.5: The genetic composition of *mer* operon and the respective proteins/enzymes encoded and their function (adapted from Mathema et al., 2011 and Silver and Phung, 2005).

MerP encodes for a periplasmatic protein that binds to Hg^{2+} via two cysteine residues and is the first protein that binds to Hg^{2+} (Silver and Phung, 2005). MerP interacts with a membrane protein encoded by *merT*. The MerT structurally consists of three helices and two pairs of cysteines. The proximal helices pair, located on membrane, receives Hg^{2+} from MerP and the distal pair of cysteines, located in the cytoplasmic "loop",

carries Hg^{2+} across the cell membrane into the cell (Brown et al., 2002). Both *merP* and *merT* are essential for the resistance to Hg^{2+}; however, studies have shown that the depletion of *merP* is less harmful than the depletion of *merT* (Barkay and Wagner-Dobler, 2005). Other membrane proteins involved in Hg^{2+} transport are MerC and MerF - membrane-bound proteins that are essential for Hg^{2+} resistance in case of MerT absence (Silver and Phung, 2005).

The *merA* and *merB* genes encode for mercuric reductase and organomercurial lyase, respectively. The mercuric reductase is a homo-dimeric cytoplasmic flavoprotein constituted by three domains (Barkay and Wagner-Dobler, 2005). Once inside the cell, Hg^{2+} is transferred to the mercuric reductase N-terminal (Silver and Phung, 2005). Then Hg^{2+} is transferred to cysteine-pair into the C-terminal (Cys557/Cys558) and follows by rapid SH/SH exchange to the Cys135/Cys140 pair of the other monomer (Figure IA.5) (Silver and Phung, 2005). After, the Hg^{2+} is transferred to the cysteine-pair of the active site. The three dimensional structure reveals that the active site of this enzyme is formed by the interaction of the central domain of one subunit to the C-terminal of the other domain (Figure IA.5) and is similar to others oxidoreductases, such as GR (Barkay and Wagner-Dobler, 2005). It is in the central domain that the catalysis occurs involving the transfer of two electrons from NADPH via FAD cofactor (Flavin Adenine Dinucleotide) to Hg^{2+} (Barkay and Wagner-Dobler, 2005).

The organomercurial lyase is a small monomeric enzyme that cleaves the Hg–CH_3 bond, releasing Hg^{2+} (the substrate of mercuric reductase) and reduces the organic components such as methyl or phenyl radicals to methane or benzene (Silver and Phung, 2005). Crystallography studies indicated that this enzyme activity depends on two highly conserved cysteine residues, Cys96 and Cys159 (Pitts and Summers, 2002); a SH-Hg covalent bond is formed with the invariant Cys159, while a proton from the Cys96 (also bounded to mercury through cysteine SH-bond) attacks the Hg-CH_3 bond. The Hg^{2+} released is transferred to the C-terminal cysteine of mercuric reductase (Figure IA.5) (Silver and Phung, 2005).

In the absence of Hg^{2+} the operon is repressed, while in its presence the operon is induced in different magnitudes. The expression of *mer* operon is controlled by the

regulatory proteins MerR and MerD. The MerR is the major regulator component of *mer* operon, repressing its own transcription and regulates the transcription of the structural genes (Barkay and Wagner-Dobler, 2005). MerR is a dimeric enzyme that binds to the operon's operator/promoter region (O/P), recruiting RNA polymerase to form a complex, that in the absence of Hg^{2+} represses the transcription of structural genes due to misalignment of -10 and -35 sequences. When Hg^{2+} is in the cell environment, it binds to MerR in the metal-binding domain of C-terminal and allosterically modifies it (Bruins et al., 2000), i.e. promotes the alignment of the regions -10 and -35 (Barkay and Wagner-Dobler, 2005). This rearrangement opens the way for RNA polymerase to bind to the promoter region and initiate transcription of *mer* operon gene products (Bruins et al., 2000). MerD is a co-repressor that releases MerR-Hg complex from the O/P region and frees MerR that further occupies the O/P region to repress transcription ("down-regulation") (Champier et al., 2004).

IA.4. IMPACTS OF METHYLMERCURY FORMATION

IA.4.1. Bioaccumulation and Biomagnification

MeHg formed in aquatic systems becomes available to be absorbed by the microalgae of phytoplankton and/or the phytobenthic organisms. In phytoplankton cells, MeHg binds to cytosolic proteins becoming available for the next trophic level (zooplankton) (Morel et al., 1998). Thus, MeHg undergoes bioaccumulation in the food chain through the ingestion of zooplankton by the fish and ingestion of small fish by large predators (Figure IA.6) (Barkay and Wagner-Dobler, 2005; NCR, 2000; Yu, 2005).

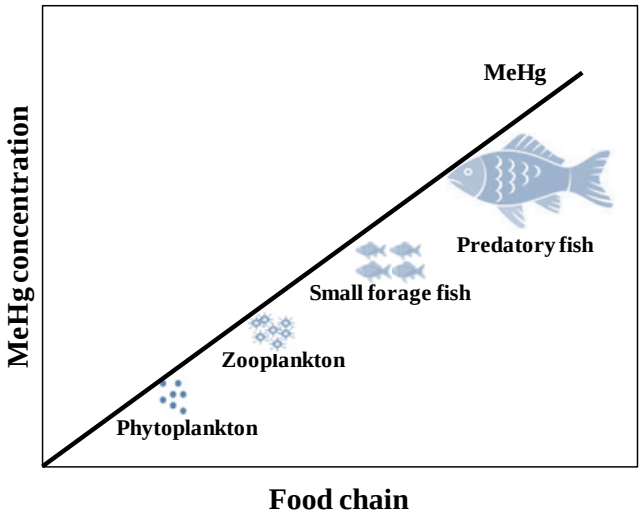

Figure IA.6: Trophic transfer of MeHg from the bottom of the food chain (phytoplankton) to top-predatory fish (adapted from Morel et al., 1998).

MeHg suffers a 10^9 magnification in concentration from the water column, where it exists at concentrations of few picograms per liter (ppq), to top-predators, where its concentration may reach several micrograms per gram (ppm). Due to this biomagnification potential, the highest levels of MeHg are found in fish that are apical predators, such as mackerel, pike, shark, swordfish, walleye, barracuda, large tuna, scabbard and marlin (WHO and UNEP, 2008). This remarkable biomagnification in the food chain is only observed in case of mercury (Monteiro and Furness, 1995).

Fish is quite important for their beneficial nutritional elements, such as proteins, omega-3 fatty acids and various vitamins and minerals. Nonetheless, since fish consumption is the dominant pathway of MeHg exposure for most human populations, many governments provide dietary advice to limit consumption of fish where mercury levels are elevated. The Codex Alimentarius Commission recommendations and EU legislation limit the concentration to 0.5 mg MeHg/kg for non-predatory fish, crustaceans and mollusks and 1 mg MeHg/kg to predatory fish species (e.g. tuna, swordfish, shark), while Japan and US EPA allow 0.3 mg MgHg/kg in fish (CEC, 2006; WHO and UNEP, 2008). Table IA.4 shows the concentration of MeHg found in some fish and seafood species in European countries.

Overall, MeHg is very important in ecological risk assessment as once formed, it undergoes rapid bioaccumulation and biomagnification in aquatic food chain (Barkay and Poulain, 2007), becoming available to humans and wildlife through the consumption of contaminated fish.

Table IA.4: Methylmercury concentrations in seafood and fish (adapted from EFSA, 2015).

Seafood and fish	**MeHg (µg/kg)**
Salmon/trout	33
Herring	36
Fish products	38
Squid	46
Carp	55
Plaice	64
Sole	76
Whitefish	85
Cod/Whiting	94
Mackerel	107
Hake	136
Perch	165
Fish meat	166
Redfish	189
Lophiiformes	195
Bass	202
Bream	225
Tuna	290
Lobster	302
Pike	394
Swordfish	1212

IA.4.2. Impacts in environment

Exposure to MeHg may cause adverse effects on living organisms both in aquatic and terrestrial environments (EPA, 1997). Several adverse effects were observed in

mammals (Muccillo-Baisch, 2012), fish (Ung et al., 2010) and birds (Burger and Gochfeld, 1997; Heinz and Hoffman, 2003; Herring et al, 2012), such as behaviour alterations, malformation and/or malfunctioning of the neurological system, deficient development and abnormalities in reproduction, such as malformation of foetus and most important inability for reproduction (Burger and Gochfeld, 1997; Herring et al., 2012; Heinz and Hoffman, 2003). For instance, Korbas et al. (2008) found that high levels of MeHg in visual system, brain, liver and kidneys of the zebra fish larva caused adverse effects to the neurological system and ocular tissue, even in the larvae stage. Burger and Gochfeld (1997) observed adverse effects on bird reproduction related to eggs hatch, while Herring et al. (2012) found a deficit in the ability of youth bird to respond to various types of environmental stress. Mammals also can suffer from MeHg toxic effects due to their milk-based diet, being the juveniles mostly affected. The neurotoxicity of MeHg is the most common effect among mammals, however, there are significant differences between species; for instance primates metabolize MeHg similar to humans, while mice have the ability to metabolize it to a less toxic inorganic form (Nordberg, 1976).

From the ecological point of view, the effects of MeHg formation are not limited to effects on individual organisms, but affect entire populations (EPA, 1997) and this disturbs also the community, namely through the decrease of species diversity and changes on the species composition. At large scale, these effects affect the ecosystems, via the negative impact in the community diversity and natural nutrient cycles (EPA, 1997).

IA.5. CONCLUSIONS

This review discusses the biological and chemical transformations that mercury undergoes in aquatic environments. All mercury compounds have toxic effects for both human and wildlife. Notwithstanding, the presence of neurotoxic MeHg in aquatic systems has a serious impact for both human and environmental health as it undergoes bioaccumulation and biomagnification in the food chain.

Microorganisms in anoxic environments are the main responsible for MeHg production from inorganic mercury forms, while chemical processes contribute only with a small fraction of the total amount. Besides the formation rate, the amount of MeHg present in aquatic systems depends on the degradation processes. Microorganisms are also responsible for processes that mitigate MeHg production, such as Hg^{2+}-reduction and MeHg-demethylation, both affecting the net and fate of MeHg, reducing its presence in aquatic environments, with important implications for risk assessment. Overall, this review highlights the importance of integrating the study of these processes in mercury-contaminated aquatic systems aiming their bioremediation.

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CHAPTER I - GENERAL INTRODUCTION

PART B - ENVIRONMENTAL IMPORTANCE OF ESTUARIES: TAGUS ESTUARY (PORTUGAL) AS A CASE-STUDY†

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ABSTRACT CHAPTER IB

Estuaries are areas in the interface between land and sea with unique characteristics and high ecological value related to their biological productivity and high species diversity. Estuaries also offer many advantages for the human population, by providing sustainable yields of fish, shellfish, and wildlife, as well as, shoreline protection, navigation routes and areas for recreation. However, due to the anthropogenic activities, namely urban and industry's discharges, many estuarine systems are threatened.

In this study, the ecological importance of estuaries including their current state (in particular the case of the Tagus Estuary) is reviewed. This estuary is one of the most important estuaries in Europe and the largest in Portugal, which provides wetland habitats for wintering migratory birds in Western Europe and nursery areas for commercial fish. Despite its ecological importance, since 1985, the Tagus has been reported to be contaminated by metals such as Pb, Zn, Hg, Cr, Cu and Cd, as a result of past and present industrial activity, located mainly in north and south margins of estuary. High levels of mercury in sediments, suspended matter, water and plants remain until the present time and are a concern.

Some research groups have evaluated the pollution evolution as well as its consequences for biota and their works are reviewed. Mitigation strategies including bioremediation are also discussed.

IB.1. INTRODUCTION

Estuaries are ecotone areas between marine and freshwater, characterized by the unique combination of physical, chemical and biological features, and distinguished by the exceptionally high productivity (Telesh and Khlebovich, 2010). The most outstanding characteristics of an estuary are the structural shape and the variability in salinity and tide, which are the main basis for the estuary classification.

Since the early ages, estuaries have been very important for humans. For instance, most cradles of civilization arose in deltaic and lower floodplain areas, such as valley of the Nile, Tigris-Euphrates, Yellow and Indus Rivers, Tabascan lowlands of Mexico, etc. (Day et al., 2012). Nowadays, human population still takes advantage on the facilities these systems offer, such as the abundance of natural biota, excellent opportunities for transport, natural dilution and dumping of wastes (Cardoso et al., 2008), rich bottomland soils resulting from flooding cycles, available freshwater supplies on which agriculture can flourish and also the offer of recreational opportunities (Day et al., 2012; Levin et al., 2001). All of these uses lead to an intensely exploitation of estuaries by industry and urbanization (Serafim et al., 2013). These anthropogenic activities are in the origin of estuaries deterioration caused by the historical pollution that represents a risk for public health and biota that routinely use estuaries.

After the Minamata Bay disaster, which caused the death of 46 people (Ekino et al., 2007) as a result of mercury contamination, many studies in estuaries have been carried out to assess their environmental quality. Estuarine sediments are an important sink for a variety of pollutants, in particular heavy metals (Wang et al., 2002), that under certain circumstances can be remobilized and released back to the water column, increasing their availability to the biota (Chapman et al., 2002).

The Portuguese coast has several estuaries, which play a recognized role as nursery areas for several commercially available fish species (Cabral et al., 2007; Vasconcelos et al., 2010). However, there is a crescent vulnerability of several of these estuaries due to anthropogenic stress, mainly chemical pollution resulted from industrial and urban discharge of organic and inorganic contaminants (Cardoso et al., 2008). Tagus estuary,

the largest estuary in Portugal and among the most important estuaries in Europe (Elliott et al., 2007), is one of the most problematic cases of deterioration of water and sediments quality due to the historical input of effluents from agricultural, industrial and urban sources. The pollution of the Tagus came mainly from two industrial areas situated in the northern and southern areas (Figuères et al., 1985). Since then, the main concern has been the presence of metals, namely mercury (Figuères et al., 1985; Canário et al., 2003, 2005).

This study reviews the ecological importance of estuaries in general and the current situation of the Tagus Estuary.

IB.2. CHARACTERIZATION AND THE IMPORTANCE OF ESTUARIES

IB.2.1. Definition

To understand estuaries ecological importance, firstly we must define what an estuary is. The term estuary comes from the Latin *aestus*, which means heat, boiling or tide. More specifically, the adjective *aestuarium* means tidal. The Oxford Dictionary defines estuary as the "tidal mouth of great river, where the tide meets the stream" (2015) and Webster's Dictionary defines it more specifically as "a passage, as the mouth of a river or lake, where the tide meets the river current, more commonly, an arm of the sea at the lower end of a river" (2015). These definitions pointed the tide as a keyword for estuary definition, but there are many nontidal or minimal tidal seas, such as the Mediterranean Sea and the Black Sea, where fresh water and salt water mix (Day et al., 2012). A classic often quoted scientific definition of estuary is the one given by oceanographer Donald Pritchard in 1967: "a semi-enclosed coastal body of water which has a free connection with the open sea and, within which, seawater mixes and usually is measurably diluted with freshwater from land runoff" (Good, 1999). Once again tide and mixing between salt and fresh water are the main characteristics. However, there are estuaries that may not receive fresh water for long periods or may be blocked from the sea due to a longshore sand drift, for example in the Pacific coast. Most of the

primary definitions of estuary reflect its geological and physical characteristics (Day et al., 2012). Two things can explain this: first, the most salient feature of estuaries is physically and geomorphic and second, the first persons who defined and classified estuaries were geologists and physical oceanographers. Nowadays, improvement in estuaries knowledge has been done, namely taking into account the complexity of the interaction involving many scientific areas, such as geology, hydrology, physics, chemistry and biology, which have been extended the concept for estuaries definition in order to cover all/or the main remarkable characteristics of these ecosystems. Here, we will use Day et al., (2012) version, which defines estuaries very broadly as "a portion of the Earth's coastal zone where there is interaction of ocean water, freshwater, land and atmosphere".

IB.2.2. Estuary characterization

Since the sea levels reached near their present levels, followed the last glaciation (approx. 20,000 year ago), estuaries formation arose (Day et al., 2012). Their subsequent maturity happened as result of the two processes - sedimentation and erosion. Characteristics such as rise and fall of the tide, complex water movements, high turbidity levels and different salt concentrations are in the origin of estuaries characterization (Day et al., 2012). Based on this, estuaries can be classified in several types (Habsen and Rattray, 1966).

Structurally, estuaries are characterized by the existence of a large bay, where at one end enters a river and at the other end a barrier island separates the bay from the ocean (Day et al., 2012) (Figure IB.1). This water body can be partially enclosed or completely enclosed. According to Pritchard, an estuarine system can be subdivided into 3 regions: 1º - a tidal river zone, 2º - a mixing zone and 3º - a near shore turbid zone - (Day et al., 2012). The tidal zone is the fluvial zone, characterized by the lack of ocean salinity, however subject to tidal rise and fall of sea level. The mixing zone is the estuary properly, characterized by the water mass mixing and existence of a large amount of physical, chemical and biotic gradient, reaching from the tidal river zone to the seaward location of the river-mouth bar or ebb-tidal delta. The near shore turbid

zone is the zone between the mixing zone and the seaward edge, i.e. the connection to the open ocean (Day et al., 2013).

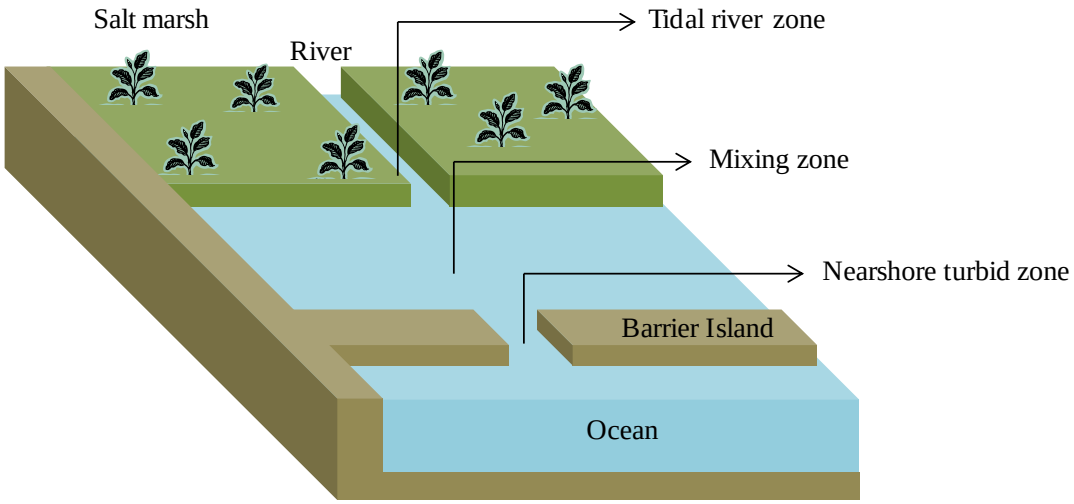


Figure IB.1: Schematic view of a typical estuarine system (adapted from Day et al., 2012).

Based on their geomorphologic characteristics, Pritchard (1967) classified estuaries in four types: coastal-plain estuaries, lagoon estuaries, fjord estuaries and tectonically-caused estuaries (Day et al., 2012; Chapman and Wang, 2001; Perillo, 1995). Coastal plain estuaries are basically the conventional idea of what an estuary should be. They were formed from gradual rise of sea level into Pleistocene-Holocene river valleys during the Flandrian transgression (Perillo, 1995), thus these estuaries exhibit the geomorphic characteristics of the river channels and flood plain (drowned river valley estuary) (e.g. Chesapeake Bay, US) (Day et al., 2012). Lagoon or bar-built estuaries is very similar to coastal plain estuaries, however the lagoon are oriented parallel to the coastline and have small tidal ranges and minimal freshwater inflow, which often creates high salinity levels (e.g. Laguna de Terminos in Mexico, Balize barrier reef lagoon in Caribbean and St. Lucia Lagoon in South Africa) (Day et al., 2012; Perillo, 1995). Fjords also resulted from the glacial cycle, but on the contrary of the coastal plain estuaries which have developed in low and middle latitudes, fjords are associated to high latitudes (Perillo, 1995). The outstanding feature of the most fjords is the steep

slope of adjacent lands and a very deep valley (e.g. Norwegian and British Columbia Coastlines (Canada)) (Chapman and Wang, 2001; Perillo, 1995). Tectonic caused estuaries are the estuaries created by tectonic processes such as landslides, faulting or volcanic eruptions (e.g. San Francisco Bay in U.S, Valdivia in Chile and Itamaracá in Brazil) (Chapman and Wang, 2001; Perillo, 1995).

Based on salinity stratification, i.e. how thoroughly the freshwater and saltwater mixes to create brackish water, estuaries can be: salt wedged or highly stratified, partially mixed or moderately stratified and well-mixed or vertically homogeneous (Hansen and Rattray, 1966). In salt-wedge estuaries, freshwater moves over saltwater in a wedge shape at the mouth (e.g. Mississippi River estuary). These estuaries are the most stratified and require high input of freshwater (Hansen and Rattray, 1966). The vertically homogenous or well-mixed estuaries are characterized by low input of fresh water and strong tidal influence currents (Hansen and Rattray, 1966). Intermediate estuaries are partly mixed, exhibiting circulation patterns between those of salt-wedge or vertically homogenous estuaries, but with less freshwater input or more tidal influence (Hansen and Rattray, 1966).

Notwithstanding these simplified classifications, there are many other variables, which constantly affect estuarine systems; among them we have ocean influence and water circulation - tidal and wave action, winds prevalence and/or changing, local and distant weather systems, variations of rainfall runoff and river discharge, the depth of the estuary, etc. (Day et al., 2012).

IB.2.3. Ecological importance of estuaries

Estuaries have been offered many goods and resources to humans and other living organisms. Due to their exceptional characteristics in the interface of sea-land, estuarine ecosystems offer highly productive habits that accommodate well-structured populations and communities (Good, 1999). Regarding to estuary productivity, Schelske and Odum (1962) defended that "estuaries are among the most important productive natural ecosystems in the world" (Day et al., 2012) and they pointed out their high productivity as a result of: 1 – three types of primary production units (marsh

grass, benthic algae and phytoplankton); 2 – outgoing tide and flow of water movements resulting from tidal action; 3 – abundant supplies of nutrients; 4 – rapid regeneration and conservation of nutrients as a result of microorganisms and filter feeders (Day et al., 2012). Concerning habitat richness, estuaries can comprise physical habitats (e.g. beaches, passes, intertidal and shallow, subtidal flats, deeper areas and deltas) and biological habitats (e.g. in intertidal zone: salt marsh, algal flats, mudflats, oyster reefs and mussel beds, and in subtidal areas: seagrass beds, sand shoals, soft muddy bottoms or mollusk beds). Additionally, the productivity and habitats are interconnected, i.e. productivity provides a diversity of habitats that enhances even more the productivity.

The high productivity and habitat richness of estuarine systems is the basis for all their important functions, namely as spawning and nursery areas, rest and feeding areas for migratory and resident animals, for fish and shellfish production and habitat diversity for many organisms, which play a vital role in ecosystem processes (decomposition, nutrient recycling, water quality improvement) (Good, 1999; Levin et al., 2001; Elliott et al., 2007). Organic detritus from a variety of plant sources are an important food source for many organisms. These groups include bacteria, fungi, phytoplankton, deposit and suspension feeders (e.g. polychaetes, bivalve mollusks, crustaceans, cnidarians, bryozoans, ascidians, sponges etc.), shredders, (e.g. gastropod or insects) and algal (Levin et al., 2001). In turn, this biota is crucial for decomposition and nutrients recycling, for instance, bacteria and fungi are important for organic matter mineralization and also for the decomposition process (Levin et al., 2001), which supports higher plants and animals biomass.

Estuarine systems are also important for many *diadromous* fish that migrate between the sea and fresh water using different habitats for migration, for spawning, as overwintering or nursery areas or residence in adult life (Day et al., 2012). For example, salmon requires fresh water for spawning and juvenile rearing, but they spend their adult life in ocean. Estuaries are well known as nursery areas, offering to juveniles' better feeding conditions, optimal growth, refuge opportunities and high connectivity with other habitats (Courrat et al., 2009). Besides the fish species, some migratory birds also use estuaries for habitat and food during their migration (Catry et al., 2012).

IB.3. CASE STUDY: TAGUS ESTUARY

IB.3.1. Description and ecological value

The Tagus Estuary is one of the largest estuaries on Atlantic coast of Europe and the second most important for waders in Iberia (Catry et al. 2012; Caçador et al., 2009). This estuary is located in the south part of Portuguese coast (38^0 45' N, 09^0 50' W) and is among estuaries of Boreal-Atlantic region of Europe. It is a well-mixed estuary with irregular river discharge (Chainho et al., 2008) which comprises an area of 320 km^2 (Figure IB.2), from Forte do Bugio up to 80 km upstream (Muge) (Figure IB.2). During spring tides, the wet area is reduced from 320 km^2 at high tide to 130 km^2 at low tide, being that the tidal range varies between 1.2 meters at neap tides and 4.2 meters at spring tides (average ≈2m) (Santos et al., 2006). About 128 km^2 (≈ 40%) of the estuary is composed of intertidal mudflats (Caçador et al., 2009).The Tagus Estuary receives the main inputs of freshwater from the river Tagus that flows from Serra de Albarracin, crosses Spain and Portugal, and discharges in the Atlantic Ocean (Barros, 1995). In terms of salinity, the Tagus can be classified as a partially stratified estuary (Rilo et al., 2014). Morphologically, it is characterized by an extensive bay shallow in the inner (5-10 meters depth) and a narrow and deep channel (15 km length, 2 km width and 32 m depth), which flows into the Atlantic Ocean. Structurally, the estuary can be subdivided into four distinct areas: zones A to D, (Canário, 2004) (Figure IB.2). The zone A is the area which encompasses the Natural Reserve, an area of high biological richness and an important area for nursery. The zone B is an area of high industrial and port activities. The zone C is an area that integrates a considerable part of two cities (Lisboa and Almada) and also has high port activity. Finally, zone D is the area where the estuary connects with the sea (Figure IB.2).

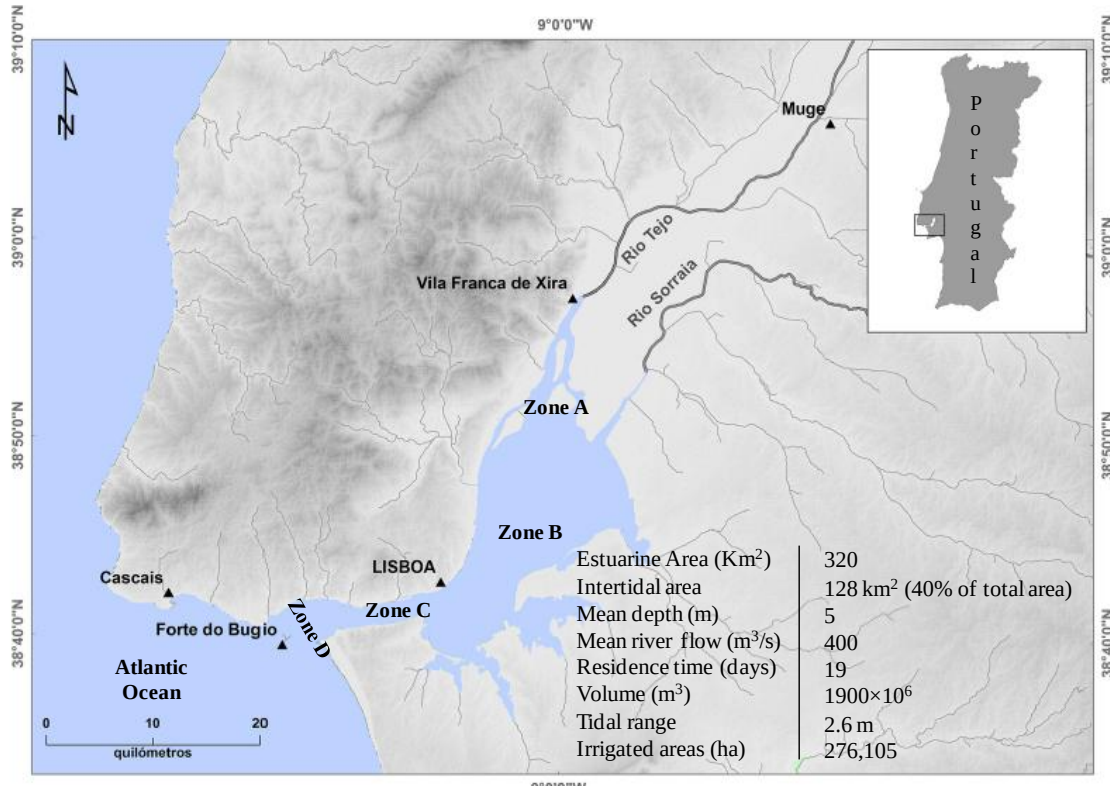


Figure IB.2: Tagus Estuary areas (zones A-D) and its morphologic, hydrologic and geomorphologic characteristics (adapted from Chainho et al 2008; Rilo et al., 2014; Vasconcelos et al., 2007).

The extensive bay of the Tagus Estuary provides high diversity of habitat (marshes, mud, shallow waters, salt marshes, pastures and rice fields), in particular in the southern and eastern part of the estuary where there is an extensive area of salt marshes (20 km^2) (Canário, 2004). These salt marshes are colonized mainly by: *Spartina maritima*, *Halimione portulacoides*, *Sarcocornia perennis*, *Sarcocornia fruticosa*, *Scirpus maritimus* (Caçador et al., 2009, ARH do Tejo and GOT, 2009) (Figure IB.3). *Arthrocnemum glaucum Salicornia vitens*, *Puccinellia marítima Inula crithmoides*, *Polygonum aviculare*, *Suaeda vera*, *Typha domingensis* and reeds are also very common in this estuary (ARH do Tejo and GOT, 2009). The saltmarsh has an important ecological role, for several reasons: (1) the vegetation is a source of food for a diversity of animal species; (2) exerts a scrubbing action through the accumulation of some heavy metals; (3) is a niche for the development of larvae and (4) functions as shelter for waterfowl (ARH do Tejo and GOT, 2009). The diversity of habitats contributes to the

presence of a highly diverse fauna, including polychaetes, bivalves, cephalopods and crustaceans. Some common species found in this estuary are the bivalve *Scrobicularia plana*, the polychaete *Nereis diversicolor*, gastropod *Hydrobia ulvae* and crustacean *Palaemon elegans* (Cardoso et al., 2008; Rosa et al., 2008) and these are the feeding for migratory and resident avifauna (Catry et al., 2012; Martins et al., 2013; Rosa et al., 2008).

The richness of avifauna in the Tagus Estuary is one of the outstanding patterns of this estuary. There are about 50,000 birds using this area as a stopover site during their winter migration (Catry et al., 2011), especially birds coming from the North of Europe, which use this estuary as a wintering area (e.g. *Recurvirostra avosetta*) (ARH do Tejo and GOT, 2009). Others waders such as *Pluvialis squatarola; Tringa totanus; Limosa limosa Charadrius alexandrinus; Charadrius hiaticula; Calidris alpina; Limosa lapponica; Numenius arquata e Calidris* sp. also use this area for wintering and breeding (Figure 3). For example, dunlins (*Calidris* sp.) represent 1% of the population (10,000 individuals) and make use of the Tagus Estuary for wintering (Martins et al., 2013). It is also frequent to find Anatidae (family of birds that includes ducks, geese and swans) in this estuary; examples of these are: *Anas crecca*, *Anas Penelope*, *Anas acuta*, *Anas platyrhynchos*, *Platalea leucorodia* and *Anser anser*. Some species of storks and herons (e.g. *Bubulcus ibis*) and predatory birds (e.g. *Elanus caeruleus* and *Circus aeruginosus*) are attracted to this area due to its humidity, cultivated land and pine woods located within its boundaries (RNET, 2015). Flamingos (*Phoenicoptherus ruber*) also come from North of Africa looking for better weather conditions and food (Canário, 2004). Altogether determined that an area of 145 km^2, located in northern of the estuary, was classified as Nature Reserve in 1976 (Catry et al., 2011), and in 1988 this area plus the intertidal areas were designated as Special Protection Area under the European Union legislation (Catry et al., 2011; Rosa et al., 2008).

<table>
<tr>
<td>Plant</td>
<td>Species
Spartina maritima (A)
Halimione portulacoides (B)
Sarcocornia perennis (C)
Sarcocornia fruticosa (D)</td>
<td colspan="3"> 
(A) (B) (C) (D)</td>
</tr>
<tr>
<td rowspan="2">Benthic community
(Chainho et al. 2008)</td>
<td>Class
Amphipoda
Bibalvia

Nemertea
Oligochaeta

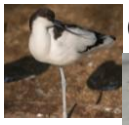
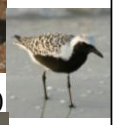
Polychaeta</td>
<td>Dominant species
Corophium achersicum
Scrobicularia plana (A)
Corbula gibba
Tetrastemmatidae n.i.
Tubificoides sp.
Limnodrilus hoffmeisteri
Chaetozone setosa
Streblospio shrubsolii
Polydora cornuta
Boccardiella redeki
Cossura coasta
Nereis diversicolor (B)</td>
<td colspan="2"> 
(A)
 
(B)</td>
</tr>
<tr>
<td colspan="2">Number of taxa = 143</td>
</tr>
<tr>
<td rowspan="2">Fish
(Cabral et al., 2007; Elliot and Hemingway, 2008; Infopédia, 20015; Vasconcelos et al., 2010)</td>
<td colspan="2">Species
Mullus surmuletus (surmullet)
Pseudopleuronectes americanus (winter flounder)
Mugil cephalus (flathead mullet)
Solea solea (sole) (A)
Sparus aurata (sea bream) (B)
Halobatrachus didactylus (lusitanian toadfish)
Dicentrarchus labrax (C)
Solea senegalensis (sole)
Diplodus sp. (sea bream)
Pomatoschistus microps (D)</td>
<td colspan="2">Usage
Nº of species (% ; n=84)
Spawning: 12 (14.3%)
Nursery: 76 (90.5%)
Feeding: 28 (33.3%)
Diadromy: 3 (3.6%)
 
(C)
 
(D)
 
(A)
 
(B)</td>
</tr>
<tr>
<td colspan="3">Total species = 70-80 species</td>
</tr>
<tr>
<td>Birds
(Catry et al., 2012)</td>
<td colspan="2">Species
Avocet (*Recurvirostra avosetta*) (A)
Black-tailed Godwit (*Limosa limosa*)
Grey Plover (*Pluvialis squatarola*) (B)
Dunlin (*Calidris alpina*) (C)
Ringed Plover (*Charadrius hiaticula*)
Bae-tailed Godwit (*Limosa lapponica*)
Black-winged Stilt (*Himantopus himantopus*)</td>
<td colspan="2">Usage
Wintering
Wintering
Wintering
Wintering
Wintering
Wintering
Breeding
  (A)
  (B)
  (C)</td>
</tr>
</table>

Figure IB.3: Species of plants, benthic community organisms, fishes and birds found in the Tagus Estuary.

Among 84 fish species using Tagus, 76 (90.5% of species) use it as nursery areas (Figure IB.3) being some commercially important species, such as flounder and sole (*Solea solea* and *Solea senegalensis*), sea bass (*Dicentrarchus labrax*), sea breams (*Sparus aurata* and *Diplodus sp.*) (Cabral et al., 2007; Vasconcelos et al., 2010; Vinagre

et al., 2006). Besides nursery, the Tagus Estuary is also an important area for feeding, being that 28 fish species (33%) use this area for that purpose (Elliot et al., 2007) (Figure IB.3).

IB.3.2. Anthropogenic impacts and pollution history

In virtue of their location – between land and ocean – estuaries are important sources of food and provide important routes for navigation (Day et al., 2012), and these are the main reasons why estuaries have become places of election for a variety of human activities. Human population has been settling nearby estuaries since the early ages. Post and Lundin concluded that more than 60% of the world's population lives within 60 km of the coast (Courrat et al., 2009). It is noticeable that many current large cities in the world, such as New York, London, Amsterdam, Venice, Calcutta, Alexandria and Shanghai, are located near estuaries and deltas (Day et al., 2012). The Tagus Estuary is located next to the capital city of Portugal (Lisboa), the most populated region of the country and the main national metropolitan area (Rilo et al., 2014). This localization is in the origin of the high pressure coming from human usage.

IB.3.2.1. Anthropogenic pressures

The Tagus Estuary is the most pressured estuary in Portugal due to its location in an urban area and human activities that include waste treatment, industry, agriculture and port uses (Vasconcelos et al., 2007; Freire et al., 2012). Figure IB.4 shows the anthropogenic activities that impact estuarine systems and their consequences, namely the pollution coming from urban and industrial discharge.

The marginal area that circumscribes the Tagus Estuary comprises eleven municipalities, such as Oeiras, Lisboa, Loures, Vila Franca de Xira, Benavente, Alcochete, Montijo, Moita, Barreiro, Seixal and Almada (from north margin to south margin). Population living nearby rounds 2,810 thousands and industry units round 294 (Vasconcelos et al., 2007), which produce per year circa 75.5×10^6 m^3 industrial loads (Chainho et al., 2008; Vasconcelos et al., 2007). The general trend of the area occupied by this activities is agriculture (35%) > urban area (34%) > industrial activity (15%) >

port (9%) > natural area (7%) (Freire et al., 2012), thus 93% of margins is occupied by anthropogenic activities and only 7% is natural area. On the north margin, urban, port and maritime infrastructures are dominant, while in south margin settled urban areas, with some recreational green area, and some important industrial areas, such as Alfeite arsenal, the Quimiparque and recent areas of business parks (Freire et al., 2012). Agricultural areas are mainly in Benavente and Vila Franca de Xira, such as the Company of Lezírias that has been active since 1837 (Rilo et al., 2014).

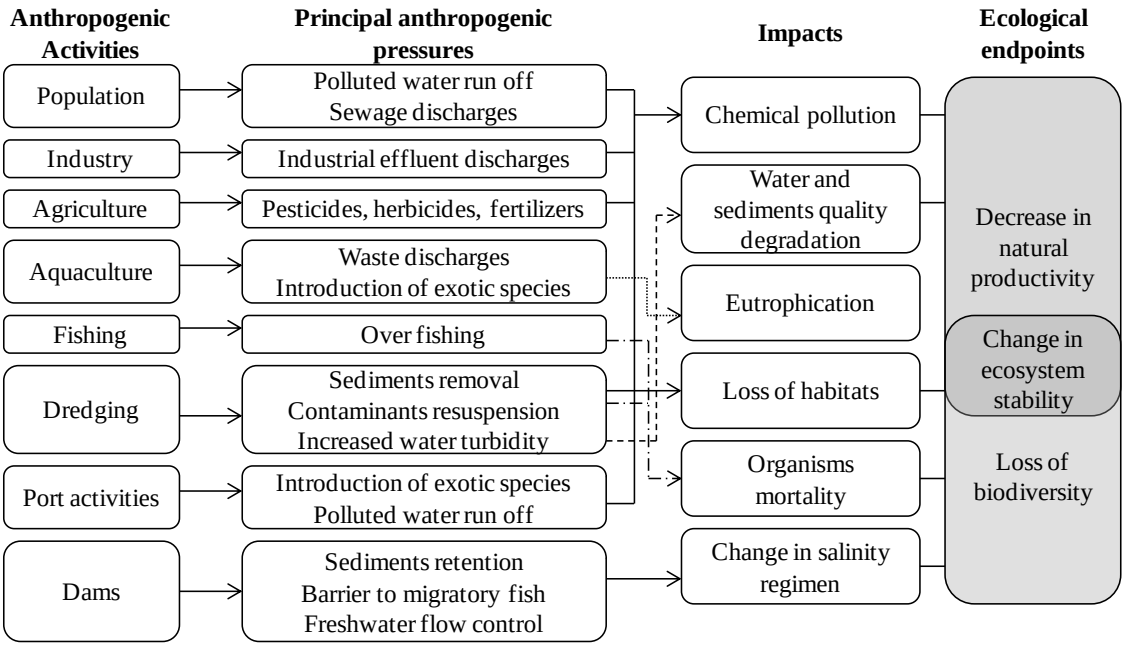


Figure IB.4: Diagram of the main anthropogenic activities causing estuarine pressure and the resulting impacts as well as the ecological endpoints of these activities (Adapted from Vasconcelos et al., 2007).

Nowadays, one of the main human activities impacting the Tagus Estuary is the commerce (Vasconcelos et al., 2007), mainly related to port activities. The port of Lisbon, which is situated in the Tagus Estuary is the second biggest in Portugal and has an intensive commercial traffic, rounding 3,689 ships per year (37×10^6 tons gross tonnage) (Vasconcelos et al., 2007). This activity may lead to chemical pollution; for instance in the past a decrease in large scale of oyster beds due to contamination by Tributyltin (TBT), an antifouling paint to keep boat hulls free of marine organisms, was

found (De Bettencourt et al., 1999). Moreover, commercial ports have been pointed out as a source for the introduction of opportunistic exotic species that are transported in ship ballast waters, which lead to the loss of indigenous species due to the habitat and food competition and reduction of diversity therefore, compromising the ecosystem equilibrium (Goldberg, 1995).

IB.3.2.2. Historical pollution

Portuguese estuaries vulnerability has been increasing as a result of chemical pollution with organic and inorganic contaminants from industrial and urban discharge (Vasconcelos et al., 2007). When comparing the Tagus Estuary with other 4 Portuguese estuaries, Tagus is the most affected by higher levels of toxic metals. This pollution comes from the inflow of effluents from industries (chemicals, steelmaking and shipbuilding) plus effluents from about 2.5 million of inhabitants of Lisbon area (Caçador et al., 1996).

Studies to evaluate the Tagus Estuary' pollution started in the late 60's and by 1980 the United Nations Development Programme (UNDP) sponsored an integrated project entitled "Environmental Study of the Tagus Estuary" (Unesco, 1984). This study aimed "to contribute to achievement of rational planning and management of the Tagus Estuary water resource, harmonizing the existing multiplicity of water use with socio-economic development of the region and the safeguard of public health" (Unesco, 1984).

Since the 80's the pollution sources were identified in the southern area (Vila Franca de Xira) and within the estuary area; 15 outfalls of urban and small industries were located within the urban center whereas 11 outfalls of large industries or industrial complexes were located in Vila Franca de Xira, Santa Iria, Sacavém, Lisboa, Barreiro and Seixal (Barros, 1985). The first estimate of total input, considering the most hazardous industrial and urban pollutants, was elaborated in 1981; the main contaminants identified in industrial effluents were nitrogen, phosphorus and metals (cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), cobalt (Co), copper (Cu), zinc (Zn), nickel (Ni), chromium (Cr) and manganese (Mn)) (Barros, 1985). However, others such as PCBs

and organochlorine insecticides were also detected in sediments and biota at levels indicating local pollution (Unesco, 1984). Table IB.1 shows a list of contaminants that show the historical background of pollution in the Tagus Estuary

Table IB.1: List of contaminants found in the Tagus Estuary.

Group	Contaminants	Analyzed samples	References
Pharmaceutically active compounds (PhACs)	[a]Endocrine-disrupting compounds	Water, macroalgaes, bivalves and fish.	Álvarez-Muñoz et al., 2015; Rocha et al., 2015.
Microbial pollution	Multiresistant *Escherichia coli* *Salmonella* spp. *Vibrio* spp.	Water and sediments	Pereira et al., 2013; Anacleto et al., 2013.
Metals	Heavy metal [b](e.g. Cd, Pb, As, Co, Cu, Zn, Ni, Cr)[a]	Sediments, benthic invertebrates, fish, seafood[e] and plants[f] .	Caçador et al., 2000, 2009; Caetano et al., 2008; Duarte et al., 2010; França et al., 2005; Maulvault et al., 2015; Mil-Homens et al., 2013; Pereira et al., 2007, 2013; Quevauviller et al., 1996; Raimundo et al., 2011; Santos et al., 2014.
	Mercury	Sediments, water and plants[f] .	Canário and Vale, 2004; Canário et al., 2003, 2005, 2007a,b, 2010; Maulvault et al., 2015; Pereira et al., 2013; Santos et al., 2014.
	Organotin	*Nassarius reticulatus*	Rato et al., 2008; De Bettencourt et al., 1999.
	Platinum	Sediments and water	Cobelo-García et al., 2011; Almécija et al., 2015.
	Osmium	Sediments and water	Almécija et al., 2015
	Microplastics	*Mytilus galloprovincialis*	Vandermeersch et al., 2015
[c]Organic compounds	PAHs	*Mytilus edulis*	Martins et al., 2012; Villeneuve et al., 2000.
	LAS	Sediments	Hampel et al., 2009
	PHs	Sediments	Villeneuve et al., 2000
[d]Organochlorine compounds	HCHs	Sediments	Mil-Homens et al., 2016;
	DDT	Sediments	Mil-Homens et al., 2016;
	HCB	Sediments	Mil-Homens et al., 2016; Magalhães and De Barros; 1987;
	PCB	Sediments and fish	Martins et al., 2012; Mil-Homens et al., 2016; Villeneuve et al., 2000.

[a]*Example: oestrogens, octylphenols, nonylphenols, mono and diethoxylates, bisphenol A, phytoestrogens (biochanin A, daidzein, formononetin, genistein), and phytosterol (sitosterol);*
[b]*Cadmium (Cd), lead (Pb), mercury (THg), methylmercury (MeHg), arsenic (As), cobalt (Co), copper (Cu), zinc (Zn), nickel (Ni), chromium (Cr);*
[c]*Polycyclic aromatic hydrocarbons (PAHs), Linear alkylbenzene Sulfonates (LAS) and Petroleum hydrocarbons (PHs);*
[d]*Hexachlorocyclohexanes (HCHs), Dichlorodiphenyltrichloroethane (DDT), Hexachlorobenzene (HCB) and Polychlorinated biphenyls (PCB);*
[e]*Mytilus galloprovincialis*, *Chamelea gallina*, *Liza aurata*, *Platichthys flesus*, *Laminaria digitata* and *Saccharina latíssima*);
[f]*Halimione portulacoides Sarcocornia fruticosa*, *Sarcocornia perennis* and *Spartina marítima;*

Nowadays, despite the cessation of industrial activities in the south part, the analyses performed in the Tagus Estuary still indicate traces of the past pollution (Table IB.1), namely high levels of nitrogen and phosphorus (23,639 ton/year and 6294 ton/year, respectively) (Vasconcelos et al., 2007) and sediments contamination levels by metals, such as Hg (Chainho et al., 2008), Zn (Vasconcelos et al., 2007; Duarte and Caçador, 2012) Cu, Pb, Cd, Co, Ni and Cr (Duarte and Caçador, 2012) (Table IB.2). Furthermore, in the case of Zn and Cd their concentration increased from 1980 to 2010 (Duarte and Caçador, 2012) probably due to the intensification of agricultural activities throughout the Tagus basin. In fact, Hg and Zn concentrations in sediments are above the Effects Range Medium (ERM) guideline value (Tables IB.2 and IB.3), and Cd, Cr, Cu and Pb concentrations are above the Effects Range Low (ERL) guideline value (Tables IB.2 and IB.3) (Vasconcelos et al., 2007). Metal concentrations in sediments below ERL values may cause minimal effects, concentrations between ERL and ERM values should be considered to be moderately toxic, leading to occasional effects, and metal concentrations higher than ERM may have deleterious effects (Long et al., 1995). Thus, the levels of metals in sediments of Tagus Estuary may cause moderate to significant toxicity and are indicative of poor sediments quality (Long et al., 1995). In good agreement, Chainho et al (2008) classified 30% of the sampled areas of Tagus Estuary in the vicinity of industrial areas as poor and bad status, reflecting the poor water and sediments quality, which in turn affects the biota.

Table IB.2: Concentration of the main metals measured in sediments, benthic invertebrates, plants and fishes from the Tagus Estuary.

Contaminants	Samples/Species	Levels	Source
Hg	Sediments	HgT: max. 66.7µg/g MeHg: max. 43 ng/g	Canário et al., 2007a
	Halimione portulacoides	HgT: 0.12-0.22 µg/g AB[a] HgT: ≈ 0.5-9 µg/g BB[b] MeHg: ≈ 0.0-900 ng/g BB[b]	Canário et al., 2007b
	Crustacean (3 species)[1]	0.14-0.50 µg/g	Canário, 2004
	Fish (4 species)[2]	0.07-0.54 µg/g	Cabral et al., 2001
	Fish (7 species)[3]	0.14-1.40 µg/g (2002)	Canário, 2004
Zn	Sediments	Max: 2854 µg/g in Barreiro	Vale et al., 2008
	Nereis diversicolor	143-197 µg/g	Cardoso et al., 2008 França et al., 2005
	Scrobicularia plana	831.4 µg/g	Cardoso et al., 2008
	Halimione portulacoides	275-963 µg/g BB[b]	Caçador et al., 1996
	Solea senegalensis	92.6 µg/g	França et al., 2005
Cd	Sediments	0.01-0.78 µg/g Max: 11 µg/g (industrial area)	Vale et al., 2008
	Nereis diversicolor	0.001-1.2 µg/g	Cardoso et al., 2008 França et al., 2005
	Scrobicularia plana	0.31 µg/g	Cardoso et al., 2008
	Solea senegalensis	0.9 µg/g	França et al., 2005
Cr	Sediments	5.3-592 µg/g	Vale et al., 2008
	Halimione portulacoides	5-6 µg/g BB[b]	Caçador et al., 1996
Cu	Sediments	2.3-593 µg/g	Vale et al., 2008
	Nereis diversicolor	11.35- 48.6 µg/g	Cardoso et al., 2008 França et al., 2005
	Scrobicularia plana	15-67.2 µg/g	Cardoso et al., 2008 França et al., 2005
	Halimione portulacoides	60-274 µg/g BB[b]	Caçador et al., 1996
	Solea senegalensis	1.4 µg/g	França et al., 2005
Pb	Sediments	Max: 2858 µg/g in Barreiro	Vale et al., 2008
	Nereis diversicolor	6.06-19.2 µg/g	Cardoso et al., 2008 França et al., 2005
	Scrobicularia plana	16.7 µg/g	Cardoso et al., 2008
	Halimione portulacoides	235-840 µg/g BB[b]	Caçador et al., 1996
	Solea senegalensis	2.9 µg/g	França et al., 2005

[a]*AB: Aboveground biomass;*
[b]*BB: Belowground biomass;*
[1]*Cragon cragon, Carcinus maenas* and *Parapenaeus longirostirs.*
[2]*Dicentrarchus labrax, Solea solea, Engraulis encrasicolus* and *Anguilla anguilla*
[3]*Halobatrachus didactilus, Solea solea, Pamatochistus minutus, Sepia officinalis, Argirosomus regius, Conger conger* and *Anguilla anguilla.*

Table IB.3: Effects Range Low (ERL) and Effects range medium (ERM) guideline values for metals according to Long et al., (1995).

Metal	ERL (µg/g, dry wt)	ERM (µg/g, dry wt)
Mercury	0.15	0.71
Cadmium	1.2	9.6
Chromium	81	370
Lead	46.7	218
Zinc	150	410

IB.3.2.2.1. Mercury contamination

The Tagus Estuary contamination by mercury is well documented and inclusive it was classified in the report prepared by Unesco (1984) as one of the most mercury-polluted estuaries that have been studied in the world. Unesco (1984) estimated that annual discharge of mercury was approximately 5 ton/year, which reflected in a contamination of sediments 10 times higher than the natural background levels (0.05 µg/g). In 1985, Figuères et al. estimated that the contamination of the whole estuary rounded 1.0 µg/g, i.e. 20 times higher than the natural background value. At the time, it was found that the Tagus contamination was only exceeded by Minamata Bay and was close to those found in Bellingham Bay (USA), Mersey estuary (UK) and Bombay Harbour (India) (Figuères et al., 1985).

Mercury pollution in Tagus resulted from past industrial activities, mainly from pyrite-roasting plant (Complexo Quimigal) and smelter (Siderurgia Nacional) located on the southern shore (Barreiro) and from a chloralkali plant (Soda Povoa) located in the northeastern part of the estuary (Figuères et al., 1985; Unesco, 1984). Other sources were a cinnabar treatment plant and a battery manufacturer situated in Vila Franca de Xira and the petroleum pier of Martinha (Figuères et al., 1985). The estimated total mercury (HgT) in deposited sediments ranged from 0.44 to 42.5 µg/g; being the highest values found near the Barreiro's chemical complex (Figuères et al., 1985).

Notwithstanding the inactivation of the most critical industrial units in the north and south areas, mercury contamination persists as demonstrated by reports of high levels of mercury in sediments, suspended matter, water (Canário et al., 2003, 2005) and biota (Canário, 2004). Inventories carried out in the first 5 cm of the sediments have estimated 21 tons of total mercury and 23 Kg of MeHg in the estuary (Canário et al., 2005), being the highest concentration found in the southeast margin (max. 67 µg/g HgT and 43 ng/g MeHg) (Canário et al., 2007a) and north channel (max. 12 µg/g HgT and 5.82 ng/g MeHg) (Canário et al., 2003) (Table IB.2). Lower concentrations have been registered in sediments of Vila Franca de Xira (0.50 µg/g) and in the Natural Reserve of Alcochete (0.39 µg/g) (Serafim et al., 2013). Several studies also reported mercury levels in fish (Cabral et al., 2001; Canário, 2004), crustaceans (Canário, 2004) and plants (Canário et al., 2007b) (Table IB.2) and concluded that the mercury levels in fish and crustaceans were above the maximum allowed by the European Commission (Regulation No 466/2001) that limits mercury content to 0.5 µg/g in prey fish and to 1 µg/g in top-predators (European Community, 2001).

Comparing to others estuaries, Tagus' mercury levels are similar or higher than those determined in a worrisome contaminated estuary as Chesapeake Bay (US), where HgT concentration in sediments exceed 1 µg/g dry wt and was considered a region of concern by U.S. Environmental Protection Agency (USEPA) (Madison et al., 1999).

IB.3.2.3. Major ecological problems resulting from anthropogenic activities

IB.3.2.3.1. Habitat loss

Habitat loss and destruction has a substantial ecological impact, as it affects important areas of nursery and feeding for estuarine biota, namely fish and birds, such as mudflats, sea grass and oyster beds, salt marshes, etc. (Vasconcelos et al., 2007). The evidence of this impact is the mortality of benthic fauna and high juvenile fish mortality, including soles and sea bass, and high discards of other non-profitable species (Vasconcelos et al., 2007). Furthermore, fish community changes have been associated with pollution, namely changes in nursery function (Cabral et al., 2001). Changes in salinity conditions, as a result of damming, is another preoccupant problem affecting fish and leading to the loss of the natural conditions that promote juvenile growth and survival (Vasconcelos et

al., 2007). Another evidence of habitat loss and destruction effects is the notorious reduction of migratory species. In the past, many species colonized most of the watershed, but presently only a few remain (Vasconcelos et al., 2007) as a consequence of loss and degradation of several roosting sites. For instance, over the past 30 years three of the five most abundant wintering waders have significantly declined, including Dunlin and Grey Plover (Catry et al., 2011). One possible explanation is the abandonment of salt exploitation and transformation of saltpans into aquacultures since saltpans in northern of the Tagus Estuary were important roosting sites for waders and other migratory species (Catry et al., 2011).

IB.3.2.3.2. Risks associated to mercury pollution

From the ecological point of view, mercury pollution represents a major risk to aquatic and terrestrial biota as well as for humans. Adverse effects have been observed in mammals (Muccillo-Baisch, 2012), fish (Ung et al., 2010) and birds (Burger and Gochfeld, 1997; Herring et al., 2012; Heinz and Hoffman, 2003), and include behaviour disturbances, neurological system deformity and abnormal function, defective development and reproductive impairment, such as malformation of fetus and even the inability for reproduction (Burger and Gochfeld, 1997; Herring, et al., 2012; Heinz and Hoffman, 2003). The analysis of fishes from the Tagus Estuary indicates that organic mercury represents ≥ 90% of total (Canário, 2004). Furthermore, the evaluation of bioaccumulation factor among aquatic organisms of Tagus, Canário (2004) concluded that *Anguilla anguilla* has the major factor, being the top predator in this estuary. This clearly indicates that mercury contamination in this estuary is already reflected in the food web.

Among mercurials, MeHg is one of the most severe toxicants as it undergoes bioaccumulation and biomagnification in aquatic food webs and causes severe neurotoxic effects especially during nervous system development. From the human health point of view, mercury affects the nervous, motor, renal, cardiovascular, immune and reproductive systems (Zahir, et al., 2005). MeHg represents a serious risk for offsprings of women exposed during pregnancy (NRC, 2000). Therefore, MeHg

bioaccumulation and biomagnification has also to be considered in human health risk assessment.

IB.4. STRATEGIES TO RECOVER ESTUARIES

IB.4.1. Management strategies

There have been significant efforts to evaluate antropogenic impacts in estuaries status, identify the problems and thus, to reduce or mitigate them. Some legislation has been adapted for the restoration of degraded aquatic habitats which covers estuarine ecosystems; among them are the Clean Water Act in USA, the Water Framework Directive (WFD), the Marine Strategy Framework Directive (Borja et al., 2006) and the Convention for the Protection of the Marine Environment of the North-east Atlantic (OSPAR convection) (Rogers and Greenaway, 2005). For example, WDF establishes guidelines for water resources management with the objective of protect groundwater, inland, estuarine and coastal water. The WDF requires the Member States to assess the ecological Quality Status of transitional and coastal waters by 2006 and to achieve at least good ecological status in all water bodies by 2015 (Borja et al., 2006).

Early efforts to attain the recovery included the monitoring of the estuaries quality by using physical and biological indicators (Rogers and Greenaway, 2005), in order to bring out and to measure the impact of anthropogenic pressure on the ecological functions of the estuaries and reduce it when necessary (Courrat et al., 2009; Vasconcelos et al., 2007). For coastal and offshore waters, the focus has been placed on nutrients and hazardous substances. However, to assess the effect of the contaminants in the environment, the so-called effects indicators, organisms' assessment have been included, for phytoplankton/zooplankton, benthic invertebrates, fishes and seabirds (Rogers and Greenaway, 2005; Courrat et al., 2009). Phytoplankton/zooplankton and benthic organisms are fundamental for the productivity of higher trophic levels, and their alteration is indicative of both environmentally driven changes as well as anthropogenic undesirable disturbances (Rogers and Greenaway, 2005). Fish stock

status, as well as non-target fish species, are well-known pressure indicators (Rogers and Greenaway, 2005; Courrat et al., 2009). Seabird population is also used as an indicator of anthropogenic impacts such as toxic contaminants, plastic particles, food resource and habitat quality (Rogers and Greenaway, 2005; Catry et al., 2011).

Recently, management has become more comprehensive and focused on the ecosystems-based approach leading to the development of the ***Drivers-Pressures-State Change-Impact-Response*** (DPSIR) framework (Figure IB.5) (Rogers and Greenaway, 2005).

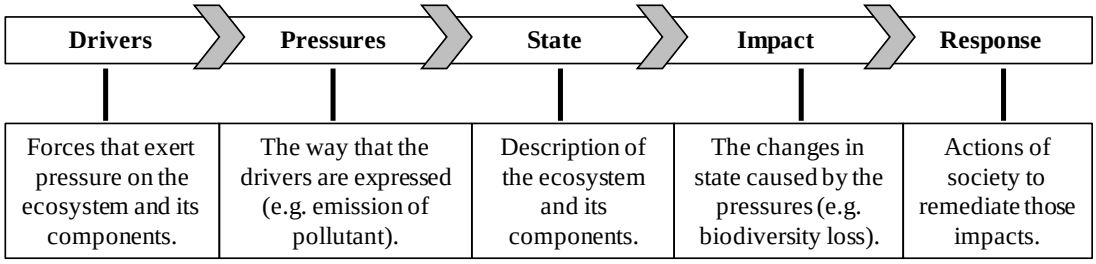


Figure IB.5: The explanation of the ecosystems-based approach Drivers-Pressures-State Change-Impact-Response (DPSIR) framework according to Rogers and Greenaway (2005).

This systems-based approach captures key relationships between society and environment, having as philosophy the structuring and communicating policy-relevant research about the environment (Atkins, et al., 2011). The goal is to produce sustainable ecosystems, which means to keep ecosystem processes and at the same time maintain ecosystem services in order to deliver societal benefits such as producing fisheries or improving water quality (Borja et al., 2006).

OSPAR convention, which is the convention formed by fifteen Governments of the western coasts (Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and United Kingdom) together with the European Union, have been cooperating to protect the marine environment of the North-East Atlantic by adopting an ecosystem-based

approach (OSPAR, 2014). This convention started with the objective of protecting and preserves ecosystems and biological diversity, and co-orientating the development of the Ecological Quality Objective (EcoQO) for the marine environments (Rogers and Greenaway, 2005). OSPAR Commission outlined a strategy for the years 2010-2020 as: "*Using Ecosystem Approach to manage human activities affecting the maritime area*", being the overall goal "*to conserve marine ecosystems and safeguard human health and, when practicable, restore marine areas which have been adversely affected in the North-East Atlantic by preventing and eliminating pollution and by protecting the maritime area against the adverse effects of human activities*". To reach this, the main strategic guidelines for OSPAR marine areas are: 1) halt and prevent by 2020 further loss of biodiversity; 2) to combat eutrophication; 3) to prevent and eliminate pollution of hazardous substances and offshore oil and gas activities and from ionising radiation; 4) to ensure integrated management of human activities in order to reduce impacts on the marine environment, and 5) to facilitate and coordinate the work to achieve good environmental status under the EU Marine Strategy Framework Directive by 2020 (Annex 25 Ref M5.2) (OSPAR, 2014).

IB.4.2. Remediation strategies

Nevertheless, to face the environmental contamination problem, the management procedures include, not only the control of the pollution sources, but also remediation strategies to recover the contaminated area. The remediation strategies deal effectively with the pollution problem by reducing the amount and toxicity of hazardous substances in environmental media (sediments and water) and include physical, chemical and biological (bioremediation) processes (see Table IB.4) (Hamby, 1996; Khan et al., 2004; NIEHS, 2016).

Among physical remediation strategies, capping and dredging, which involves removal of benthic sediments and placement of a layer of proper isolating materials (e.g. sand) between the layer of contaminated sediments and overlying water, respectively, are two widely used solutions for contaminated sediments in aquatic systems (Wang 2004). However, these techniques only concentrate the contaminants in smaller volumes and are, thus useful before chemical, thermal, or other remediation processes (Mulligan et

al., 2001). Indeed, some techniques, such as soil washing, vapor extraction, solidification/stabilization, pump-and-treat technology; combine physical and chemical processes (Khan et al., 2004).

Thermal remediation is also a physical treatment and there are several kinds of *in situ* thermal remediation methods (Table IB.4) (Hamby, 1996; NIEHS, 2016). These techniques are applicable for a wide variety of metals; for instance, heating up to 800°C has been used for mercury, arsenic and cadmium and its compounds evaporation (Mulligan et al., 2001). Vitrification is a special case of thermal remediation based on the heating of sediments electrically, reaching temperature as high as 1600-2000°C (Hamby, 1996; Mulligan et al., 2001).

Chemical methods (Dabrowski et al., 2004; Navarro et al., 1996; NIEHS, 2016) have specific purposes; for instance, solvent extraction enables to desorb the contaminants (e.g. used in the cleanup of chemicals such as PCBs), while chemical oxidation/reduction helps to break down the contaminants into harmless substances, such as water and carbon dioxide (NIEHS, 2016). Among oxidation processes, there are treatments with chlorine dioxide and hydrogen peroxide additives, photolysis (e.g. UV radiation in PCBs, dioxins and PAHs degradation in surface and groundwater remediation) and reductive dechlorination (e.g. remediation of soil contaminated with PCBs) (Hamby, 1996). Oxidation/reduction together with precipitation are important techniques for the remediation of heavy metals contaminated areas; for example the technology called TR-DETOX involves the reduction of heavy metals to their lowest valence state and form stable organometallic complexes using inorganic and organic reagents (e.g. sodium polythiocarbonate) (Mulligan et al., 2001). Solidification/stabilization processes reduce the mobility of hazardous substances and contaminants through both physical and chemical means, with solidification responsible for the encapsulation of waste materials in a monolithic solid (e.g. lime, fly ash, cement) and stabilization is responsible for the conversion of the contaminants into less soluble, immobilized and less toxic forms (Khan et al., 2004; Mulligan et al., 2001). This technique is useful for the remediation of organic and heavy metal contaminants in soil (Khan et al., 2004; Mulligan et al., 2001). Adsorbents are important in water purification and especially in industrial wastewater treatment (e.g. chemically modified

clay minerals with 2-mercaptobenzothiazole impregnated for the removal of some heavy metal ions) (Manohar et al., 2002).

To avoid the aggressive remedial methods and their costs, bioremediation is an option by providing a cost-effective and environmentally friendly alternative to clean up contaminated sites. Among them, phytoremediation, which consists in the usage of plant to clean up contaminated soil and groundwater, is the most common (Khan et al., 2004). This process relies on naturally occurring processes within certain plant species to uptake, accumulate, and/or degrade contaminants from soil and water environments and has been applied to a number of contaminants, such as heavy metals, radionuclides, chlorinated solvents, petroleum hydrocarbons, PCBs, PAHs, organophosphate insecticides, surfactants, etc. (Khan et al., 2004).

Although the metabolic potential of microorganisms in remediation strategies is also well recognized, it has been implemented only in few cases. One example was the elimination of cyanide, zinc and copper at Homestake Mine plant (United States), by using aerobic bacteria, namely *Pseudomonas*, and microbial biomass (Wagner-Döbler, 2003). Other full scale treatment systems, such as Thiopaq system (Netherlands), Metex anaerobic sludge reactor (Germany) and Bio-Substrat anaerobic micro-carrier reactor (Germany), include the usage of sulphate-reducing bacteria to precipitate metals, such as Sn, Cu, Pb, Ni, Zn, Fe, Cr and trace metals, from contaminated groundwater or industrial effluents (Wagner-Döbler, 2003). The usage of microorganisms is based mainly on their genetic characteristics as genes that encode for contaminant-degrading enzymes or other with interest in remediation process are desired. This is of great interest in genetic engineer as tool for the development of genetically engineered organisms (bacteria and plant); for example a model plant, *Arabidopsis thaliana*, has been engineered to express a modified bacterial gene (*merBpe*) involved in mercury demethylation.

Table IB.4: Techniques for remediation of environmental pollution (soil and water).

Technique		**Definition**	**Examples**
Physical	**Mechanical**	Physical removal of the contaminated environmental media (soil or water), followed by its treatment at a plant or on off-site, or the installation of wells and pipes in the environmental media, throughout the contaminants are extracted.	Capping Dredging
	Thermal	Heating of contaminated groundwater or sediments to push chemicals toward a collection wells, where they are then pushed up to the ground surface for clean up or in alternative destroyed or evaporated.	Steam heat injection, incineration, vitrification, thermal desorption, radio frequency heating, and thermal conduction.
Chemical		Usage of chemicals to extract pollutants from contaminated media.	Solvent extraction, oxidation/reduction, chemical precipitation, ion exchange, immobilization, adsorbents, solidification/stabilization, etc.
Bioremediation		Usage of organisms, including microbes and plants, to convert bioavailable hazardous substances to less toxic or more easily degraded products - biotransformation and biodegradation.	Phytoremediation

Table IB.5 shows some examples of different recovered estuaries around the world and the strategies employed.

Table IB.5: Examples of estuaries impacted by anthropogenic activities and their current situation after the implementation of recovery strategies.

Estuaries	Pollution		Recovery strategies	Current situation	Source
	Origin	Contaminants			
Mersey Estuary (U.K.)	Industrial activity	Metals (As, Cr, Cu, Hg, Zn and Pb) PAH DDT PCBs	Usage of percolating filters for sewage treatment; Infrastructures for the treatment and disposal of domestic and industrial wastes; Pressure on industries to reduce pollution.	Besides considerable improvements, organic and inorganic contaminants are still present.	Ridgway and Shimmield, 2002; Burton et al., 2003; Jones et al., 2006
Chesapeake Bay (USA)	Urban and industrial activity	Metals (e.g. Hg)	Wastewater treatment plant; Cattle exclusion; Limiting livestock access to streams.	Extensive restoration efforts in the past 25 years, but the estuary continues to have poor water quality and is considered a region of concern by US EPA.	Manson et al., 1999; US EPA, 2013, 2014
Nervión River Estuary (Spain)	Waste; Industry (iron and steel); Urban effluents.	Cyanide Heavy metals Fluorides Phenols	Physico-chemical water treatment plant; Iron and steel industries were closed.	Progressive improvement in physico-chemical properties, benthic and fish assemblages, but the estuary continues to be moderately polluted by metals and organic compounds.	Borja et al., 2010
Hudson-Raritan Estuary (USA)	Dredging; Channelization for navigation; Commercial and residential development of coastal margins; Chemical contamination from runoff; Wastewater treatment facilities; Industrial plants; Illegal dumping; etc.	Heavy metals (Cu, Zn, Pb Cd, Cr and Hg) PCBs PAHs	Implementation of dredged material (e.g. oyster reefs, artificial reefs, seagrass beds, intertidal mud flats, salt marshes, etc.); Introduction of clay and geotextile landfill liners to contain potential contaminants; Leachate collection and treatment; Disposal systems.	Successful restoration program that counter the extensive habitat losses; Capped landfills are being transformed into recreational areas or natural upland sites.	Wolfe et al., 1996; Heyes et al., 2004

IB.4.3. Portuguese estuaries recovery

In Portugal, there are some regulations aiming at protecting vulnerable estuaries. It is the case of Decree-Law No 173/2008 (implemented under the Directive 96/61/EC), which aims to ensure the establishment of actions to prevent or when that is not possible, to reduce the emissions to air, water or soil, and prevent/control of waste

production, in order to achieve a high level of environmental protection (ARH do Tejo and GOT, 2009). Other example is the Decree-Law No. 254/2007, transposing Directive 96/82/EC, which seeks to prevent the occurrence of accidents with dangerous substances likely to cause significant damage to the environment and to human health (ARH do Tejo and GOT, 2009). Following the Decree-Law No. 129/2008, which established Estuaries Management Plans, in 2009, the Tagus Estuary Management Plan (POE Tejo) was created. The main objective of this Plan is the protection of water source, through a holistic approach focused on water, wetlands and aquatic/ terrestrial ecosystems (ARH do Tejo and GOT, 2009). However, besides all the efforts, Chainho et al. (2008) concluded that the recovery numbers are still far below the records prior the onset of commercial exploration.

Nevertheless, Portuguese estuaries have not yet been comprehensively assessed and there has been little or no application of management plan to fulfill the WFD requirements (Vasconcelos et al., 2007).

In case of the Tagus Estuary, from the analysis performed in 1984, it was concluded that despite the pollution, the ecology, growth and reproductive cycle of fish, mollusks and crustacean were similar to undisturbed environments. Vasconcelos et al. (2007) observed in their analysis that although the Tagus Estuary is one of the most pressured estuaries in Portugal it is among the less vulnerable systems in Portuguese estuarine systems. However, some visible toxics effects of the pollutants have been noticed since 80's. Management action has been taken to improve water quality through several water treatment plants (Cabral et al., 2001) and also recover projects that have been implemented, such as project Arco Ribeirinho Norte and Arco Ribeirinho Sul, which have been engaged in the recovery of north and south margin, respectively (ARH do Tejo and GOT, 2009).

IB.5. CONCLUSIONS

Estuaries are important areas from ecological point of view because of their high biological productivity and high species diversity and also are essential areas for many human population's activities. However, despite their biological importance, estuarine systems are impacted by human's usage, being modified drastically, mainly due to pollution. The Tagus Estuary is an example of an impacted estuary in Portugal. It has been extensively studied during the past 20 years, which resulted in a considerable amount of technical and scientific data. However, besides all the data obtained and the capacity to analyze and predict processes, it is necessary a better assessment of data and a characterization based on a conceptual framework, such as DPSIR (ARH do Tejo and GOT, 2009), in order to implement the recovery actions more effectively and robustly.

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CHAPTER II

ISOLATION AND CHARACTERIZATION OF MERCURY-RESISTANT BACTERIA FROM SEDIMENTS OF TAGUS ESTUARY (PORTUGAL): IMPLICATIONS FOR ENVIRONMENTAL AND HUMAN HEALTH RISK ASSESSMENT†

†Figueiredo et al., 2014. J. Toxicol. Environ. Health A, 77:155-16

ABSTRACT CHAPTER II

Mercury (Hg) contamination of aquatic systems has been recognized as a global and serious problem affecting both human and environmental health. In the aquatic ecosystems, mercurial compounds are microbiologically transformed with methylation responsible for generation of methylmercury (MeHg) and subsequent biomagnification in food chain, consequently increasing the risk of poisoning for humans and wildlife. High levels of Hg, especially MeHg, are known to exist in Tagus Estuary as a result of past industrial activities. The aim of this study was to isolate and characterize Hg-resistant bacteria from Tagus Estuary. Mercury-resistant (Hg-R) bacteria were isolated from sediments of two hotspots (Barreiro and North Channel) and one reserve area (Alcochete). Mercury contamination in these areas was examined and bacterial susceptibility to Hg compounds evaluated by determination of minimal inhibitory concentrations (MIC). The isolates characterization was based on morphological observation and biochemical testing. Bacteria characteristics, distribution, and Hg resistance levels were compared with metal levels. Barreiro and North Channel were highly contaminated with Hg, containing 126 and 18 μg/g total Hg, respectively, and in Alcochete, contamination was lower at 0.87 μg/g total Hg. Among the isolates there were aerobic and anaerobic bacteria, namely, sulphate-reducing bacteria, and Hg resistance levels ranged from 0.16 to 140 μg/mL for Hg^{2+} and from 0.02 to 50.1 μg/mL for MeHg. The distribution of these bacteria and the resistance levels were consistent with Hg contamination along the depth of the sediments. Overall, results show the importance of the characterization of Tagus Estuary bacteria for ecological and human health risk assessment.

II.1. INTRODUCTION

Mercury (Hg) is among the metals with highest toxicological importance and is widespread in the human environment (Domingo, 1994; Nichols et al., 1999). Naturally occuring Hg results from degassing of earth's crust and evaporation from oceans (Boening, 2000). Mercury exists in different chemical species, with the major forms in water being mercuric mercury (Hg^{2+}) and methylmercury (MeHg) (Morel et al., 1998). However, the increase of aquatic systems with mercury contamination, resulting from human activities, was recognized as a global and serious problem, affecting both wildlife and human health (Campbell et al., 2003; Marvin-DiPasquale and Agee, 2003; Sweet and Zelikoff, 2001). Mercury pollution and its effects through bioaccumulation in food web is a serious problem because of the high toxicity associated with its forms (Mathema et al., 2011). Ecologically, mercury pollution affects both high predators and microbial community. Adverse effects were observed in mammals (Ni et al., 2012; Sweet and Zelikoff, 2001), amphibians (Davidson et al., 2011), fish (Ung et al., 2010), and birds (Burger and Gochfeld, 1997; Herring et al., 2012; Heinz and Hoffman, 2003), and include behavioural disturbances, immunotoxicity, neurological system deformity and abnormal functions, developmental and reproductive abnormalities, such as fetal malformation or inability of reproduce (Burger and Gochfel, 1997; Davidson et al., 2011; Herring et al., 2012; Heinz and Hoffman, 2003 Sweet and Zelikoff, 2001). The most important case of human poisoning by mercury pollution involved the population of Minamata Bay (Japan), who suffered severe neurological disorders termed Minamata disease as a consequence of contamination on Minamata Bay by industrial activity (Ekino et al., 2007).

Mercury toxicity derives from the formation of highly toxic organomercurial complexes, predominantly MeHg, which is highly neurotoxic (Mathema et al., 2011; Ni et al., 2012). In humans, mercury affects the central nervous system, motor system, and renal, cardiovascular, immune and reproductive systems (Ratcliffe et al., 1996; Zahir et al., 2005), by disrupting cell function due to the affinity to thiol groups of proteins (Ung et al., 2010). Among mercurial compounds, MeHg is one of the most severe toxic forms as it passes through biologic membranes and, once inside the cell, produce irreversible

damage to nucleic acids, altering the normal configuration and biological cell activities (Mathema et al., 2011; NRC, 2000).

Although mercury compounds are toxic to all living organisms, constant exposure to mercurial compounds has enable bacterial community to develop several types of resistance mechanisms, which allow them to deal with the adverse effects of metal-mediated toxicity (Mathema et al., 2011). In water and sediments, these mechanisms are responsible for bacterial mercury transformation, namely, reduction of Hg^{2+}, mercury methylation and MeHg demethylation. These conversion processes may occur as the result of diverse biological pathways (Barkay and Wagner-Dobler, 2005). The best known pathway is the reductive demethylation followed reduction of Hg^{2+} - a process mediated by enzymatic activity of proteins codified by a cluster of genes organized in the *mer* operon (Nascimento and Chartone-Souza, 2003).

The methylation process is considered of great concern, as it yields the highly toxic compound MeHg. Among bacterial community, mercury methylation was found to predominantly be associated to sulphate-reducing bacteria (SRB) (King et al., 2000, 2001, 2002). SRB are anaerobic bacteria that use sulphate as a terminal electron acceptor for degradation of organic compounds, resulting in production of sulphide (Muyzer and Stams, 2008). Bacterial transformation of mercury is influenced by several factors, such as bacterial community composition and the availability of reactive mercury species (Macalady et al., 2000; Rasmussen et al., 1998).

Estuaries are important for their ecological potential that offers adequate conditions for the development of both animal and plant species, promoting biodiversity. However, these systems are often impacted by anthropogenic activities, namely, industrial discharge (Ferreira, 1988). Tagus Estuary, one of the most important estuaries in Europe, covering an area of 325 Km^2, was reported to be contaminated since 1985 due to two industrial areas located in north and south margins, North Channel and Barreiro, which led to high levels of mercury in sediments, suspended matter and water (Figuères et al., 1985). Despite the inactivation of the most critical industrial units in these areas, contamination still persists. Several studies reported high levels of mercury in fish (Lima et al., 1982) and microalgae (Ferreira, 1988) and in sediments (Canário et al., 2003, 2005) of several areas of Tagus Estuary. Inventories in sediments estimated 21

tons of total mercury and 23 Kg of MeHg in the estuary (Canário et al., 2005). Despite the knowledge of contamination levels, there is still a lack of information on the biogeochemical processes involved in the mercury cycle in Tagus Estuary, namely the reduction/methylation/demethylation processes. Thus, the aim of this study was to isolate and characterize Hg-resistant (Hg-R) bacteria found in sediments of two industrial sites and one natural reserve area Tagus Estuary to assess the role of bacteria in mercury cycling.

II.2. MATERIAL AND METHODS

II.2.1. Study area and sample collection

According to previous studies (Canário et al., 2003, 2005), three sites of Tagus Estuary were sampled (Figure II.1): two highly mercury contaminated areas, Barreiro (Lat: 38º40´45.40´´N; Long: 9º3´1.70´´W) and North Channel (Lat: 38º51´21.21´´N; Long: 9º3´40.51´´W), and Alcochete (Lat: 38º45´41.58´´N; Long: 8º56´49.93´´W), which was used as a control area due to low contamination (Figure II.1). Barreiro station had an intensive industrial activity since 1960s decade, which now stopped, while North Channel industrial activity started around 1940-1950 and continued to the present (LNEC, 2008). In contrast, Alcochete station belongs to a natural reserve of high ecological value having an important role in conservation of plant and animal species.

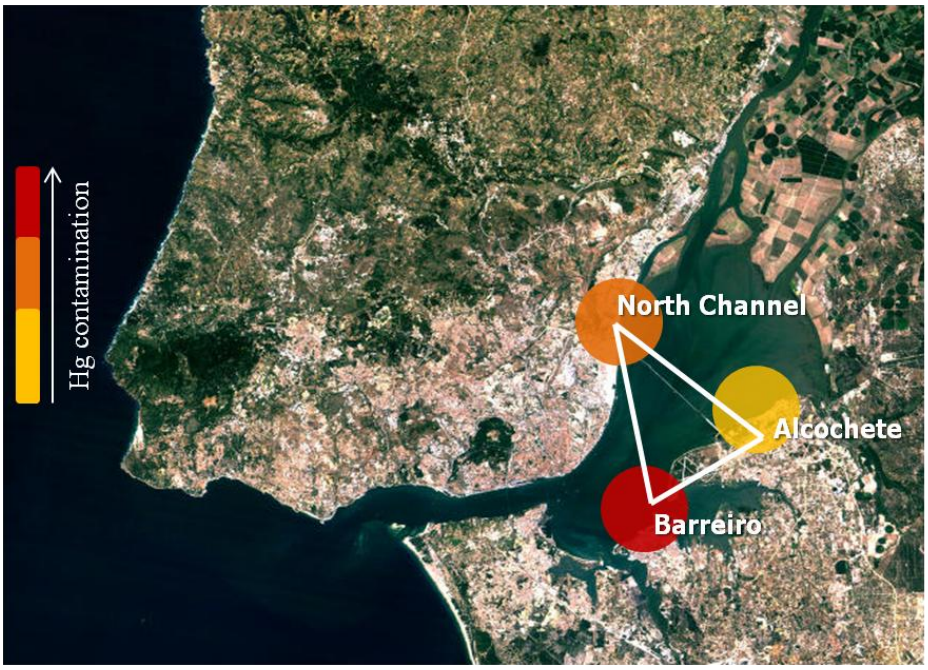

Figure II.1. Map of Tagus Estuary with the location of the sampled areas (Barreiro, North Channel, and Alcochete).

Sediments samples were collected during the summer. Sediments cores, approximately 50 cm long, were collected twice (one for mercury analysis and other for bacterial isolation) and rapidly sliced in layers of 3 cm along the depth (Figure II.2). Samples were stored in sealed tubes, in a refrigerated environment, and transported to the lab for microbial isolation.

II.2.2. Analysis of Total Hg (HgT) and MeHg on sediments

Mercury contamination levels of total mercury (HgT) in the solid portion and pore water, along the depth of sediment core, were determinated. HgT of solid fraction and pore water was determined following sediment centrifugation by atomic absorption spectrometry using a silicon ultraviolet (UV) diode detector LECO AMA-254 after pyrolysis of each sample in a combustion tube at 750^{o}C under an oxygen atmosphere and collection on a gold amalgamator (Costley et al., 2000).

MeHg was determined in dry sediments by alkaline digestion (KOH/MeOH), organic extraction with dichloromethane (DCM) and preconcentration in aqueous sulphide

solution, back-extraction into DCM and quantification by gas chromatography with atomic fluorescence spectroscopy (GC-AFS) using an Agilent chromatograph coupled with a pyrolizer unit and a PSA fluorescence detector (Canário et al., 2004). Recoveries and possible MeHg artifact formation were assessed by spiking several samples with Hg^{2+} and MeHg standard solutions with different concentrations. Recoveries varied between 97 and 103% and no artifact MeHg formation was observed during the procedure. Precision, expressed as relative standard deviation of 4 replicate samples, was less than 4% (p<0.05). Certified reference materials for MeHg (IAEA-405; 54.9 ± 5.3 ng/g) and for HgT (BCR-580; 132 ± 3 µg/g) were used to ensure the accuracy of the procedure, and obtained values 55.9 ± 5 ng/g for IAEA-405 and 131 ± 2 µg/g for BCR-580 were not statistically different.

II.2.3. Isolation and morphological characterization of bacteria strains

Inoculums were prepared through the dilution of sediments samples with 20 mL of distilled sterile water. Figure II.2 shows the scheme of different techniques used for the isolation of different Hg-R bacteria: aerobic Hg-R bacteria and anaerobic Hg-R bacteria, including Hg-R SRB.

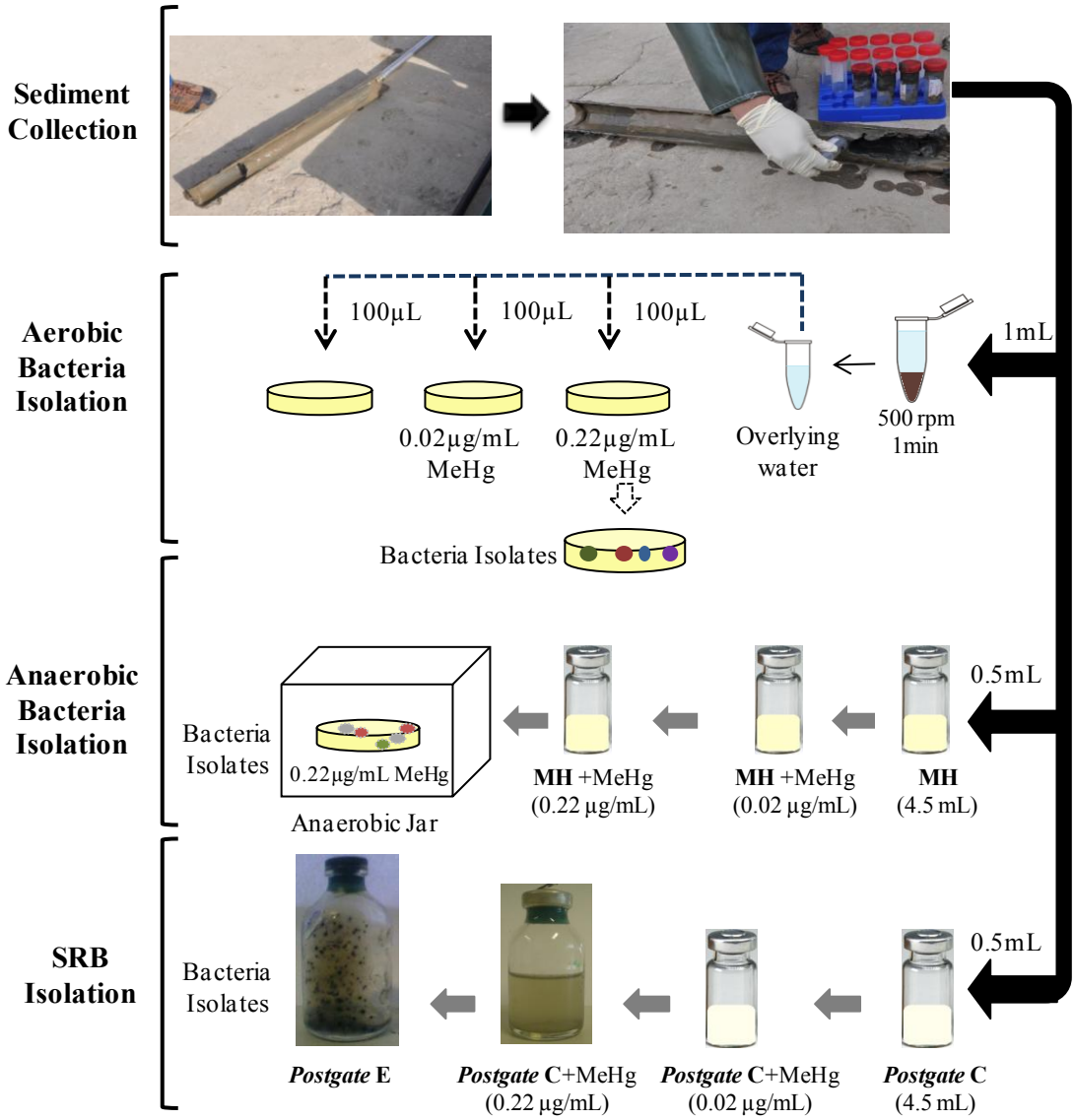

Figure II.2. Schematic representation of samples collection and different type of Hg-R bacteria isolation from sediments of sampled areas of Tagus Estuary. Aerobic Hg-R bacteria and anaerobic Hg-R bacteria, including Hg-R SRB, were isolated.

II.2.3.1. Aerobic Hg-R bacteria isolation

The samples were shaken and after centrifugation at 2650 × g for 1 min (4°C); 100 µL of supernatant was plated on Mueller-Hinton (MH) agar media without and with MeHg selective pressure in a concentration of 0.022 µg/mL and 0.22 µg/mL (Figure II.2). MeHg was used to establish a selective pressure since the main objective of the study

was focused on bacteria responsible for methylation/demethylation of Hg. Colony numbers were counted in all media for colony-forming units (CFU) quantification. Different colonies were selected on MH agar plus 0.22 μg/mL MeHg and after were stored in MH broth plus 15% of glycerol and 0.022 μg/mL MeHg at –80°C.

II.2.3.2. Anaerobic Hg-R bacteria isolation

Washed sediment, 0.5 mL, was inoculated in serum bottles (Belco Glass, Inc.) containing 4.5 mL MH and closed with rubber stoppers with a crimped metal seal. Media were prepared under nonsterile conditions and added to serum bottles. Serum bottles were gassed with N_2 during and after media addition and then sealed as they were withdrawn from gassing needles. Metal seals were then crimped and the bottled media were autoclaved (Figure II.2). To avoid O_2 contamination, all inoculations were performed with a hypodermic syringe and needle washed with N_2 in anaerobic chamber (with N_2 flux). After 3 d of growing at 22°C, 0.5-mL inoculums were transferred to a new bottled medium supplemented with 0.022 μg/mL MeHg and then to bottled medium supplemented with 0.22μg/mL MeHg, in order to select Hg-R bacteria. After 3 d of growth, aliquots were inoculated on solid MH (0.22 μg/mL MeHg) in an anaerobic chamber and incubated in anaerobic jars (Oxoid) (anaerobic conditions were obtained using AnaeroGen sachet [Oxoid]). Single colonies were selected and stored in MH broth plus 15% of glycerol added by 0.022 μg/mL MeHg at –80°C.

II.2.3.3. Hg-R SRB isolation

The isolation of SRB was performed using the same methodology already described, with the exception of colony isolation techniques, which were different (Figure II.2) and involved the use of a selective medium, Postgate. This medium contains sulphate that is reduced by SRB to sulfide, forming a black precipitate that indicates SRB growing. Postgate C (liquid medium) supplemented with MeHg (0.022 and 0.22 μg/mL) was used to select Hg-R SRB. Single colonies were isolated according to the roll tube method described by Miller and Wolin (1974), with some modifications. Fifty-milliliter serum bottles containing 6 mL melted agar medium (Postgate E with 0.22 μg/mL MeHg) were inoculated with 0.1 mL Postgate C inoculum with added 0.22 μg/mL MeHg and

adjusted to 10^{-5} CFU/mL. For soft mix, bottles were manually rolled prior to inoculation and placed in cold water to solidify. After 5–7 d of growth, single colonies were selected and submitted again to isolation process just described. After 3 repetitions, single colonies were incubated into Postgate C medium containing 0.22 μg/mL MeHg and stored at 4°C.

II.2.4. Morphological and biochemical characterization

Cells at the early growth stages were examined with respect to morphology and gram staining characteristics. Biochemical characterization was carried out following standard methodologies, such as lactose, glucose, and mannitol fermentation, enzymatic activity detection (catalase, oxidase, amylase, casease, phosphatase, and lipase), salt tolerance, or ability to growth on media (MH) containing 8% (w/v) NaCl. Commercial micromethods for the biochemical identification were also applied and included BBL Crystal Identification Systems (BD) for aerobic and anaerobic bacteria identification and API20E/20NE (bioMérieux sa) comprising tests for fermentation, oxidation, degradation, and hydrolysis of various substrates. Some organic compounds (electron donors), such as formate, lactate, fumarate, and acetate, and electron acceptors (sulphate and nitrate) were used to characterize SRB.

II.2.5. Mercury susceptibility testing

Aerobic and anaerobic isolates susceptibilities to Hg compounds were determined by minimal inhibitory concentration (MIC) determination, using a modified micro dilution broth method described by CLSI (2006). Microorganism cultures in brain heart infusion broth (BHI) at a concentration of 10^8 CFU/mL (OD 0.5 at 595 nm) were diluted in MH in order to obtain 3×10^6 CFU/mL. To achieve concentrations ranging from 1.33×10^{-3} to 1358 μg/mL HgCl2 (4.88×10^{-6} to 5.00 μM) and from 1.23×10^{-5} to 125.5 μg/mL MeHgCl (4.88×10^{-8} to 0.50 μM), 100 μL of aqueous solution of these compounds (2.72 to 2715 μg/mL HgCl2 and 0.025 to 251.1 μg/mL MeHgCl) was diluted with 100 μL of bacterial suspension into the first well of a sterile 96-well microplate and then sequentially diluted 1:2 in the following 10 wells. Bacterial suspension in the absence of mercurial compounds was used as a control in the 12^{th} well. Duplicate samples were

performed for each concentration tested. After incubation at 37°C for 24 h under aerobic and anaerobic conditions, bacterial growth or its absence was observed. The MIC was defined as the minimum concentration of test compound that inhibited visible growth. All data points represent the mean ± standard deviation (STD) of two to three independent determinations.

SRB susceptibility to mercurial compounds was determined following the same criteria already described, adjusting it into a macro dilution broth method using Postgate C bottled and gassed with N_2. An adequate volume of sterile Postgate C medium was added to each sealed and sterile serum bottle using a sterile and N_2-washed syringe. After 24 h, bacterial growth was determined by spectrophotometric reading at 595 nm. It was only possible to determine MIC_{50} (minimal inhibitory concentration that inhibited 50% of bacterial growth) once high concentrations of $HgCl_2$ led to formation of colored precipitate, probably due to cinnabar (HgS) formation.

II.3. RESULTS

II.3.1. Isolation and characterization of Hg-R bacteria in sediments

In total, 93 different Hg-R bacteria from sediments collected in Tagus Estuary were isolated on media (MH and Postgate) in the presence of 0.22 μg/mL MeHg, a concentration closely associated to resistance, according to the results of Sadhukhan and co-workers (1997), which indicated 2.5μg/mL as the frontier for Hg^{2+}. Also François et al., (2011) corroborated this edge by using 2.72 μg/mL of Hg^{2+} as a typical concentration for the isolation of resistant strains. For MeHg the concentration was decreased by one order of magnitude according to the normal tolerance ratio that isolates present for Hg^{2+}/MeHg. Among the Hg-R isolates, 43 were isolated from Barreiro, 24 from North Channel, and 26 from Alcochete sediments (Table II.1).

The Hg-R isolates were mostly gram-positive rods (44%). According to the BBL test, the aerobic gram-positive rods belong mainly to *Bacillus* sp. and anaerobic gram-

positive rods mainly to *Clostridium* sp. Bacilli were mainly isolated from Barreiro sediments (41%, 17/43). Gram-negative bacteria were predominately isolated from the two highly contaminated areas (Barreiro and North Channel) (Table II.1). Among the gram-negative isolates, genera such as *Vibrio, Aeromonas,* and Enterobacteriaceae species were identified through BBL and API 20E/20NE systems (data not shown). For SRB characterization, biochemical evaluation indicated that all isolates were able to use lactate as an electron donor and sulphate as an electron acceptor. Further, some bacteria had the capability of using formate, fumarate, and acetate as electron donor and nitrate as electron acceptor (Table II.1).

Table II.1: Microbiological and biochemical characterization of the bacteria isolated from the three sampled areas in Tagus Estuary: Barreiro, North Channel, and Alcochete (Natural Reserve).

Sampled area		Barreiro		North Channel		Alcochete	Biochemical characterization
Total no. of Hg-R isolates[a] (% of total)		43 (46%)		24 (26%)		26 (28%)	
Isolates	%	Gram/Morfology	%	Gram/Morfology	%	Gram/Morfology	Genera identification[c]
Aerobic bacteria	51	Gram$^+$ rod: 41% Gram$^+$ coccus: 2% Gram$^-$ rod: 5% Gram$^-$ vibrio: 5%	58	Gram$^+$ rod: 13% Gram$^-$ rod: 13% Gram$^-$ vibrio: 33%	54	Gram$^+$ rod: 54%	*Bacillus sp.* *Aeromonas sp.* Enterobacteriacea sp *Vibrio sp.*
Anaerobic bacteria	40	Gram$^+$ rods: 2% Gram$^+$ coccus : 12% Gram$^-$ rod: 26%	21	Gram$^+$ rod: 16% Gram$^+$ coccus: 4%	46	Gram$^+$ rod: 19% Gram$^+$ coccus: 8% Gram$^-$ rod: 19%	*Clostridium sp.*
SRB[b]	7	Gram$^-$ vibrio: 7%	21	Gram$^-$ vibrio: 21%	0	-	Electron Donor Acceptor Lactate, Sulphate Formate Nitrate Fumarate Acetate

[a]*Isolated bacteria in presence of 0.22 µg/mL MeHg, which was considered the cutting edge for resistance.*
[b]*Sulphate-reducing bacteria (SRB).*
[c]*Identification based on BBL and Api test, as described in materials and methods section.*

II.3.2. Hg-R bacteria distribution and Hg contamination profile

Figures II.3–II.5 exhibit Hg contamination and Hg-R bacteria isolates distribution profile along the depth in different areas of collection. The distribution of Hg

contamination in Barreiro sediments (Figure II.3) showed that HgT was spread along the depth at mainly between 8 and 22 cm, with a peak (126 μg/g) at 13 cm (Figure II.3a) whereas MeHg concentrated in the first 20 cm with the peak (201 ng/g) also at 13 cm (Figure II.3b). In the case of North Channel, results demonstrated a contamination with MeHg also in the first 20 cm (peak at 7 cm [87 ng/g]), whereas HgT was spread along the depth maintaining a concentration of 15–18 μg/g between 9 and 48 cm (Figures II.4a and II.4b). Alcochete, the less contaminated station, displayed levels of Hg contamination two orders of magnitude lower (HgT peak [0.87 μg/g] at 15 cm) than values registered in Barreiro and North Channel, with MeHg contamination located more at the superficial layer of sediments (MeHg peak [4.1 ng/g] at 5 cm) (Figures II.5a and II.5b).

Table II.2: MIC values range exhibited by the isolates for mercurial compounds depending on the sampled area of Tagus Estuary.

Sampled areas	MIC values range (μg/mL)				[a]MIC_{50} values range (μg/mL)	
	Aerobic Bacteria		Anaerobic Bacteria		SRB	
	Hg^{2+}	MeHg	Hg^{2+}	MeHg	Hg^{2+}	MeHg
Barreiro	0.16 - 9.87	0.02 - 0.54	8.11 - 92.73	0.10 - 50.14	3.69 - 140.3	1.12 - 32.75
North Channel	1.13 - 10.01	0.11 - 1.12	0.65 - 4.06	0.07 - 1.75	36.93 - 81.25	5.19 - 15.18
Alcochete	0.41 – 1.13	0.04 - 0.19	0.87 - 46.36	0.05 - 5.01	-	

[a]*MIC_{50}: Minimal inhibitory concentration that inhibits 50% of bacterial growth.*

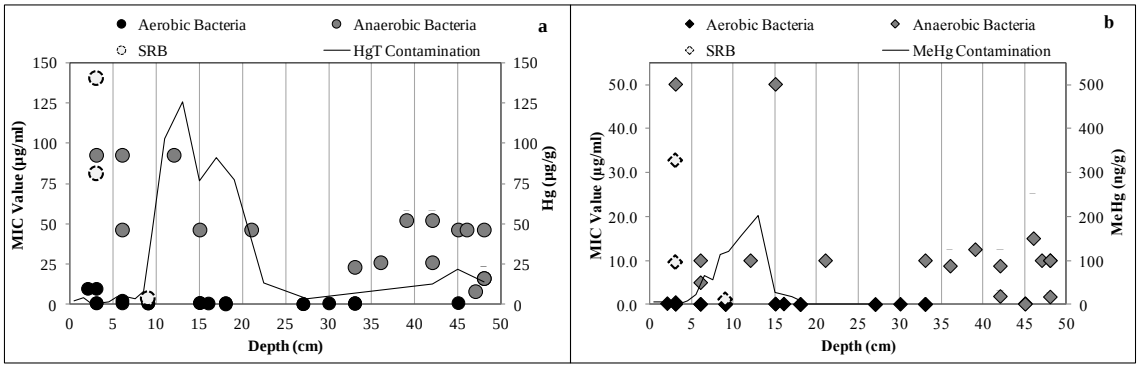

Figure II.3: Distribution of Hg^{2+} (a) and MeHg (b) MIC values for the bacteria isolated from sediments and the profile of HgT (a) and MeHg (b) contamination in Barreiro

station. Standard deviation (STD) values were calculated from three independent experiments.

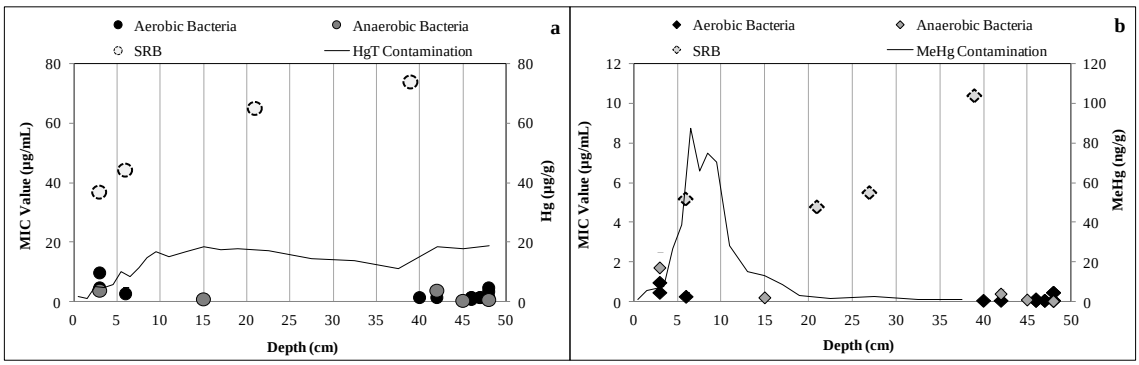

Figure II.4: Distribution of Hg^{2+} (a) and MeHg (b) MIC values against the bacteria isolated from sediments and the profile of HgT (a) and MeHg (b) contamination in North Channel station. Standard deviation (STD) values were calculated from two independent experiments.

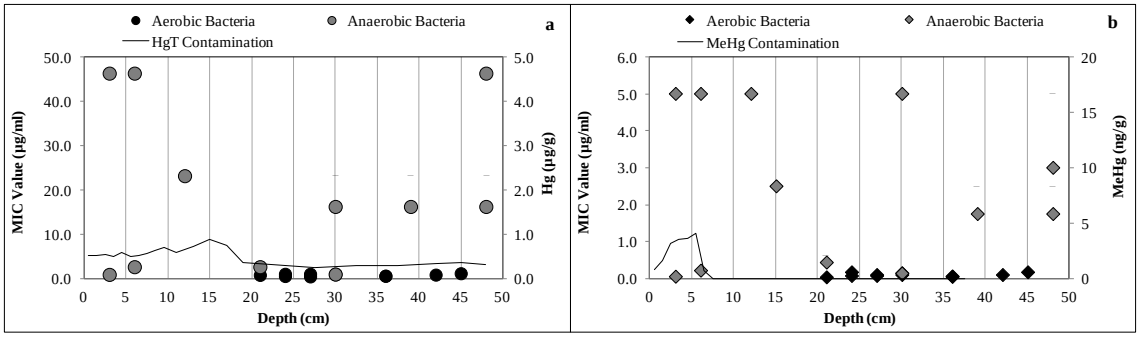

Figure II.5: Distribution of Hg^{2+} (a) and MeHg (b) MIC values against the bacteria isolated from sediments and the profile of HgT (a) and MeHg (b) contamination in Alcochete station. Standard deviation (STD) values were calculated from two independent experiments.

The distribution profile of the Hg-R isolated is in agreement with the contamination profile, as seen in Figures II.3–II.5. In Barreiro, the majority of Hg-R bacteria were isolated in the first 15 cm, indicating that resistant bacteria were concentrated on sediments surface close to contaminated layers (Figures II.3a and II.3b). In North

Channel, resistant bacteria distributed more evenly with a numerical increase in depth (40–48 cm) (Figure II.4). In Alcochete, Hg-R were located along the depth (Figure II.5) without a clear separation between aerobic and anaerobic bacteria and Hg-R SRB were absent. These differences in the distribution between the three sampled areas showed that bacterial communities are influenced differently by levels of contamination. As observed in Figures II.3–II.5, Hg-R bacteria are predominantly in sediments layers adjacent to high Hg content layers, and normally there are only anaerobic bacteria or SRB - highly Hg-R bacteria - adjacent to Hg, especially MeHg peak concentrations.

II.3.3. Mercury resistance levels

The resistance levels of the isolates to Hg were evaluated by determining MIC values of inorganic mercury ($HgCl_2$) and methylmercury (MeHgCl). Data are summarized in Table II.2. The isolates exhibited MIC values ranging from 0.16 to 140 μg/mL for Hg^{2+} and 0.02 to 50.1 μg/mL for MeHg (Table II.2). In general, the isolates were 10-fold more resistant to Hg^{2+} than to MeHg, and the magnitude of resistance was aerobic bacteria < anaerobic bacteria < SRB (Table II.2). The most resistant bacteria, including aerobic, anaerobic, and SRB, were isolated from sediments of Barreiro and North Channel, and the highest MIC values for Hg^{2+} and MeHg were observed on Barreiro isolates (140 and 32.8 μg/mL, respectively) (Table II.2).

II.4. DISCUSSION

The sampled areas of Tagus Estuary showed high Hg contamination, in particular MeHg. Sediments of Barreiro and North Channel were highly contaminated, presenting HgT average 33.2 μg/g (0.31–126 μg/g) and 11.7 μg/g (0.99–18 μg/g), respectively (Figures II.3 and II.4). These levels of contamination are in agreement with the tendency reported in 2003 and 2005 by Canário et al. (2003, 2005) and 20 years ago by Figuères et al. (1985). Barreiro contamination is comparable with that reported in highly contaminated ecosystems such as Fort Churchill (Carson River, Sierra Nevada), which presented Hg levels of 29.95 μg/g (Oremland et al., 1995).

Data indicated that Hg contamination was exerting metal tolerance/resistance within the bacterial community as evidenced by the ratio between total number of bacteria capable of tolerating 0.02 μg/mL MeHg and total number of bacteria (7/9 in Barreiro and 4/5 in North Channel and Alcochete) (Table II.3). Further, the number of bacteria resistant to 0.22 μg/mL represented 77% of total CFU in Barreiro, while in North Channel and Alcochete less than 5% of the total CFU were resistant to the same concentration of MeHg (Table II.3). In addition, a significant number of Hg-R bacteria was found in sediments of Barreiro (46% of total number of bacteria isolated) when compared to North Channel and Alcochete (26% and 28%, respectively) (Table II.1). Thus, colonization of sediments by resistant bacteria was influenced by the chronic high level of Hg contamination in Barreiro. The high levels of mercurial compounds also affected Hg-R bacteria prevalence in the neighborhood of Hg contamination peaks (Figures II.3–II.5). These results are in accordance with several studies that reported that the number of Hg-R bacteria in soil and aquatic environment depended upon Hg content of the environment (Barkay, 1987; Batten and Scow, 2003; Ruggiero et al., 2011; Sadhukhan et al., 1997; Summers, 1986; Timoney et al., 1978).

Table II.3: Mercury contamination in the three sampled areas of Tagus Estuary and analysis of total number of Hg-Tolerant bacteria and Hg-R bacteria.

Sampled areas	Total number of bacteria (CFU/cm[a])	Total no of Hg-tolerant and Hg-resistant bacteria (CFU/cm[a])		Mercury contamination range	
		0.02μg/mL MeHg[b]	0.22μg/mL MeHg[b]	HgT (μg/g)[c]	MeHg (ng/g)[c]
Barreiro	9.45×10^3	7.28×10^3	7.28×10^3	0.31-126	0.76-201
North Channel	5.08×10^4	4.19×10^4	9.80×10^2	0.99-18	1.0 - 87
Alcochete	1.69×10^4	1.40×10^4	7.70×10^2	0.24-0.87	0.0-4.10

Note: CFU, colony-forming units.
[a]*cm of sediment.*
[b]*Incorporated in MH agar.*
[c]*Mercury content was quantified until ± 40 cm of depth.*

The isolates included aerobic and anaerobic bacteria, and among anaerobic bacteria there were SRB (Table II.1). Biochemical identification indicated that most of the isolates belonged to *Bacillus, Aeromonas, Vibrio,* Enterobacteriaceae, and *Clostridium*

species, which are typical freshwater bacteria (Ivanova et al., 2001; Matyar et al., 2000). *Bacillus* spp. were the most common Hg-R bacteria found, which is not surprising, as *Bacillus* usually predominate in sediments, including contaminated ecosystems such as sediments of Minamata Bay (Nakamura and Silver, 1994), which led to these bacteria being considered important indicators for Hg resistance monitoring (Summers, 1986). Among Barreiro and North Channel anaerobic isolates detected, SRB are normally associated with Hg methylation. Some SRB are more likely to methylate Hg, especially SRB, which also use acetate as an electron donor (King et al., 2000). These SRB were detected, suggesting their probable involvement in Hg methylation processes. Other bacteria described as an Hg methylator are *Clostridium* sp. (Yamada and Tonomura, 1972), frequently found among the anaerobic isolates identified.

In general, in Barreiro and North Channel there was more diversity among the Hg-R, while in Alcochete the isolates were mostly gram-positive rods (Table II.1). This might be related to the fact that chronic exposure to high Hg concentrations selectively affects different bacterial communities and increases Hg-R bacteria diversity (Ruggiero et al., 2011; Vishnivetskaya et al., 2011). However, the influence of other factors, such as temperature, pH, organic matter content, and eutrophization (Macalady et al., 2000), as well as other contaminants including antibiotics (Lima-Bittencourt et al., 2007) and chemical compounds, such as heavy metals (Nascimento and Chartone-Souza, 2003), needs to be considered. For instance, high levels of As, Pb, Zn, Cr, Cu (Vale, 1990; Caçador et al., 1996; Vale et al., 2008), and cadmium (Cd) (Vale, 1990) were noted in sediments of Tagus Estuary, which may also affect bacterial diversity. In addition, Hg contamination influenced Hg-R bacterial distribution. Mercury contamination in Barreiro was high, between 10 and 20 cm (Figures II.3a and II.3b), with higher levels of Hg-R bacteria in this segment than in adjacent layers (0–8 and 25–45 cm). On the other hand, in North Channel contamination started close to the surface, with MeHg contamination increasing sharply at 3 cm and peaking at 7 cm, and therefore exerting a selective pressure for Hg-R bacteria that are scarce on the first sediment slices and more frequent with depth (40–45 cm) (Figures II.4a and II.4b). In Alcochete sediments, Hg-R bacteria were isolated along the depth; however, the most resistant bacteria were isolated on the surface, where Hg contamination was higher (Figures II.5a and II.5b). Thus, Hg contamination exerted a selective pressure on Hg-R bacteria in these areas,

affecting their distribution along the depth, which is in agreement with a study from Ruggieno and coworkers (2011) that concluded that Hg contamination altered microbial community.

This is also supported by the isolates susceptibility to Hg compounds. The most resistant Hg-R bacteria were isolated from Barreiro and North Channel; resistance levels reached 9.87 μg/mL and 10 μg/mL among aerobic bacteria and 140 μg/mL and 81.3 μg/mL among anaerobic bacteria (SRB) (Table II.2). The resistance levels correlated positively with contamination levels, with high contamination in North Channel and Barreiro exerting strong selective pressure for generation and persistence of Hg-R bacteria, while low contamination in Alcochete station did not favor selection. The comparison of bacteria isolated in Tagus Estuary and bacteria isolated in other polluted places indicate that in our study resistance was of the same magnitude or higher. For example, Hg^{2+} MIC for *Bacillus* spp. was found by others to range from 0.01 to 36.9 μg/mL (Oslon et al., 1979, Timoney et al., 1978, Pan Hou, 2010), and bacteria from Tagus showed values between 0.18 and 5.01 μg/mL. Austin and coworkers (1977) studied a mixture of bacteria isolated from estuary sediments and water and MIC of Hg^{2+} was 0.01 μg/mL, whereas our data demonstrated Hg^{2+} MIC averages on the order of 1.8 μg/mL for aerobic bacteria and 38.69 μg/mL for anaerobic bacteria including SRB.

Thus, the results presented in this study support the contention that chronic and high Hg contamination in some areas of Tagus Estuary (Barreiro and North Channel) exerts a selective effect on Hg-R bacteria, which may be involved in Hg cycling. The different chemical forms of Hg are not confined, and various processes affect the environmental cycling of Hg. Concentrations of Hg in water may be extremely low but microbial activity in aquatic ecosystems converts a small proportion of deposited Hg in inorganic form to MeHg, which bioaccumulates to relatively high concentrations in fish and especially in top predatory fish. Conversion of inorganic Hg into MeHg is responsible for neurotoxic, cardiotoxic, and teratogenic effects. The MeHg concentrations result from a balance between methylation and demethylation processes, which is not fully understood, but sediments are potential sources of MeHg for food webs. From a human health perspective, exposure to MeHg rather than total Hg is more important, since

MeHg is readily available for absorption into the bloodstream and crosses the blood–brain barrier, producing neurotoxicity.

Bacteria as a community has a rather complex role in Hg conversion, as bacteria such as SRB and *Clostridium* sp. were shown to methylate Hg (King et al., 2000; Eckstrom et al., 2003; Yamada and Tonomura, 1972) whereas *Bacillus* sp. has the ability to reduce the metal and volatilize it in the form of Hg^0 (Sadhukahan et al., 1997). Most studies focused on identifying bacteria with methylation capacities; although for remediation purposes and human health protection Hg-R bacteria perform reduction and volatilization, which is crucial. Therefore, isolates need to be individually tested to evaluate enzymatic conversion capacity and detoxifying processes.

II.5. CONCLUSIONS

This study is the first that identifies and characterizes Hg-R bacteria from Tagus Estuary and investigates their involvement in Hg cycling in this polluted ecosystem. Data showed that Hg-R bacteria exist and are frequent in Tagus Estuary sediments. Further, this study provided evidence that contamination by Hg compounds influenced significantly not only diversity of Hg-R bacteria but also their distribution, and resistance levels to mercurial compounds. Overall, data indicate involvement of these bacteria in Hg conversion (reduction/methylation/demethylation) and importance of this for aquatic ecosystems for the biosphere in general and human health risk in particular.

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CHAPTER III

MERCURY-RESISTANT BACTERIA FROM SALTMARSH OF TAGUS ESTUARY: THE INFLUENCE OF PLANTS PRESENCE AND MERCURY CONTAMINATION LEVELS†

†Figueiredo et al., 2014. J. Toxicol. Environ. Health A, 77:959-9

ABSTRACT CHAPTER III

Mercury (Hg) contamination of aquatic systems has been recognized as a global, serious problem affecting both wildlife and humans. High levels of Hg, in particular methylmercury (MeHg), were detected in surface sediments of Tagus Estuary. MeHg is neurotoxic and its concentration in aquatic systems is dependent upon the relative efficiency of reduction, methylation, and demethylation processes, which are mediated predominantly by the microbial community, in particular mercury-resistant (HgR) bacteria. Plants in contaminated ecosystems are known to take up Hg via plant roots. Therefore, the aims of this study were to (1) isolate and characterize HgR bacteria from a salt marsh of Tagus Estuary (Rosário) and (2) determine HgR bacteria levels in the rhizosphere and, consequently, their influence in metal cycling. To accomplish this objective, sediments samples were collected during the spring season in an area colonized by *Sacocornia fruticosa* and *Spartina maritima* and compared with sediments without plants. From these samples, 13 aerobic HgR bacteria were isolated and characterized morphologically, biochemically, and genetically, and susceptibility to Hg compounds, Hg^{2+}, and MeHg was assessed by determination of minimal inhibitory concentration (MIC). Genetically, the *mer* operon was searched by polymerase chain reaction (PCR) and 16S rRNA sequencing was used for bacterial identification. Results showed that the isolates were capable of growing in the presence of high Hg concentration with MIC values for $HgCl_2$ and MeHgCl in the ranges of 1.7–4.2 μg/mL and 0.1–0.9 μg/mL, respectively. The isolates from sediments colonized with *Sacocornia fruticosa* displayed higher resistance levels compared to ones colonized with *Spartina maritima*. Bacteria isolates showed different capacity of Hg accumulation but all displayed Hg volatilization capabilities (20–50%). *Mer* operon was found in two isolates, which genetically confirmed their capability to convert Hg compounds by reducing them to Hg^0. Thus, these results are the first evidence of the relevance of interaction between bacteria and plants in Hg cycling in Tagus Estuary.

III.1. INTRODUCTION

Salt marshes are recognized globally for being an important source of biological productivity and suitable habitat for fish and wildlife (Castro, et al., 2009). However, salt marshes also serve as ecosystems with a significant role in metal recycling, as these may act as sources, sinks, or fields for transformation of chemicals (Válega et al., 2008a). Being between land and sea, salt marshes receive large inputs of pollutants from urban and industrial sewage, as they are often situated in the vicinity of highly populated and industrialized areas (Reboreda and Caçador, 2007). Once present in salt marshes, metal contaminants are distributed among sediments, pore water, and plants, which are able to capture metals from the soil through their roots, accumulating and/or translocating them to the stems and leaves (Alberts et al., 1990; Caçador et al., 2000; Weis et al., 2002; Windham et al., 2003).

Mercury is a heavy metal and appears naturally as the result of degasification of the earth's crust and oceans (10,000 tons/yr). However, as a result of fossil fuel combustion (Morel et al., 1998), 20,000 tons/yr of metal from anthropogenic sources (Hansen and Dasher, 1997) is added to naturally released Hg. After the discovery of high levels of Hg, particularly methylmercury (MeHg), in Minamata Bay (Japan), which led to neurotoxic effects and the deaths of a large number of people (Robinson and Tuovinen, 1984), the presence of Hg in the environment has been the subject of considerable attention. Mercury in the environment exists in different oxidation states - elemental (Hg^0), mercurous ion (Hg_2^{2+}), and mercuric ion (Hg^{2+}), all of them involved in a continuous cycle of chemical transformations. In this biogeochemical cycle, Hg^0 released is transported through atmosphere and oxidized to Hg^{2+} (Morel et al., 1998). The Hg^{2+} is found predominantly in aquatic systems and appears bound to or associated with chlorides, sulfides, and organic acids, generating the inorganic mercury compounds (Morel et al., 1998), or establishes a covalent bond with the carbon atoms, generating organic forms, such as MeHg (Tchounwou et al., 2003). Inorganic mercury can also be reduced to Hg^0 (Morel et al., 1998). Once formed, organic forms accumulate in the food web or undergo the reverse process, demethylation.

In the mercury cycle, abiotic and biotic factors mediate Hg transformations (Morel et al., 1998). Among biotic factors, mercury-resistant bacteria (HgR) are predominantly responsible for the three critical transformations: reduction, methylation, and demethylation (Barkay and Wagner-Dobler, 2005). Reduction and demethylation by HgR bacteria are normally associated with a cluster of linked genes organized in an operon, the *mer* operon, which encodes proteins with functions related to regulation (*merR*), transport (such as *merT*, *merP*, and *merC*), and reduction of mercurial compounds (e.g., *merA* and *merB*). The presence of *mer* operon provides resistance either to inorganic or to organic mercurials (Narita et al., 2003). Plants have been also recognized as an important biotic factor in Hg cycle due to phytofiltration, phytoextraction, phytostabilization, and phytovolatilization potential (Raskin et al., 1997). Plants assimilate both inorganic and organic Hg through their root systems, where these may be accumulated and transported to the foliage and undergo volatilization (Leonard et al., 1998). Several studies reported the potential role of vegetation of salt marsh in the Hg cycle, promoting metal species conversion and availability through (1) changes of the redox state, (2) bioaccumulation into plant tissue underground or aerial organs, (3) mineralization of senescent plant material, and (4) enhancing microbial MeHg production in sediments associated with the root system (Caçador et al., 1996; Válega et al., 2008a, 2008b, 2008c, 2009). However, all these studies noted mostly the flux between plants and sediments without taking into account the influence of these fluxes on the microbial community associated with vegetation.

Thus, considering the importance of mercury-resistant (HgR) bacteria role in metal cycling (Barkay and Wagner-Dobler, 2005) and knowledge that salt marshes possess a high microbial activity (Válega et al., 2008b), it is essential to evaluate the interactions between naturally occurring HgR bacteria and plants. The aims of this study were to (1) isolate and characterize HgR bacteria from salt marsh of a highly Hg contaminated estuary (Canário et al., 2005), Tagus Estuary, and (2) evaluate the influence of rhizosphere in the HgR bacteria community.

III.2. MATERIALS AND METHODS

III.2.1. Study area and sample collection

Samples were collected from sediments of Rosário salt marsh, Tagus Estuary, Portugal, (38◦40'15.42"N; 9◦0'45.07" W), colonized by *Sarcocornia fruticosa* and *Spartina maritima* and from unvegetated sediments (UvS) in the same area, during the spring season (Figure III.1). Vegetated cores were approximately 10 m away and the distance from unvegetated core was approximately 30 m. From each vegetated area and unvegetated area two sediments cores (one for Hg analysis and the other for bacterial isolation) were collected over the rhizosphere area. Layers along the depth were sliced and stored in sealed tubes, refrigerated, and transported to the lab for bacteria isolation and metal analysis.

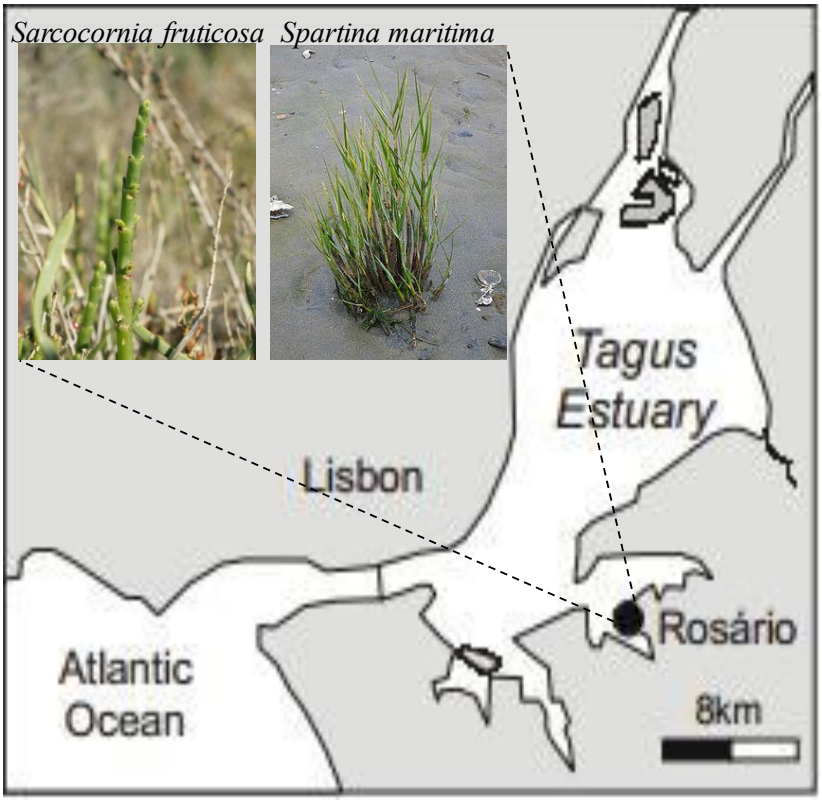


Figure III.1: Sampled area of Tagus Estuary (Rosário) colonized by *Sarcocornia fruticosa* (Hans Hillewaert, http://commons.wikimedia.org/wiki/File:Sarcocornia_fruticosa.jpg) and *Spartina maritima* (Miguel Sanz Alcántara, http://commons.wikimedia.org/wiki/File:Plant%C3%B3n_de_Spartina_maritima.jpg).

III.2.2. Analysis of Total mercury (HgT)

Mercury contamination level was evaluated by determination of HgT in sediments and pore water obtained through sediments centrifugation, and in plant root (dried at 40°C during 1 wk), along the depth of sediment core. Total Hg was measured by atomic absorption spectrometry using a silicon ultraviolet (UV) diode detector LECO AMA-254 as previously described (Figueiredo et al., 2014). Precision, expressed as relative standard deviation of 4 replicate samples, was less than 4%. Certified Reference Materials (PACS-2 and MESS-3) were used to ensure the accuracy of the procedure. PACS-2 (3.04 ± 0.20 mg/kg) and MESS-3 (0.09 ± 0.01 mg/kg) are marine sediments acquired from National Research Council Canada (NRCC). The obtained and certified

values were not statistically different (PACS-2 [3.02 ± 0.32 mg/kg] and MESS-3 [0.09 ± 0.01 mg/kg]).

III.2.3. HgR bacteria isolation and characterization

HgR bacteria were isolated from sediments of Tagus Estuary salt marsh colonized by *Sarcocornia fruticosa* (*S. fruticosa*) and *Spartina maritima* (*S. maritima*), two of the most common plants of south European salt marshes, which also possess the ability to phytostabilize contaminants in rhizosediment (Duarte et al., 2010). Inoculums for bacteria isolation were prepared by diluting sediments samples with 20 mL distilled sterile water following vigorous shaking. Supernatants at 100 μL, obtained following a centrifugation at 2,650 × g for 1 min (4°C), were plated on Mueller–Hinton (MH) agar media without and with MeHg (0.22 μg/mL) and incubated in aerobic growth conditions at room temperature (26°C). MeHg selective pressure was used to isolate HgR bacteria. After 3 d of incubation, different colonies were picked on selective medium (0.22 μg/mL MeHg) and stored at -80°C.

Isolated cells in early growth stages were evaluated following standard methodologies of microbiology, including morphological examination, gram staining characterization, lactose fermentation, and detection of catalase and oxidase enzymatic activities. Commercial micro methods for biochemical identification (BBL Crystal Identification Systems, BD), comprising tests for fermentation, oxidation, degradation, and hydrolysis of various substrates, were used for preliminary identification of isolates.

III.2.4. Mercury susceptibility testing

Isolates' susceptibility to Hg compounds was ascertained by minimal inhibitory concentration (MIC) determination using a modified micro dilution broth method described by CLSI (2006). Microorganism cultures in brain heart infusion broth (BHI) at a concentration of 10^8 colony-forming units (CFU)/ml (OD 0.5 at 595 nm) were diluted in MH in order to obtain 3×10^6 CFU/mL. Mercury concentrations were incubated with 100 μL bacterial suspension in order to obtain concentrations ranging

from 1.33×10^{-3} to 1.36 μg/mL $HgCl_2$ and from 1.23×10^{-5} to 126 μg/mL MeHgCl. Bacterial suspension in the absence of Hg compounds was used as a control. Duplicate samples were performed for each concentration tested. After incubation at 37°C for 24 h under aerobic conditions, bacterial growth or absence was determined. The MIC was defined as the minimum concentration of test compound that inhibited visible growth. All data points represent mean ± standard deviation (STD) of at least two independent determinations.

III.2.5. Quantification of mercury reduction potential

Mercury reduction potential was analyzed following two parameters: Hg^0 volatilization observation and total mercury (HgT) concentration measurement after bacterial action. The volatilization of Hg^0 was assayed by a modification of a protocol described by François et al. (2011) and Sadhukhan et al. (1997). Bacterial strains were cultivated on BHI liquid broth containing 0.5 μg/mL $HgCl_2$. After overnight incubation, cells were adjusted to 10^6 CFU/ml in BHI broth and placed into a 12-well microplate. $HgCl_2$ solution was added to a final concentration of 0.5 μg/mL. BHI plus $HgCl_2$ at 0.5 μg/mL was used as negative control and one *mer* positive strain was used as positive control (*Citrobacter* sp.). The microplate was covered with a sensitive silver-containing film and incubated for 48 h at 37°C in the dark. The observation of foggy or whitish areas on the film as a result of the reduction of silver of the film by the Hg vapor was interpreted as a positive result for Hg^0 volatilization. Subsequently, cells were harvested by centrifugation at 15,300 × g for 5 min, and supernatant after each cell harvesting was taken separately. Harvested cells were washed with sterile deionized water and weighed. Quantification of Hg reduction was performed through measurement of HgT in the supernatant and in the pellet by atomic absorption spectrometry using a silicon UV diode detector LECO AMA-254. Two independent experiments were performed.

III.2.6. Genetic characterization

Bacterial DNA was extracted directly from boiled bacterial suspension after 10 min at 95°C through centrifugation at 13,000 × g for 4 min and supernatant was stored at -20°C to be subsequently used for PCR reaction. *Mer* gene amplification was performed

using primers P1-P4, whereas the 16S rRNA gene amplification was fulfilled with P5 universal primer (Table III.1). Polymerase chain reaction (PCR) reactions were carried out in 25 μL volume containing 12.5 μL of PCR master mix (50 mM Tris-HCl, pH 9, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.2 U/μL of NZYTaq DNA polymerase), 8.5 μL of nuclease-free water, 0.1-1 μM of primers, and 0.05-0.5 μg of template DNA. PCR mixtures were amplified by initial holding at 98°C for 30 s and then 20–35 cycles of denaturing at 98°C for 10 s, annealing at 59-64°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The sizes of amplicons were confirmed by gel electrophoresis. PCR products were purified using the NZYGelpure kit (NZYTech), following the protocol instructions. Sequencing was performed by STAB-Vida (Lisbon, Portugal), using the same primers used for amplification. All sequences were subjected to a BLAST search (http:// www.ncbi.nlm.nih.gov/BLAST) for comparison with published sequences. Multiple alignments with known 16S rRNA gene sequence from GenBank (http://www.ncbi.nlm.nih.gov) were performed by CLUSTAL W2 algorithm.

Table III.1: List of primers used for genetic characterization based on *mer* operon search (P1–P4) and 16S rRNA sequencing for bacteria identification (P5).

Number	Forward	Reverse	Amplified region
P1	GCGGATTTGCCTCCACGTTGA	CCAGGCAGCAGGTCGATGCAAG	*merR-merT* (225 pb)
P2	ACGGATGGTCTCCACATTG	CGAGGCAGCAAGCCGAGGCG	*merR-merT* (225 pb)
P3	GGCTATCCGTCCAGCGTCAA	GTCGCAGGTCATGCCGGTGATTTT	*merP-merA* (134-500 pb)
P4	GGCATGACTTGCGACTCGT	GCGTAGATGTTCGGGTGC	*merA* (1178 pb)
P5	CCTACGGGAGGCAGCAGT	CGTTTACGGCGTGGACTAC	16S rRNA (500 pb)

III.3. RESULTS

III.3.1. Mercury contamination levels in Rosário salt marsh

Sediments samples collected in salt marsh of Tagus Estuary colonized by *S. fruticosa* and *S. maritima* and UvS were all contaminated with Hg, and the level of contamination along the core depth is shown in Figure III.2. Mercury contamination levels in sediments among the three sampled areas were: UvS HgT mean 0.94 mg⁄kg (0.04-1.41 mg⁄kg) < *S. fruticosa* HgT mean 1.85 mg⁄kg (0.15–3.18 mg⁄kg) < *S. maritima* HgT mean 2.16 mg⁄kg (0.16-4.46 mg⁄kg) (Figure III.2). The distribution of Hg contamination depicts that sediments colonized by plants (*S. fruticosa* and *S. maritima*) are more contaminated in intermediate layers (5-15 cm and 9-19 cm, respectively) (Figure III.2). In UvS, the distribution of Hg contamination shows a constant level (approximately 1.2 mg⁄kg) until 17 cm and then declines (Figure III.2). HgT mean values in pore water were UvS 2.43 ng/L (0.9–6.8 ng/L) < *S. maritima* 2.82 ng/L (0.9–4.6 ng⁄L) < *S. fruticosa* 2.96 ng/L (0.4–6.1 ng/L), being the highest value registered in UvS (Figure III.2). Both plants (*S. fruticosa* and *S. maritima*) accumulated high amounts of Hg in their roots, and despite the accumulation peak displayed by *S. maritima* (19.5 mg⁄kg at 9 cm) taking the overall accumulation in roots, *S. fruticosa* accumulated more than S. *maritima* (HgT mean 5.59 mg⁄kg (1.2–10.3 mg⁄kg) and 4.46 mg⁄kg (0.2–19.5 mg⁄kg), respectively).

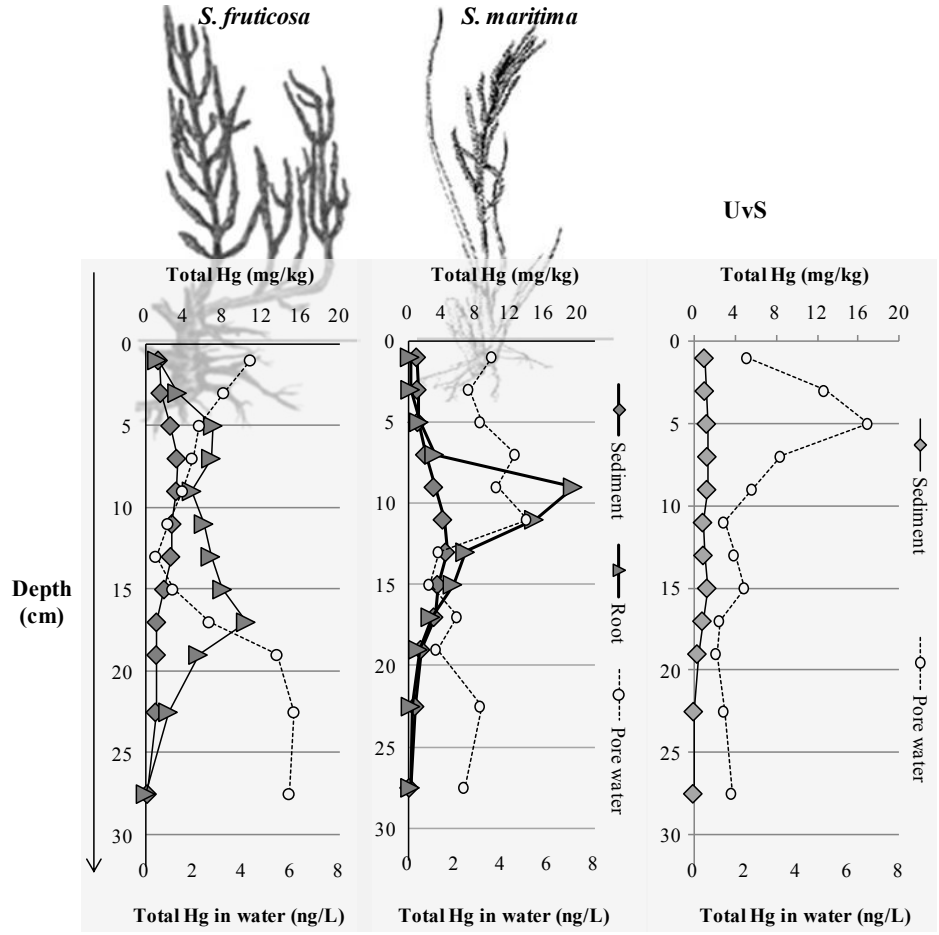


Figure III.2: Schematic representation of the plants studied and sediments position containing the plots of HgT contamination levels in sediments, pore water and plant roots of areas colonized by *S. fruticosa* and *S. maritima* and an area of unvegetated sediment (UvS). The scheme combines results of environmental conditions to which bacteria are subject with illustrative figures of plants (adapted from Duarte, 2010) and does not intend to represent real rizhosphere conditions.

III.3.2. HgR bacteria isolates from salt marsh and their characterization

In total, 13 HgR isolates were isolated from salt marsh of Tagus Estuary: 5 from sediments colonized by *S. fruticosa*, 2 from sediments colonized by *S. maritima*, and 6 from UvS. The isolates were all gram-positive bacilli, oxidase negative and lactose nonfermentative. All isolates with exception of two were catalase positive. The isolates

were *Bacillus* sp., with the exception of one that was *Micrococcus* sp. (Table III.2). The biochemical identification was confirmed with 16S rRNA sequencing as indicated in Table III.2.

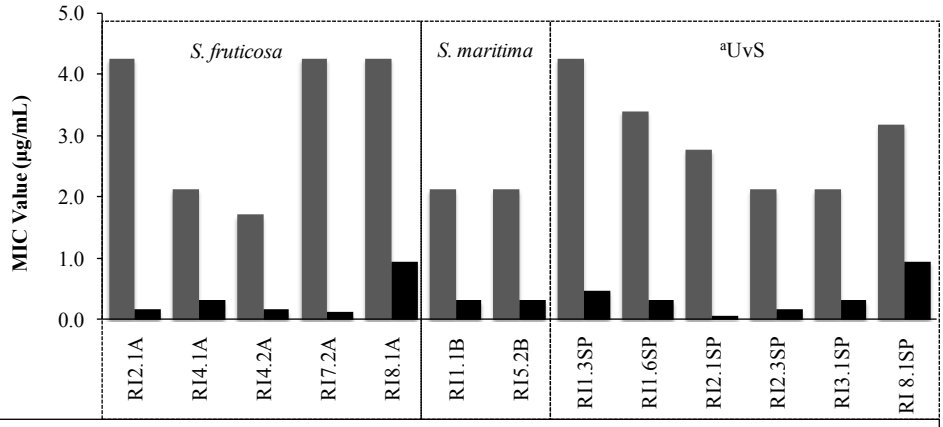
Table III.2: HgR bacteria isolates from salt marsh of Tagus Estuary: morphological, biochemical, and genetic characterization.

Strain isolates[b]		Gram	Morphology	Catalase	Oxidase	Lactose fermentation	Identification	
							Biochemical test (≥90%)*	16S rRNA) (≥99%)**
S. fruticosa	RI2.1A	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	*Bacillus* sp.
	RI4.1A	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	-
	RI4.2A	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	*Bacillus megaterium*
	RI7.2A	Gram$^+$	Bacilli	-	-	No	*Bacillus* sp.	-
	RI8.1A	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	
S. maritima	RI1.1B	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	*Bacillus megaterium*
	RI5.2B	Gram$^+$	Bacilli	+	-	No	*Micrococcus* sp.	-
UvS[a]	RI1.3SP	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	-
	RI1.6SP	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	-
	RI2.1SP	Gram$^+$	Bacilli	-	-	No	*Bacillus* sp.	-
	RI2.3SP	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	*Bacillus megaterium*
	RI3.1SP	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	-
	RI8.1SP	Gram$^+$	Bacilli	+	-	No	*Bacillus* sp.	-

*Note. *Percentage of identity found for genera, using BBL cristal test. **Percentage of identity found for species, by 16S rRNA sequences subjected to a BLAST search.*
[a]Strains isolated from Mueller–Hinton (MH) agar media plus MeHg selective pressure (0.22 μg/mL) in aerobic condition at room temperature (26◦C). [b]UvS, unvegetated sediment.

III.3.3. Bacterial mercury resistance levels

The resistance levels to Hg compounds of isolates were evaluated through MIC determination, using inorganic mercury ($HgCl_2$) and MeHg. The isolates exhibited MIC values ranging from 1.7 to 4.2 μg/mL for $HgCl_2$ and from 0.1 to 0.9 μg/mL for MeHg (Figure III.2). All isolates were 3-20-fold more resistant to $HgCl_2$ than to MeHg (Figure III.3). The most resistant bacteria were isolated from sediments colonized by *S. fruticosa* and UvS (Figure III.3).



MIC Range (µg/mL)			
$HgCl_2$	1.7 – 4.2	2.1	2.1 – 4.2
MeHgCl	0.1 – 0.9	0.3	0.1 – 0.9

Figure III.3: MIC values exhibited by bacteria isolates for mercury compounds (inorganic form, $HgCl_2$, and organic form, MeHgCl). Bacteria were isolated from plant-growing and unvegetated sediments (UvS) in the salt marsh area of Rosário, Tagus Estuary, Portugal.

III.3.4. Mercury reduction potential of HgR bacteria

All 13 isolates were capable of volatilizing Hg as indicated by whitish areas on the x-ray film covering the cultured microplate due to reduction of Ag^+ by vapor mercury (Hg^0) (data not shown). Figure III.4 shows the distribution of Hg in bacterial cells and supernatant after 48 h of growth. All isolates removed between 20 and 50% metal from medium with remaining Hg distributed between cell pellet and supernatant (Figure III.4). Comparing the three sediment sampled areas, the removal percentages were: UvS (27–47%) < *S. maritima* (22–48%) < *S. fruticosa* (32–50%) (Figure III.4).

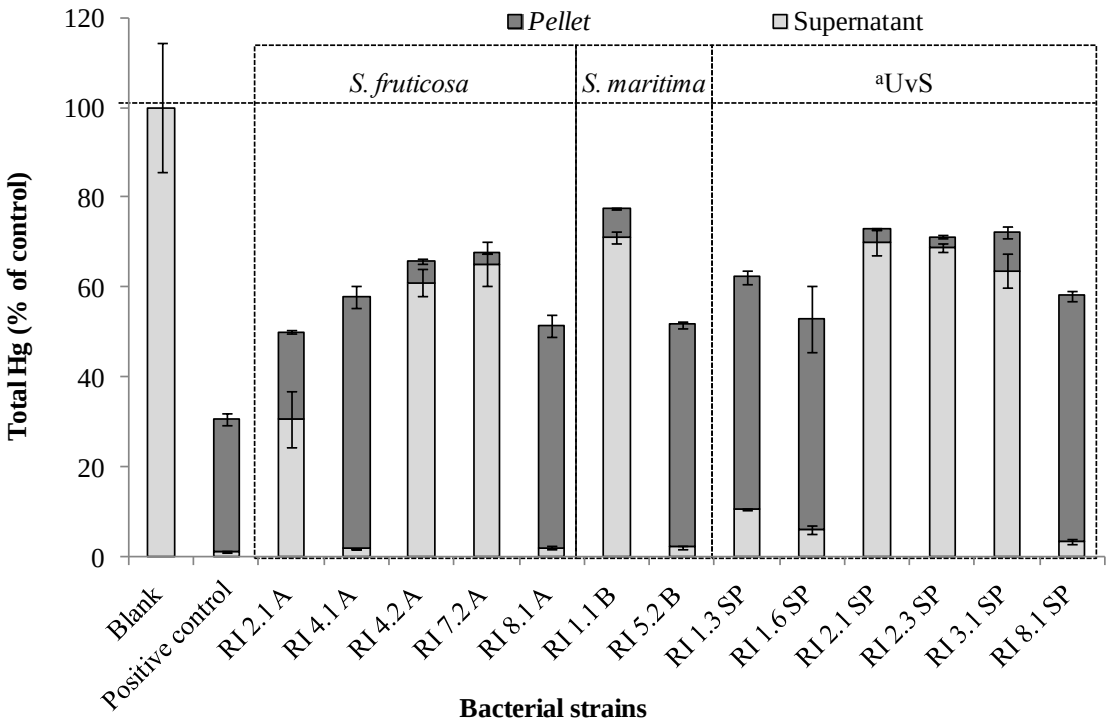


Figure III.4: Distribution of total mercury among bacterial cells and supernatant after 48 h of bacterial growth in BHI media containing an initial concentration of 0.47 μg/mL $HgCl_2$. Media plus $HgCl_2$ was used as blank (negative control) and a bacterial strain containing *mer* operon was used as positive control. Percentages were calculated using as a control the concentration of the blank (negative control). UvS, unvegetated sediment.

III.3.5. Genetic characterization for mercury resistance

PCR amplifications were positive for four isolates: two from sediment colonized by *S. fruticosa*, one from sediment colonized by *S. maritima*, and one from UvS (Figure III.5). The amplifications revealed the presence of *mer* genes in the two isolates of sediment colonized by *S. fruticosa* (strain RI2.1A and strain RI4.2A) (Figure III.5). The *mer* genes found were *merT*, *merR*, and *merP*, which codified proteins responsible for *mer* regulation (merR) and Hg transport into and out of cells (merT and merP) (Figure III.5). Although the strain RI2.3SP yielded a positive result for PCR amplification, no homology with *mer* gene was found for its amplicon sequence, being a cold-shock

protein (Sequence ID: ref|NC_014103.1|). Strain RI1.1B also yielded a positive result for PCR amplification with primer P2, but it was not possible to sequence its amplicon.

***Mer* operon composition (Proteins expressed)**					
Regulation	Transport			Transformation (enzymes)	
merR	merT	merP	merC	merA	merB
				Hg^{2+} Reduction	MeHg Demethylation

***Mer* operon search results**		
PCR Positive	Primer	**Sequence homology (Blast)**
I - RI 2.1A	P1	*mer*T (100%)[a]
II - RI 4.2A	P1 P3	*mer*R (87%)[a] *mer*P (90%)[a]
III - RI 1.1B	P2	-
IV - RI 2.3SP	P1	no homology with *mer*

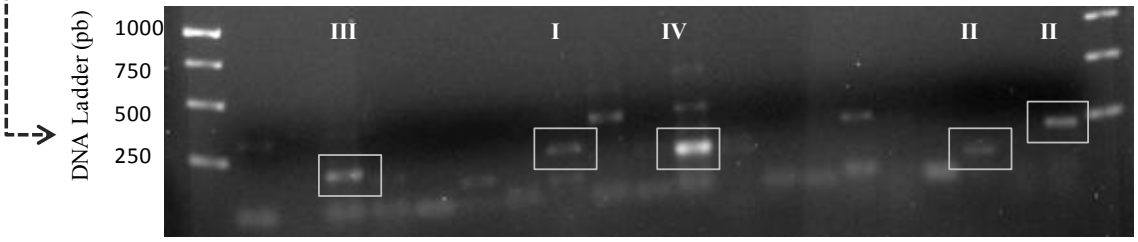


Figure III.5: *Mer* operon composition: proteins expressed, including enzymes responsible for mercury transformation (merA and merB). These are results for *mer* operon search among the isolates, showing PCR amplification obtained for four isolates (gel electrophoresis) and result for homology search in http://www.ncbi.nlm.nih.gov/BLAST. [a]Percentage of identity found.

III.4. DISCUSSION

III.4.1. Mercury contamination levels in Rosário salt marsh

In the present study, HgR bacteria were isolated from a moderately Hg-contaminated salt marsh of Tagus Estuary (Rosário, Portugal). The Hg content in sediments of these areas ranged from 0.04 mg/kg to 4.46 mg/kg. Compared with a normal value for

unpolluted soil and water (10–20 ng/g and 0.1–1.2 ng/L Hg, respectively) (Sadhukahan, et al., 1997), the values found in these salt marsh are considered high. However, comparing the two hotspots of Hg contamination in Tagus Estuary, Barreiro and North Channel, reaching metal contamination levels of 126 and 18 mg/kg, respectively (Figueiredo et al., 2014), this salt marsh is far below that contaminated by Hg. Thus, the number of HgR bacteria isolated is also low compared with the number of isolates obtained from two highly contaminated areas (Figueiredo et al., 2014).

III.4.2. HgR bacteria isolates from salt marsh and their characterization

The isolates were mostly *Bacillus* sp., in particularly *Bacillus megaterium* (Table III.2). *Bacillus* sp. is a common genus found in other Portuguese salt marsh (Ria de Aveiro), including the species *B. megaterium* (Osório, 2009) that is also considered an important indicator for Hg resistance monitoring (Summers, 1986). A similar number of different HgR bacteria isolated were found in sediment colonized by *S. fruticosa* and UvS (five and six, respectively), while in sediment colonized with *S. maritima*, only two different HgR were detected. In Rosário, Santos et al. (2006) assessed the influence of salt marsh in bacteria abundance and concluded that the existence or absence of plants did not affect bacterial community composition or its abundance, although contaminants level were not examined.

III.4.3. Bacterial mercury resistance levels

These isolates resistance levels to Hg compounds ranged from 1.7 to 4.2 μg/mL for $HgCl_2$ and from 0.1 to 0.9 μg/mL for MeHgCl (Figure III.3). Compared with bacteria considered highly resistant to mercury (25 μg/mL $HgCl_2$) (De et al., 2008), these isolates were far less highly resistant; however, compared with the MIC range found for *Bacillus* sp. isolated from sediment of the New York Bight (in the United States) (2–50 μg/mL $HgCl_2$), these isolates are within the range of Hgresistant bacteria (Timoney et al., 1978). This level of resistance reflected in bacterial potential to transform Hg, particularly reduction (see further discussion).

III.4.4. Relationship between mercury content and HgR bacteria characteristics

Mercury content in water and sediments were higher in areas colonized by plants than in UvS: *S. fruticosa* > *S. maritima* > UvS. This is in agreement with previous observations of Canário et al. (2010) for Alcochete salt marsh, also in Tagus Estuary, suggesting that Hg is taken up by roots (Canário et al., 2007). Mercury distribution was quite similar in the different compartments, and the pattern was roots > sediments > pore water, independent of the plant species. In addition, it was confirmed that *S. fruticosa* in total accumulated more Hg in roots than *S. maritima*, which is in agreement with Canário and coworkers (2010), which estimated Hg levels in the rooting zone to be 9.3 g/m^2 for *S. fruticosa* and 2.2 g/m^2 for *S. maritima*. This might be related to the slow flux of metal between sediments and roots in *S. maritima* and also to a lower specific area of *S. maritima* roots occurring at this period of the year (April); *S. fruticosa* has approximately double the root biomass of *S. maritima* (Caçador et al., 2009).

The kinetics of Hg mobility between plant and roots may be the main factor influencing HgR selection, once the presence of dense root in salt marsh sediment is responsible for creation of a dynamic subsurface that modulates the biogeochemical cycles (Oliveira, 2008). Thus, high root bioaccumulation of Hg noted for *S. fruticosa* may result in a return of large amounts of metal to sediment matrix due to necromass generation and mineralization processes (Duarte et al., 2010). All of these aspects allow us to conclude that *S. fruticosa* has a higher capacity of accumulation and generates more rapid Hg fluxes that act as selective pressure for HgR bacteria living in their vicinity. Consequently, those bacteria present higher MIC values when compared to bacteria from *S. maritima* sediments.

Several studies reported that the number and diversity of HgR bacteria in soil and aquatic environment varied according to its Hg content (Summers, 1986; Sadhukhan et al., 1997; Rugiero et al., 2011; Vishnivetskaya et al., 2011). In our previous study in Hg contaminated areas of Tagus Estuary, a strong relationship was also found between Hg contamination levels and HgR bacteria resistance levels to Hg compounds. However, no clear relation was noted in salt marsh between metal content and number and diversity of HgR bacteria. This suggests that in salt marsh, there are environmental factors such

as salinity that decrease bacteria diversity in vegetative forms and increase the sporulated forms, namely, for *Bacillus* spp., which may be the determinant for bacterial community composition.

III.4.5. Mercury reduction potential of HgR bacteria

Bacteria from the rhizosphere were shown to be involved in Hg remediation by promoting accumulation in plant tissues (de Souza et al., 1999); however, microbial activity in salt marsh sediments might also participate in Hg methylation, once salt marshes reside under ideal conditions such as availability of organic carbon and sulphate (Válega et al., 2008b). Our data show that HgR bacteria from Rosário salt marsh have the ability to reduce Hg^{2+} to Hg^{0}, removing metal from aquatic environment through volatilization of Hg^{0} (Figure III.4). This is the first evidence revealing that microbial communities of Tagus Estuary salt marsh are involved in Hg transformation, particularly reduction.

HgR bacteria cells were reported to volatilize metal from Hg-containing liquid media and also to bind Hg with cell constituents (Nakamura et al., 1986; Sadhukhan et al., 1997; Zhang et al 2011). Data indicated that all HgR isolates were able to remove Hg from liquid media and volatilize it (Figure III.4), but efficiency was different. Analyses of cell metal content revealed that there is an uptake of Hg into cell and/or cell-bound Hg, but it seems that the isolates that presented high levels of metal in cell fraction (strains 2.1A, strain 5.2B from *S. maritima*, and strains 1.3SP, 1.6SP, and 8.1SP from UvS) were slightly more efficient in Hg removal from media. These differences might be due to different efficiency of Hg detoxifying systems in different HgR bacteria (Sadhukhan et al., 1997). Thus, to better understand the cellular mechanisms responsible for this conversion, *mer* operon genes were searched.

III.4.6. Genetic characterization for mercury resistance

Most HgR gram-positive and gram-negative bacteria possess *mer* operons on transposons, plasmids, or bacterial chromosomes as their Hg-resistant determinants (Nascimento and Chartone-Souza 2003). *Mer* operon mechanisms of resistance to Hg^{2+}

involve uptake of Hg^{2+} into bacterial cytoplasm by MerT and MerP and then reduction of Hg^{2+} to Hg^0 by MerA (mercuric reductase enzyme). The Hg^0 formed then diffuses out of the bacteria cell through the cell membrane without the need of a specialized transport system (Barkay and Wagner-Dobler, 2005).

Although PCR amplification of *mer* genes was positive for four isolates, two isolates from *S. fruticosa* sediments (strain 2.1A and 4.2A), one from *S. maritima* sediments, and one from UvS; analysis of these amplicons sequence gave a positive match for *merT*, *merR*, and *merP* only for the two isolates from *S. fruticosa*. In general, *merR*, *merT*, *merP*, and *merA* (mercury reductase gene) are commonly conserved as core *mer* operon genes, and additional genes responsible for regulation (*merD*), transport (*merC*, *merE*, and *merF*), and transformation (*merB*, organomercury lyase gene; and *merG*, phenylmercury resistance gene) can be inserted (Narita et al., 2003). Thus, the presence of *merT*, *merR*, and *merP* genes in the two isolates from *S. fruticosa* sediment may account for Hg resistance as well as reduction potential. However, all of the isolates behave in the same way in media containing Hg^{2+} by undergoing volatilization, thus leading to the possibility that the Hg^{2+} removed was transformed into Hg^0 by the *mer* operon proteins and then Hg^0 diffused out of the culture media. The lack of genetic evidence confirming this may be due to the existence of other detoxifying systems or to genes' structural arrangement or even genetic variability (Nascimento and Chartone-Souza, 2003), which may lead to a nonspecificity of the primers used.

III.5. CONCLUSIONS

Overall, this study highlights that the HgR bacteria present in salt marsh of Tagus Estuary may play a vital role in detoxifying Hg as well as in modulating metal release from these environments. Further studies are needed to detail the exact balance between processes of methylation/detoxification of Hg in salt marsh, in order to find bioremediation applications for the isolates identified.

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CHAPTER IV

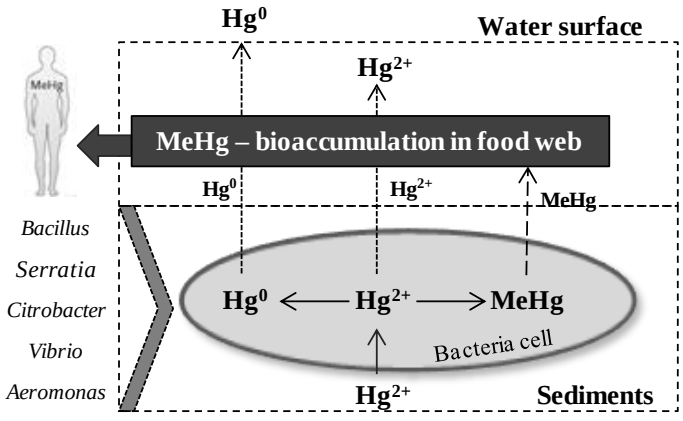
AEROBIC MERCURY-RESISTANT BACTERIA ALTER MERCURY SPECIATION AND RETENTION IN THE TAGUS ESTUARY (PORTUGAL)†

†Figueiredo et al., 2016. Ecotox. Environ. Safe., 124:60-67.

ABSTRACT CHAPTER IV

Aerobic mercury-resistant bacteria were isolated from the sediments of two highly mercury-polluted areas of the Tagus Estuary (Barreiro and Cala do Norte) and one natural reserve area (Alcochete) in order to test their capacity to transform mercury. Bacterial species were identified using 16S rRNA amplification and sequencing techniques and the results indicate the prevalence of *Bacillus* sp. Resistance patterns to mercurial compounds were established by the determination of minimal inhibitory concentrations. Representative Hg-resistant bacteria were further tested for transformation pathways (reduction, volatilization and methylation) in cultures containing mercury chloride. Bacterial Hg-methylation was carried out by *Vibrio fluvialis*, *Bacillus megaterium* and *Serratia marcescens* that transformed 2-8 % of total mercury into methylmercury in 48 h. In addition, most of the HgR bacterial isolates showed Hg^{2+}-reduction and Hg^{0}-volatilization resulting 6-50% mercury loss from the culture media.

In summary, the results obtained under controlled laboratory conditions indicate that aerobic Hg-resistant bacteria from the Tagus Estuary significantly affect both the methylation and reduction of mercury and may have a dual face by providing a pathway for pollution dispersion while forming methylmercury, which is highly toxic for living organisms.



Graphical abstract: Schematic representation of mercury cycle in a polluted environment (adapted from Clarkson et al., 2003) highlighting the impact of the transformations performed by the isolated bacteria of Tagus Estuary in this cycle.

IV.1. INTRODUCTION

Mercury is distributed throughout the environment by both natural processes, such as degasification of the earth's crust and volcanic activity (Hansen and Dasher, 1997), and anthropogenic processes including fossil fuel combustion and mining (Morel et al., 1998; Selin, 2009; Strode et al., 2009). The primary forms of mercury are: (1) elemental mercury (Hg^0); (2) divalent mercury (Hg^{2+}) associated with various ligands; and (3) organic alkyl compounds of mercury (e.g. methylmercury CH_3Hg^+). As these forms move through environmental compartments, they undergo a continuous cycle of chemical conversions (O'Driscoll et al., 2005).

Methylmercury exposure is a particular concern as it undergoes bioaccumulation and biomagnification in aquatic food webs (Chadhain et al., 2006; Corrales et al., 2011) and is neurotoxic (NRC, 2000).

In contaminated environments microbial transformations are one of the main mechanisms determining mercury speciation (Barkay et al., 2003). Research has shown that prolonged exposure to mercury compounds in polluted aquatic environments can result in bacterial populations with resistance/tolerance mechanisms (Nithya et al., 2011). Here, the resistance mechanisms refers to the genetically-encoded detoxification mechanisms as a response to mercurial compounds, while the tolerance mechanisms refer to the detoxification mechanism mediated by normal cell metabolism and not specifically induced, in accordance to Baldi (1997) and Glendinning et al., (2005). Thus, resistance mechanisms include enzymatic reduction and biomethylation (Barkay et al., 2003) while tolerance mechanisms include binding of mercury to cell wall constituents (Glendinning et al., 2005; Sadhukhan et al., 1997) and precipitation of insoluble inorganic complexes - HgS or Hg-sulphur complex – (Essa et al., 2002). The genetic machinery associated with resistance is usually located in transposons inserted in chromosomal DNA or in plasmids (Mindlin et al., 2001), and confers resistance to Hg^{2+}/organomercurials. Additionally, it is common to find the association of mercury resistance phenotypes with multiple antibiotics resistance (Allen et al., 1977; Sadhukhan et al., 1997; Zhang et al., 2011) located on the same mobile genetic elements (Wireman et al., 1997). Among the antibiotics, resistance to ampicillin,

chloramphenicol, tetracycline and kanamycin have been widely found in mercury-resistant bacteria (Allen et al., 1977, Sadhukhan et al., 1997; Zhang et al., 2011).

Mercury-resistant bacteria (HgR bacteria) are the main organisms responsible for three critical transformations in mercury cycle: 1) reduction of Hg^{2+}; 2) methylation of Hg^{2+} and 3) demethylation of MeHg (Barkay and Wagner-Dobler, 2005; Chadhain et al., 2006). Reduction and demethylation are normally associated with a cluster of genes organized in the *mer* operon, which can be classified into two types: 1) narrow spectrum, conferring resistance only to inorganic compounds, and 2) broad spectrum, providing resistance to inorganic and organic compounds (Nascimento and Chartone-Souza, 2003). The narrow spectrum involves the mercuric reductase enzyme to reduce Hg^{2+} to Hg^0 whereas in the broad spectrum there is the additional participation of the organomercurial lyase enzyme to break the carbon-mercury (Hg-C) bond, thus releasing Hg^{2+} and CH_4 (Nascimento and Chartone-Souza, 2003; Parks et al., 2009; Schaefer et al., 2004). The methylation, i.e. the transfer of a methyl group to the Hg^{2+} ion, resulting in the formation of MeHg, has primarily been associated with sulphate-reducing bacteria (SRB) (King et al., 2002), iron-reducing bacteria (Kerin et al., 2006), and methanogens (Hamelin et al., 2011) present in anoxic aquatic environments. Recently the identification of two genes (*hgcA* and *hgcB*) in methylating bacteria provided a genetic basis for mercury methylation (Parks et al., 2013).

Estuaries are transitional zones between land, freshwater habitats and sea, which included both vegetated (mangroves, salt marshes, and seagrass beds) and unvegetated habitats (mudflats and sand beaches) (Levin et al., 2001). For this reason, they are dynamic areas for organic matter cycling, which may enhance microbial activity (Levin et al., 2001). The Tagus Estuary covers an area of 325 km^2 and provides wetland habitat for wintering migratory birds in Western Europe. It is a Nature Reserve and a Special Protection Area under the European Birds Directive (Rosa et al., 2008). This estuary is also an important nursery area for commercial fish species, such as flatfish (Cabral et al., 2007). Despite its ecological importance, contamination by heavy metals such as Hg; Zn; Cd; Cr; Cu and Pb is well documented (Vasconcelos et al., 2007). For instance, mercury concentrations found in sediments - 126 µg/g (Figueiredo et al., 2014a) and 66.7µg/g (Canário et al., 2007) - and plants roots - 19.5 mg/kg (Figueiredo et al., 2014b) have been related to long-term industrial activity. However, to predict mercury

movement and speciation we need a better understanding of microorganisms including *Archeae* that have been shown to be capable of methylating mercury.

In previous work we described the characterization of 46 aerobic HgR bacteria from sediments of Tagus Estuary (Figueiredo et al., 2014a), and found mercury resistance in several bacterial genera colonizing these sediments. In this work we selected representative aerobic HgR bacteria in controlled experiments to measure the transformation of mercury species in oxic conditions through biotic reduction and methylation mechanisms. Overall, the objective of the study is to investigate if the bacteria present in Tagus sediments alter mercury speciation and if so, their contribution for mercury cycle.

IV.2. MATERIAL AND METHODS

IV.2.1. HgR bacteria isolation

Aerobic HgR bacteria were isolated from the mudflat sediments within three areas of Tagus Estuary (Barreiro (B) - Lat: 38º40´45.40”N; Long: 9º3´1.70”W –, Cala do Norte (CN) - Lat: 38º51´21.21”N; Long: 9º3´40.51”W - and Alcochete (A) - Lat: 38º45´41.58”N; Long: 8º56´49.93”W) as shown in Figure IV.1. Procedures used for sediment sampling and bacteria isolation, maintenance and biochemical characterization have been previously described (Figueiredo et al., 2014a). Briefly, bacteria isolation was performed in Mueller-Hinton (MH) medium, a testing medium recognized as standard by NCCLS, once the usage of minimal medium drastically reduced bacteria colonies and limited their growth. MH media containing mercury selective concentration (0.02 and 0.22 µg/mL methylmercury chloride (99.9% CH_3HgCl from Sigma)) were used to isolates HgR bacteria as previously explained (Figueiredo et al., 2014a) and according to the sensitivity to these two concentration, the isolates were classified in three groups: (1) mercury susceptible bacteria , i.e. bacteria unable to grow in media containing ≥ 0.02 µg/mL MeHg; (2) mercury tolerant bacteria i.e. bacteria able to grow in media containing 0.02 µg/mL MeHg but unable to grow at

concentration of 0.22 µg/mL MeHg, and (3) mercury-resistant bacteria (HgR), i.e. bacteria able to grow in media containing 0.22 µg/mL MeHg.

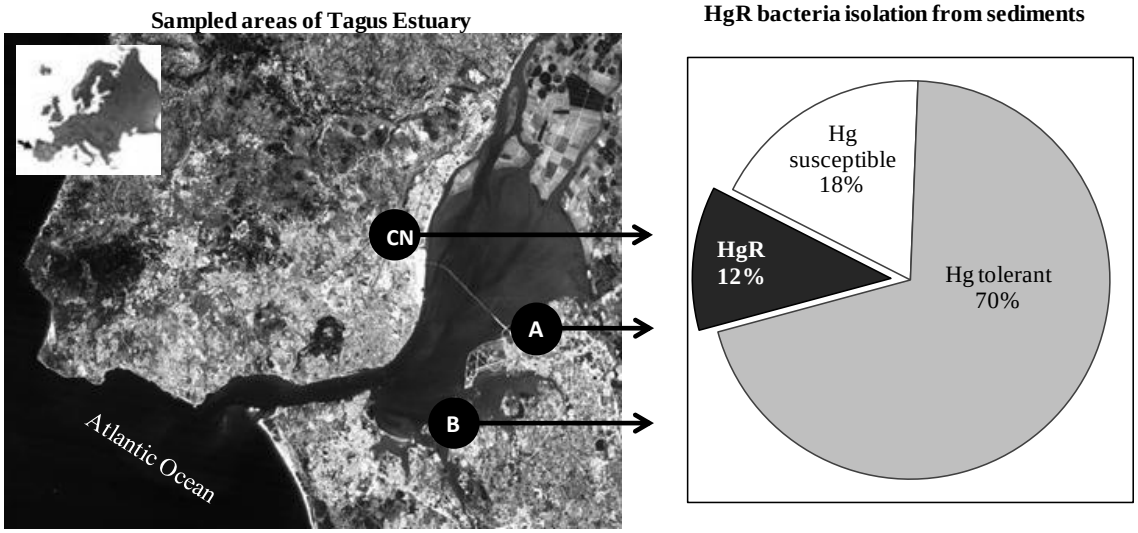


Figure IV.1: Sample sites in mercury-polluted areas of Tagus Estuary (Portugal): Barreiro (B), Cala do Norte (CN) and Alcochete (A). The chart represents the total percentage of bacteria isolates classified into three groups, according to their mercury resistance: mercury susceptible bacteria, mercury tolerant bacteria and mercury resistant bacteria (HgR).

IV.2.2. HgR bacteria characterization

IV.2.2.1. Bacteria identification

IV.2.2.1.1. 16S rRNA amplification and sequencing

Bacterial DNA was extracted from boiled bacterial suspension (10 minutes at 95ºC) through centrifugation at 13,000 g for 4 min and was used for PCR reaction. The 16S rRNA gene was PCR amplified using primers P1-P8 (supplemental Table IV.S1) in 25 µL volume (12.5 µL of PCR master mix (NzyTech), 8.5 µL of nuclease-free water, 0.1-1µM of primers and 0.05-0.5 µg of template DNA). PCR conditions were as follows:

initial holding at 98ºC for 30 seconds, 20-35 cycles of denaturing at 98ºC for 10 seconds, annealing at 59-64ºC for 30 seconds and extension at 72ºC for 30 seconds, and a final extension at 72ºC for 10 min. After the confirmation of amplicons size by gel electrophoresis, PCR products were purified using NZYGelpure kit (NZYTech) and sequencing was performed by STAB-Vida (Lisboa, Portugal), using the specific primers for amplification. All sequences were subjected to a BLAST search (NCBI, 2013) for comparison with published sequences. Multiple alignments with known 16S rRNA gene sequence from GenBank (NCBI, 2013) were performed by CLUSTAL W2 algorithm (EMBL-EBI, 2013).

IV.2.2.1.2. Identification criteria

Identification to the genus or species level was performed using 16S rRNA as follows: (a) when the comparison of the 16S rRNA sequence determined with a reference sequence of a classified species yielded a similarity score ≥99%, the isolate was assigned to the respective species; (b) when the 16S rRNA similarities were <99% and >96% the isolate was assigned according to the classification obtained by BBL (BD) and API (bioMérieux) multi-test identification systems (O'Hara, 2005) with a score (% id) ≥99 %; (c) when 16S rRNA similarities were ≤96%, the unknown isolate was assigned to genera level.

IV.2.2.2. Determination of mercury resistance

The minimal inhibitory concentration (MIC) of isolates was determined, using a modified micro dilution broth method described by CLSI (2006). MIC determinations were performed for mercury chloride ($HgCl_2$) (Riedel-de Haën) (99.9%), and MeHg (CH_3HgCl) (Sigma) (99.9%), ethylmercury chloride (C_2H_5HgCl herein referred as EtHg) (Alfa Aesar), and phenylmercury chloride (C_6H_5HgCl herein referred as PhHg) (Sigma) (99.9%). A solution containing cultured microorganisms in brain heart infusion broth (BHI) at the concentration of 10^8 CFU/mL (OD 0.5 at 595 nm) was diluted in MH broth (considered by NCCLS a testing medium suitable for bacterial susceptibility studies as stated above)) in order to obtain 10^6 CFU/mL . In a sterile 96-well microplate 100 µL of these bacterial suspensions were added with 100 µL of aqueous solution of

each mercury compound into the first well and sequential dilution (1:2) was performed in the following 10 wells, thus achieving nominal concentrations ranging from 0.01 to 136 µg/mL $HgCl_2$, 1.23×10^{-4} to 1.26 µg/mL CH_3HgCl, 1.29×10^{-4} to 1.33 µg/mL C_2H_5HgCl and 1.53×10^{-4} to 1.57 µg/mL C_6H_5HgCl. The 12^{th} well was used as a positive control of bacterial growth. Determinations of MIC were carried out in duplicate at each concentration tested. After incubation at 37°C for 24 hours in dark and under aerobic conditions, bacterial growth detection was performed. The MIC was defined as the minimum concentration of test compounds that inhibited visible growth. All data points represent the mean ± standard deviation (STD) of 2 independent determinations (each one also performed with duplicates).

IV.2.2.3. Determination of antibiotic susceptibility

Antibiotic susceptibility test were performed by the diffusion method on MH agar with antibiotic discs, according to CLSI (2010). Bacterial suspensions equivalent to 0.5 McFarland standard were evenly dispersed in MH agar and then antibiotic discs (BD) were placed as follows: nalidixic acid (30 µg), rifampicin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), kanamycin (30 µg) and streptomycin (10 µg). These antibiotics were chosen as they are the ones mostly associated to mercury resistance (Allen et al., 1977; Sadhukhan et al., 1997; Zhang et al., 2011). After 24 h incubation at 37ºC, the diameter of inhibition zone was measured and interpreted according to the zone diameter standard (CLSI, 2010).

IV.2.2.4. Genetic identification of *mer* operon mechanisms for mercury resistance

The presence of the *mer* operon was investigated through the amplification of *mer* genes: (1) encoding for functional proteins for regulation (*merR*), for mercuric reductase (*merA*), and for organomercurial lyase (*merB*), using internal and external primers P9-P26 (supplemental Table IV.S1). The amplifications were performed as described above, respecting the annealing temperature of the primers (50-66ºC). PCR products treatment was also the same described for 16S rRNA, using for the multiple alignments with *mer* genes sequence from GenBank (NCBI, 2013).

IV.2.3. Evaluation of Hg^{2+} transformation by HgR bacteria

Among the HgR bacteria isolated, ten representative bacterial strains (RBS) were selected to study their Hg^{2+}-transformation capacity, namely reduction and methylation. RBS included bacteria belonging to the most frequent genera identified in the bacterial community and with high *versus* low resistance.

Overnight cultured bacteria were adjusted to 10^6 CFU/mL and, to this sub-MIC concentrations of $HgCl_2$ (0.40-1 µg/mL) were added. The bacteria suspensions and controls (MH plus $HgCl_2$) were incubated at 37°C on an orbital shaker (100 rpm/min) for 24, 30 and 48 hours and when necessary at 60 hours. At each time point, the optical density was measured at 595 nm (Hitachi spectrophotometer) and the cells were harvested by centrifugation at 15,300 g for 5 min with the supernatant separated for further analysis. Harvested cells were washed with sterile deionized water and weighed. Mercury concentrations were analyzed in the different fractions as described below. All the experiments were carried out in Teflon fluorinated ethylene propylene (Teflon FEP) tubes that showed 100% mercury recovery (1.16±0.15 µg/mL) in controls (growth media in the absence of bacteria) added by 1.00 µg/mL Hg^{2+}.

IV.2.4. Mercury speciation

IV.2.4.1. Quantification of organomercurial species

Total mercury (HgT) was determined after acidic digestion in microwave acid digestion bombs (Parr) of 0.5 mL of each sample in 2.5 mL HNO_3 (65%) to bring all the mercury into its inorganic form. Sealed bombs were heated for 30s at 600 watts using a regular microwave. After digestion, all the liquid content was collected from the digestion bombs and diluted to 10 mL before analysis by CV-AFS. The concentration of mercuric inorganic species and HgT was determined by external calibration of the signal obtained by the continuous Hg CV-AFS for 7 standard solutions.

Mercuric inorganic species were extracted according to the methodology described by Carvalho et al. (2006) replacing HCl by HNO_3 that showed similar extraction results. In

brief, 0.5 mL of sample (supernatant and washed cell pellet) was extracted with 2.5 mL 65% HNO_3 and then, homogenized before filtration through 0.4 µm Acrodisc® filters. The liquid phase was diluted to 10 mL before direct analysis by cold vapour atomic fluorescence spectroscopy (CV-AFS).

OrgHg was calculated by subtracting the mercuric inorganic fraction to the HgT. The OrgHg fraction denomination includes different mercuric organic species such as alkylated mercurial compounds (e.g. MeHg) and stable mercuric complexes with protein or non-protein thiol complexes among others.

IV.2.4.2. Analysis of MeHg

The presence of MeHg in samples where OrgHg was detected was verified by GC-AFS of diluted (1/100) samples after ethylation by previously described procedures (Cai et al, 1997; Edmonds et al., 2012). An aliquot of 20-40 µL of each sample was injected into a glass reaction bubbler for isolation and quantification of Hg species by aqueous phase ethylation, purge and trap, and gas chromatography followed by detection with atomic fluorescence spectrometry using a Brooks Rand Model III (EPA, 2001). DOLT-4 CRM was analysed and the values obtained were within the interval (1.33 ± 0.12 mg/kg) indicated by NRC of Canada (2013).

IV.2.4.3. Qualitative determination of Hg^0 volatilization

Mercury volatilization was verified according to the protocol described by François et al. (2011) with some modifications. After overnight growth in MH liquid broth, cells were adjusted to 10^6 CFU/mL in MH broth into a 12-well microplate. $HgCl_2$ solution was added to achieve sub-MIC concentrations as in section 2.3. MH plus $HgCl_2$ solution was used as negative control. A layer of sensitive X-ray film was inserted in the microplate followed by incubation at 37ºC for 48h in the dark. The observation of foggy areas on the X-ray film, due to the reduction of Ag^+ by mercury vapor (Hg^0), was interpreted as a positive result for Hg^0 volatilization.

IV.2.4.4. Quantification of mercury reduction

Growth media plus $HgCl_2$ was prepared at sub-MIC concentrations and split in two tubes, one inoculated with bacteria and the other kept as prepared and used as control. To calculate mercuric mercury reduction HgT was quantified in control media (0 and 60h), in supernatant and in washed cell pellet after 60 hours of growing. HgT determination in samples was performed by pyrolytic reduction and atomic absorption spectrometry using a LECO AMA-254 gold amalgamator (Costley et al., 2000), a method that is very effective and suitable for non-acidic samples. The values obtained were used to calculate the percentage of reduction: Reduction (%) = (HgT Control - ($HgT_{Supernatant}$ + $HgT_{Cell\ pellet}$))/ (HgT Control) ×100.

IV.3. RESULTS

IV.3.1. Isolation and identification of HgR bacteria

Sediments from Tagus Estuary - the two hotspots (Barreiro (B) and Cala do Norte (CN)) and one less impacted area (Alcochete (A)) - were sampled for aerobic HgR bacteria isolation. Forty six bacteria (B:21; CN:14 and A:11), representing 12% of the total number of the isolates (Figure IV.1) were considered mercury-resistant (HgR) and subjected to 16S rRNA identification. These isolates belong to *Bacillus*, *Vibrio*, *Aeromonas*, *Micrococcus*, *Citrobacter*, *Serratia* and *Pseudomonas* genera. *Bacillus*, the most common genus found (61%; n=28), was dominant in Barreiro and Alcochete and included *B. megaterium, B. cereus, B. subtilis* and *B. soli* (Gram positive). Among Gram negative bacteria *Vibrio* represented 22% (n=10) of the isolates and the species *Vibrio metschnikovii* was frequent in Cala do Norte. The remaining 17% (n=8) of the isolates belong to *Aeromonas* spp. (Barreiro), *Citrobacter freundii*, *Pseudomonas putida* and *Serratia marcescens* (Cala do Norte).

IV.3.2. Mercury and antibiotics resistance

Twelve per cent of the isolates (n=46) described above were found to be able to growth in MH agar media containing 0.22 µg/mL MeHg, being classified as mercury-resistant bacteria. However, prior to start with mercury speciation studies the isolates resistance was evaluated by determining MIC values for Hg^{2+}, $MeHg^{+}$ and other organomercurials. Among all 46 isolates, Gram positive bacteria showed MIC for Hg^{2+} and $MeHg^{+}$ from 0.16 to 5.05 µg/mL and 0.02 to 0.50 µg/mL whereas for Gram negative, MICs ranged from 0.55 to 10.0 µg/mL and 0.07 to 1.01 µg/mL, respectively for Hg^{2+} and MeHg. Moreover, mercury compounds and antibiotic resistance patterns were evaluated in detail for selected isolates, herein referred as RBS - a group of ten bacterial strains encompassing the most representative genera identified (see a more complete definition in section IV.2.3 of materials and methods). In general the mercurial resistance pattern for this group was: $Hg^{2+} >> MeHg^{+} \geq EtHg^{+} \approx PhHg^{+}$ (Table IV.1). Resistance to ampicillin was the most common among the antibiotics tested, followed by nalidixic acid (Table IV.1). Resistance to antibiotic normally associated to mercury resistance, such as rifampicin, ampicillin tetracycline, kanamycin and streptomycin, were found in the top HgR strains (*Vibrio metschnikovii* strain 1.3BvA, *Citrobacter freundii* strain 1.1SvA and *Serratia marcescens* strain 1.2SvA) (Table IV.1). However, our data failed to prove the presence of these antibiotic and mercury resistances in the same genetic element.

Table IV.1: Mercury and antibiotic resistance for selected HgR bacteria isolated from two highly-contaminated areas of Tagus Estuary – Barreiro and Cala do Norte.

Sampled areas	Strains	MIC (µg/mL)[a]				Antibiotic resistance[b]
		Hg^{2+}	MeHg[c]	EtHg[c]	PhHg[c]	
Barreiro	*Aeromonas media* 1.1BvA	9.87±0.00	0.25±0.00	0.19±0.06	0.11±0.01	AM
	Vibrio metschnikovii 1.3BvA	9.87±0.00	0.50±0.00	0.11±0.01	0.11±0.01	K and S
	Vibrio fluvialis 3.1BvA	1.00±0.00	0.08±0.03	0.08±0.02	0.02±0.00	NA
	Bacillus megaterium 5.1BvA	1.13±0.13	0.12±0.01	0.38±0.13	0.19±0.06	AM
	Bacillus sp. 9.1BvA	0.50±0.00	0.03±0.02	0.11±0.01	0.09±0.04	NA
Cala do Norte	*Citrobacter freundii* 1.1SvA	10.03±0.00	1.00±0.00	0.56±0.06	1.03±0.00	NA, RA, K, AM
	Serratia marcescens 1.2SvA	5.02±0.00	0.50±0.00	0.38±0.13	0.50±0.00	NA, RA,TE, AM
	Bacillus subtilis 16.3SvA	5.02±0.00	0.50±0.00	0.25±0.00	0.11±0.01	NA
	Bacillus cereus 16.10SvA	1.75±0.75	0.10±0.00	0.11±0.01	0.08±0.02	AM
	Bacillus cereus 16.11SvA	3.76±1.26	0.50±0.00	0.06±0.00	0.06±0.01	AM

[a]*Minimum inhibitory concentration values. All the concentrations are referred to mercury content;*
[b]*AM: ampicillin (10 µg); K: kanamycin (30 µg); S: streptomycin (10 µg); NA: nalidixic acid (30 µg); RA: rifampicin (30 µg), and TE: tetracycline (30 µg);*
[c]*MeHg – Methylmercury; EtHg – Ethylmercury, and PhHg – Phenylmercury.*

IV.3.3. Genetics factors conferring mercury resistance

Mer genes were searched in all 46 isolates, but positive results were found only in 2 isolates, *Aeromonas media* (strain 1.1BvA) and *Citrobacter freundii* (strain 1.1SvA). These genes included *merR-A* (strain 1.1SvA), *merC-A* (strain 1.1SvA) and *merA-D* (strain 1.1BvA), identified through multiple alignment with *mer* genes.

The highest identities (93-98%) were found with *merR*, *merC*, *merA* and *merD* genes of *Enterobacter cloacae* subsp. *cloacae* ATCC 13047 (GenBank CP001918) (NCBI, 2013). No *merB* was found among the isolates.

IV.3.4. Potential for mercury species transformation

The formation of other mercurial forms, such as alkylated mercurial forms and/or complexes with bacterial proteins (OrgHg) was evaluated for RBS (Figure IV.2). In the

presence of $HgCl_2$ for 48 hours, all these bacteria showed the same pattern: mercuric inorganic species decreased in liquid media and increased in cell pellet and, for most strains, removal of mercury was already observed after 24 hours of growth (Figure IV.2). Moreover, OrgHg was produced in cell pellet and supernatant fractions of strains 1.3BvA, 3.1BvA, 5.1BvA, 1.2SvA and 16.3SvA, (Figure IV.2). After evaluating the formation of organic alkyl compounds of mercury we used a more specific method (GC-AFS after ethylation) to investigate and quantify MeHg formation in supernatant samples. Data showed that MeHg is only a fraction of OrgHg (Table IV.2). MeHg concentrations (1-26% of the initial $HgCl_2$) were found in supernatant after incubation. Methylation was only performed by *Vibrio fluvialis* strain 3.1BvA, *Bacillus megaterium* strain 5.1BvA and *Serratia marcescens* strain 1.2SvA (Table IV.2).

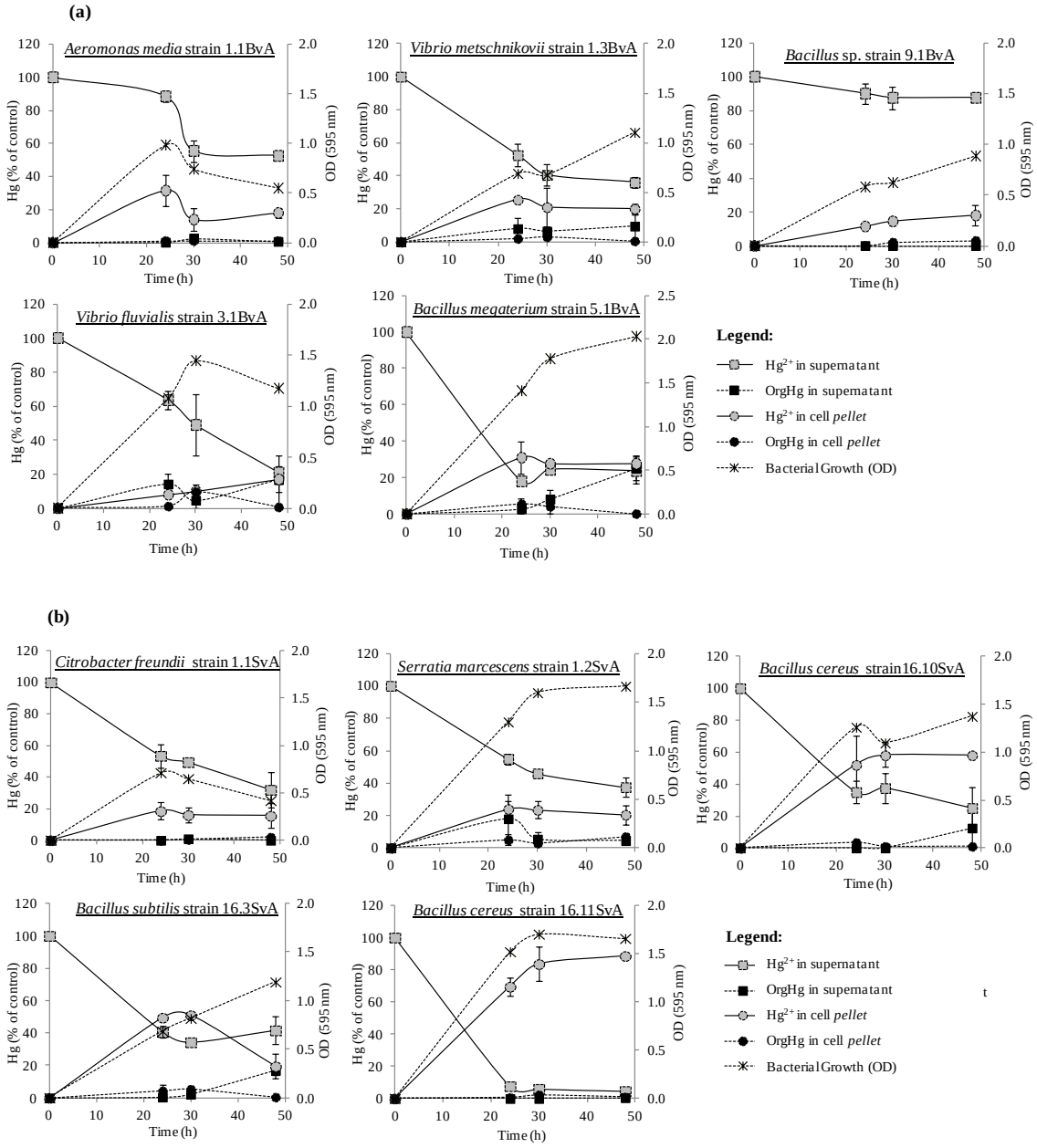


Figure IV.2: Evaluation of Hg^{2+} transformations by RBS (ten representative bacteria strains) selected: (a) 5 bacteria from Barreiro and (b) 5 bacteria from Cala do Norte. Bacteria were incubated in MH media containing sub-MIC concentration of $HgCl_2$; mercury species were determined using CV-AFS. Bacterial growth was controlled by the measurement of optical density (595 nm). Standard deviation (STD) values were calculated from 2-3 independent experiments.

Table IV.2: MeHg produced by representative selected strains displaying high OrgHg formation. Supernatant samples were analyzed by GC-AFS after ethylation and MeHg concentration is indicated in ng/mL.

Areas	Strains	% of MeHg (concentration in ng/mL)		
		Time (h)		
		24	30	48
Barreiro	1.3BvA	<C[a]	<C[a]	<C[a]
	3.1BvA	0	1 (11.3)	4 (37.2)
	5.1BvA	0	0	2 (15.5)
Cala do Norte	1.2SvA	8 (76.5)	26 (260)	8 (76.2)
	16.3SvA	<C[a]	<C[a]	<C[a]

[a]*C – Control media without bacteria.*

IV.3.5. Hg^0 volatilization and Hg^{2+} reduction

As shown in Figure IV.2 for the RBS, data indicate a loss of mercury from reactional media for all the tested isolates with the exception of strain 9.1BvA, resulting in the removal of 6-50% mercury (Figure IV.2). A plausible hypothesis is that upon Hg^{2+} reduction to Hg^0, this compound volatizes subsequently and is released from media. It should be stressed that the amount of mercury volatilized corresponds to the amount of mercury lost from the system and not adsorbed onto the cells or concentrated on the pellet. To verify the volatilization of mercury from liquid media, an experiment based on the reaction between Hg^0 and Ag was carried out and the results confirmed the release of mercury vapor (Supplemental Figure IV.S2). Thereof, it was assumed that removal from supernatant without correspondent accumulation in pellet is a result of Hg^0 volatilization.

As Hg^0 volatilization was confirmed, an experiment was designed to ascertain the percentage of reduction among the mercury-resistant bacteria isolated from the three sampled areas. It was verified that in the presence of $HgCl_2$, not only RBS but most of the 46 isolates were able to remove mercury from growth media. Through the analysis of mass balance, it was possible to assign the potential of bacterial isolates to reduce Hg^{2+} through the calculation of % of reduction (see materials and methods section

IV.2.4.4). On average the reduction observed among the isolates of the three sampled areas was Barreiro (41%) > Cala do Norte (32 %) > Alcochete (15%) and the isolates presenting the highest % of reduction (≥ 60%) had been collected in Barreiro and Cala do Norte (Figure IV.3).

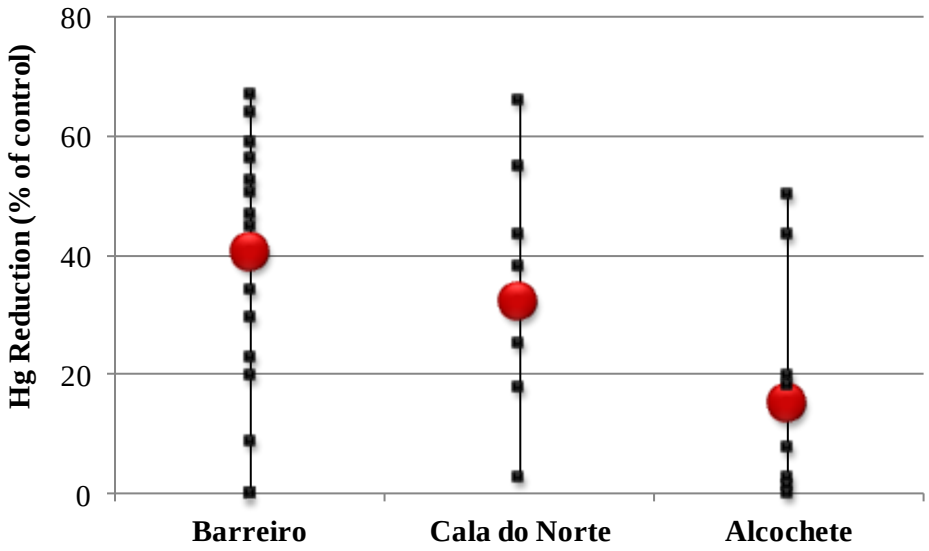


Figure IV.3: Potential of HgR bacteria isolated from three mercury-polluted areas of Tagus Estuary (Barreiro (n=18), Cala do Norte (n=9) and Alcochete (n=10)) to reduce Hg^{2+} from the contaminated growth media. Averaged results of bacteria isolates belonging to the same sampled site are represented by the symbol (●). Sub-MIC HgT concentrations in controls ranged from 0.13 to 0.94 µg/mL (t=0 hours). Controls were sampled at the same time-points as the bacterial isolates.

Figure IV.4 integrates all the results obtained in this study for mercuric mercury transformation, summarizing the processes and transformations that Hg^{2+} undergoes in presence of aerobic mercury resistant bacteria of Tagus Estuary.

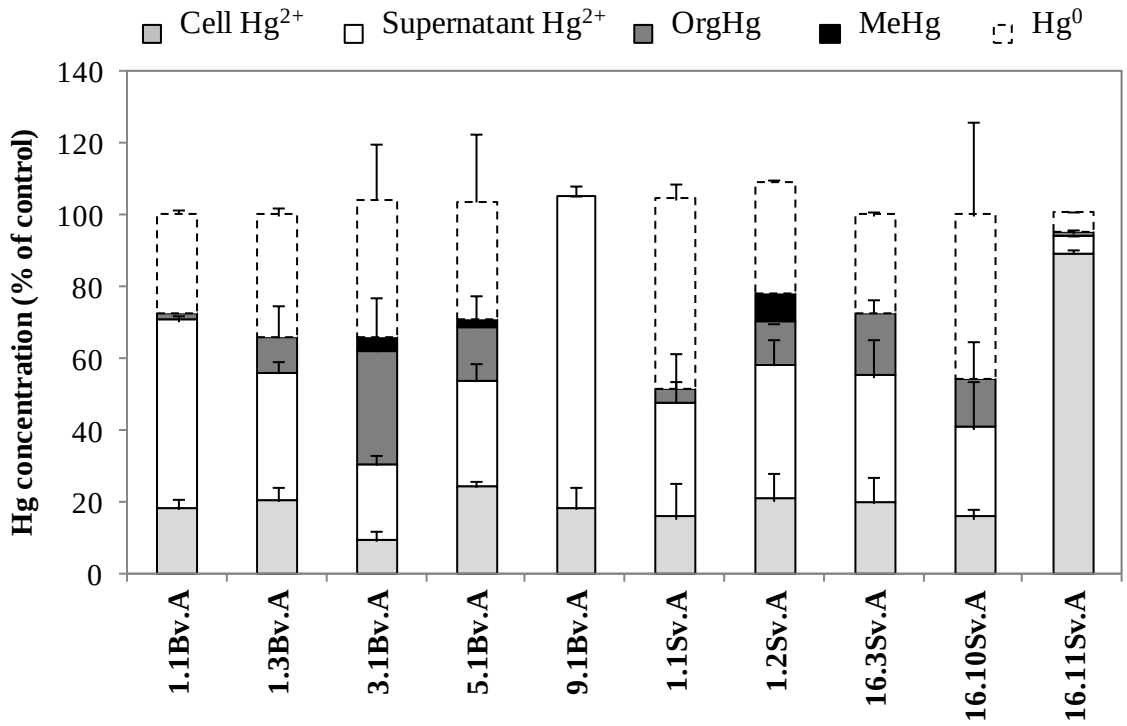


Figure IV.4: Final balance of the different species of mercury after 48h of (RBS) bacterial growth in presence of sub-MIC $HgCl_2$. The graphic represents the percentage of Hg^0 volatilized, Hg^{2+} remaining in growth media and cell pellet and organomercurial species formed, including the amount of MeHg produced.

IV.4. DISCUSSION

IV.4.1. Characterization of HgR bacteria isolates

In Tagus Estuary 12% of the aerobic bacteria isolated from sediments of the three studied areas were found to be Hg-resistant (Figure IV.1). The HgR isolates included both Gram positive and Gram negative bacteria. Gram positive isolates belong to the genus *Bacillus*, the dominant genus found in Tagus estuary, as well as in other mercury contaminated sites such as Minamata Bay (Japan) (Nakamura and Silver, 1994) and Hudson Shelf Valley (USA) (Timoney et al., 1978) where *B. megaterium, B. soli*, *B. cereus* and *B. subtilis* were isolated (Narita et al., 2003). Gram negative bacteria such as *Vibrio*, *Aeromonas*, *Pseudomonas*, *Citrobacter*, and *Serratia* were also found in Tagus

Estuary as well as in Hudson Shelf Valley (USA) (Timoney et al., 1978) and in marine (Zhang et al., 2011).

The ten most representative strains, RBS, showed multiple resistances to organomercurial compounds (broad spectrum) and antibiotics. The general trend for mercury resistance was $Hg^{2+} >> MeHg^{+} \geq EtHg^{+} \approx PhHg^{+}$, being the isolates resistant to 0.5-10.0 µg/mL Hg^{2+} (mean 4.78 µg/mL) and 0.03-1.0 µg/mL MeHg (mean 0.36 µg/mL), which classifies these HgR strains as moderate to highly resistant. Good examples of high resistance to mercury are *Aeromonas media* 1.1BvA, *Vibrio metschnikovii* 1.3BvA, *Citrobacter freundii* 1.1SvA and *Serratia marcescens* 1.2SvA (Table IV.1). Among the 10 selected RBS, 2 strains (*Citrobacter freundii* strain 1.1SvA and *Aeromonas media* strain 1.1BvA) were positive for *mer* operon genes (*merR*, *merC*, *merA* and *merD*), thus conferring mercury-resistant phenotypes to these strains through the expression of mercury reductase. Negative results for *mer* genes in other strains may be related with absence of these genes or to other constraints, such as non-specificity of the primers used, since wide genetic variation within each gene also occurs (Narita et al., 2003). Overall, molecular mechanisms used by these aerobic isolates that justifying high mercury resistance still need further investigation.

IV.4.2. Hg^{2+} transformation by HgR bacteria

The detailed study of the most significant strains (RBS) (Figure IV.2) showed that these bacteria in presence of Hg^{2+} were able to perform several transformations (Figures IV.4) including the production of OrgHg species (Figure IV.2) and mercury reduction followed by volatilization of Hg^{0} (Figures IV.3 and IV.S2). Among OrgHg species we have shown the production of MeHg (Table IV.2). With the exception of *Bacillus* sp. strain 9.1BvA, all RBS lessen inorganic mercury species in growth media, with removal of 39-95% from supernatant (Figure IV.2). There was also some adsorption to the bacterial biomass (Figure IV.2), and a significant amount of mercury that undergoes reduction to Hg^{0} followed by subsequent volatilization (up to 50%) (Figures IV.3 and IV.4). Similar results have been reported for aerobic bacteria (Chadhain et al., 2006), resulting in 34-89% mercury loss (De et al., 2008; Sadhukhan et al., 1997; Summers and Lewis, 1973; Vetriani et al., 2005; Zhang et al., 2011). The results obtained

strengthen the reduction potential of Tagus' bacteria that is more prone to occur among isolates of Barreiro (Figure IV.3). For *Citrobacter freundii* strain 1.1SvA and *Aeromonas media* strain 1.1BvA, Hg^{2+}-reduction with the production of Hg^0 can be explained as a result of mercury reductase activity whereas for other bacteria with negative results for *mer* genes, it may be hypothetically related to the presence of putative non-*mer* mediated mercury reduction (De et al., 2008) or eventual mismatches during *merA* gene amplification, as mentioned above. Moreover, one cannot exclude that dimethylation can also promote mercury loss by volatilization (Baldi et al., 1995); however, this is more common among SRB such as *Desulfovibrio desulfuricans* strain LS (Baldi et al., 1995). On the other hand, non-reducer bacteria such as *Bacillus* sp. strain 9.1BvA may use other mechanisms to keep Hg^{2+} ions outside the cell, such as binding to proteins, amino acids, peptone, glutathione or H_2S (Hamdy and Noyes, 1975).

Isolates of *Vibrio fluvialis* strain 3.1BvA, *Bacillus megaterium* strain 5.1BvA, and *Serratia marcescens* strain 1.2SvA, were found to produce MeHg (Table IV.2). Mercury methylation has been mainly associated with anaerobic bacteria (Lin et al., 2014), although methylation of Hg can be mediated in oxic conditions by microorganisms (Montperrus et al., 2007). In fact aerobes such as *Bacillus megaterium* (Ramamoorthy, et al., 1982) *Klebsiella* sp. (Achá et al., 2012) and *Enterobacter aerogenes* (Hamdy and Noyes, 1975) were also reported to act as methylators providing that bacteria possess methyl transfer enzymes like coenzymes N5-methyltetrahydrofolate, S-adenosylmethionine and methylcobalamine (vitamin B12) (Wood et al., 1968; Robinson and Touvinen, 1984).

The formation of MeHg was in the range 2-8% of initial HgT (1000 ng/mL) present in media. This is in good agreement with the outcome found by Monperrus et al. (2007) in oxic surface seawater and mediated mainly by microorganisms (0.3-6.3%), considering that in the environmental conditions the percentage of methylation is expected to be slower than the one showed in laboratory conditions. Nevertheless, it should be stressed that some bacteria perform simultaneously methylation and demethylation as has been observed by several authors (Achá et al., 2012; Ramamoorthy et al., 1982; and Spangler et al., 1973) therefore what we report is the net formation of MeHg, which is relevant for environmental and human mercury risk assessment.

IV.4.3. Environmental implication of these bacterial transformations

Our study is the first contribution to understand mercury conversion by bacteria of Tagus Estuary. Clearly, the aerobic bacteria isolates change mercury speciation through methylation and reduction. These two reactions have great environmental significance, since one of the most important factors affecting mercury bioavailability and uptake by aquatic organisms is its redox transformation (Lin et al., 2014). Since these bacteria were isolated from sediments of mercury-contaminated areas, it is expected that these conversions will happen in oxic sediments of these areas, albeit at a lower rate than the observed in laboratory conditions. Furthermore, this also suggests that anaerobes, namely SRB, are not the only mercury methylators in these areas.

IV.5. CONCLUSIONS

In conclusion, aerobic bacteria have impact in mercury dynamics in Tagus Estuary and possibly in other aquatic environments worldwide. These bacteria affect the biogeochemical cycle of mercury, as they are contributing for the decrease of mercury available for methylation and mobilization of Hg^0 to the atmosphere and its global circulation (Barkay et al., 2003). Furthermore, MeHg production, even in a low percentage, is always a risk for aquatic and human health due to its bioaccumulation in fish and this may justify future investigation about the methylation rate in sediments of Tagus Estuary. Finally, knowing that inorganic mercury (II) is often the most abundant mercury species resulting from pollution, the prevalence of reduction among the isolates studied will open new perspectives for bioremediation.

Table IV.S1: List of primers used for amplification of 16S rRNA gene and *mer* operon genes.

	No.	Forward/ Reverse (5'-3')	Source	Reference
16S rRNA	**P1**	CTCGCGTCAGGATATGCCC/ CGTTAGCTCCGAAAGCCAC	*Vibrio cholerea* (AJ554204)	This study[a]
	P2	GGACGGGTGAGTAATGCCT/ TCACCGCGACATTCTGATTCG	*A. hydrophilia (*AB680394)	This study[a]
	P3	TGATGAAGGTTTTCGGATCG/ GGGTTGTCAGAGGATGTCAAG	*B. licheniformis* (JQ700450)	This study[a]
	P4	TATGACGTTAGCGGCGGACG/ GGCACTGATGATTTGACTTCC	*B. megaterium* (AB703264)	This study[a]
	P5	ACATGCAAGTCGAGCGGAC/ TTCACCCCAATCATCTGCC	*B. pumilus* (AJ494730)	This study[a]
	P6	ATGCAAGTCGAGCGATGGA/ CGTGTGTAGCCCAGGTCAT	*Bacillus* sp. (BSU11551)	This study[a]
	P7	ACATGCAAGTCGAGCGAAC/ CACCCCAATCATCTGTCCC	*B. cereus* (NC011969)	This study[a]
	P8[b]	CCTACGGGAGGCAGCAGT/ CGTTTACGGCGTGGACTAC	*E. coli* (J01859)	Chiang et al., 2006
***Mer* genes**	**P9**	GCGGATTTGCCTCCACGTTGA/ CCAGGCAGCAGGTCGATGCAAG	Plasmid NR1 (*merR*)	Liebert et al., 1997
	P10	ACGGATGGTCTCCACATTG/ CGAGGCAGCAAGCCGAGGCG	Transposon Tn501 (*merR*)	Liebert et al., 1997
	P11	GGCTATCCGTCCAGCGTCAA/ GTCGCAGGTCATGCCGGTGATTTT	Transposon Tn501 (*merA*)	Liebert et al., 1997
	P12	ACCATCGGCGGCACCTGCGT/ ACCATCGTCAGGTAGGGGAACAA	Plasmid NR1 (*merA*)	Liebert et al., 1997
	P13	GCGGATTTGCCTCCACGTTGA/ ACCATCGTCAGGTAGGGGAACAA	Plasmid NR1 (*merA*)	Liebert et al., 1997
	P14	ACGGATGGTCTCCACATTG/ ACCATCGTCAGGTAGGGGAACAA	Plasmid NR1 (*merA*)	Liebert et al., 1997
	P15	GGTTGTGTGCCGTCCAAGATC/ CCATCGTCAGGTAGGGGAACA	Plasmid 4 (*merA*)	This study[a]
	P16	CCTGTGTCGTGCATGTGAAAG/ AGCACTTCGATGCCCTCCATA	*Pseudomonas putida SP1 (merA)*	This study[a]
	P17	GGACTGCACGTCGCCGTTATT/ GGCTTGCGTGTGTTCCAATAC	Plasmid R934 (*merA*)	This study[a]
	P18	GAACGCCTACTGTGACAACGA/ GCATGGTGGACAGATCCAGTT	Plasmid 1 (*merA*)	This study[a]
	P19	TCGCCCCATATATTTTAGAAC/ GTCGGGACAGATGCAAAGAAA	Plasmid NR1 (*merB*)	Liebert et al., 1997
	P20	GCGGATTTGCCTCCACGTTGA/ GTCGGGACAGATGCAAAGAAA	Plasmid NR1 (*merB*)	Liebert et al., 1997
	P21	ACGGATGGTCTCCACATTG/ GTCGGGACAGATGCAAAGAAA	Plasmid NR1 (*merB*)	Liebert et al., 1997
	P22	GAACGTCTCACTTCGGTCAATC/ TGTCCTAGATGACATGGTCTGC	*A. salmonicida* p4 (*merB*)	This study[a]
	P23	GAAATCGTAACCCGACTTGAC/ ATGTCACAGCATGACCCATTG	*B. cereus* Tn5084 (*merB*)	This study[a]
	P24	CTGCCTAAAGAATCAGTGCCT/ TTCGTTTCCAAAAGAGAGAGC	*B. megaterium* MB1 (*merB*)	This study[a]
	P25	GGTTTGCCAGAAGAAGGATTT/ CCAAAAACAGCAGCCCCAACC	*B. megaterium* MB1 (*merB2*)	This study[a]
	P26	GCCGACCAGTTGTTCCCCTACCTGACG/ CGCACGATATGCACGCTCACCC	Plasmid NR1 (*merA-B*)	Liebert et al., 1997

[a]*Primers designed using Genamics Expression software (Genamics Expression 1,100©, 2000);*
[b]*Universal primer.*

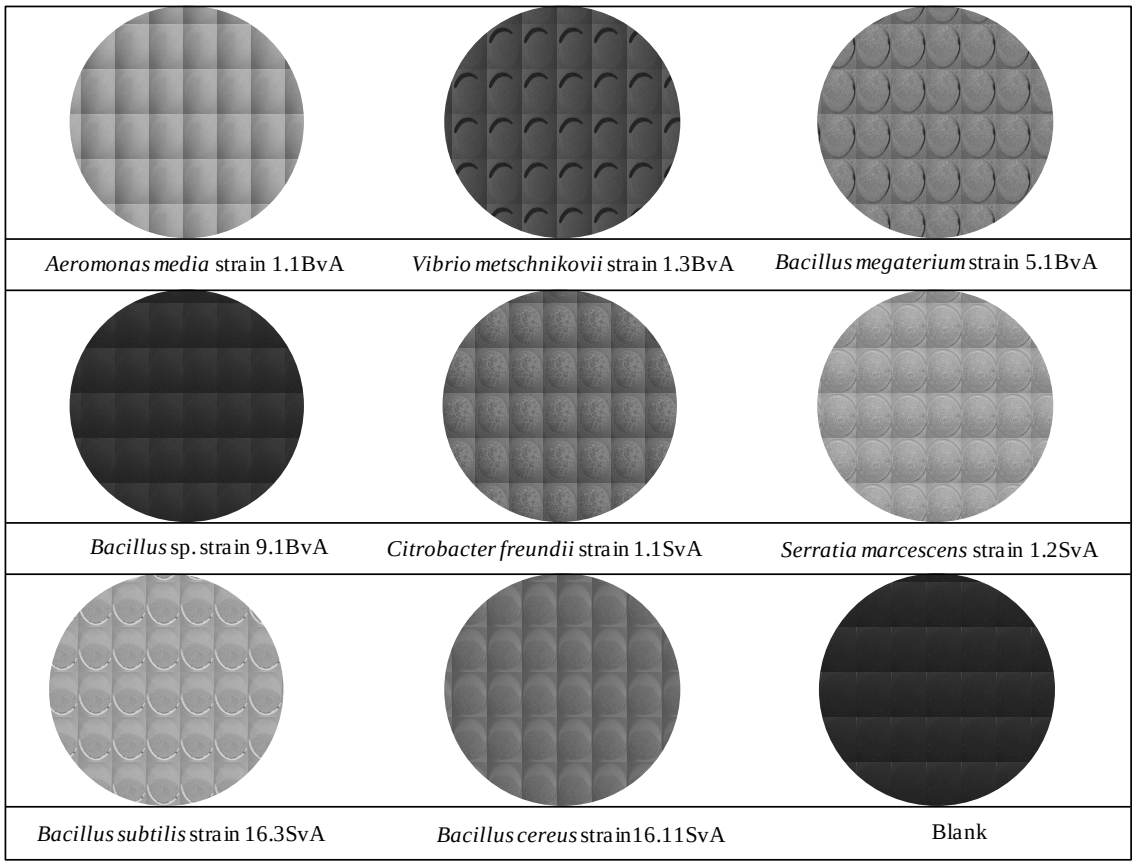


Figure IV.S2: Capture of volatilized Hg^0 by X-ray film. Bacterial strains were inoculated into a 12-well microplate containing MH liquid media plus sub-MIC concentration of $HgCl_2$ (covered with X-ray film) and incubated in the dark at 37°C for 48h. The foggy areas result from the reaction between Hg^0 and Ag^+ of X-ray film.

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CHAPTER V

EVIDENCE OF MERCURY METHYLATION AND DEMETHYLATION BY THE ESTUARINE MICROBIAL COMMUNITIES OBTAINED WITH ENRICHED STABLE Hg ISOTOPES†

†Figueiredo et al., 2016 (*In prep to be submitted*)

ABSTRACT CHAPTER V

Microbial activity is a critical factor controlling methylmercury formation in aquatic environments. Microbial communities were isolated from sediments of two highly mercury polluted areas of the Tagus Estuary (Barreiro and Cala do Norte) and differentiated according to their dependence on oxygen into three groups – aerobic, anaerobic and sulfate-reducing microbial communities. Their potential to methylate mercury and demethylate methylmercury was evaluated through incubation with isotope enriched Hg species (^{199}HgCl and $CH_3{}^{201}$HgCl). The results showed that the isolated microbial communities are actively involved in methylation and demethylation processes. The production of $CH_3{}^{199}$Hg was positively correlated with sulfate reducing microbial communities, methylating up to 0.07% of the added ^{199}Hg within 48h of incubation. A high rate of $CH_3{}^{201}$Hg degradation by aerobic microbial community was observed and > 20% of $CH_3{}^{201}$Hg was transformed. Mercury removal of inorganic forms was also observed.

The results confirm the simultaneous occurrence of microbial methylation and demethylation processes and suggest that microorganisms are mainly responsible for methylmercury formation and accumulation in the polluted Tagus Estuary.

V.1. INTRODUCTION

Methylmercury (MeHg) is one of the most toxic forms of mercury (Barkay and Poulain, 2007) and extensively studied for its neurotoxic effects such as blindness, loss of balance and in severe cases, death (Barkay and Wagner-Dobler, 2005). Humans are mainly exposed to MeHg via the consumption of contaminated fish and marine mammals (Clarkson et al., 2003). The release of mercurial compounds by industrial activities has been the cause of two large epidemics disasters related to the consumption of contaminated fish in Japan (Minamata Bay and Agano River) (Clarkson et al., 2003).

The Tagus Estuary (Portugal) has high levels of mercury contamination as a result of past industrial activity (Canário et al., 2003, 2005; Figuères et al., 1985). Two areas in the North (Cala do Norte) and the South (Barreiro) are most contaminated, with reported levels for total mercury of up to 11 and 33 mg/kg and for MeHg of up to 28 and 47 μg/kg, respectively (Figueiredo et al., 2014a).

MeHg is produced environmentally when the oxidized mercury species react with a methylgroup (Barkay and Wagner, 2005). Methylation of mercury can occur under aerobic and anaerobic conditions by abiotic or biotic mechanisms. The abiotic methylation occurs by transmethylation reactions, i.e. the transfer of a methyl group by the action of ultraviolet radiation or by reaction with humic and fulvic acids (Morel et al., 1998). However, in aquatic environments, biomethylation of mercuric mercury is the major pathway responsible for the appearance of high concentrations of MeHg (Celo et al., 2006). The biomethylation of mercury has been associated with anaerobic microorganisms in aquatic sediments (Barkay and Wagner, 2005). It was first described by Jensen and Jernelov in 1969, who at the time assumed that the methyl group was transferred to Hg^{2+} by the carbon monoxide dehydrogenase (CODH) pathway in sulfate-reducing bacteria (SRB) (Barkay and Wagner, 2005). Therefore, the production of MeHg was primarily associated with SRB (King et al., 2002). Later, iron-reducing bacteria (Kerin et al., 2006) and methanogens (Hamelin et al., 2011) were also associated with MeHg production in anoxic environments. Recently, a genetic basis for mercury methylation was provided through the identification of two genes (*hgcA* and *hgcB*) in methylating bacteria (Parks et al., 2013).

Numerous microorganisms have been reported to convert MeHg into less toxic forms (Oremland et al., 1991) by cleaving the carbon-mercury bond (Hg-CH_3) (Nascimento and Chartone-Souza, 2003). Biotic demethylation can be oxidative or reductive. Oxidative demethylation is mediated by anaerobic bacteria and probably related to carbon metabolism (C1), with release of CO_2 and Hg^{2+} (Barkay and Poulain, 2007). Reductive demethylation leads to the formation of Hg^{2+} and CH_4 as end products and is usually genetically encoded by a cluster of genes organized in the *mer* operon (Barkay and Wagner, 2005). The encoded enzymes break the Hg-C covalent bound (organomercurial lyase) and reduce Hg^{2+} to Hg^0 (mercuric reductase) (Parks et al., 2009; Schaefer et al., 2004). However, MeHg degradation can be also performed by abiotic factors, such as the photodegradation of MeHg mediated by the action of ultraviolet light (Barkay and Wagner, 2005).

The concentration and bioaccumulation of MeHg in aquatic environments depends on the balance between both methylation and demethylation MeHg (Celo et al., 2006; Hintelmann et al., 2000). The efficacy of biomethylation is influenced by the microbial activity and the concentration of bioavailable mercury species. In turn, demethylation processes control the net increase of MeHg. Thus, the integrated study of these two simultaneous processes is important to understand the dynamics of production and degradation of MeHg and for future remediation management in contaminated environments. In this context, the objective of this work is to evaluate mercury methylation together with MeHg demethylation, performed by microbial communities of sediments of Tagus Estuary, using isotope enriched Hg species combined with inductively coupled plasma (ICP-MS) detection.

V.2. MATERIALS AND METHODS

V.2.1. Studied areas and sampling

Two areas of the Tagus Estuary were sampled: Barreiro - 38º40´45.40”N; 9º3´1.70”W and Cala do Norte - 38º51´21.21”N; 9º3´40.51”W. Sediment samples were collected

during spring. Sediment cores of approximately 24 cm length were collected and rapidly sliced in layers of 3 cm (Figure V.1). Samples were stored refrigerated in sealed tubes, and transported to the lab for mercury-resistant microbial communities' isolation.

V.2.2. Microbial communities' isolation

Inoculums were prepared through the dilution of sediment samples with 20 mL of distilled and sterile water. After vigorous shaking, 5 mL was taken from each suspension and added to a new tube, creating a mixture of the 24 cm sediment core (Figure V.1A). The mixture was shaken and after centrifugation at 5,000 rpm for 1 min (4ºC), 2-5 mL of supernatant was inoculated in liquid media containing 2 µg/mL Hg^{2+}. Figure V.1A schematizes the techniques used for the isolation of different Hg-resistant microbial community - Aerobic Microbial Community (AMC), Anaerobic Microbial Community (AnMC) and Sulfate-reducing Microbial Community (SO_4-RMC), which are also described below.

Aerobic community: To isolate AMC, 2 mL of washed sediment supernatant were inoculated in 20 mL of Mueller-Hinton (MH) broth and incubated under aerobic conditions. After 24 hours of growing at 37ºC, 2 mL of the inoculums were transferred to a new MH broth containing 2 µg/mL Hg^{2+} and incubated in aerobic condition.

Anaerobic community: To isolate AnMC and SO_4-RMC, 5 mL of the supernatant were inoculated in serum bottles (Belco Glass inc.) containing 50 mL of MH broth and *Postgate* C medium, respectively. Media were prepared under nonsterile conditions and added to N_2 gassed serum bottles and closed with rubber stoppers with a crimped metal seal, after which the bottled media were autoclaved. To avoid O_2 contamination, all inoculations were performed with a hypodermic syringe and needle washed with N_2 in an anaerobic chamber (with N_2 flux). After 3 days of growing at 37ºC, 5 mL of the inoculum was transferred to new bottled medium supplemented with 2 µg/mL Hg^{2+}.

All three communities were stored in the respective media (MH broth or *Postgate* C) plus 15% of glycerol containing 2 µg/mL Hg^{2+} at -80ºC.

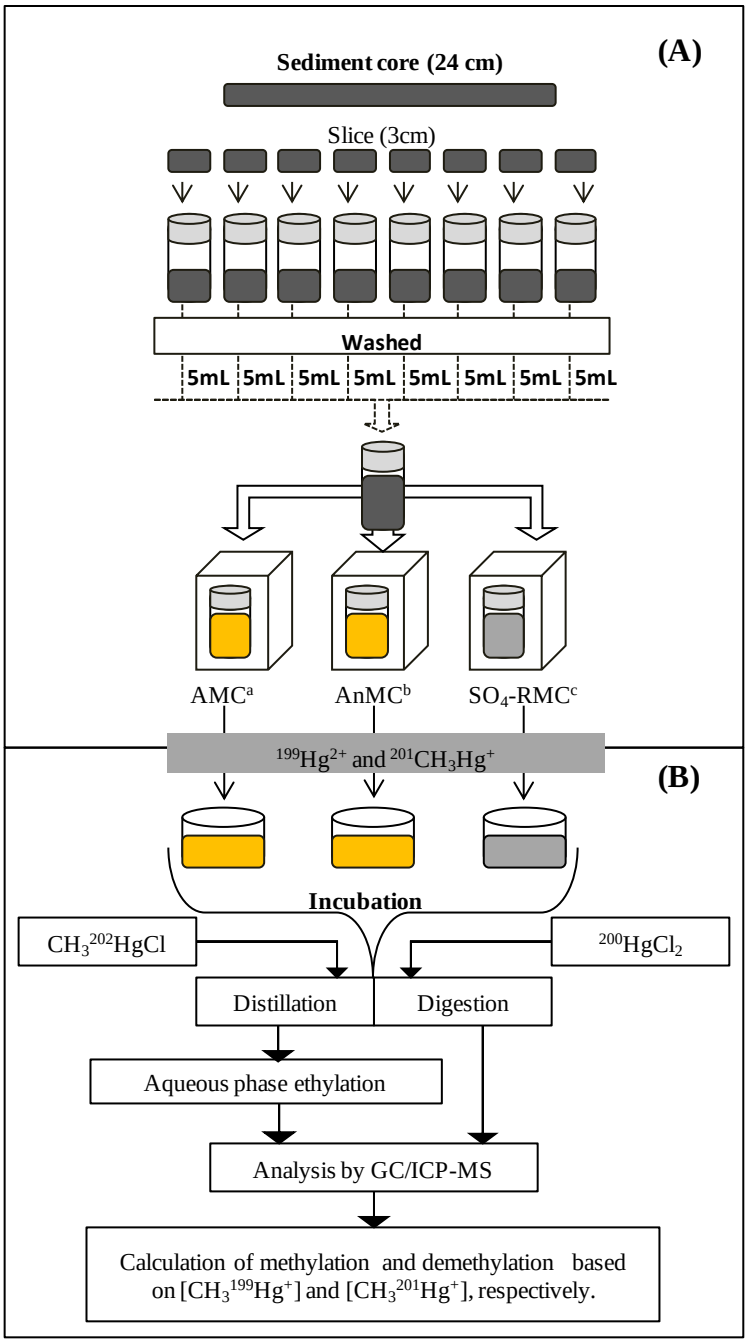

Figure V.1: Illustration of the process for the isolation of microbial communities (A) (AMC – Aerobic Microbial community, AnMC – Anaerobic Microbial Community, and SO_4-RMC – Sulfate-reducing bacteria Microbial Community) and subsequent incubation with isotope enriched Hg species to evaluate microbial potential to methylate and demethylate mercury (B). The $CH_3{}^{201}Hg$ degradation and $CH_3{}^{199}Hg$ production were monitored using GC/ICP-MS analysis.

V.2.3. Determination of mercury resistance

Mercury resistance levels of each microbial community were determined, as described before for individual bacteria (Figueiredo et al., 2014b). Mercury resistance determinations were performed for mercuric mercury ($HgCl_2$) (Riedel-de Haën) and MeHg (CH_3HgCl), (Sigma, Portugal) using nominal concentrations ranging from 0.01 to 1003 µg/mL Hg^{2+} and 0.01 to 100 µg/mL CH_3Hg^+. Determinations of mercury resistance were carried out in duplicate at each concentration tested. After incubation at 37°C for 24 hours in the dark and under aerobic and anaerobic (anaerobic jars with AnaeroGen sachet (Oxoid)) conditions, bacterial growth was monitored. The mercury resistance was registered as the lowest concentration of test compounds where there was no visible growth. All data points represent the mean ± standard deviation (STD) of 2 independent determinations (each one also performed in duplicates).

V.2.4. Mercury methylation and demethylation evaluation

Methylation and demethylation potential were evaluated simultaneously for the three isolated microbial communities as illustrated in Figure V.1B. A spike solution containing isotope enriched $^{199}HgCl_2$ and $CH_3{}^{201}HgCl$ in a proportion of approximately 100:1, was prepared (see below V.2.4.1) and added to the growth media, where the microbial communities (AMC, AnMC and SO_4-MC) were placed. After incubation, MeHg analysis was performed as described below.

V.2.4.1. Preparation of the spike solution

A stock solution of $^{199}HgCl_2$ (880 µg/mL^{199}Hg) was obtained by dissolving ^{199}Hg enriched (91.95% purity) HgO (Oak Ridge National Laboratories) in 1 mL of hydrochloric acid (10 mM). To prepare stock solution of $CH_3{}^{201}HgCl$ in toluene (800 µg/mL), ^{201}Hg enriched (96.17% purity) HgO (Oak Ridge National Laboratories) was synthesized using the methylcobalamin method (Hintelmann and Evans, 1997). $CH_3{}^{201}HgCl$ was prepared for the demethylation assay. The spike solution was prepared by adding 60 µL of ^{199}Hg stock and 15 µL of $CH_3{}^{201}HgCl$ (80 µg/mL) to deionized

water (final volume of 5 mL). Thus, the spike solution was constituted by 0.205 µg/mL of $CH_3{}^{201}Hg$ and 10.56 µg/mL of ^{199}Hg. This solution was used for the subsequent methylation and demethylation assays.

V.2.4.2. Microbial community incubation with mercury isotopes

To the overnight culture suspensions adjusted to 10^6 CFU/mL, mercury spike solution was added to achieve 0.106 µg/mL of ^{199}Hg and 0.002 µg/mL of $CH_3{}^{201}Hg$. The microbial community suspensions and controls (MH broth and *Postgate* C medium plus spike solution) were incubated at 37°C under aerobic conditions for AMC and anaerobic condition for AnMC and SO_4-RMC. Anaerobic condition were achieved using serum bottle prepared as describe above (section V.2.1 of Material and Methods). Samples were taken after 6 and 28 of microbial growth and in case of SO_4-RMC, an additional time point was taken at 48 h. After each experimental end point, the optical density was measured using absorption spectrophotometer (595 nm) and the microbial suspension was filtered using syringe filters 0.4 µm (Acrodisc) to separated supernatant for further methylmercury analysis. Two independent experiments were carried out for each experimental condition.

V.2.4.3. Analysis of MeHg

The analysis of total MeHg was performed via distillation/ethylation. MeHg was extracted from supernatant samples using water vapor distillation. Supernatant aliquots (250 µL) were transferred into Teflon distillation vials with flat bottom containing 10 mL deionized water, 200 µL KCl (20% v/v) and 500 µL 9M H_2SO_4. The samples were distillated under a nitrogen gas flow of 80 mL/min at 135ºC. The distillate was collected into Teflon distillation vials containing 5 mL of deionized water. After collection of approximately 90% of the distillate, the distillation was stopped. Blanks were prepared following the same procedure. Total MeHg was measured on the Tekran 2700 MeHg Auto Analysis System, using a method based on EPA method 1630.

The concentration of isotopes ($CH_3{}^{201}Hg$ and $CH_3{}^{199}Hg$) was quantified after gas chromatographic separation using inductively coupled plasma mass spectrometry - ICP/MS - (X-Series II ICP-MS, Thermo Fisher Scientific Inc., Waltham,

Massachusetts). In order to correct for procedural losses, $CH_3{}^{202}HgCl$ was added to the samples as an internal standard, before the distillation (Figure V.1B). The measurement procedure and the scheme to calculate the tracer concentrations are described in detail elsewhere (Ogrinc and Hintelmann, 2003). The following isotopes of Hg were measured: ^{199}Hg (Hg methylation), ^{201}Hg (MeHg demethylation), ^{202}Hg (internal standard) and ^{200}Hg (representing ambient MeHg).

V.2.4.4. Analysis of total Hg

Total mercury was determined in digested samples of supernatant using cold-vapor atomic fluorescence spectrometry (CV-AFS). To bring all the mercury into its ionic form, filtered samples were treated with an oxidant agent (0.5% of 0.2N bromine monochloride solution - BrCl) plus 0.5% HCl overnight. The digestion was stopped with the addition of $NaH_2OH.HCl$ (20µL to 40mL). To correct for procedural losses, $^{200}HgCl$ was added to the samples as an internal standard, before the digestion (Figure V.1B). To the overnight digested samples, 0.05% of 20% hydroxylamine hydrochloride in deionized water was added in order to stop the digestion.

The concentration of Hg isotopes in the digest was quantified using continuous-flow cold-vapor generation and ICP/MS detection (X-Series II ICP-MS, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The acidified sample was continuously mixed with a solution of stannous chloride 3% (w/v) in 10% HCl (v/v) by means of a peristaltic pump. The formed mercury vapor was separated from the liquid using an in-house made gas-liquid separator and the elemental mercury swept into the plasma of the ICP/MS. The following isotopes of mercury were measured: ^{199}Hg (from $^{199}HgCl$ added for methylation essay), ^{201}Hg (from $CH_3{}^{201}Hg$ added for demethylation essay), ^{200}Hg (internal standard) and ^{202}Hg (to calculate ambient total mercury).

V.2.4.5. Determination of methylation and demethylation rates

The formation of MeHg was evaluated by measuring the amount of MeHg production ($CH_3{}^{199}Hg$) from the inorganic spike (^{199}Hg) and the rate was calculate as: Methylation

(%) = ([$CH_3{}^{199}Hg$]$_{Final}$×100)/ [^{199}HgT]$_{Initial.}$ The percentage of $CH_3{}^{201}Hg$-demethylated were calculated as following: Demethylation (%) = ([$CH_3{}^{201}Hg_{Initial}$- $CH_3{}^{201}Hg_{Final}$] ×100)/ [$CH_3{}^{201}HgT$]$_{Initial}$.

V.2.5. Evaluation of microbial Hg-reduction potential

Mercury reduction and subsequent volatilization of Hg^0 was verified, according to the protocol described by François et al. (2011), with some modifications. To the overnight microbial community adjusted to 10^6 CFU/mL in MH into a 12-well microplate, $HgCl_2$ solution was added to achieve 2µg/mL Hg^{2+}. A sensitive X-ray film layer was inserted in the micro-plate followed by incubation at 37ºC in the dark for 48 hours. The Hg^0 volatilization was observed through the foggy areas on the X-ray film, due to the reduction of Ag^+ by mercury vapor (Hg^0). The optical density was measured at 595 nm (Hitachi spectrophotometer) and the cells were harvested by centrifugation at 15,300 g for 5 min with the supernatant and cell pellet separated for total mercury (HgT) analysis. Harvested cells were washed with sterile deionized water and weighed.

Determination of HgT was performed by pyrolytic reduction and atomic absorption spectrometry using a LECO AMA-254 gold amalgamator (Costley et al., 2000). The experiment was performed in duplicate with uninoculated control run in the same conditions. The percentage of reduction was calculated as: Reduction (%) = ($HgT_{Initial}$-($HgT_{Supernatant}$ + $HgT_{Cell\ pellet}$))/($HgT_{Initial}$) ×100.

V.3. RESULTS

V.3.1. Microbial community characterization

Sediments from two mercury polluted areas of the Tagus Estuary Barreiro and Cala do Norte, were sampled (Figure V.1) to isolate three microbial communities exhibiting mercury resistance: AMC, AnMC and SO_4-RMC. AMC was a group of microorganisms capable to grow in a typical microbiological medium (MH) in presence of oxygen,

while AnMC and SO_4-RMC were groups of microorganisms capable to grow in absence of oxygen. The difference between AnMC and SO_4-RMC is the medium used, whereas AnMC were grown in a typical microbiological medium (MH), SO_4-RMC were grown in a selective medium for SRB (*Postgate* C). Mercury resistance levels found for these communities were higher for SO_4-RMC (Table V.1).

Table V.1: Mercury ($HgCl_2$ and CH_3HgCl) resistance levels of the microbial communities isolated from two mercury contaminated areas of the Tagus Estuary – at Cala do Norte and Barreiro.

Sampled areas	Hg (II) (µg/mL)			MMHg(µg/mL)		
	AMC[a]	AnMC[a]	SO_4-RMC[a]	AMC[a]	AnMC[a]	SO_4-RMC[a]
Cala do Norte	10	8	50-100	2.5	0.5	2.5
Barreiro	13	50	50-100	2.5	10	2.5

[(a)]AMC: Aerobic microbial community, AnMC: Anaerobic microbial community and SO_4-RMC: Sulfate-reducing microbial community.

V.3.2. Mercury content after incubation

Figure V.2 shows the percentage of $CH_3{}^{201}Hg$ and total ^{199}Hg (% of initial) after 6h and 28h of incubation in the presence of bacteria. $CH_3{}^{201}Hg$ decreased over time, with 10-69% remaining after 28 hours. Among the three microbial evaluated communities, the highest demethylation rates were registered in AMC samples of isolated communities from Cala do Norte (Figure V.2). Total ^{199}Hg also decrease along the time (Figure V.2).

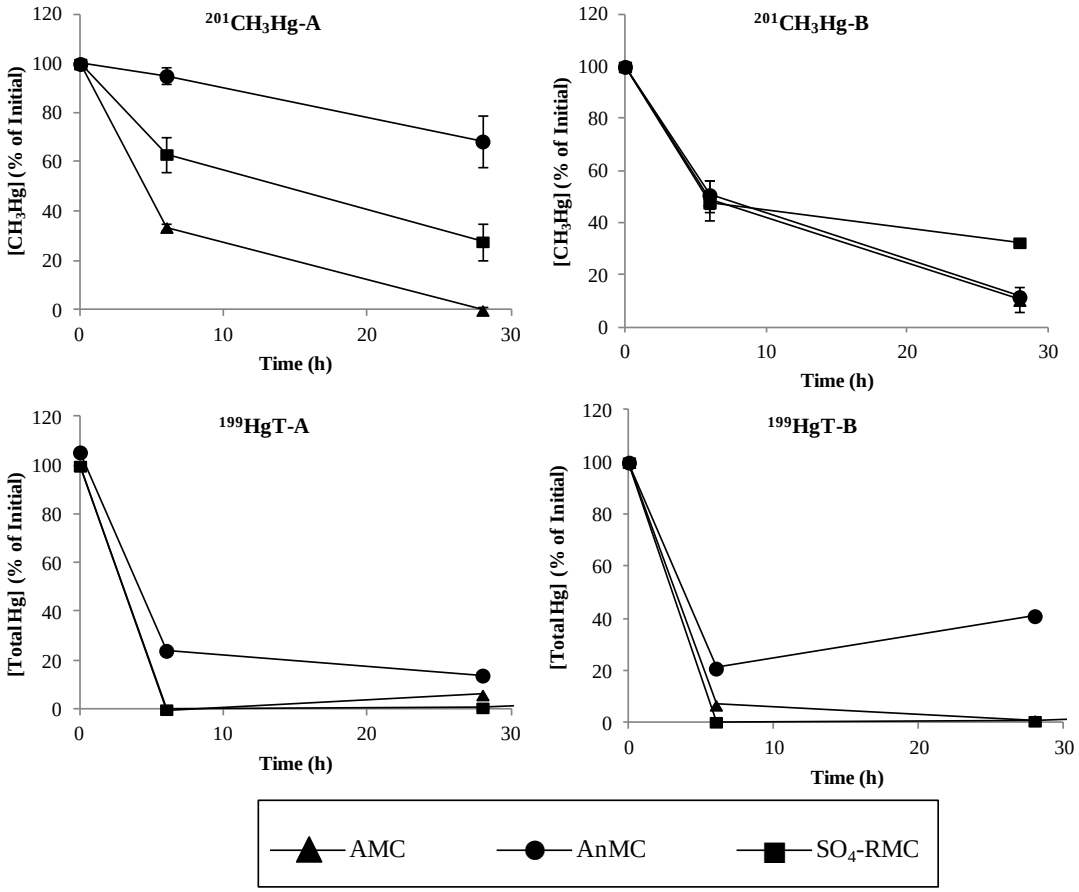

Figure V.2: $CH_3{}^{201}Hg$ and total ^{199}Hg content in supernatant samples after 6 and 28 hours of incubation of three different microbial communities of aerobic microbes (AMC), anaerobic microbes (AnMC) and sulfate-reducing microbes (SO_4-RMC) after initial addition of 2.05 ng/mL of $CH_3{}^{201}Hg^+$ and 105.6 ng/mL of $^{199}Hg^{2+}$. The three microbial communities were isolated from two areas of the Tagus Estuary in Cala do Norte (A) and Barreiro (B).

V.3.3. MeHg formation

Figure V.3 shows MeHg concentrations in liquid media samples, discriminating the methylated $CH_3{}^{199}Hg$ from ^{199}HgT. In SO_4-RMC media, between 0.02% and 0.07% of the initial ^{199}Hg (0.02-0.07 ng/mL) was methylated. Methylation was also observed among AnMC (0.01 % of the initial ^{199}Hg) (Figure V.3A). The highest percentage of methylation was registered in medium containing SO_4-RMC from Barreiro (Figure

V.3B and Table V.2); MeHg formed after 28 and 48 hours ranged from 3.47 to 13.9% of HgT determined after 6 hours (data point where $^{199}Hg^{+}$ available stabilized) (Table V.2).

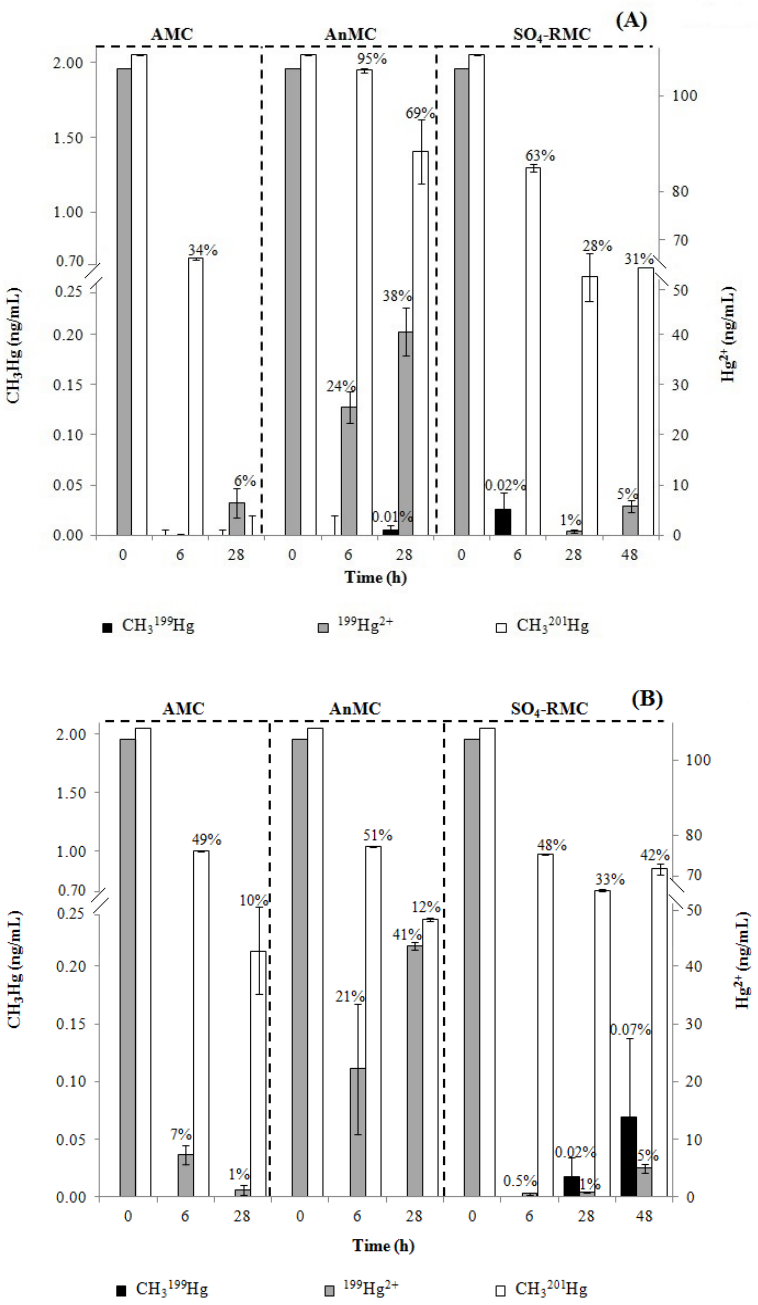

Figure V.3: Representation of isotopes concentration in supernatant of media containing different microbial communities. The formation of $CH_3{}^{199}Hg$ from the initial 105.6 ng/mL $^{199}Hg^{2+}$ added and the decrease of the initial 2.05 ng/mL of $CH_3{}^{201}Hg$, is represented at different incubation time (6, 28 and 48 hours). The three different microbial communities: aerobic microbial community (AMC), anaerobic microbial community (AnMC) and sulfate-reducing microbial community (SO_4-RMC), were isolated from two mercury contaminated areas of the Tagus Estuary – Cala do Norte (A) and Barreiro (B).

Table V.2: Percentage of MeHg formed from the isotope ^{199}Hg added to the bacterial communities isolated from the two mercury-contaminated areas of the Tagus Estuary.

Sampled areas	Samples	% of $CH_3{}^{199}Hg$ from ^{199}Hg Spike	
		% of HgT initial[(a)]	% of HgT 6h[(b)]
Cala do Norte	SO_4-MRC* 6h	0.02	-
	AnMC* 28h	0.01	0.02
Barreiro	SO_4-MRC* 28h	0.02	3.47
	SO_4-MRC* 48h	0.07	13.9

[(a)] Initial ^{199}Hg added - 105.6 ng/mL;

[(b)] % of HgT 6h were calculated using the total ^{199}Hg determined at 6 hours for the same sample;

[*] SO4-MRC – sulfate-reducing microbial community, and AnMC – anaerobic microbial community

V.3.4. MeHg degradation

The $CH_3{}^{201}Hg$ concentration decreased during incubation (Figures V.2-V.4). This decrease was more accentuated in media containing AMC of Cala do Norte, where only 0% to 10% of the initial $CH_3{}^{201}Hg$ added to media remained after 28 h. Figure V.4 shows the percentage of ^{201}Hg demethylated over time. In the non-inoculated control, a decrease in $CH_3{}^{201}Hg$ concentration was also observed for aerobic control (AMC) as a consequence of abiotic demethylation, however to a lesser extent than the inoculated homologous (Figure V.4C). No decrease in $CH_3{}^{201}Hg$ in control media of AnMc and SO_4-RMC was observed (Figure V.4C).

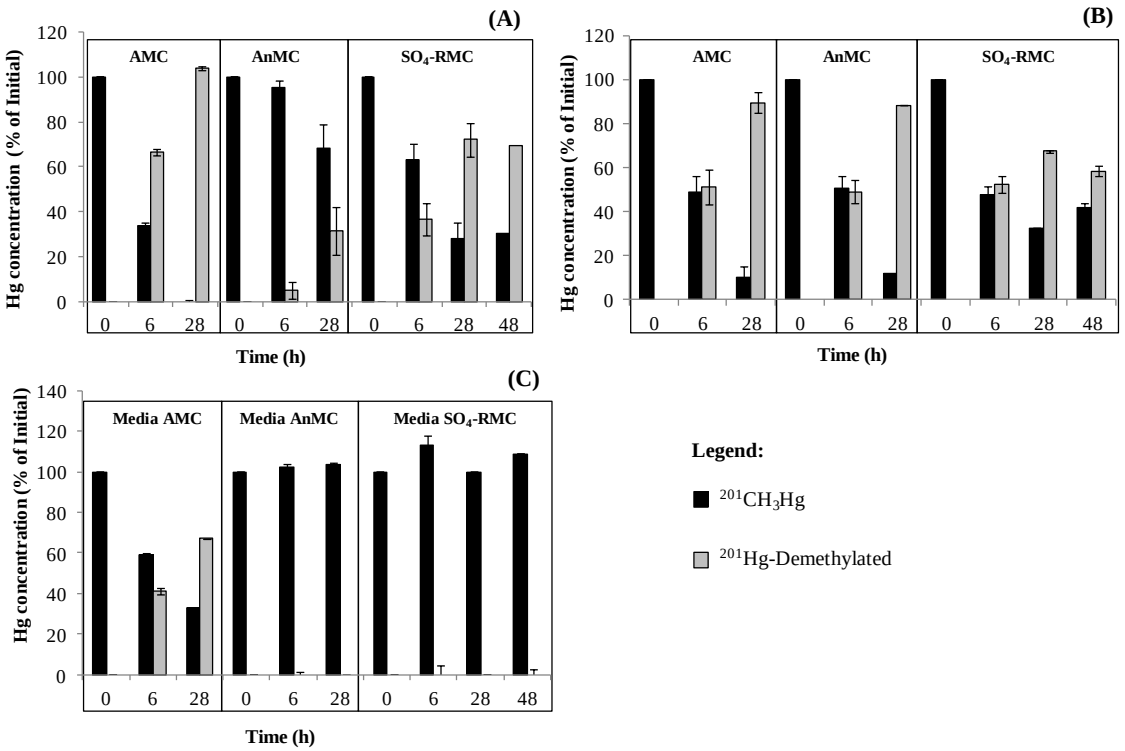

Figure V.4: Concentration of $CH_3{}^{201}Hg$ after 6, 28 and 48 hours of incubation with three microbial communities of aerobic microbes (AMC), anaerobic microbes (AnMC) and sulfate-reducing microbes (SO_4-RMC) isolated from Cala do Norte (A) and Barreiro (B). The control media were also evaluated (C).

V.3.5. Hg^{2+}-reduction and Hg^0-volatilization

Figure V.4 shows that in the presence of Hg^{2+}, both aerobic and anaerobic communities were able to remove mercury from liquid media by cell uptake and also by the reduction Hg^{2+} with subsequent volatilization of Hg^0. The percentage of Hg^{2+} reduced was higher among aerobes (40 and 49 % by aerobes and 16 and 37% by anaerobes of Cala do Norte and Barreiro, respectively) (Figure V.5).

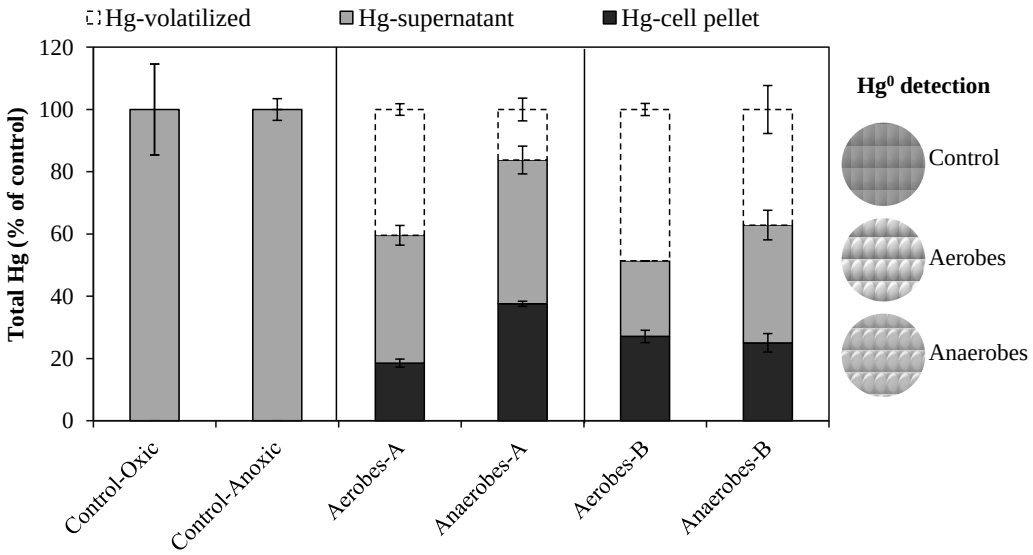

Figure V.5: Final balance of total mercury after incubation of aerobic and anaerobic microbial communities isolated from two areas of the Tagus Estuary (A – Cala do Norte and B – Barreiro) with $HgCl_2$ during 48 hours. The graphic representation shows the percentage of Hg^0 volatilized and total mercury remained in cell pellet and supernatant). Hg^0 volatilization was detected by the foggy area resulted from the reaction between Hg^0 and Ag, using an X-ray film.

V.4. DISCUSSION

MeHg net concentrations in aquatic environment depend on methylation/demethylation processes, mostly occurring in sediments. Several authors investigated these processes using sediments and pure cultures of isolated microorganisms under anaerobic and aerobic conditions (Hintelmann et al., 2000; Martín-Doimeadios et al., 2004; Heyes et al., 2006; Kerin et al., 2006; Hamelin et al., 2011; Figueiredo et al., 2016). The objective of our work was to investigate communities of bacteria, which is a more natural situation condition and establish the contribution of aerobic, anaerobic and SRB microbial communities for the processes of mercury methylation and demethylation.

Methylation of mercuric mercury (i.e. $CH_3{}^{199}Hg$ production) was observed in media containing anaerobes, especially SRB (Figure V.3). Although, methylation was

observed previously for a few aerobic bacteria isolated from the Tagus Estuary (Figueiredo et al., 2016), the study of the aerobic community did not detect Hg methylation, suggesting that this type of bacteria does not significantly contribute to MeHg formation.

The highest percentage of observed MeHg formation ranged from 0.02% to 0.07% of the initially $^{199}Hg^{2+}$ added (105.6 ng/mL) to the media containing SRB from Cala do Norte and Barreiro, respectively (Figure V.3). Likewise, other groups (Barkay and Wagner-Dobler, 2005; Batten and Scow, 2003), also reported that methylation is a process promoted by anaerobes and that over 95% of the mercury methylation occurs in anoxic sediments (Compeau and Bartha, 1995), pointing out sulfate reducers as the main methylators (Heyes et al., 2006; King et al., 2000).

The observed methylation can be related to the presence of *Desulfovibrio desulfuricans* and *Clostridium* sp., namely *Clostridium difficile*, which we identified among anaerobes (data not shown) and that are well-known for their methylation potential (Achá et al., 2012; Compeau and Bartha, 1995; King et al., 2000; Pak and Bartha, 1998; Pan-Hau and Imura, 1982; Spangler et al., 1973).

Comparing the two sampled areas, data show that the percentage of methylation was higher in Barreiro (0.07%) than in Cala do Norte (0.02%). Possible explanations for this observation may be differences in bacteria species composition related to the higher and long-term mercury contamination in Barreiro causing a selective pressure for methylators. On average, sediments of Cala do Norte have 11.7 µg/g total Hg, including 28.4 ng/g of MeHg, and sediment of Barreiro have 33.2 µg/g total Hg, including 47.2 ng/g of MeHg (Figueiredo et al., 2014a). Applying the rate of methylation observed in this study (mentioned above) to ambient field concentrations of total Hg, up to 2.34 ng/g day^{-1} and 11.62 ng/g day^{-1} of MeHg could originate from microbial methylation in Cala do Norte and Barreiro, respectively. It is important to stress that in the Estuary physic-chemical conditions may differ from the laboratory, therefore, these estimates, still need confirmation in the field.

Demethylation was observed for all inoculated media, indicating that demethylation is very common among both, aerobes and anaerobes (Baldi et al., 1993; Oremland et al.,

1991; Pak and Bartha, 1998) and that both biotic and abiotic mechanisms may be involved (Martín-Doimeadios et al., 2004). Comparing the proportion of mercury methylation and demethylation in the present study, it is clear that in estuarine sediments containing CH_3Hg^+ and Hg^{2+}, microorganisms are responsible for CH_3Hg^+ demethylation occurring in oxic and anoxic sediments and for methylation taking place in anoxic sediments.

The analyses of ^{199}HgT (Figure V.2) revealed that there is also a removal of Hg^{2+} from liquid media along the time of incubation with the microbial communities, being this removal between 59-99% of ^{199}HgT, after 28 hours. This removal may be explained as a result of two mechanisms – cell uptake and/or reduction of Hg^{2+} into Hg^0 and its subsequent volatilization from medium. Both phenomena, i.e. cell uptake and reduction followed by volatilization, were previous observed among individual microorganisms, such as *Bacillus*, *Vibrio*, *Aeromonas* and *Enterobacteriaceae* (Figueiredo et al., 2014; 2016), and also here for aerobic and anaerobic communities (Figure V.4) isolated from sediments of the Tagus Estuary.

V.5. CONCLUSIONS

Overall, microbial communities of mercury contaminated sites of the Tagus Estuary are performing both methylation of mercuric mercury species and demethylation of MeHg, being demethylation the predominant process. Besides demethylation, microbial communities in the Tagus Estuary are also capable of removing Hg^{2+} from the aquatic environment which is important for bioremediation of the Estuary.

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CHAPTER VI

OPTIMIZATION OF MICROBIAL DETOXIFICATION FOR AN AQUATIC MERCURY-CONTAMINATED ENVIRONMENT†

†Figueiredo et al., 2016 (*In prep to be submitted*)

ABSTRACT CHAPTER VI

Mercury reduction performed by microorganisms is well recognized as a biological way for the remediation of contaminated environment. Recently, we found that mercury resistant microorganisms of Tagus estuary are involved in mercury reduction processes. In the present study, aerobic microbial community isolated from a highly mercury-contaminated area of Tagus was used to study the optimization of the reduction process in conditions similar to the contaminated ecosystem. Factorial design methodology was used to study the effect of glucose, sulfate, iron and chloride on mercury reduction. In the presence of several concentrations of these elements, microbial community reduced mercury in a range of 37-61% of the initial 0.1 mg/mL of Hg^{2+}. The response prediction through central composite design showed that the increase of sulfate concentration leads to an optimal response in mercury reduction by microbial community, while the increase of chloride decreases mercury reduction sharply. Iron can have antagonistic effects depending on the media composition. These results are important for bioremediation strategies planning.

VI.1. INTRODUCTION

Mercury is a toxic metal dispersed throughout the ecosystems. Environmental contamination by mercury is caused by both natural and anthropogenic sources. The natural sources include volcanic activity, erosion of sediments containing mercury and gaseous emissions from the earth's crust (Yu, 2005). However, the majority of mercury comes from anthropogenic sources, such as mining, combustion of fossil fuels, usages in agriculture and industries (Yu, 2005). In addition, mercury and mercurial compounds usage in industry, especially the chloro-alkali industry, are the main reasons for the severe mercury pollution in aquatic systems and soils (Chang and Law, 1998; Wagner-Dobler, 2003).

Due to its high mobility, mercury discharged into the environment disperses widely and undergoes complex physical, chemical and biological transformations (Chang and Law, 1998; Wagner-Dobler, 2003). Among the transformations, methylation of Hg^{2+} with methylmercury (MeHg) formation is a process mainly mediated by microorganisms. Due to the bioacummulation and biomagnification of the neurotoxic MeHg in the food chain, it represents a risk to fish consumers (Barkay and Wagner-Dobler, 2005; Clarkson, 2002; Wagner-Dobler, 2003).

In Portugal, the Tagus Estuary has been reported to be contaminated with mercury since 1985, as a result of industrial activity (Figuères et al., 1985; Canário et al., 2003, 2005, 2007). High levels of mercury have been found in sediments, suspended matter and water (Canário et al., 2003, 2005, 2007; Figueiredo et al., 2014a,b). Inventories in sediments have estimated 21 tons of total mercury and 23 Kg of MeHg in the estuary (Canário et al., 2005). Regardless of the inactivation of the most critical industrial units located in north (Cala do Norte) and south (Barreiro) margins, mercury released in the past is still a threat to aquatic organisms, animals and human populations.

The environmental contamination by mercury remains a major concern worldwide. Efforts to reduce mercury level in industrial waste waters using various technologies are underway (Sinha and Khare, 2011). Conventional processes to reduce mercury levels involve physical and chemical approaches, such as carbon adsorbents, ion exchange,

reverse osmosis, precipitation and electrochemical treatment (Chiarle et al., 2000; Dabrowski et al., 2004; Sinha and Khare, 2011; Yardim et al., 2003; Zhang et al., 2005). These techniques are usually expensive, non-specific and inefficient at low mercury concentrations and sometimes generate hazardous by-products (Chiarle et al., 2000; Manohar et al., 2002; Zhang et al., 2005). To overcome these drawbacks, biological processes have been considered as alternative approaches that combine low cost and better efficiency (Wagner-Dobler, 2003).

Several studies have been made to exploit the application of microbial cell to remediate mercury (Chien et al., 2010; Das et al., 2007; De et al., 2008; Essa et al., 2002; Glendinning et al., 2005; Oremland et al., 1991; Pepi et al., 2011; Sadhukhan et al., 1997; Siciliano et al., 2002; Sugio et al. 2003; Wagner-Dobler, 2000; Wiatrowski et al., 2006; Zhang et al., 2012). The potential application of microorganisms in mercury remediation is related to enzymatic reduction of Hg^{2+} and MeHg degradation (demethylation), bioprecipitation of insoluble mercurial forms (such as HgS), biosorption and intracellular accumulation. Although all these processes are promising, the cell adsorption and accumulation produces a large volume of mercury-loaded biomass (Nascimento and Chartone-Souza, 2003) being together with bioprecipitation more suitable for industrial applications then for the environmental context. On the other hand, the enzymatic transformations can be used in both applications since they promote the detoxification of mercury through the reduction of Hg^{2+} into volatile Hg^{0} (Hg-reduction), which passively diffuses out of the cell and its environment (Nascimento and Chartone-Souza, 2003).

In contaminated environments, microorganisms have developed resistance to mercury mediated by enzymes encoded by the *mer* operon genes (Barkay and Wagner-Dobler, 2005). This genetic system confers resistance to inorganic and sometimes also to organomercurial compounds, depending on the transformation enzymes that are encoded. *MerA* gene encodes for mercuric reductase that reduces Hg^{2+} to Hg^{0} whereas *merB* gene encodes for organomercurial lyase that breaks the carbon-mercury (Hg-C) bond, thus, releasing Hg^{2+} and CH_4 (Barkay and Wagner-Dobler, 2005; Nascimento and Chartone-Souza, 2003; Schaefer et al., 2004; Parks et al., 2009).The potential use of the *mer* system for bioremediation purposes has been recognized since 1984 (Barkay and

Wagner-Dobler, 2005), due to its high level of efficacy and specificity (Nascimento and Chartone-Souza, 2003).

The application of mercury-resistant bacteria with Hg-reduction capacity on mercury remediation in the Tagus Estuary has also been considered (Figueiredo et al., 2014b, 2016). The current work describes the optimization of Hg-reduction potential exhibited by aerobic community, using conditions that mimicry the Tagus Estuary environment. Therefore, the entire aerobic microbial community was used and water collected from the estuary was used as growth medium. Factorial design methodology was used to optimize the community reduction potential. This approach allows obtaining information about the influence of the selected factors as well as their interactions on the mercury reduction with fewer experiments (Box, 1995). The factors were chosen either for their importance on mercury cycling and on bacterial growth and the basal concentrations corresponded to field conditions in the Tagus Estuary. Therefore, the effects of glucose, sulfate, iron and chloride on microbial reduction were evaluated.

VI.2. MATERIALS AND METHODS

VI.2.1. Culture

The culture used in this study was an aerobic microbial community isolated from sediment of a highly contaminated area of the Tagus Estuary - Barreiro (Lat: 38º40´45.40”N; Long: 9º3´1.70”W). This microbial community exhibits resistance to mercurial compounds (minimal inhibitory concentration: 13 μg/mL Hg^{2+}).

VI.2.2. Growth media and chemicals

Microorganisms were isolated in Mueller-Hinton (MH) broth. The optimization of microbial Hg-reduction was performed in water collected from Rosário, a moderate mercury-contaminated site of the Tagus Estuary (38°40'15.42"N; 9°0'45.07"W). After

the sterilization process, the absence of viable microorganisms in the water was confirmed through the inoculation of an aliquot in MH agar. Non visible growth was detected in the plate after 10 days of incubation.

Mercury content in the water media was evaluated, and was found to be 1.2±0.0005 ng/mL before sterilization and 1.0±0.0000 ng/mL after sterilization. Sulfate, iron, and chloride and dissolved organic carbon (DOC) in estuary' water were evaluated using methodologies described before by Canário et al. (2003 and 2008). DOC content was 20.3 mg/L and the content in sulfate, iron and chloride was 10 mM, 0.01 μM and 125 mM, respectively.

D(+)-Glucose anhydrous (Scharlau), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Merck), iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) (Merck) and sodium chloride (NaCl) (Merck) were used in this study as the sources of glucose, sulfate, ferrous iron and chloride, respectively. The mercury was used as $HgCl_2$ (Riedel-de Haën) (99.9%).

VI.2.3. Preliminary evaluation of microbial Hg-reduction potential

Mercury reduction and subsequent volatilization of Hg^0 was verified, according to the protocol described by François et al. (2011), with some modifications. Overnight microbial community was adjusted to 10^6 CFU/mL in Mueller-Hinton (MH) broth into a 12-well microplate. $HgCl_2$ solution was added to achieve 2 μg/mL Hg^{2+}. A layer of sensitive X-ray film was inserted in the micro-plate followed by incubation at 37ºC in the dark. The observation of foggy areas on the X-ray film, corresponding to the reduction of Ag^+ by mercury vapor (Hg^0), was interpreted as a positive result for Hg^0 volatilization. After 48 hours of incubation, the optical density was measured at 595 nm (Hitachi spectrophotometer) and the cells were harvested by centrifugation at 15,300 g for 5 min being the supernatant and cell pellet separated for further analysis of total mercury (HgT). Harvested cells were washed with sterile deionized water and weighed. HgT determination was performed by pyrolytic reduction and atomic absorption spectrometry using a LECO AMA-254 gold amalgamator (Costley et al., 2000), a method that is very effective and suitable for non-acidic samples. The experiments were

performed in duplicate with uninoculated control runs in the same conditions. HgT values obtained were used to calculate the percentage of reduction:

Reduction (%) = ($HgT_{Initial}$- ($HgT_{Supernatant}$ + $HgT_{Cell\ pellet}$))/($HgT_{Initial}$) ×100.

VI.2.4. Factorial design methodology

To study the effects of glucose, sulfate, iron and chloride in the reduction of mercury from media containing the mercury-resistant microbial community, a 2^4 factorial design was planned (Appendix 1), accommodating 4 variables, each one at two levels (-1/+1) comprising 16 experiments. The design was further expanded to a central composite design (CCD) by the introduction of the extreme levels -2/+2 (Barker, 1985). Besides this, a medium point was also considered (level 0) and 6 independent replicates were run in order to estimate the standard deviation that is assumed to extend to all the experiments. The different factors and their concentrations at each level are shown in Table VI.1.

Microbial community suspensions were adjusted to 10^8 CFU/mL in 5 mL of sterile water collected from Rosário plus 0.1 µg/mL Hg^{2+}, and to them aliquots of D(+)-Glucose anhydrous, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and NaCl solutions were added, to achieve the concentrations for experiments.

The microbial suspensions and uninoculated controls were incubated at room temperature. After 16 hours of incubation, the optical density was measured using absorption spectrophotometry (595 nm) (Hitachi), then the supernatant and the cell pellet were prepared for HgT determination as described above. Microbial Hg-reduction potential was also confirmed in sterile water in the same conditions referred above without the addition of glucose, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and NaCl, solutions. All the experiments were performed in polypropylene tubes (Sarstedt) to prevent mercury adsorption.

The experimental data was then submitted to the algorithm of Yates to calculate the effects of each factor and important interactions (Box et al., 1985). Besides the

algorithm the experimental data were also used to calculate the coefficients of a second-order polynomial equation in order to predict the response in relation to factor variations. Through the application of this equation, two variables can be represented at the same time in response surfaces while others are kept constant. The correlation between the experimental results and the modeled results (CCD) was R = 0.90. See more details in Appendix 1.

Table VI.1: Parameters studied for the optimization of Hg^{2+}-reduction. Some parameters were kept constant: room temperature (≈ 20°C) and no stirring.

Factor	Level				
	-2*	**-1**	**0**	**+1**	**+2**
1. Glu (mM)	**0**	0.5	1	1.5	2
2. SO_4 (mM)	**10**	14	18	22	26
3. Fe (µM)	**0.01**	0.61	1.01	1.61	2.01
4. Cl (mM)	**125**	127	129	131	133

**Level -2 represents the baseline concentrations, without any addition of the compounds.*

VI.3. RESULTS

VI.3.1. Mercury reduction

The microbial community promoted the reduction of Hg^{2+} into Hg^0 as seen through the visualization of foggy areas on the X-ray film (pictures not shown). The percentage of reduction was tested by using water from the Tagus Estuary after sterilization, being 40% Hg^{2+} reduced from the initial 0.1µg/mL, while in the control it was only 4%.

The effects of 4 parameters: 1 - glucose, 2 - sulfate, 3 – ferrous iron and 4 - chloride, over mercury reduction, was studied through a 2^4 factorial design. Table VI.2 shows the effects estimated of each individual factor as well as of their interactions on mercury reduction by microbial community. These effects represent the change in the response when the

concentration of a factor was moved from –1 to +1, being the levels of each parameter studied as indicated in Table VI.1. All parameters affected the mercury reduction potential by microbial community (Table VI.2) as explained in detail in sections VI.3.2 and VI.3.3 as well as mercury cellular uptake and cell growth (section VI.3.4).

Table VI.2: Estimated effects of each factor (1 – glucose, 2 – sulfate, 3 – ferrous iron and 4 – chloride) and their correspondent interactions (combining pairs of all factors).

Parameters[b]	Effects estimation[a] for:		
	Hg-reduction (% of initial)	**Hg-Cell[c] (% of initial)**	**Microbial growth (cel/mL)**
Average	44.70	41.40	3.25E+08
1	-0.68	-0.29	2.40E+06
2	0.05	0.48	-1.38E+07
3	4.93	-3.46	-8.40E+06
4	-90.77	-69.13	-4.81E+08
12	-46.29		
13	-40.03		
14	-90.99	-	-
23	-39.33		
24	-89.56		
34	-83.76		

[a]*Effects calculated according to the algorithm of Yates;*
[b]*Parameters: 1 – glucose; 2 – sulfate; 3 – ferrous iron; and 4 –chloride.*
[c]*Hg-cell – mercury associated to cellular fraction, including mercury adsorbed to cells and inside the cells.*

VI.3.2. Factors with positive effects on mercury reduction: sulfate and iron

The interaction between sulfate and chloride showed a range of mercury reduction between <10-100% (Figure VI.1A). The reduction is enhanced with the increase of sulfate, even when chloride is at high concentration. The optimal conditions are achieved when the concentration of sulfate is high (26 mM) and chloride is low (125 mM) (Figure VI.1A).

The interaction between ferrous iron with chloride shows a reduction ranging from 0-54%, being that values up to 10% were observed when the chloride concentration was high and when the ferrous iron concentration was low (Figure VI.1B). The highest reduction (54%) takes place when chloride concentration is low and [Fe] ≤ 1.01µM. This reveals that despite iron negative effects, the increase in iron counteracts the detrimental effects of chloride at high concentrations (Figure VI.1B).

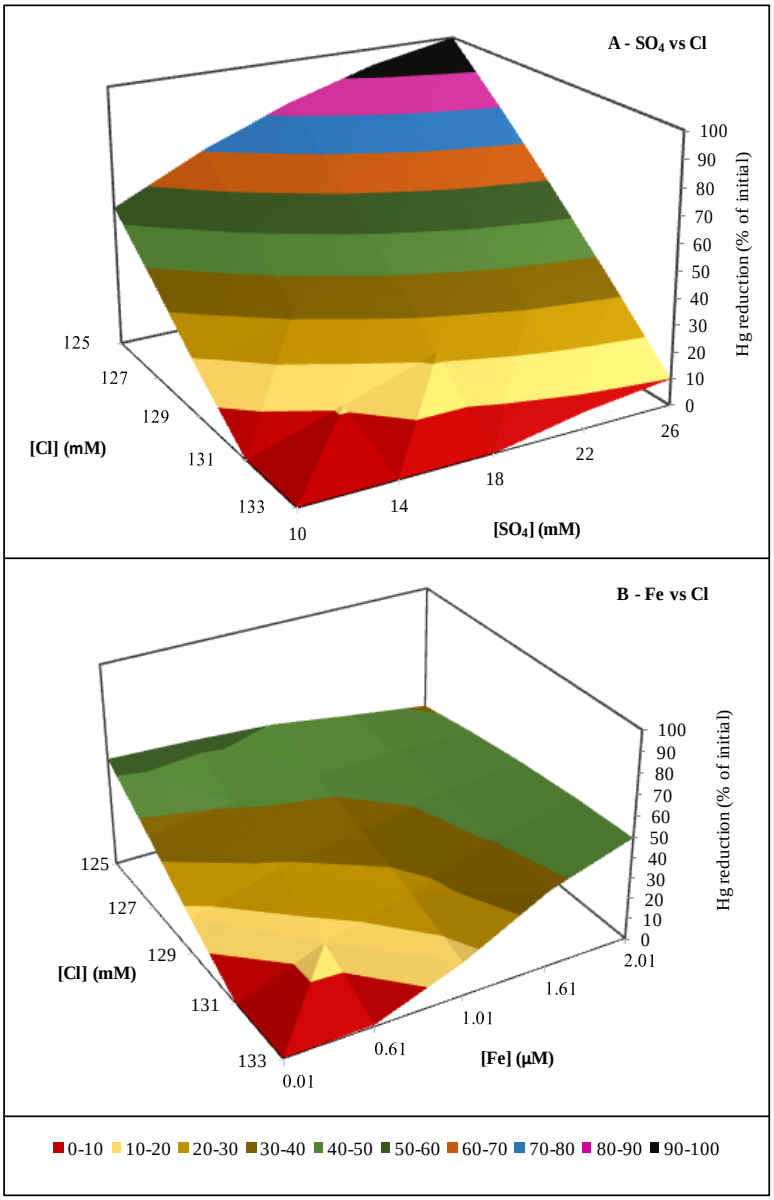

Figure VI.1: Effect of sulphate and chloride concentrations (A) and ferrous iron and chloride concentrations (B) on microbial reduction of Hg^{2+}. (A) $[Fe^{2+}]$ = 0.01µM without glucose addition and (B) $[SO_4^{2-}]$ = 10 mM in the absence of glucose.

Figure VI.2 shows the interaction between sulfate and ferrous iron, when glucose and chloride concentrations are kept at the levels found in the Tagus Estuary. It can be visualized that high concentrations of both sulfate and ferrous iron drastically decrease the reduction of mercury (Figure VI.2). The surface response shows an increase in reduction over 60% when [Fe] ≤ 0.61 µM and [SO_4] ≥ 14 mM and the optimal condition (OC) is achieved when [SO_4] ≥ 22 mM and [Fe] = 0.01 µM (Figure VI.2).

Thus, these observations show that the optimization of mercury reduction in the Tagus Estuary involves the increase of sulfate concentration ≥ 22 mM, while ferrous iron, chloride must be maintained at natural ambient levels.

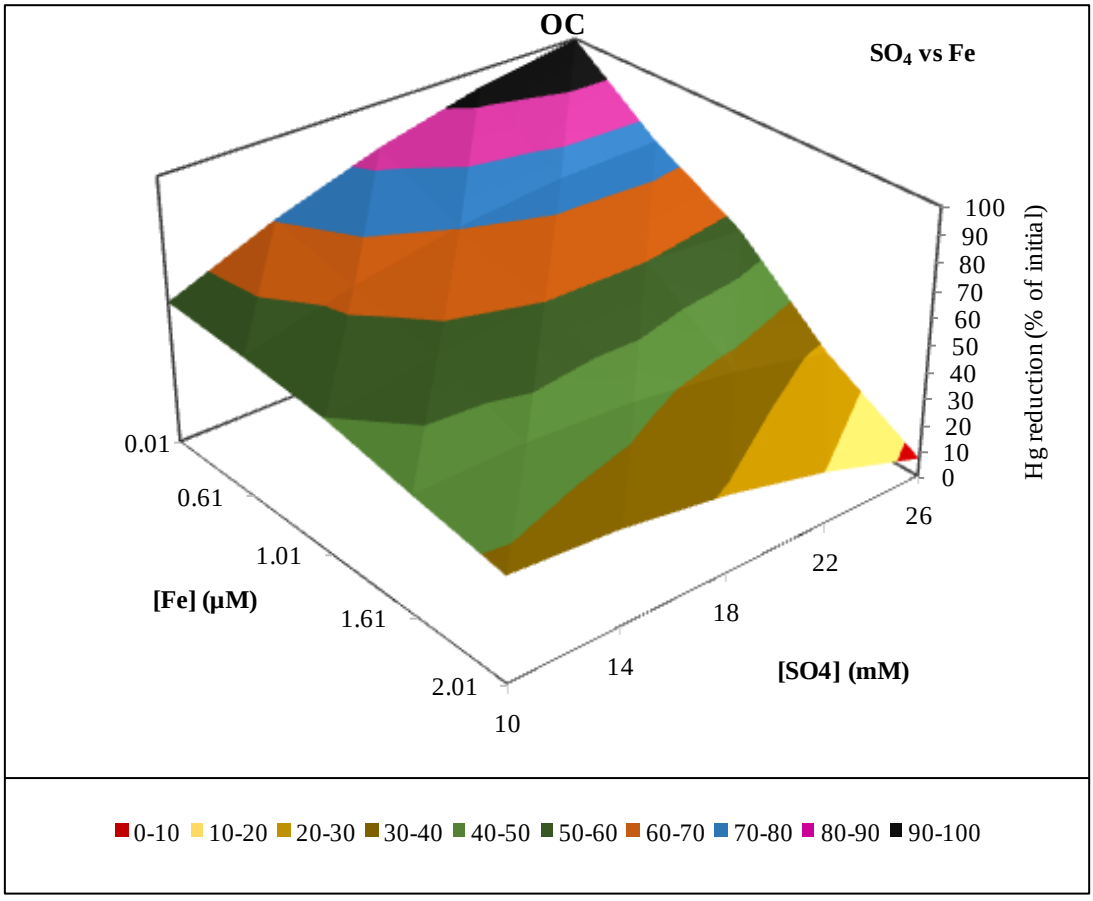

Figure VI.2: Effect of sulfate and iron concentrations on microbial reduction of Hg^{2+}. Chloride concentration was 125 mM and no glucose was added to water media. OC – Optimal condition.

VI.3.3. Factors with negative effects on mercury reduction: glucose and chloride

The effect of glucose over mercury reduction by microbial community was studied using concentrations ranging between 0-2 mM, being this amount added to the DOC (20.3 mg/L) already existing in the Tagus Estuary water. The main objective of this evaluation was to predict the effect of organic matter on the microbial reduction. As shown in Table VI.2, glucose had a moderate negative effect on the mercury reduction by microbial community. However, its interaction with others factors shows negative effects, especially with chloride (14) (Table VI.2). The detailed study of the interaction between glucose and chloride is shown in Figure VI.3. It can be seen that the highest mercury reduction rate (54%) occurs when both glucose and chloride are in the lowest concentrations (Figure VI.3). These results show that glucose addition does not improve by itself the microbial reduction potential.

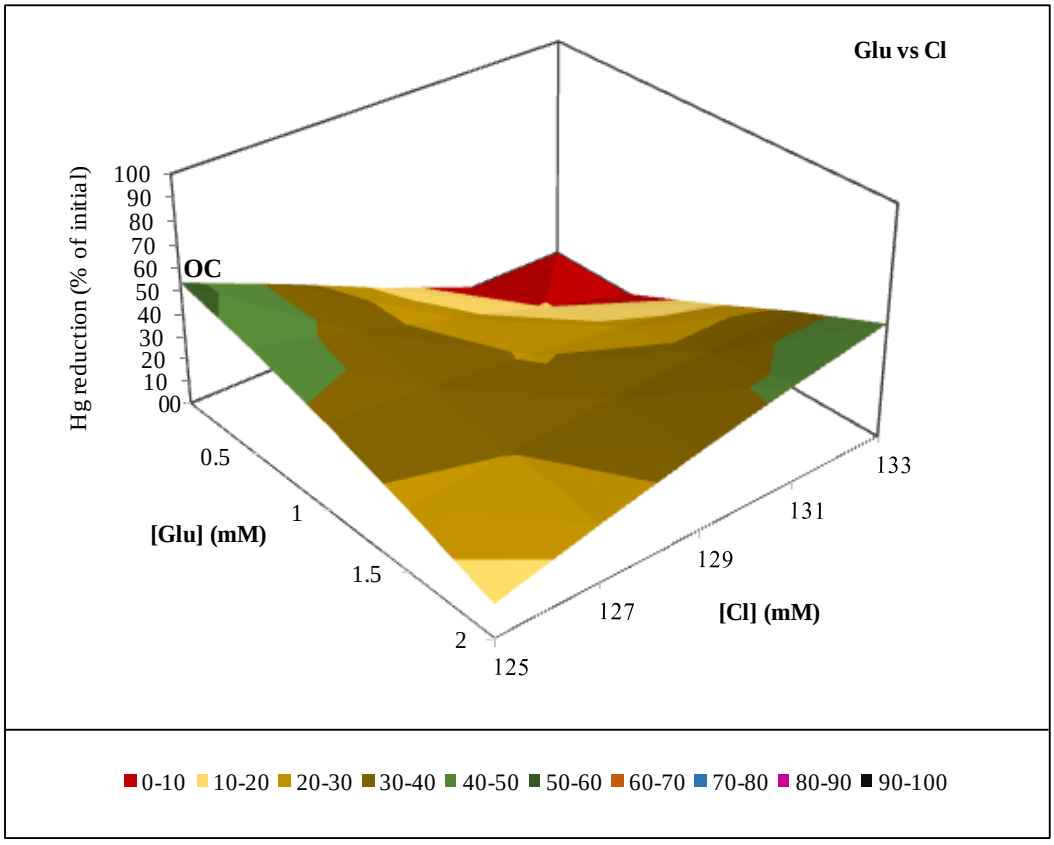

Figure VI.3: Effect of glucose and chloride concentrations on microbial reduction of Hg^{2+}, maintaining sulfate at 10 mM and ferrous iron at 0.01 µM. OC – Optimal condition.

Chloride is the compound at higher concentration in the Tagus Estuary, comparing with sulfate and iron. Comparatively to all others studied parameters, chloride increase exhibited the greatest negative effect over Hg-reduction (Table VI.2 and Figures VI.1 and VI.3). This inhibitory effect was observed in every response surface involving chloride, being that small increases in the chloride concentration decrease the mercury reduction potential by microbial community below 40% (Figures VI.1A, VI.1B and VI.3).

Overall, Figure VI.3 shows that when sulfate and ferrous iron are maintained in environmental levels, an increase on glucose and/or chloride concentrations reduces mercury reduction by microbial community. Thus, this result stressed that for the optimization of mercury reduction in the Tagus Estuary, it is important to maintain the concentrations of simple dissolved carbon sources (such as glucose) and chloride in the ambient levels.

VI.3.4 Effects on Mercury uptake and cellular growth

To better understand the effects of the parameters studied in mercury concentration associated to microbial cell fraction (Hg-cell), which includes mercury adsorbed to cells and mercury internalized by cells, and in microbial growth were also estimated (Table VI.2). Regarding mercury associated to the cellular fraction, only sulfate had a positive effect, while glucose, iron and especially chloride showed negative effects (Table VI.2).

Microbial growth was also positively and negatively affected; glucose was responsible for the positive effect while sulfate, iron and mostly chloride showed detrimental effects as indicated in Table VI.2.

VI.4. DISCUSSION

Microbial sulfur and iron cycles likely control the reactivity of inorganic mercury by changing its speciation (Slowey et al., 2007). Our results indicate that sulfate enhances Hg-reduction by the microbial community. This may be explained based on the fact that sulfate complexes quite slowly with Hg^{2+} thus, not interfering with its bioavailability. Another hypothesis may be related to the Hg^{2+} uptake by microbial cells, as sulfate can reduce the electrostatic repulsion caused by the positive charge that Hg^{2+} founds in the environment (Kim et al., 2004). This hypothesis was tested for Hg^{2+} uptake by mineral sorbents, such as goethite, bayerite and γ-alumina (Kim et al., 2004). The mild positive effect that ferrous iron showed in some circumstances on mercury reduction may be related with its oxidation (Slowey et al., 2007). Ferrous iron can be abiotically or biologically oxidized, namely some microorganisms such as *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, Sulfolobus* spp., *Acidianusbrierleyi, Sulfobacillus thermosulfidooxidans, Gallionella ferruginea* and *Leptothrix* spp., can oxidize ferrous iron enzymatically, when their metabolic activities alter the microenvironment (Gadd et al., 2010). Mercury reduction in iron-oxidizing bacterium has been observed for *Acidithiobacillus ferrooxidans*, involving cytochrome c and with Fe^{2+} as electron donor (Sugio et al., 2006, 2008; Iwahori et al., 2000). Regardless some positive effects of ferrous iron, at high concentrations it affects mercury reduction negatively (Figures VI.1). This may be related with the formation of hydrous iron oxides, since its formation in aqueous environments may cause coprecipitation of metal ions (Gadd et al., 2010).

Glucose enhances the microbial growth (Table VI.2); however, the increase of its concentration in the presence of low concentration of chloride does not increase mercury reduction. This can be justified since glucose forms a very stable complex with Hg^{2+} (Daoud et al., 2012), which may affect mercury availability for reduction. Accordingly, the analysis of effects estimation for mercury concentration associated to cell fraction shows that glucose slightly affects it, which may be related with a decrease in mercury uptake by cell. Another explanation may rely on the fact that the enhancement of microbial growth does not mean an increase in reduction potential, if the amount of mercury available for this purpose is the same.

Emphasis should also be given to the fact that glucose being a good source of energy for bacterial growth is substantially different from the complex dissolved organic carbon components that mostly originate from humic matter existing in the natural environment. Therefore, this source of carbon might be replaced in future studies.

Chloride increase exhibited the greatest negative effect over mercury reduction. Chloride may interfere with mercury uptake by microbial cell as it forms stable $HgCl_2$ complexes. Furthermore, Kim et al. (2004) concluded in their study that high concentration of chloride (≥1 mM) may facilitate photoreduction of Hg^{2+} to Hg^{+} and the formation of $Hg_2Cl_{2(s)}$ or $Hg_2Cl_{2(aq)}$, which interferes with mercury bioavailability.

Overall, the optimization of mercury reduction in the Tagus Estuary involves the increase of sulfate concentration ≥ 22 mM, while ferrous iron, chloride and simple dissolved carbon sources (such as glucose) concentrations must be maintained in the ambient levels.

VI.5. CONCLUSIONS

The present study evaluated the optimal conditions for the mercury reduction process using the isolated aerobic microbial community of a highly polluted area of the Tagus Estuary. The data obtained showed that sulfate and to a lesser extent ferrous iron enhance the microbial Hg-reduction, while chloride inhibits it. These results help to understand the persistence of mercury contamination in the Tagus Estuary after the inactivation of critical industrial units, and are also useful for the development of new bioremediation strategies either in the Tagus Estuary as well as in others mercury contaminated aquatic environments.

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CHAPTER VII

GENERAL CONCLUSIONS AND FUTURE WORK PERSPECTIVES

GENERAL CONCLUSIONS

Mercury contamination of aquatic systems has been recognized as a global and serious problem, mainly because of the neurotoxicity associated to methylmercury. Methylmercury is produced mainly by microbial activity in aquatic sediments. Once formed, it undergoes bioaccumulation and biomagnification in food webs, representing a risk for human health. Microorganisms are also responsible for the detoxification processes that affect the rate of MeHg production and also its accumulation. These processes include microbial activity promoting mercuric mercury reduction to form the elemental mercury and methylmercury demethylation. Therefore, it is important to understand the dynamics of microbial activity and the processes of mercury species transformation that determine methylmercury production and degradation.

Mercury pollution is a concern in the Tagus Estuary (Portugal), where high levels of mercury contamination have been registered.

In this work, mercury-resistant microorganisms as well as microbial communities were isolated from sediments of the Tagus Estuary to deeply investigate their activities on mercury cycling. The main conclusions resulting from this work are:

(1) Through the isolation processes, it was concluded that mercury-resistant microorganisms are widely distributed in the Tagus Estuary and the mercury contamination of this estuary has influenced significantly the microbial communities. The comparison between high and low contamination sampled areas showed clear differences in microbial community composition and in their resistance levels. From these results, it was possible to conclude that mercury contamination is:

a) Selecting mercury tolerant and resistant phenotypes among microbial community;
b) Influencing microbial community composition;
c) Increasing resistance levels of microorganisms to mercurial compounds.

(2) Through the processes of the identification of the isolates, it was possible to verify that:

a) Microbial community exhibiting mercury-resistance in the Tagus Estuary includes both, aerobic bacteria and anaerobic microorganisms;
b) The mercury-resistant aerobes of the Tagus Estuary encompass *Bacillus*, *Vibrio*, *Aeromonas* and *Enterobacteriaceae* species,
c) *Bacillus* is the most common genera found in the Tagus Estuary;
d) The anaerobic microbial community exhibiting mercury resistance is mainly composed by *Clostridium* sp., *Enterobacteriaceae* sp. and SRB;
e) Among SRBs, the species *Desulfovibrio desulfuricans* was found to exist in the Tagus Estuary.

(3) Through the investigation of the isolates resistance level and the mechanisms conferring this resistance, it was observed that:

a) The isolates are 10 to 100 times more resistant to mercuric mercury than to methylmercury;
b) Anaerobic microorganisms are 20 to 90 times more resistant to mercury compounds than the aerobic microorganisms;
c) Highly resistant microorganisms exist in the vicinity of mercury contamination peaks;
d) The occurrence of *mer* operon among the isolates is low and its genes are more likely to be found among the aerobes.

(4) The study of microbial role on mercury cycle in the Tagus Estuary showed that mercury-resistant microorganisms of the Tagus Estuary are involved in processes of mercury conversion in this estuary. The figure below is a graphical abstract summarizing all microbial-mediate conversions observed in this study, which include the reduction and methylation of mercuric mercury and demethylation of methylmercury.

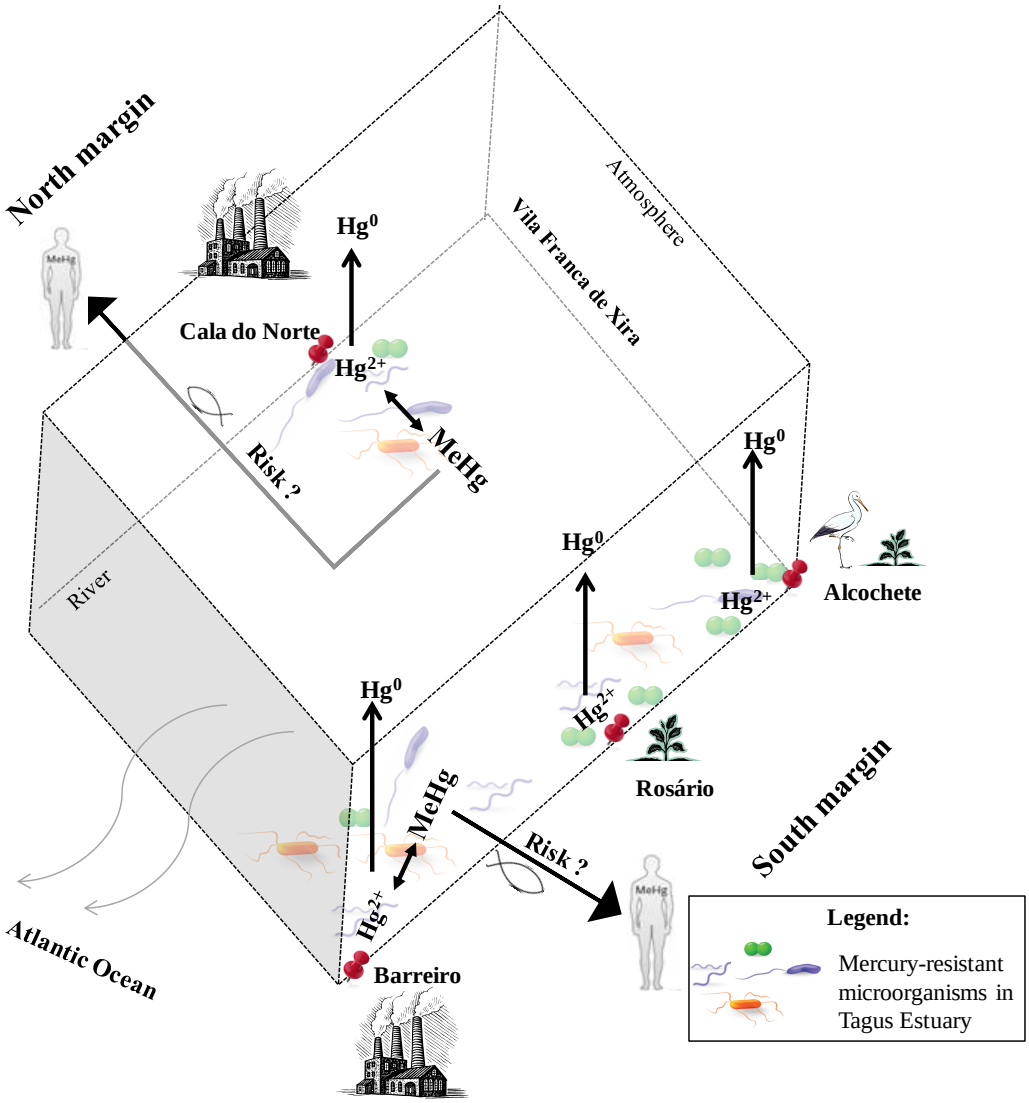

Figure VII.1: Graphical resume of the results showing microbial-mediated conversions of mercury in the Tagus Estuary.

Thus, from these investigations, it was concluded that:

a) Microorganisms of the Tagus Estuary are promoting Hg^{2+} reduction into Hg^{0} and its subsequent volatilization from this ecosystem; additionally, the aerobic mercury-resistant bacteria have a critical role in this process;
b) The reduction rates rounded 50% and this was observed for the microorganisms isolated from the three sampled areas;

c) Microbial communities of Barreiro and Cala do Norte are actively involved in methylation and demethylation processes in the Tagus Estuary;
d) Methylation is positively correlated with anoxic environments and with the presence of SRBs;
e) Demethylation is positively correlated with oxic environments and with the presence of aerobic bacteria;
f) There is the simultaneous occurrence of methylation and demethylation processes catalysed by microbial community.

(5) Bioremediation hypothesis

A final objective of this work was obtaining information that could be used to remediate the estuary pollution, namely mercury contamination. Using the factorial design methodology for the optimization of mercury reduction by microbial community it was concluded that:

a) Sulphate enhances mercury reduction rate by microbial community;
b) Chloride decreases mercury reduction rate by microbial community;
c) The optimization process indicates that for bioremediation one should focus our attention on sulphate concentration effects on microbial communities' activity.

From the study of mercury-resistant bacteria of salt marsh of the Tagus Estuary it was concluded that:

a) *Sacocornia fruticosa* accumulates more mercury than *Spartina maritima*;
b) *Sacocornia fruticosa* promotes the selection of highly mercury-resistant microorganisms;
c) Microorganisms found in vicinity of *Sacocornia fruticosa* rhizosphere are actively involved in mercury reduction process.

Therefore, as a bioremediation strategy, a combined strategy using sulphate enrichment and promotion of *Sacocornia fruticosa* plants to enhance mercury reduction potential by microbial community can be a hypothesis to be tested in the pilot scale (Figure VII.2). However, it must be stressed that, before implementing this hypothesis to an ecological

system such as Tagus Estuary, all the risks associated to sulphate enrichment must be carefully addressed.

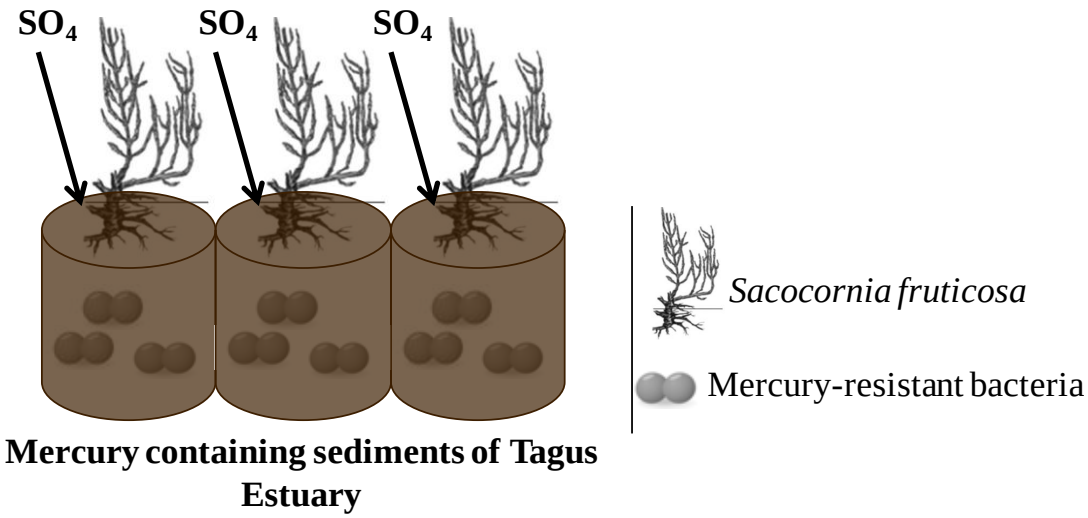

Figure VII.2: Shematic representation of the bioremediation hypothesis based in the usage of plants and bacteria to enhance mercury reduction in a pilot scale.

Overall, the results obtained in this work demonstrate the existence of mercury resistant microorganisms in the Tagus Estuary areas and their active involvement in mercury cycling in the ecosystem. Their role includes mercury reduction and methylmercury formation and degradation. The demethylation and reduction processes represent an ecological pathway for the remediation of the Tagus Estuary, by promoting the degradation and decreasing the rate of formation of the neurotoxic methylmercury and the overall removal of mercury from this ecosystem. On the other hand, methylation represents a risk for aquatic organisms and human health, as it bioaccumulates and through the consumption of contaminated fish or seafood, humans become highly exposed. Therefore, the risk for aquatic organisms and human health must be assessed. Thus, this study presents a set of data useful for risk assessment associated with the mercury pollution in the Tagus Estuary and for the development of future remediation strategies to be implemented.

FUTURE WORK PERSPECTIVES

The results presented in this study show that mercury resistant microorganisms exist in the Tagus Estuary and they are promoting the interconversion of mercury forms. Although *per se*, this study gives an insight about microbial role in mercury cycling in the Tagus Estuary, it also raised interesting questions that must be addressed:

(1) One of the most challenging points along this work was to determine the mechanisms used by mercury-transforming microorganisms of the Tagus Estuary. In particular, the mismatch found between the high rate of reduction and low occurrence of *mer* genes among the isolates puts a question about the mechanisms used that can justify their high mercury resistance and the detoxification potential observed. Thus, a deep research to understand other possible mechanisms (such as Fe^{2+}-dependent mechanism) used by these microorganisms still needs to be performed;

(2) Methylation was observed among some aerobic isolates; however, methylmercury formation was not observed for the aerobic community as a whole. This showed that the reaction of microorganisms in community with mercury is different from their reaction when isolated. Thus, there is a need to better understand the organization of microorganisms in community and to study the factors controlling the expression of different phenotypes among the diverse mercury-transforming microorganisms;

(3) The integrate study using isotope enriched Hg species was performed for the microbial community, however it would be interesting to use this same approach to better understand the dynamics of mercury transformations in microbial community, namely the cell uptake and the subsequent transformations. Such integrate study would be a valuable tool for remediation of mercury contamination.

(4) The factorial design methodology optimization used to investigate bioremediation strategies showed interesting results involving sulphate positive effects over microbial mercury reduction and the study of salt marsh of thte Tagus Estuary showed the effect of plant in mercury cycling. Based on these two investigations, a bioremediation strategy was proposed. However, there is the need to study in the field the viability of this proposal, to prove the benefits of using sulphate and *Sacocornia fruticosa* to

enhance microbial reduction potential in mercury-contaminated environments; additionally the study will allow the determination that an increase in sulphate would have in anaerobic community and especially in SRB methylating activity;

(5) Bacterial thioredoxin has many important functions, as summarized in the figure below (Figure VII.3). Thioredoxin, glutathione and catalase are the major antioxidant systems in bacteria. Moreover, in some bacteria the glutathione system is lacking, which confers to the thioredoxin system an essential role for growth and survival under oxidative conditions (Lu and Holmgren, 2014). Recent studies showed that drugs such as ebselen inhibit the growth of glutathione-lacking bacteria through the inhibition of TrxR (Lu et al., 2013). Since mercurials are known to inhibit the thioredoxin system in mammals it would be very interesting to investigate if bacterial TrxR/Trx activities are affected in mercury-contaminated environments and, if so, how the mercury-resistant bacterial cells respond to this effect.

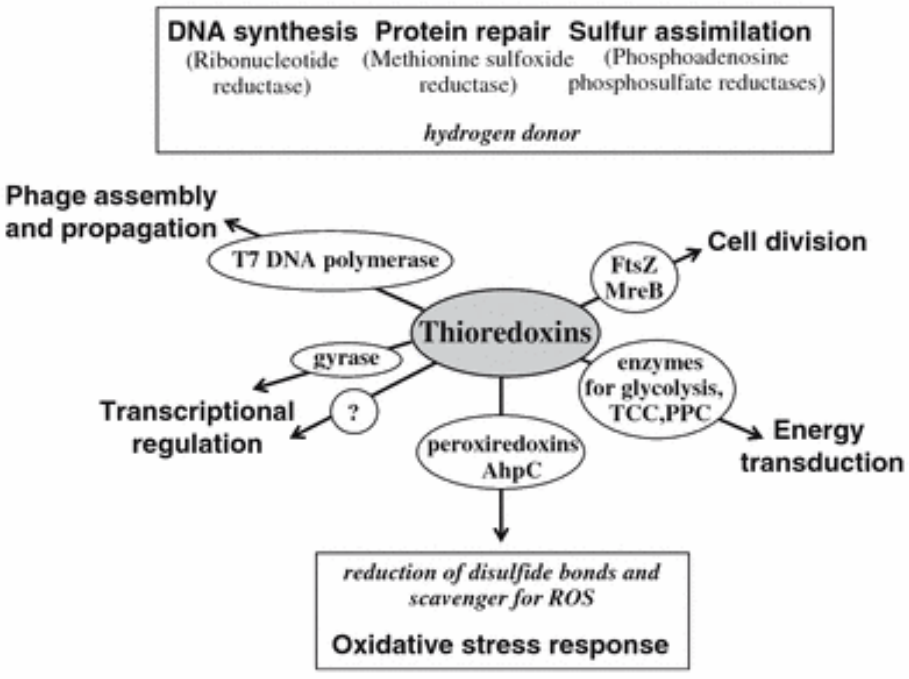

Figure VII.3: Thioredoxin role in bacterial cells (Zeller and Klug, 2006).

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APPENDIX 1

FACTORIAL DESIGN METHODOLOGY

A1. Factorial design for the optimization of mercury reduction by microbial community

To optimize mercury reduction by microbial community, the factorial 2^4 design methodology following the Central Composite Design (CCD) was used. Four variables with effects on mercury reduction by microbial community were selected: glucose, sulfate, ferrous iron and chloride. Following, an experimental plan was design for the study of these four variables (see Table A1.1). Variation in the concentrations -/+ of each variable was performed according to the Table A1.1. The medium point experiment (7 repetitions) was also performed.

Table A1.1: The experimental plan of 2^4 factorial design for the evaluation of the effects of glucose (Glu), sulphate (SO_4), ferrous iron (Fe) and chloride (Cl) on mercury reduction by microbial communities.

Experiments	Study levels				Concentrations			
	Glu	SO4	Fe	Cl	Glu (mM)	SO_4 (mM)	Fe (µM)	Cl (mM)
1	-1	-1	-1	-1	0,5	14	0.61	127
2	+1	-1	-1	-1	1,5	14	0.61	127
3	-1	+1	-1	-1	0,5	22	0.61	127
4	+1	+1	-1	-1	1,5	22	0.61	127
5	-1	-1	+1	-1	0,5	14	1.61	127
6	+1	-1	+1	-1	1,5	14	1.61	127
7	-1	+1	+1	-1	0,5	22	1.61	127
8	+1	+1	+1	-1	1,5	22	1.61	127
9	-1	-1	-1	+1	0,5	14	0.61	131
10	+1	-1	-1	+1	1,5	14	0.61	131
11	-1	+1	-1	+1	0,5	22	0.61	131
12	+1	+1	-1	+1	1,5	22	0.61	131
13	-1	-1	+1	+1	0,5	14	1.61	131
14	+1	-1	+1	+1	1,5	14	1.61	131
15	-1	+1	+1	+1	0,5	22	1.61	131
16	+1	+1	+1	+1	1,5	22	1.61	131
Extreme level								
1	-2	0	0	0	0	18	1.01	129
2	+2	0	0	0	2	18	1.01	129
3	0	-2	0	0	1	10	1.01	129
4	0	+2	0	0	1	26	1.01	129
5	0	0	-2	0	1	18	0.01	129
6	0	0	+2	0	1	18	2.01	129
7	0	0	0	-2	1	18	1.01	125
8	0	0	0	+2	1	18	1.01	133
Medium point								
1	0	0	0	0	1	18	1.01	129
2	0	0	0	0	1	18	1.01	129
3	0	0	0	0	1	18	1.01	129
4	0	0	0	0	1	18	1.01	129
5	0	0	0	0	1	18	1.01	129
6	0	0	0	0	1	18	1.01	129

A1

The responses obtained (Y), i.e. mercury reduction percentages, on the levels -1/+1, were used to build an algorithm of Yates in order to estimate the influence of each variable and their respective interactions on the Y (Tables A1.2 and A1.3).

Table A1.2: Formulary to calculate the Yates' algorithm (column I to IV).

Experiments	G	H	I	J	K	Sum of square	Half effect	Effect
	Y	I	II	III	IV			
1	47.80	G41+G42	H41+H42	I41+I42	J41+J42	(K41^2)/16	K41/16	average
2	45.88	G43+G44	H43+H44	I43+I44	J43+J44	(K42^2)/16	K42/16	1
3	61.02	G45+G46	H45+H46	I45+I46	J45+J46	(K43^2)/16	K43/16	2
4	40.67	G47+G48	H47+H48	I47+I48	J47+J48	(K44^2)/16	K44/16	12
5	49.03	G49+G50	H49+H50	I49+I50	J49+J50	(K45^2)/16	K45/16	3
6	44.36	G51+G52	H51+H52	I51+I52	J51+J52	(K46^2)/16	K46/16	13
7	39.10	G53+G54	H53+H54	I53+I54	J53+J54	(K47^2)/16	K47/16	23
8	38.02	G55+G56	H55+H56	I55+I56	J55+J56	(K48^2)/16	K48/16	123
9	36.67	G42-G41	H42-H41	I42-I41	J42-J41	(K49^2)/16	K49/16	4
10	41.59	G44-G43	H44-H43	I44-I43	J44-J43	(K50^2)/16	K50/16	14
11	38.03	G46-G45	H46-H45	I46-I45	J46-J45	(K51^2)/16	K51/16	24
12	50.55	G48-G47	H48-H47	I48-I47	J48-J47	(K52^2)/16	K52/16	124
13	45.78	G50-G49	H50-H49	I50-I49	J50-J49	(K53^2)/16	K53/16	34
14	48.32	G52-G51	H52-H51	I52-I51	J52-J51	(K54^2)/16	K54/16	134
15	45.65	G54-G53	H54-H53	I54-I53	J54-J53	(K55^2)/16	K55/16	234
16	48.18	G56-G55	H56-H55	I56-I55	J56-J55	(K56^2)/16	K56/16	1234

Table A1.3: Formulary leading to effects estimation.

Factor	Effects estimation
Average	SOMA(H41:H56)/16
1	+(-H41+H42-H43+H44-H45+H46-H47+H48-H49+H50-H51+H52-H53+H54-H55+H56)/8
2	+(-H41-H42+H43+H44-H45-H46+H47+H48-H49-H50+H51+H52-H53-H54+H55+H56)/8
3	+(-H41-H42-H43-H44+H45+H46+H47+H48-H49-H50-H51-H52+H53+H54+H55+H56)/8
4	(-H41-H42-H43-H44-H45-H46-H47-H48+H49+H50+H51+H52+H53+H54+H55+H56)/8
12	+(-H41-H42-H43+H44-H45-H46-H47+H48-H49-H50-H51+H52-H53-H54-H55+H56)/8
13	+(-H41-H42-H43-H44-H45+H46-H47+H48-H49-H50-H51-H52-H53+H54-H55+H56)/8
14	+(-H41-H42-H43-H44-H45-H46-H47-H48-H49+H50-H51+H52-H53+H54-H55+H56)/8
23	+(-H41-H42-H43-H44-H45+H46+H47-H48-H49-H50-H51-H52-H53+H54+H55+H56)/8
24	(-H41-H42-H43-H44-H45-H46-H47-H48-H49-H50+H51+H52-H53-H54+H55+H56)/8
34	(-H41-H42-H43-H44-H45-H46-H47-H48-H49-H50-H51-H52+H53+H54+H55+H56)/8

After knowing the effects of each variable and their interactions, a matrix was elaborated as shown in Table A1.4.
The matrix results were then used to calculate the coefficients of the second-order equation, following equation A1.1.

Equation A1.1

$$\{= \text{MATRIX.MULT (MATRIX.INVERSE(MTRIX.MULT(TRANSPOSE(A),A),MATRIX.MULTI(A),Y)}\}$$

A: area of the result calculated in the matrix (Table A1.4).
Y: the obtained response by the 2^4 factorial design experimental plan.

Using the obtained coefficients, the response values were predicted (Y^) by the model, according to the equation A1.2.

Equation A1.2.

$$= \text{MATRIX.MULT (Ai, PC)}$$

Ai: area of the result calculated in the matrix (Table A1.4) for i experiment
PC – polynomial coefficient (Equation A1.1)

The surfaces response were build up based on the Y^.

The statistical validation of the model was peformed through the calculation of the correlation coefficient, by knowing that:
R^2 is R^2 = (SQfact/SQcorr), with

SQ fact= Sum $(Yi\text{^}-Ymed)^2$

Sqcorr= Sum$(Yi-Ymed)^2$

Table A1.4: Matrix contructed for the factorial design.

Exp.	1:Glu	2: SO_4	3: Fe	4: Cl	1*1	2*2	3*3	4*4	1*2	1*3	1*4	2*3	2*4	3*4	1*2*3	2*3*4	1*3*4	1*2*3*4
1	0.50	14.00	0.61	127.00	0.25	196.00	0.37	16,129.00	7.00	0.31	63.50	8.54	1,778.00	77.47	4.27	1,084.58	38.74	542.29
2	1.50	14.00	0.61	127.00	2.25	196.00	0.37	16,129.00	21.00	0.92	190.50	8.54	1,778.00	77.47	12.81	1,084.58	116.21	1,626.87
3	0.50	22.00	0.61	127.00	0.25	484.00	0.37	16,129.00	11.00	0.31	63.50	13.42	2,794.00	77.47	6.71	1,704.34	38.74	852.17
4	1.50	22.00	0.61	127.00	2.25	484.00	0.37	16,129.00	33.00	0.92	190.50	13.42	2,794.00	77.47	20.13	1,704.34	116.21	2,556.51
5	0.50	14.00	1.61	127.00	0.25	196.00	2.59	16,129.00	7.00	0.81	63.50	22.54	1,778.00	204.47	11.27	2,862.58	102.24	1,431.29
6	1.50	14.00	1.61	127.00	2.25	196.00	2.59	16,129.00	21.00	2.42	190.50	22.54	1,778.00	204.47	33.81	2,862.58	306.71	4,293.87
7	0.50	22.00	1.61	127.00	0.25	484.00	2.59	16,129.00	11.00	0.81	63.50	35.42	2,794.00	204.47	17.71	4,498.34	102.24	2,249.17
8	1.50	22.00	1.61	127.00	2.25	484.00	2.59	16,129.00	33.00	2.42	190.50	35.42	2,794.00	204.47	53.13	4,498.34	306.71	6,747.51
9	0.50	14.00	0.61	131.00	0.25	196.00	0.37	17,161.00	7.00	0.31	65.50	8.54	1,834.00	79.91	4.27	1,118.74	39.96	559.37
10	1.50	14.00	0.61	131.00	2.25	196.00	0.37	17,161.00	21.00	0.92	196.50	8.54	1,834.00	79.91	12.81	1,118.74	119.87	1,678.11
11	0.50	22.00	0.61	131.00	0.25	484.00	0.37	17,161.00	11.00	0.31	65.50	13.42	2,882.00	79.91	6.71	1,758.02	39.96	879.01
12	1.50	22.00	0.61	131.00	2.25	484.00	0.37	17,161.00	33.00	0.92	196.50	13.42	2,882.00	79.91	20.13	1,758.02	119.87	2,637.03
13	0.50	14.00	1.61	131.00	0.25	196.00	2.59	17,161.00	7.00	0.81	65.50	22.54	1,834.00	210.91	11.27	2,952.74	105.46	1,476.37
14	1.50	14.00	1.61	131.00	2.25	196.00	2.59	17,161.00	21.00	2.42	196.50	22.54	1,834.00	210.91	33.81	2,952.74	316.37	4,429.11
15	0.50	22.00	1.61	131.00	0.25	484.00	2.59	17,161.00	11.00	0.81	65.50	35.42	2,882.00	210.91	17.71	4,640.02	105.46	2,320.01
16	1.50	22.00	1.61	131.00	2.25	484.00	2.59	17,161.00	33.00	2.42	196.50	35.42	2,882.00	210.91	53.13	4,640.02	316.37	6,960.03
1	0.00	18.00	1.01	129.00	0.00	324.00	1.02	16,641.00	0.00	0.00	0.00	18.18	2,322.00	130.29	0.00	2,345.22	0.00	0.00
2	2.00	18.00	1.01	129.00	4.00	324.00	1.02	16,641.00	36.00	2.02	258.00	18.18	2,322.00	130.29	36.36	2,345.22	260.58	4,690.44
3	1.00	10.00	1.01	129.00	1.00	100.00	1.02	16,641.00	10.00	1.01	129.00	10.10	1,290.00	130.29	10.10	1,302.90	130.29	1,302.90
4	1.00	26.00	1.01	129.00	1.00	676.00	1.02	16,641.00	26.00	1.01	129.00	26.26	3,354.00	130.29	26.26	3,387.54	130.29	3,387.54
5	1.00	18.00	0.01	129.00	1.00	324.00	0.00	16,641.00	18.00	0.01	129.00	0.18	2,322.00	1.29	0.18	23.22	1.29	23.22
6	1.00	18.00	2.01	129.00	1.00	324.00	4.04	16,641.00	18.00	2.01	129.00	36.18	2,322.00	259.29	36.18	4,667.22	259.29	4,667.22
7	1.00	18.00	1.01	125.00	1.00	324.00	1.02	15,625.00	18.00	1.01	125.00	18.18	2,250.00	126.25	18.18	2,272.50	126.25	2,272.50
8	1.00	18.00	1.01	133.00	1.00	324.00	1.02	17,689.00	18.00	1.01	133.00	18.18	2,394.00	134.33	18.18	2,417.94	134.33	2,417.94
1	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22
2	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22
3	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22
4	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22
5	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22
6	1.00	18.00	1.01	129.00	1.00	324.00	1.02	16,641.00	18.00	1.01	129.00	18.18	2,322.00	130.29	18.18	2,345.22	130.29	2,345.22