**Universidade de Aveiro** *Departamento de Biologia*
**2013**

**Francisco José Riso da Costa Coelho**

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*Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Maria Ângela Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e do Doutor Newton Carlos Marcial Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM).*

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*Ao meu Pai, Vasco Coelho*

**o júri**

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*Doutor Newton Carlos Marcial Gomes*
*Investigador Auxiliar do CESAM – Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.*

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**palavras-chave**

*Bactérias degradadoras de hidrocarbonetos de petróleo, microcamada superficial marinha, alterações climáticas, sedimento, metagenómica*

**resumo**

*A capacidade dos microrganismos utilizarem hidrocarbonetos de petróleo como fonte de carbono e energia é crucial na reciclagem destes compostos nos meios marinhos. Apesar da importância deste processo muitos dos aspectos da degradação microbiana de hidrocarbonetos em alguns habitats específicos e na perspetiva de diferentes cenários de alterações climáticas são ainda desconhecidos.*

*Na primeira fase deste trabalho, a fracção cultivável das bactérias com capacidade para degradar hidrocarbonetos de petróleo na microcamada superficial marinha de um sistema estuarino (Ria de Aveiro) foi caracterizada. Na segunda fase, o impacto da contaminação por petróleo nas comunidades microbianas em diferentes cenários de alterações climáticas foi estudado.*

*O género Pseudomonas revelou ser preponderante na fracção cultivável das bactérias degradadoras de hidrocarbonetos na microcamada superficial marinha. Além disso, os métodos independentes de cultivo revelaram que a abundancia relativa e diversidade da classe Gammaproteobacteria, na qual se inclui o género Pseudomonas, é diferente ao longo de um gradiente estuarino de contaminação.*

*De forma a caracterizar o impacto da contaminação por petróleo nas comunidades microbianas sob diferentes cenários de alterações climáticas, um sistema de suporte de vida (ELSS) foi construído com o objectivo de realizar experiências de microcosmo. O ELSS permite simular e controlar aspectos fundamentais da dinâmica de sistemas costeiros e estuarinos preservando os elementos mais representativos da comunidade bacteriana original. Com este sistema foi possível realizar uma experiência onde o impacto independente e combinado da radiação ultravioleta, acidificação da água e contaminação por petróleo sobre comunidades bacterianas foi determinado. O impacto na estrutura das comunidades foi avaliado através de pirosequenciação do 16S RNA (cDNA). A ordem Desulfobacterales sofreu uma redução de abundância relativa muito significativa em resposta à contaminação por petróleo para uma redução de pH estimada para 2100. Uma vez que os membros desta ordem desempenham um papel crucial na degradação de hidrocarbonetos de petróleo, a reciclagem destes compostos em ambientes costeiros e estuarinos pode ser alterada em cenários ambientais futuros. A previsão do metagenoma a partir da base de dados do 16S RNA indica que várias vias de degradação de hidrocarbonetos de petróleo podem ser afectadas. Os resultados obtidos neste trabalho acescentam novas pistas sobre o papel das bactérias degradadoras de hidrocarbonetos em ambientes costeiros e estuarinos nos contextos ambientais actuais e futuro.*

**keywords**

*Oil hydrocarbon bacteria, sea surface microlayer, climate change, microcosm experiments*

**abstract**

*The ability of microorganisms to use oil hydrocarbons as a source of carbon and energy is crucial for environmental oil detoxification. However, there is still a lack of knowledge on fundamental aspects of this process on specific habitats and under different climate scenarios.*

*In the first phase of this work, the culturable fraction of the oil hydrocarbon (OH) degrading bacteria from the sea surface microlayer (SML) of the estuarine system Ria de Aveiro was characterized. In the second phase, the impact of oil contamination on the active bacterial community was studied under climate change scenarios. Pseudomonas emerged as the prevailing genera among OH degrading bacteria in the SML. Moreover, culture-independent methods revealed that the relative abundance and diversity of Gammaproteobacteria, in which Pseudomonas is included, varies along an estuarine gradient of contamination.*

*In order to access the impact of oil contamination on microbial communities under climate change scenarios, an experimental life support system for microcosm experiments (ELLS) was developed and validated for simulation of climate change effects on microbial communities. With the ELSS it is possible to simulate, in controlled conditions, fundamental parameters of the dynamics of coastal and estuarine systems while maintaining community structure in terms of the abundance of the most relevant members of the indigenous bacterial community. A microcosm experiment in which the independent and combined impact of ultraviolet radiation, ocean acidification and oil contamination on microbial communities was conducted. The impact on bacterial communities was accessed with a 16S RNA (cDNA) based barcode pyrosequencing approach. There was a drastic decrease of Desulfobacterales relative abundance after oil contamination under the reduced pH value estimated for 2100, when compared to present values. Since members of this order are known OH degraders, such a significant decrease may have consequences on OH detoxification of contaminated environments under the pH levels of the ocean expected for the future. Metagenome predictions based on the 16S RNA database indicated that several degradation pathways of OH could be affected under oil contamination and reduced water pH. Taken together, the results from this work bring new information on the dynamics of OH degrading bacteria in coastal and estuarine environments under present and future climate scenarios.*

# Contents

## List of publications

**Chapter II** - Coelho F.J.R.C., Sousa S., Santos L., Santos A.L., Almeida A., Gomes N.C.M., Cunha A. (2011) Exploring hydrocarbonoclastic bacterial communities in the estuarine surface microlayer. *Aquatic Microbial Ecology. 64, 185-195.*

**Chapter III** - Coelho FJRC, Rocha RJM, Pires ACC, Ladeiro B, Castanheira JM, Costa R, Almeida A, Cunha Â, Lillebø AI, Ribeiro R, Pereira R, Lopes I, Marques C, Moreira-Santos M, Calado R, Cleary DFR, Gomes NCM (in press) Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities. *Global Change Biology*.

**Appendix** - Coelho F.J.R.C., Santos A.L., Coimbra J., Almeida A., Cunha A., Cleary D.F.R., Calado R., Gomes N.C.M. (in press) Interactive effects of global climate change and pollution on marine microbes: the way ahead. *Ecology and Evolution.*

## List of abbreviations

| | |
|---|---|
| cDNA | Complementary deoxyribonucleic acid |
| DAPI | 4 ,6-diamidino-2-phenylindole |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| DOC | Dissolved organic carbon |
| CCMs | Carbon-concentrating mechanisms |
| DOM | Dissolved organic matter |
| ELSS | Experimental life support system |
| FISH | Fluorescence *in situ* hybridization |
| KEGG | Kyoto encyclopedia of genes and genomes |
| KO | KEGG orthologs |
| OH | Oil hydrocarbons |
| OTU | Operational taxonomic units |
| PAH | Polycyclic aromatic hydrocarbons |
| PCR | Polimerase chain reaction |
| PCO | Principal coordinates analysis |
| PICRUSt | Phylogenetic investigation of communities by reconstruction of unobserved states |
| POC | Particulate organic carbon |
| QIIME | Quantitative insights into microbial ecology |
| RDP | Ribossomal database project |
| RNA | Ribonucleic acid |
| rRNA | Ribossomal ribonucleic acid |
| ROS | Reactive oxygen species |
| SRB | Sulphate reducing bacteria |
| SML | Sea Surface Microlayer |
| UVR | Ultraviolet radiation |
| UW | Underlying water |
| TEP´S | Transparent exopolymer particles |

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**Supporting information**

# Justification, objectives and thesis outline

Microbial communities have been shown to play a key role in pollutant detoxification in marine environments. The environmental importance of this process is such that the world´s ocean would be literally covered with oil, if bacteria were not catabolising these compounds (Head *et al.*, 2006). The ability of bacteria to use petroleum hydrocarbons as energy and carbon source has been studied in detail (Bamforth *et al.*, 2005). However there is still a lack of knowledge on fundamental aspects of this process in specific habitats and under different environmental scenarios. For example, although the sea surface microlayer (SML) covers almost 70% of the earth surface and oil hydrocarbons tends to concentrate there (Wurl *et al.*, 2004), little is known about the structure of the hydrocarbon degrading bacterial community inhabiting this particular water compartment.

Concurrently, the impact of climate change scenarios, such as ocean acidification and increase ultraviolet radiation (UVR), on microbial interactions with oil hydrocarbons is not known. Ocean acidification and UVR have the potential to significantly impact microbial community structure and function. Among other effects, modifications can impact microbial mediated detoxification processes, with unpredictable effects on metabolic pathways and toxicity of anthropogenic pollutants.

There is an urgent need for a mechanistic understanding of the complex interactions involving bacteria, pollutants and climate factors. This knowledge can ultimately lead to more accurate predictions of shifts in microbial communities and in pollutant toxicity under climate change scenarios and to the development of new microbial-based remediation technologies. However, the complexity of biological, chemical and environmental interactions in natural environments restricts our ability to establish cause-effect relationships. Understanding the interactions of global climate change and pollutants on living organisms is a complex task that entails the study of a multitude of interactions. This demands innovative and statistically robust experiments conducted under controlled conditions during which biological and non-biological markers of environmental function can be identified and measured.

The general objective of this work was to gain fundamental knowledge on the interactions of microbial communities with oil hydrocarbon pollutants in marine environments. First, from a phenomenological perspective, the hydrocarbon degrading

microbial communities were characterized in a model environment. Second, from a more mechanistic view, the effects of a crude oil spill in the microbial communities of estuarine sediment were assessed under controlled climate change scenarios.

Three specific objectives were formulated in order to accomplish the general objective:

To characterize the hydrocarbon degrading bacteria of the sea surface microlayer in a chronically oil polluted estuarine system;
To develop an innovative framework that would enable statistically robust microcosm simulations of climate change scenarios in marine environments;
To characterize the synergistic effects between ocean acidification, ultraviolet radiation and anthropogenic pollutants on microbial communities.

In **Chapter 1** a brief introduction that focus the relevance of oil hydrocarbon bacterial degradation and the current knowledge on the effects of climate change and anthropogenic pollution interactions on microbial communities is presented.

**Chapter 2** addresses objective 1 by examining for the first time the oil hydrocarbon degrading bacteria in a estuarine surface microlayer. *Pseudomonas* was the dominant group among the culturable polycyclic aromatic hydrocarbon (PAH) degrading bacteria. Screening for specific genes involved in PAH degradation revealed that identical genes were present in two phylogenetically distinct isolates, thus suggesting that horizontal transfer of genes might be fundamental in PAH degradation in the SML. Culture-independent analysis revealed that although the general structure of the bacterial community remains unchanged along a contamination gradient, the relative abundance and diversity of organisms of The *Gammaproteobacteria* group, including those of the genus *Pseudomonas*, reveals differences. These findings highlight the importance of SML-adapted oil hydrocarbon degrading bacteria with potential use in bioremediation of polluted ecosystems. This chapter was published in the journal **Aquatic and Microbial Ecology**.

**Chapter 3** describes the architecture and validation of a microcosm framework designed to study the interactive effects of climate change and anthropogenic pollution on marine benthic communities. This chapter addresses objective 2. This system, named experimental life support system (ELSS), allows simulating climate change scenarios under high controlled conditions. In this chapter, the validation of the reproducibility and

environmental quality of this system was achieved by comparing chemical and biological parameters recorded in the ELSS with those prevalent in the natural environment. Average concentration of inorganic nutrients remained stable during ELSS functioning. Although some shifts in bacterial community composition were observed between *in situ* and ELSS sediment samples, the relative abundance of the most abundant metabolically active bacterial taxa was stable. In addition, ELSS operation did not affect survival, oxidative stress and neurological biomarkers of the model organism *Hedistes diversicolor*. The validation data thus supports the use of this system to address objective 3. This chapter was published as a technical advance in the journal **Global Change Biology**, ranked third (following Nature and Science) in the list of the most-cited journals in climate change research.

**Chapter 4** addresses objective 3 by examining the interactive effects of ocean acidification and ultraviolet radiation in estuarine sediment microbial communities. The effects on the active bacterial community were characterized by using a barcoded pyrosequencing approach of the 16S rRNA (cDNA). The most drastic change detected was a reduction in anaerobic sulphate reducing bacteria under oil contamination and reduced pH values. Given the role of sulphate reducing bacteria in anaerobic oil hydrocarbon degradation we used PICRUSTs to predict the community metagenome and on focused specific contaminant degradation pathways. The predicted metagenomes revealed that under oil contamination and reduced pH, several contaminant degradation pathways were affected. These results suggest that in future ocean reduced pH conditions the impact of an oil spill can be substantially different than under current pH levels.

**Chapter 5** discusses the experimental design and the main conclusions. The **Appendix** is a review published in the journal **Ecology and Evolution** journal that combines sections of the introduction and discussion of this thesis.

## Literature cited

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Wurl O, Obbard JP (2004) A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. *Marine Pollution Bulletin,* **48,** 1016-1030.

# Chapter I

# Introduction

## Microbial degradation of oil hydrocarbons

Oil hydrocarbons (OH) comprise several persistent organic pollutants (POPs) and represent the most widespread pollutant in oceans worldwide. Natural seepage alone introduces about 600000 metric tons of natural crude oil per year in our oceans representing 47% of crude oil entering the marine environment. The remaining 53% results from anthropogenic activities (accidental oil spills, transport activities, refining, storage, and others) (Kvenvolden *et al.*, 2003).

Crude oils are complex mixtures of many different hydrocarbons and organic molecules. Petroleum components, for example, can be classified by thin-layer chromatography as saturate, aromatic, resins and asphaltenes (Garrett *et al.*, 1998). The saturated fraction includes the linear and branched chain alkanes and cycloalkanes, while the aromatic fraction consists of the aromatic mono- or polycyclic compounds (Garrett *et al.*, 1998)

Polycyclic aromatic hydrocarbons (PAH) are among the most common compounds associated to OH pollution and probably the most recalcitrant and therefore categorized as POPs. They are widespread in soil, water and air and are known to be genotoxic, carcinogenic and susceptible to bioaccumulation (Newsted *et al.*, 1987). The solubility of these pollutants is generally low and decreases as the molecular weight increases. Due to their chemical characteristics, PAH tend to adsorb to solid particles such as soils and sediments, or to concentrate at the SML (Wurl *et al.*, 2004, Zhou *et al.*, 2009). Very often, adorption contributes to their stability and persistence in the environment.

Microorganisms have developed the ability to metabolize or degrade oil hydrocarbons as a source of energy and carbon. The oil-catabolic versatility of marine microorganisms, especially bacteria, is such that the world´s oceans would be literally covered with a layer of oil if bacteria could not metabolize these compounds (Head *et al.*, 2006). Hydrocarbonoclastic (OH degraders) bacteria can be found in marine ecosystems in the SML, in underlying water and in sediments. Several marine bacteria capable of metabolizing petroleum hydrocarbons, namely *Alcalinovorax (*Yakimov et al., 1972) *Cycloclasticus* (Dyksterhouse *et al.*, 1995), *Oleiphilus* (Golyshin *et al.*, 2002) and *Oleispira* (Yakimov, 2003) have been isolated from marine environments. Commonly, these organisms are found in low abundance in pristine

environments and in areas exposed to low levels of OH pollution, but they can rapidly respond to OH contamination with an increase in biomass and activity (Gomes *et al.*, 2005, Yakimov *et al.*, 2005). Information on the ecology and physiology of *in situ* PAH detoxification is largely available in the scientific literature, which supports a fairly extensive understanding on the process of environmental PAH detoxification (Kasai *et al.*, 2002, Ro *et al.*, 2002, Head *et al.*, 2006, Gomes *et al.*, 2007). The metabolic pathways and enzymatic reactions involved in the microbial aerobic degradation of naphthalene have been studied in detail. The basis of these mechanisms is the oxidation of the PAH aromatic ring by the action of dioxygenase enzymes, followed by the systematic breakdown into PAH metabolites and $CO_2$ (Bamforth *et al.*, 2005). Anaerobic degradation of PAH occurs at lower rates, but it might play an essential role in the long-term detoxification of soil and sediments (Zhang *et al.*, 2011). The hydrocarbonoclastic bacteria are important players in environmental OH detoxification through a complex process in which environmental factors and biological activities interact. Also, it may involve the synergistic contribution of enzymatic pathways operated by several other functional groups rather than exclusively assigned to OH-degrading populations (Head *et al.*, 2006).

**Interactions between climate change and anthropogenic pollution**

Atmospheric concentrations of carbon dioxide ($CO_2$), mainly through the burning of fossil fuels, have increased from approximately 280 ppmv (parts per million volume) in preindustrial times (Indermühle *et al.*, 1999) to nearly 394 ppmv in 2012 (NOAA Earth System Research Laboratory, 2012). The postulated impact of elevated $CO_2$ levels on the earth's temperature is only one, although probably the best known, of the consequences of the increased $CO_2$ concentrations. Increased atmospheric $CO_2$ also causes a net air-to-sea flux of $CO_2$, reducing seawater pH and inducing modifications in the chemical balance between inorganic carbon species. This process, known as ocean acidification, is often referred to as "the other $CO_2$ problem" (Doney *et al.*, 2009, Doney, 2010). Since ocean acidification is a direct consequence of the increase of the partial pressure of $CO_2$ in the atmosphere, it does not depend on uncertainties related with other climate change predictions (Doney *et al.*, 2009).

Although international treaties have been effective in reducing atmospheric concentrations of ozone depleting substances (UNEP, 2012), increased concentrations of greenhouse gases have the potential to affect the spatial distribution of ozone and its exchange between the stratosphere and the troposphere. This, in turn, will impact ultraviolet radiation

(UVR) levels reaching the earth's surface (Bais *et al.*, 2011). Global warming, ocean acidification and changes in oceanic stratification patterns, are also expected to increase the penetration of UV radiation in the water column and enhance the exposure of aquatic organisms to UV-B in the future (UNEP, 2010). Importantly, increased UVR has been shown to disrupt aquatic food webs and reduce the biological sinking capacity of atmospheric $CO_2$ (Hader *et al.*, 2007, Fabry *et al.*, 2008).

Anthropogenic activities have been seriously and adversely affecting marine ecosystems for decades with a continuous influx of pollutants including oil hydrocarbons (OH), pesticides and heavy metals (Doney, 2010). Ocean acidification and UVR have the potential to interact synergistically with a range of natural organic compounds and environmental pollutants, with deleterious effects on oceanic ecosystems. An alert on the possibility that ocean acidification in synergy with anthropogenic stressors could lead to widespread changes in marine ecosystems was recently sent (Fabry *et al.*, 2008). However, the exact nature of such changes was not discussed.

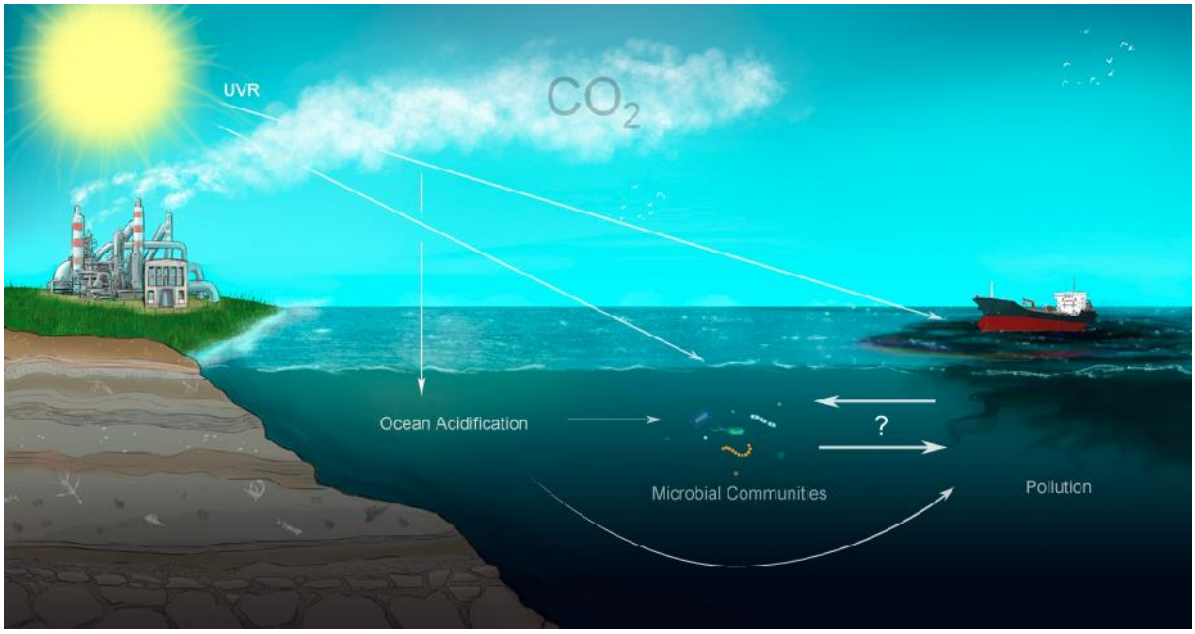


**Figure I-1:** Interactions between ultraviolet radiation, ocean acidification, anthropogenic pollution and microbial communities**.** Climate change has the potential to influence pollutant toxicity by acting directly on pollutant chemistry or indirectly by affecting microbial mediated detoxification. Both have the potential to act synergistically on microbial communities and possible on microbial mediated processes.

Despite the important role microbial assemblages play in the global recycling of anthropogenic pollutants, the potential interactive effects of UVR, ocean acidification and anthropogenic pollutants on marine microbial communities have been largely ignored. Little is known about how pollutant pathways may change under different climate change scenarios and how this will affect biogeochemical cycling. Moreover, although microbial assemblages have been shown to be affected by increased UVR and ocean acidification, nothing is known about how these changes will affect pollutant toxicity (Figure I-1).

## Ocean acidification and UVR interactions with marine microbial communities – what we know so far

### *Effects of ocean acidification in marine microbial communities*

Oceanic pH has declined by 0.1 units from pre-industrial times to present (IPCC, 2007). Under unabated $CO_2$ emissions, pH will further decline 0.3 to 0.4 units by the end of this century, and up to 0.7 units in 2300 (Caldeira *et al.*, 2003). When $CO_2$ dissolves in seawater, carbonic acid ($H_2CO_3$) is formed. It quickly dissociates into a hydrogen ion ($H^+$) and a bicarbonate ion ($HCO_3^-$). The hydrogen ion can then react with the carbonate ion ($CO_2^{-3}$) to form bicarbonate. This process leads to increased partial pressure ($pCO_2$), increased concentrations of $H_2CO_3$, $HCO^-_3$, and $H^+$, and reduced concentrations of $CO_3^{-2}$ (Fabry *et al.*, 2008).

The predicted reduction in carbonate ions has led to several studies of marine organisms such as corals, mollusks, echinoderms and crustaceans, that depend on the availability of $CO_3^{-2}$ to form biogenic calcium carbonate to produce shells and skeletons (Fabry *et al.*, 2008). Similar studies on how functionally important marine microbes will respond to ocean acidification are, however, lacking.

Recently, Joint *et al.* (2011) suggested that given that microbial assemblages have always experienced variable pH conditions, the appropriate null hypothesis to be tested is that "there will be no catastrophic changes in marine biogeochemical processes driven by phytoplankton, bacteria and archaea" (Joint *et al.*, 2011). Liu *et al.* (2010), however, performed a meta-analysis of published data and suggested that this hypothesis should be rejected (Liu *et al.*, 2010). Although few and far from consistent, studies on the impacts of ocean acidification on marine microbial communities include changes to primary production rates, phytoplankton

community structure, the nitrogen cycle and the structure and activity of bacterial communities (IPCC, 2011).

Most marine primary producers show little or no change in photosynthetic rates when grown under increased $pCO_2$ (Doney *et al.*, 2009). This appears to be related to the predominant phytoplankton species. In a mesocosm experiment, a phytoplankton community dominated by the coccolithophore *Emiliana Huxley*, increased $CO_2$ uptake by up to 39% under increased $pCO_2$ levels (Riebesell *et al.*, 2007). The $CO_2$ consumption was not balanced by increases in particulate organic carbon (POC). Instead, it was rapidly converted to DOC and TEPs, and any additional carbon incorporation into POC was lost from surface to deep waters. These results suggest that increased atmospheric $pCO_2$ may lead to increased particle flux from surface to deep water, potentially providing a negative feedback to increased $CO_2$ emissions (Arrigo, 2007, Riebesell *et al.*, 2007).

Phytoplankton responses to increased $pCO_2$ depend to a large extent, on inorganic carbon-concentrating mechanisms (CCMs) operated by cells (Gattuso *et al.*, 2011). Marine primary producers include phylogenetically diverse groups that differ in their photosynthetic apparatus and CCMs. These differences are likely to alter competitive relationships among phytoplankton and induce shifts in plankton species composition as oceanic $pCO_2$ increases (Gattuso *et al.*, 2011).

Increased concentrations of $CO_2$ may also affect the nitrogen cycle. The filamentous cyanobacterium *Trichodesmium*, a major contributor of new nitrogen in oligotrophic oceans, increased carbon and nitrogen fixation rates by 35-100% at predicted $pCO_2$ scenarios of the year 2100 when compared to present day $pCO_2$ conditions (Hutchins *et al.*, 2007). In contrast, a negative impact of high $pCO_2$ was observed for *Nodularia spumigena*. These contradictory results may be related to different ecological strategies of non-heterocystous (*Trichodesmium*) and heterocystous (*Nodularia*) cyanobacteria (Czerny *et al.*, 2009).

In addition to nitrogen fixation, other processes of the nitrogen cycle may be altered by ocean acidification. It has been suggested that ocean acidification can reduce nitrification rates by 3-44% within the next few decades (Beman *et al.*, 2010). A few studies have addressed effects of elevated $pCO_2$ on bacterial abundance and community structure coupled to phytoplankton dynamics. Abundance of bacteria varied considerably in nitrate and phosphate induced phytoplankton blooms. However, the effect of $CO_2$ on bacterial abundance could not be demonstrated (Grossart *et al.*, 2006, Allgaier *et al.*, 2008). The structure of communities of free living bacteria demonstrated significant changes under increased $pCO_2$, but this effect was

not detected in the attached fraction, that correlated more strongly with phytoplankton development (Allgaier *et al.*, 2008). Increased $pCO_2$ can also affect bacterial extracellular enzymatic activity and secondary biomass production. Protease activity was reported to be highest at elevated $pCO_2$ level (Grossart *et al.*, 2006). Piontek *et al.* (2010) found higher α and β-glucosidase in enhanced $pCO_2$ conditions, suggesting that in these circunstances, polymer hydrolysis may be diverted towards polysaccharide degradation. Polysaccharide-based dissolved organic matter (DOM) could inhibit the biological carbon pump, resulting in a positive feedback on $CO_2$ emissions (Piontek *et al.*, 2010). Bacteria production was found to be higher (Grossart *et al.*, 2006) or unchanged under future $pCO_2$ scenarios (Allgaier *et al.*, 2008).

In summary, although the underlying mechanisms of increase $pCO_2$ effects are not yet fully understood, the current knowledge suggests that increased $pCO_2$ levels will affect the structure of microbial communities and rates of several microbial mediated processes.

*Effects of UVR in marine microbial communities*

Research on increase UVR implications was stimulated 30 years ago by the realization that with the stratospheric ozone layer in danger due to anthropogenic chlorofluorocarbons emissions, there would be an increase in UV-B radiation. Since then, mainly due to the implementation of the Montreal protocol that successfully reduced ozone-depleting substances, the ozone levels in the atmosphere are no longer declining (Mckenzie *et al.*, 2011). However, the increasing concentrations of greenhouse gases are expected to interact with ozone and alter its spatial distribution and exchanges between the stratosphere and troposphere, with potential effects on UVR levels that reach the earth (Bais *et al.*, 2011).

The amount of UVR that reaches the earth surface has direct consequences to aquatic ecosystems. The UVR component of incident solar radiation is the most photochemically reactive waveband of incident radiation, being able to penetrate to considerable depths in aquatic ecosystems, with potential genotoxic, cytotoxic and ontogenetic effects in aquatic organisms (Bancroft *et al.*, 2007). UVR can be divided in three wavelength ranges: UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (<290 nm). Since DNA absorbs only weakly at longer UV wavelengths (Jones *et al.*, 1987, Wells *et al.*, 1984) the detrimental effects of UV-A are usually considered to be mostly indirect, resulting from intracellular generation of ROS, such as oxidative damage to lipids, proteins and DNA (Pattison *et al.*, 2006). UV-C

wavelengths are almost completely screened out of the atmosphere by oxygen and ozone, therefore they are generally not considered as environmentally relevant. UV-B is the highest energy wavelength of solar radiation that reaches the Earth's surface and the UV wavelength most affected by changes in the ozone layer (Andersen *et al.*, 2002). The UV-B radiation is the most relevant of the UV spectrum, since it can cause damage to nearly all biomolecules by direct absorption or indirectly as a result of enhanced formation of reactive oxygen species (Vincent *et al.*, 2000).

In the natural environment, effects of UV-B radiation are generally attenuated by protection strategies such as avoidance, photochemical quenching and repair (Vincent *et al.*, 2000). The overall stress imposed by UVR exposure reflects a balance between damage, repair and the energetic costs of protection, and may affect energy consumption and biochemical composition of cellular material, shifting survival and growth rates (Vincent *et al.*, 2000). Due to short generation times, simple haploid genomes with little or no functional redundancy and small size, UVR represents an important stressor for microorganisms in aquatic ecosystems (Garcia-Pichel, 1994). Exposure to UV-B radiation results in a general decrease in bacterial abundance (Herndl *et al.*, 1993, Santos *et al.*, 2012). Effects of UV-B radiation on bacterial community composition have been observed, which could have implications to the biogeochemical impact of enhanced UVR levels on ecosystems. For example, evidences have been found that *Alphaproteobacteria* populations sensitive to UVR are responsible for a large part of low-molecular-weight DOM uptake, while the more UVR-resistant *Bacteroidetes*, tend to be specialized in high-molecular-weight DOM uptake (Alonso-Sáez *et al.*, 2006, Cottrell *et al.*, 2000). This suggests that changes in bacterial community due to UVR pressure could lead to shifts in the pathways of DOM (Morris *et al.*, 2002).

In general, exposure to UV-B also decreases extracellular enzymatic activities (Herndl *et al.*, 1993, Miiller-niklasl *et al.*, 1995, Santos *et al.*, 2011a), oxygen consumption (Pakulski *et al.*, 1998), leucine and thymidine incorporation (Sommaruga *et al.*, 1997, Santos *et al.*, 2012), and induce shifts in carbon source use profiles by bacteria (Santos *et al.*, 2012). This can also affect DOM utilization in aquatic ecosystems quantitatively and qualitatively. However, results are sometimes contradictory between studies and vary even between bacterial isolates tested in a particular study (Fernández Zenoff *et al.*, 2006, Santos *et al.*, 2011b). Furthermore, stimulatory effects of UVR exposure on bacterial activity have also been reported as a result of phototransformation of organic matter (Pakulski *et al.*, 2007).

UVR can also affect microorganisms indirectly. Bacteria, viruses, phytoplankton and bacterivores interact closely, and therefore, if one group of organisms is directly impacted by UVR (*e.g.*, due to DNA damage) this will indirectly affect carbon flow through the other groups (Vincent *et al.*, 2000). Effects of UVR on bacteria can also be influenced by the photochemical alteration of the bioavailability of dissolved organic carbon (DOC). The irradiation of DOC can result in the production of low-molecular weight (LMW) compounds that are easily taken up by bacteria and used for growth, as well as more refractory products and ROS which might exert an overall inhibitory effect (Kieber, 2000).

Depending on the environmental properties (physical, chemical and biological) of the water body studied, UVR can exert a stimulatory or inhibitory effect on bacterial metabolism. However, most studies agree on the potential of UVR to alter the efficiency with which dissolved organic matter is transformed into bacterial biomass and thus affect the carbon cycle.

**Interactions between climate change, oil pollution and microbial communities**

Despite the fact that several studies have already shown that physical and chemical parameters directly affect the toxicity of anthropogenic pollutants, the synergistic effects of UVR and ocean acidification on the chemistry of these pollutants and their effects on marine microbial communities have received very little attention. Increased levels of UVR and changes in ocean pH levels will certainly affect the chemical structure of several natural compounds and environmental pollutants, affecting the way that they interact with marine organisms.

Polycyclic aromatic hydrocarbons (PAH) are, due to their highly conjugated π-orbital systems, ideal examples of photoactive contaminants that strongly absorb in the UV-B and UV-A spectral regions (Krylov *et al.*, 1997). It is known that the toxicity of PAH to marine organisms increases with exposure to UVR (Krylov *et al.*, 1997, McConkey *et al.*, 1997, Pelletier *et al.*, 2006, Petersen *et al.*, 2007). This increase is largely regulated by photosensitization and photooxidation reactions. Photosensitization begins with the sensitizing molecule absorbing a photon, which elevates it to an excited singlet state. From there, the molecule can be transformed to the excited triplet state, where it can react with ground triplet-state oxygen to form singlet oxygen. Singlet oxygen is capable of oxygenating or oxidizing a wide range of biomolecules, with potentially lethal effects to a wide range of

organisms (Krylov *et al.*, 1997). Photomodification (photooxidation) occurs due to the absorption of photon energy at 280–400 nm (UVR) by the aromatic molecules, which leads to the formation of new compounds. This process releases phototoxic aromatic hydrocarbons into the environment, which are more toxic than their parent compounds (Krylov *et al.*, 1997, McConkey *et al.*, 1997, Peachey, 2005, Sargian *et al.*, 2006, Petersen *et al.*, 2007).

Reduced seawater pH has the potential to affect the adsorption of metals by organic particles. Generally, organic particles are negatively charged and, as pH declines, surface sites become less available to adsorb positive charges like metals. This is particularly important because more than 99% of the total concentrations of many metals in seawater are in organic complexes (Doney *et al.*, 2009, Millero *et al.*, 2009). A small deviation in the concentration of elements like Cu and Cd can result in toxic effects to marine organisms (Millero *et al.*, 2009), including microorganisms. However, organic materials are often nonhomogeneous and have unknown structures, so the speciation of metals in organic complexes is not as well characterized as is the case with inorganic ligands (Doney *et al.*, 2009, Millero *et al.*, 2009)

Studies have already found evidences of synergistic interactions between climate change and anthropogenic pollutants. For example, the exposure to PAH and UVR treatments independently had no significant effects on larval crab mortality but the combined exposure caused an increase in mortality of up to 100% when compared to the independent treatments (Peachey, 2005). Photoenhanced toxicity of PAH due to UVR exposure has been observed in a variety of organisms, such as algae (Marwood *et al.*, 1999) and crustaceans (Nikkilä *et al.*, 1999, Peachey, 2005) and plants (Huang *et al.*, 1997). This phenomenon, has also been reported for isolated bacterial strains such as *Photobacterium phosphoreum* (McConkey et al., 1997) and *Vibrio fisheri* (El-Alawi *et al.*, 2001). However, only few studies have addressed the effect of UVR photo-modified pollutants in complex microbial assemblages.

In a microcosm experiment designed to study the effects of increased UV-B in the presence of the water-soluble fraction of crude oil, an increase in mortality was observed in the phytoplankton community in UV-B exposed microcosm. Bacterial growth was found to increase, which the authors related with organic matter release due to toxicity effects on phytoplankton (Pelletier *et al.*, 2006). More recently, a similar effect was observed in sediment microcosms (Petersen *et al.,* 2008). Algal $^{14}$C-incorporation and chlorophyll *a* content in sediments exposed to UV-light and pyrene was found to decrease when compared to sole pyrene treatments. Concurrently, increase O consumption and increase release of N and P was

detected, suggesting an increase in bacterial activity (Petersen *et al.*, 2008). Both of these studies suggest that PAH phototoxicity may be relevant to microbial communities.

Fabry et al. (2008) suggested that altered water $CO_2$ chemistry in synergy with other environmental stressors may modify organismal and even ecosystem responses to these stressors in ways that differ substantially from the action of only a single stressor (Fabry *et al.*, 2008). There have, however, been no studies on the toxicity of PAH and other persistent organic pollutants under increased $pCO_2$ scenarios on marine microorganisms.

An important question that we need to answer thus is whether ocean acidification and UVR will affect microbial communities in such a way as to alter microbial mediated detoxification of anthropogenic pollutants. In addition to directly measurable physiological effects on marine microbes, UVR and ocean acidification can also exert indirect effects on the toxicity of anthropogenic pollutants by inducing shifts in microbial community structure; they can also alter microbial mediated detoxification processes. For example, the bioavailability of inorganic nutrients required for bacterial growth, such as nitrogen and phosphorus, are key factors in successful ecosystem detoxification of PAH (Atlas *et al.*, 1972). Any factor likely to alter the nitrogen cycle, such as ocean acidification, has therefore the potential to alter microbial mediated PAH detoxification processes and consequently PAH toxicity.

Another potential effect of elevated UVR levels and ocean acidification is an alteration of metal bioavailability. Metals interact with microorganisms in various ways, and are involved in virtually all aspects of microbial growth and metabolism (Gadd, 2010). Changes in iron chemistry are particularly important, given that iron is a limiting nutrient for marine phytoplankton in large oceanic regions (Shi *et al.*, 2010). Although iron is the fourth most abundant element in the Earth´s crust, it is present in very low concentrations in oceanic water (Ducklow *et al.*, 2003). This is partially due to the low solubility of Fe (III), the thermodynamically stable redox form of iron (Sunda, 2010). Recently, it was reported that the $pCO_2$ level for the year 2100 would reduce iron uptake by diatoms and coccolithophores by 10 to 20% (Shi *et al.* 2010). The reduction in iron availability is believed to be related to pH induced binding of iron to organic ligands, thus reducing biologically available Fe(III) (Sunda, 2010, Shi *et al.*, 2010). However, the net effect of ocean acidification is still unclear. For example, although lower pH increases iron binding to organic ligands, the solubility of Fe(III) increases with water acidification (Millero *et al.*, 2009). Moreover, increased organic binding reduces iron precipitation and adsorption to particulate surfaces, two mechanisms by which iron is removed from seawater (Sunda, 2010). Lower pH levels will also increase Fe(II)

concentration, due to its ability to form carbonate complexes to a larger degree than the other free metals and by increasing rates of Fe(III) reduction to soluble Fe(II) (Millero, 2001, Millero *et al.*, 2009).

Despite the complexity of iron regulation in the ocean, the study by Shi et al. (2010) highlights potentially harmful effects of iron bioavailability due to ocean acidification. Iron is also an important factor in the detoxification of hydrocarbons, by influencing the activity of enzymes that catalyze the oxidative breakdown of PAH (Dinkla *et al.*, 2001, Santos *et al.*, 2008). Monoxygenase and dioxygenase enzymes, essential in most microbial PAH degradation pathways, require a metal cofactor which is often iron (Bugg, 2003, Dinkla *et al.*, 2003). The activity of several key enzymes involved in the degradation of the aromatic hydrocarbon toluene by *Pseudomonas putida*, including toluene monooxygenase, was found to be reduced under iron limitation conditions (Dinkla *et al.*, 2001). Furthermore, increasing iron availability stimulates PAH degradation by increasing the activities of the enzymes involved in the aerobic degradation, and by increasing PAH bioavailability due to the stimulation of biosurfactant production (Santos *et al.*, 2008).

**Microcosm coupled with molecular biology techniques as a study tool**

In order to obtain the information necessary to formulate predictive models of the combined effects of ocean acidification and UVR on pollutant toxicity and degradation, it is essential to move forward from a phenomenological approach to a mechanistic view of the effects of climate change at several scales, ranging from communities to individual organisms at cellular, genetic and biochemical levels (Reid, 2011). However, anthropogenic environmental perturbations occur over a very wide range of spatial and temporal scales and the characterization of cause-effect relationships is difficult to gauge, particularly when studying additional levels of complexity such as synergistic effects with other stressors. Small scale models, such as micro- and mesocosms, can provide useful tools to obtain an integrated view of various phenomena. The distinction between microcosms and mesocosms systems is somewhat arbitrary. Laboratory small size systems are often referred to as microcosm and larger 'outdoor' systems are usually referred to as mesocosms.

Microcosms are simplified ecosystems, constructed to mimic natural ecosystems under controlled conditions (Roeselers *et al.*, 2006). Their size, design and cost vary with the research problem to be addressed. They can range from simple flasks and glass containers (Buckling *et*

*al.*, 2002) to more elaborate controlled environments, such as those developed by Mock and colleagues to simulate the ice-water interface (Mock *et al.*, 2003). The major advantage of these experimental setups is that they enable a high degree of experimental control and replication that are important to our understanding of ecological processes in microbial populations or communities. The level of experimental control provided by microcosms is virtually impossible to obtain through field surveys or outdoor mesocosm experimentation (Benton *et al.*, 2007, Jessup *et al.*, 2004). In addition to this, microcosms permit experimental exposure to toxic substances that would not be possible in a field setting.

An important, but valid, criticism is that these models are of limited relevance in understanding large-scale processes, as opposed to field studies (Carpenter *et al.*, 1997). There is a concern that these models are too small in spatial and temporal scales to be useful. However, the goal of the microcosm is not to fully reproduce nature in a laboratory model system, but rather to simplify complex ecosystems (Jessup *et al.*, 2004). Depending on the research question, laboratory models are not only a valid approach but they can isolate variables and capture essential dynamics of ecological systems better than field studies (Cadotte *et al.*, 2005). Although only presenting a limited representation of nature, microcosm experiments allow the deconstruction of nature to explore hypotheses and test mechanisms presumed to be operating in field systems (Jessup *et al.*, 2004, Cadotte *et al.*, 2005, Benton *et al.*, 2007).

Microbial microcosms are particularly interesting since they can overcome scale related microcosm limitations. The small sizes and short generation times of microorganisms allow microcosm experiments with a representative range of spatial and temporal scales (Jessup *et al.*, 2004). Microbial microcosm systems have already allowed the study of ecological processes including the study of predator-prey coevolution (Jessup *et al.*, 2004), ecosystem level-selection (Swenson *et al.*, 2000), resource competition (Grover, 2000) and adaptive radiation (Rainey *et al.*, 1998). Simple microcosms have also been used to study the effect of global change on microbial communities and simple culture bottles were already used to study the effects of environmental warming on eukaryotic microbial communities (Petchey *et al.*, 1999).

A major concern in microcosm experiments is whether the structural and functional properties of the source ecosystem are well represented in the microcosm (Rainey *et al.*, 1998). Before extrapolating microcosm results to the source ecosystem, ecological parameters must be compared to determine whether they are within certain limits (Pritchard *et al.*, 1984, Leser, 1994). It is important to underline that laboratory model-systems cannot be the sole focus of

the research. Microcosm studies must be developed along with modeling and they can also be seen as an important complement to field studies in order to address specific hypotheses (Benton *et al.*, 2007). Many field studies, for example, only provide correlative evidence of certain phenomena. For example, the community structure of certain microbial communities may be significantly related to variation in certain environmental variables (Cleary *et al.*, 2012). Microcosm experiments can help to elucidate whether there is an actual mechanistic effect of one or more of these variables on community composition.

Another advantage of using less complex microbial models is that we now have the tools to characterize the composition and function of microbial communities with unprecedented resolution. The introduction of high-throughput "omics" technologies in microbial ecology now allows us to study complex microbial communities at several levels of biological organization without the need of prior cultivation. First restricted to genomes (metagenomics), it is now possible to detect RNA transcripts (metatranscriptomics), protein levels (metaproteomics) and metabolites (metabolomics). We are, however, still far from being able to use this information to map every biochemical path and trophic interactions so as to develop descriptive and predictive models (Raes *et al.*, 2008, Jansson *et al.*, 2012). Nevertheless, these technologies enable a thorough characterization of the structure; function and genetic potential of microbial communities and under controlled conditions can contribute to the assessment and monitoring of the effects of global climate changes on key biogeochemical and ecological processes.

Microcosm experiments paired with new "omics" technologies can provide an excellent framework to test anthropogenic pollutant toxicity under different climate change scenarios in marine environments. Such small-scale experiments help to unravel key structural and functional aspects of environmental microbiology, and may help to test hypotheses that would be virtually impossible to test in nature. As mentioned previously, microcosms have already made important contributions to our understanding of microbial ecology. However, in order to simulate global change scenarios such as those discussed above, with a high degree of experimental control, more complex microcosm systems are required.

Although there are some major technical challenges that still need to be met with respect to data acquisition and integration, the ultimate goal must be the development of integrated approaches that will enable us to test various climate change scenarios and to ascertain what effect they will have on microbial composition, nutrient cycling and the toxicity of anthropogenic pollutants.

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# Chapter II

# CHAPTER II

# Exploring hydrocarbonoclastic bacterial communities in the estuarine surface microlayer

**Francisco J. R. C. Coelho, Sara Sousa, Luísa Santos,**
**Ana L. Santos, Adelaide Almeida, Newton C. M. Gomes, Ângela Cunha**
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**Abstract:** Bacteria that degrade polycyclic aromatic hydrocarbons (PAHs) in the estuarine surface microlayer (SML) of the Ria de Aveiro, Portugal which is chronically polluted with oil hydrocarbons (OH) were isolated and characterized; *Pseudomonas* was dominant among the PAH-degrading bacteria. Screening for PAH dioxygenase genes detected almost identical *nahAc* genes (encoding the alpha subunits of naphthalene dioxygenase) in 2 phylogenetically distinct isolates: *Pseudomonas sp.* and an unknown species of the family *Enterobacteriaceae*; this suggested that horizontal transfer of *nah* genes might be involved in PAH degradation in the SML. We also investigated the effect of PAH contamination on the spatial variability of the bacterioneuston along a gradient of pollution in the estuarine system of the Ria de Aveiro. Culture-independent techniques - fluorescence in situ hybridization (FISH) and denaturing-gradient gel electrophoresis (DGGE) - revealed a similar structure among the bacterioneuston communities along the estuary. In contrast, we detected differences in the relative abundance and diversity of organisms of the *Gammaproteobacteria*, including those of the genus *Pseudomonas* (which belongs to the *Gammaproteobacteria*). This is the first insight into the hydrocarbonoclastic bacterial communities in the SML of an estuarine area polluted with hydrocarbons. Our findings highlight the importance of SML-adapted hydrocarbonoclastic bacterioneuston as a potential source of new PAH-degrading bacteria (including new pseudomonads) with potential use in the bioremediation of hydrocarbon-polluted ecosystems.

**Keywords:** Sea surface microlayer, Bacterioneuston, Polycyclic aromatic hydrocarbons, PAH degradation

## Introduction

The surface microlayer (SML) is the interface between the atmosphere and the hydrosphere. This layer is roughly defined as the uppermost millimeter of the water column, and is characterized as a unique environment with distinct chemical and physical processes when compared with the underlying water (UW) (Liss *et al.*, 1997, Cunliffe *et al.*, 2009b, Cunliffe *et al.*, 2011).

The formation of the SML results from the accumulation of particles at the air–water interface, establishing a film that extends into the UW (Cunliffe *et al.*, 2011). Recently, the classical model of the SML as a stratified structure with an upper lipid and a lower protein–polysaccharide layer (Hardy, 1982) has been reviewed. Wurl & Holmes (2008) reported that the enrichment of transparent exopolymer particles (TEPs) in the SML at several locations around Singapore appears to support the model of the SML first advanced by Sieburth (1983), *i.e.* a hydrated gelatinous film (Sieburth, 1983, Wurl *et al.*, 2008, Cunliffe *et al.*, 2009a, Cunliffe *et al.*, 2011). The organisms within the SML are known collectively as neuston; the bacterial fraction of the neuston is the bacterioneuston (Franklin *et al.*, 2005). The importance of the SML in the control of environmental processes, and in the exchange of chemicals between the water and the atmosphere (Wurl *et al.*, 2004, Obernosterer *et al.*, 2010, Cunliffe *et al.*, 2009b), has raised interest in the effects of the activities of the bacterioneuston in this ecosystem. Cunliffe et al. (2008) found evidence that the diversity of functional genes encoding subunits of methane monoxygenase (*mmox*) and carbon monoxide dehydrogenase (*coxL*) is different in the SML when compared with the UW (Cunliffe *et al.*, 2008). There is an emerging consensus that the SML harbors microbial communities distinct from those in the UW (for a full review see Cunliffe *et al.* 2011); however, there is still a lack of knowledge on both the structure and function of the SML community. For example, due to the

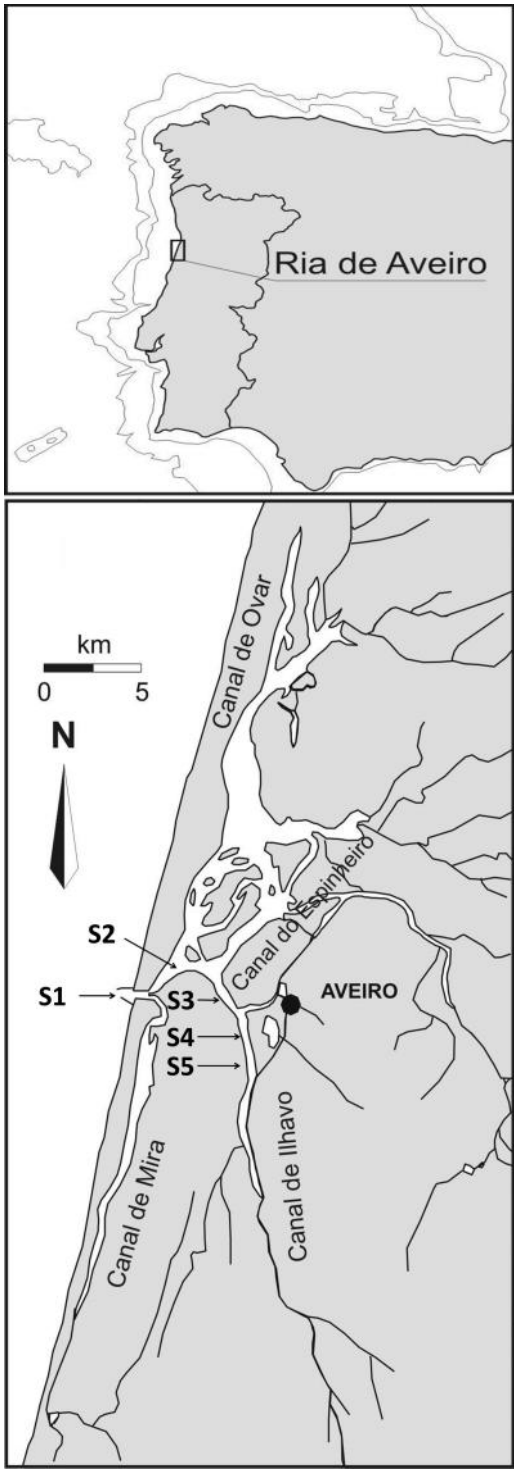


**Figure II-1:** Ria de Aveiro (Portugal). Sampling sites are indicated with arrows: Stn S1 in the Canal de Navegação, Stns S2 and S3 near the harbour facilities, and Stns S4 and S5 in the Canal de Ílhavo.

chemical nature of the SML, natural and anthropogenic aromatic compounds - *e.g.* polycyclic aromatic hydrocarbons (PAHs) - can be concentrated up to ~500Å in this layer in relation to the rest of the water column (Wurl *et al.*, 2004). High concentrations of PAHs have been found in the SML at locations chronically polluted with oil hydrocarbons (OH), such as shipping harbours (Cincinelli *et al.*, 2001, Wurl *et al.*, 2004). However, so far, no studies have exploited the potential of microorganisms in the SML to degrade PAHs, or have investigated the effect of PAHs on the bacterioneuston. It is reasonable to hypothesize that the increased levels of PAHs in the SML may stimulate the development of hydrocarbonoclastic bacterial populations in this layer. However, studies on the effects of PAH pollution on bacterioneuston are scarce, and none has specifically addressed the hydrocarbonoclastic bacterial populations of the SML. In order to obtain more information about the bacterioneuston, and the potential of these organisms to degrade PAHs, we: (1) isolated and identified representatives of the hydrocarbonoclastic population in the SML, and (2) screened the isolates for genes encoding enzymes involved in the degradation of PAHs. We also evaluated the effect of OH pollution on the spatial variability of the bacterioneuston in a gradient of contamination in the estuarine system of the Ria de Aveiro in Portugal.

## Materials and Methods

### *Study site and sampling*

Ria de Aveiro is a branched estuarine ecosystem, also described as a coastal lagoon, located in the northwest coast of Portugal. Samples for the selective isolation of hydrocarbonoclastic bacterioneuston were obtained from the SML in the middle section of the estuary, near a shipping harbour. In order to characterize the spatial variation of the bacterioneuston, 5 collecting points were chosen for sampling. Extending from the outer segment of the lagoon to the inner section of Canal de Ílhavo, the sites were designated: S1 (40° 40' 01" N, 08° 49' 24" W) in the outer section, S2 (40°39 29 N, 08°42 12 W) and S3 (40° 38' 20" N, 08° 41' 32" W) in the middle section (near the shipping harbour), and S4 (40° 37' 21" N, 08° 41' 01" W) and S5 (40° 35' 41" N, 08° 41' 21" W) in the inner section of the Canal de Ílhavo (Figure II-1). Samples were collected from the SML with plexiglass and glass plates. Both plates were 0.25 m wide, 0.35 m long, and 4 mm thick. Prior to sample collection, the plates were rinsed with ethanol and sterile distilled water. The plates were introduced vertically

through the SML and withdrawn in the same position. Excess water was allowed to drain for about 5 s. Approximately 5 ml of water were collected each time the plates were introduced into the SML. The water adhering to the plate was subsequently removed from both sides of the plate with a wiper blade system (Harvey *et al.*, 1972). The estimated thickness of the collected SML, determined from the volume of collected sample and the area of both sides of the plate, was approximately 60 μm, which is in the range reported by Harvey & Burzell (1972) for SML glass plate samplers. Salinity was determined immediately after the sampling with a WTW (Wissenschaftlich Technische Werkstätten) Cond330i/SET. Quantification of PAHs was performed by gas chromatography–mass spectrometry (gas chromatograph Varian CP-3800 with split/splitless injection and mass spectrometry detector—Ion Trap Saturn 2200) with detection limits between 20 and 40 ng $l^{-1}$. The result was calculated as the average of 2 sub-samples and expressed in ng $l^{-1}$.

*Enrichment and isolation of PAH-degrading bacteria*

A PAH, 2-methylnaphthalene, was used as the sole source of carbon and energy for the enrichment and isolation of PAH-degrading bacteria in a liquid mineral medium (MM). Selective plates were prepared by adding 2% (wt/vol) agarose to MM. The 2-methylnaphthalene (Fluka) was added by spreading ethanol solutions (50 mg $ml^{-1}$) onto the surface of the solid medium. Inoculation was carried out only when the solvent had fully evaporated, producing a film of PAH on the surface of the medium (Ma *et al.*, 2006). Bacterioneuston samples for enrichment cultures were collected near the shipping harbour. A volume of 200 ml of SML sample was added to 800 ml of sterile MM with 200 mg of 2-methylnaphthalene (Fluka) in a 1 l sterilized Erlenmeyer flask. The liquid cultures were incubated at room temperature for 2 weeks, in the absence of light, on a rotary shaker (90 rpm). After the enrichment, 20 ml of culture were transferred to fresh medium and incubated for the same period of time. After 2 similar subcultures, a serial dilution of the culture was spread onto the MM selective plates. These cultures were incubated at 25°C for up to 14 d, protected from light. Individual isolated colonies were further purified by re-streaking onto new MM selective plates. Control plates without 2-methylnaphthalene were also incubated.

*Extraction of DNA*

Genomic DNA was extracted from all the strains recovered from the isolation procedure, as previously described by Henriques et al. (2004). Environmental samples were filtered (pore size: 0.2 μm; Poretics Products). Total DNA was extracted in 3 replicates and purified using the Genomic DNA Purification Kit (MBI Fermentas) (Henriques *et al.*, 2004).

*Molecular characterization and identification of the PAH-degrading bacterial isolates*

For molecular typing of the isolates, a repetitive-sequence PCR using a BOX A1R primer (BOX-PCR fingerprinting) was followed (Rademaker *et al.*, 1998). BOX-PCR profiles were visualized after separation of PCR amplicons by electrophoresis in 2% agarose gel using 1×TAE (Trisacetate-EDTA) at 100 V for 3 h. The gels were stained with ethidium bromide and digitalized in a Molecular Imager FXTM system (Bio-Rad Laboratories). The band positions were normalized with the GeneRuler™1 kb Plus DNA ladder (75–20 000 bp) and analysed with GelCompar I software (Applied Maths) using the pairwise Pearson's product–moment correlation coefficient (r-value). Cluster analysis of the similarity matrices was performed by the unweighted pair-group method (UPGMA) using arithmetic averages. For isolates displaying distinct BOX-PCR profiles, the 16S rRNA gene was amplified by PCR using the universal bacterial primers U27 and 1492R (Weisburg *et al.*, 1991). PCR products from the amplified 16S rRNA were used as templates in the sequencing reactions - which were carried out using an ABI PRISM_BigDye_Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis. These isolates were also screened for the Pseudomonas-specific *gacA* gene using primers GACA-1F and GACA-2 (De Souza *et al.*, 2003). All PCR reagents were purchased from MBI Fermentas unless otherwise indicated.

*Detection of the dioxygenase gene*

PCR was used for detecting genes that encode enzymes involved in the aerobic degradation of PAHs in the isolated strains. Primers specific for the alpha subunit of the PAH-ring hydroxylating dioxygenases, common to Gram-negative PAH degraders (PAH-RHDαGNF and PAH-RHDαGNR primers), were used to obtain amplicons of *ca.* 306 bp (Cébron *et al.*, 2008). The PCR products were used as templates in the sequencing reactions. Sequencing was carried out as described in the previous section, and a comparison was made

with sequences available in the GenBank database by using the BLAST service to determine their closest relative. All PCR reagents were purchased from MBI Fermentas unless otherwise indicated.

*Microbial community structure*

Denaturing-gradient gel electrophoresis (DGGE): DGGE was performed on the *Pseudomonas*-specific *gacA* gene sequence amplified from bacterial isolates and from environmental samples. This technique was also applied to the 16S rRNA gene sequence amplified from environmental samples. A nested PCR approach was used to amplify the 16S rRNA gene fragments for DGGE analyses. For the first PCR, the universal bacterial primers U27 and 1492R were used to amplify *ca.* 1450 bp of the 16S rRNA gene (Weisburg *et al.*, 1991). The amount of template DNA used per reaction was *ca.* 10 ng. For the second PCR, 1 µl of the product of the first PCR was used as template with bacterial DGGE primers 968F-GC and 1401R (*ca.* 433 bp) (Nübel *et al.*, 1996). A nested-PCR approach was also applied to the amplification of *Pseudomonas*-specific *gacA* gene fragments for DGGE analysis (Costa *et al.*, 2007). For the first PCR, primers GACA-1F and GACA-2 were used to amplify *ca.* 425 bp of the *gacA* gene (De Souza *et al.*, 2003). The amount of template DNA used per reaction was *ca.* 10 ng. An aliquot of 1 µl of the product of the first PCR was used as the template for a second PCR with primers gacA1F-GC and gacA2R (Costa *et al.*, 2007). All PCR reagents were purchased from MBI Fermentas unless otherwise indicated. DGGE analysis was performed with a CBS-DGGE 2401 system (CBS Scientific). The GC-clamped amplicons were applied to a double-gradient polyacryl-amide gel containing 6 to 9% acrylamide (Rotiphorese) with a gradient of 30 to 58% of denaturant. The run was performed in Tris-acetate-EDTA buffer (0.5 M Tris-Base, Sigma, 0.05 M EDTA, Sigma; 0.1 M $CH_3CO_2Na$, Sigma, pH 8.0) at 60°C at a constant voltage of 220 V for 16 h. The DGGE gels were silver stained (Heuer *et al.*, 2001). The Shannon index of diversity (H) was used to compare the complexity of the DGGE profiles. The band position and relative intensity (abundance) of each lane (community) were used as parameters to indicate categories (Costa *et al.*, 2006). Parametric analysis of variance (Anova) was applied to assess significant differences between samples, provided that data were normally distributed. The relative abundance of members of the domain *Bacteria* and the *Gammaproteobacteria* in the SML was assessed by Fluorescence *in situ* hybridization (FISH) (Pernthaler *et al.*, 2001) using Cy3-labeled oligonucleotide probes (MWG Biotech). Triplicate

samples (1 ml) were filtered through polycarbonate filters (pore size: 0.2 μm; GE Osmonics), fixed with 4% paraformaldehyde for 30 min and rinsed with 1×PBS and MilliQ water. The filters were stored at room temperature until hybridization. The probes used in this study were EUB338 for the domain *Bacteria* (Amann *et al.*, 1990) and EUB338-II and EUB338-III to cover the phyla *Planctomycetes* and *Verrucomicrobia* (Daims *et al.*, 1999). For the *Gammaproteobacteria* we used the probes GAM42a (Manz *et al.*, 1992) and an unlabeled competitor probe specific for the *Betaproteobacteria*. A non-binding probe was also used as a control for nonspecific binding (Karner *et al.*, 1997). Samples were examined with a Leitz Laborlux K microscope equipped with the appropriate filter sets for fluorescence from 4,6-diamidino-2-phenylindole (DAPI) and CY3. At least 10 fields were counted per replicate of sample. In addition to the relative abundance of organisms of the domain *Bacteria* and of the *Gammaproteobacteria*, it was also possible to obtain a measure of total microorganisms with the DAPI counts. All reagents were purchased from Fluka, except when otherwise indicated. Total microorganisms (DAPI counts) were tested for normality (Kolmogorov–Smirnov test) before the comparison of means. Parametric Anova was performed, provided that data were normally distributed.

## Results and discussion

### *Physical and chemical parameters*

Salinity in the analyzed SML samples ranged between 31.4% (Stn S5) and 36.0% (Stn S1). Salinity is an important factor that influences the structure and composition of prokaryotic assemblages in estuarine environments (Bouvier *et al.*, 2002, Henriques *et al.*, 2004). However, in this study, the variation of salinity along the estuarine profile was very small, and did not seem to influence the distribution of bacterial communities. The PAH analyses revealed that the composition and concentration of PAHs varied along the estuary (Table II-1). Concentrations ranged from below the limit of detection of the method to 14 ± 5 ng $l^{-1}$ of naphthalene. Stn S1 showed the lowest concentration of PAH (anthracene: 7.3 ± 0.2 ng $l^{-1}$). In Stns S2 and S3, located near the shipping harbour, naphthalene reached the highest concentrations (S2: 14 ± 5 ng $l^{-1}$; S3: 9.4 ± 1.5 ng $l^{-1}$). Naphthalene is the simplest of the oil PAH compounds; it is the most easily degradable and is highly volatile (Sporstol *et al.* 1983). The 3-ring PAH compounds anthracene (6.9 ± 0.2 ng $l^{-1}$) and phenanthrene (8.1 ± 1.7

ng $l^{-1}$) were detected at the Stns S2 and S3, respectively. At Stn S4, fluorene (9.4 ± 1.5 ng $l^{-1}$) was the only PAH detected. Stn S5 showed the largest spectrum of PAH compounds (phenanthrene 5.2 ± 0.1 ng $l^{-1}$, fluoranthrene 8.5 ± 1.1 ng $l^{-1}$, benzo[a]anthracene 5.1 ± 0.8 ng $l^{-1}$ and benzo[k]fluoranthene 8.1 ± 0.6 ng $l^{-1}$).

**Table II-1:** Concentrations of individual polycyclic aromatic hydrocarbons (PAHs) in the sea surface microlayer (SML) at Stns S1, S2, S3, S4 and S5. LD = limit of detection, BDL = below limit of quantification. Values are the means ±SD of the results for each site

| PAH | | PAH concentrations (ng $l^{-1}$) at site | | | | |
|---|---|---|---|---|---|---|
| | LD | S1 | S2 | S3 | S4 | S5 |
| Naphthalene | 8.8 | BDL | 14 ± 5 | 9.4 ± 1.5 | BDL | BDL |
| Acenaphthylene | 5.0 | BDL | BDL | BDL | BDL | BDL |
| Acenaphthene | 1.7 | BDL | BDL | BDL | BDL | BDL |
| Fluorene | 4.1 | BDL | BDL | BDL | 9.4±1.5 | BDL |
| Phenanthrene | 1.9 | BDL | BDL | 8.1 ± 1.7 | BDL | 5.2 ± 0.1 |
| Anthracene | 6.2 | 7.3 ± 0.2 | 6.9 ± 0.2 | BDL | BDL | BDL |
| Fluoranthrene | 5.7 | BDL | BDL | BDL | BDL | 8.5 ± 1.1 |
| Pyrene | 3.9 | BDL | BDL | BDL | BDL | BDL |
| Chrysene | 7.5 | BDL | BDL | BDL | BDL | BDL |
| Benz[a]anthracene | 3.7 | BDL | BDL | BDL | BDL | 5.1 ± 0.8 |
| Benzo[k]fluoranthene | 8.0 | BDL | BDL | BDL | BDL | 8.1 ± 0.6 |
| Benzo[b]fluoranthene | 9.5 | BDL | BDL | BDL | BDL | BDL |
| Benzo[a]pyrene | 5.2 | BDL | BDL | BDL | BDL | BDL |
| Indeno[1.2.3-cd]pyrene | 28 | BDL | BDL | BDL | BDL | BDL |
| Dibenzo[a.h]anthracene | 38 | BDL | BDL | BDL | BDL | BDL |
| Benzo(ghi)perylene | 33 | BDL | BDL | BDL | BDL | BDL |

**PAH-degrading isolates: characterization of isolates**

In order to isolate organisms from hydrocarbonoclastic bacterioneuston populations, in the first phase of this study, our sampling efforts were focused on the region of the Ria de Aveiro near the harbour facilities (see Figure II-1, Stns S2 and S3). This area showed the highest levels of contamination by low-molecular-weight PAHs. These PAHs constitute a significant fraction of crude oil and petroleum products and are often used as indicators of recent or chronic contamination with petroleum hydrocarbons (Sporstol *et al.*, 1983). The PAH 2-methylnaphthalene, a common constituent of crude oil and fossil fuels, was used in this study as the sole source of carbon and energy for the enrichment and isolation of strains

of the hydrocarbonoclastic bacterioneuston (Kasai *et al.*, 2002). The isolation procedure recovered 42 bacterial isolates that were able to use 2-methylnaphthalene as the sole source of carbon. Genotypic diversity of the isolates, assessed by the whole-genome BOX-PCR fingerprint method (Rademaker *et al.*, 1998), assigned the isolates to 29 different genotypes. The 16S rRNA gene sequence analysis showed that all hydrocarbonoclastic isolates were Gram-negative and had high homology with 6 different taxa: *Pseudomonas*, *Klebsiella*, *Serratia*, *Acinetobacter*, *Rhizobium* and *Vibrio* (Table II-2).

*Pseudomonas* and *Klebsiella* were preponderant, accounting to 34.5 and 31.0%, respectively, of the total representative isolates (10 isolates were assigned to the genus *Pseudomonas* and 9 to *Klebsiella*). These results point to a high genotypic diversity in the populations of hydrocarbonoclastic *Pseudomonas* in the SML (Table II-2). Members of this genus have a remarkable ability to degrade a wide range of organic pollutants, including PAHs, halogenated derivates and recalcitrant organic residues (Johnsen *et al.*, 1996, Bhattacharya *et al.*, 2003). Besides their capacity to degrade toxic compounds, species such as *P. putida* can efficiently produce a range of valuable compounds with industrial uses (Wackett, 2003). These abilities, together with enhanced stress resistance, amenability to genetic manipulation and suitability as a host for heterologous expression, makes strains of *Pseudomonas* particularly useful for biotechnological applications (Puchałka *et al.*, 2008). As well as contamination with PAHs, the estuarine system of the Ria de Aveiro is also subject to chronic contamination with domestic sewage (Cunha *et al.*, 2000). This might explain the large number of isolates related to the family *Enterobacteriaceae*, such as *Klebsiella* and *Serratia*. Nonetheless, the catabolic capacity of *Klebsiella* strains to degrade hydrocarbons, including PAHs, has been previously described. A recent study suggested that *Klebsiella* can be an important part of the OH-degrading bacterial groups in estuarine areas exposed to sewage contamination (Rodrigues *et al.*, 2009). *Acinetobacter* and *Rhizobium* were also part of the culturable fraction of PAH degraders in the SML. Many environmental strains of *Acinetobacter* with hydrocarbon-degrading capacities have been isolated in terrestrial and marine environments (Vanbroekhoven *et al.*, 2004, Rodrigues *et al.*, 2009). Reports of the presence of *Rhizobium* in PAH-contaminated soils, and its hydrocarbon-degrading metabolism, are also available (Poonthrigpun *et al.*, 2006). One of the isolates was identified as *Vibrio*, which has also been reported as a PAH degrader in marine environments (Hedlund *et al.*, 2001).

**PAH-degrading isolates: detection of dioxygenase genes**

In the present study, an attempt was made to detect genes encoding the enzyme system PAH-RHDα (involved in the initial step in bacterial metabolism of PAH) in the bacterioneuston isolates. All isolates, including those having similar BOX-PCR profiles, were screened. Although the isolates were cultivated with 2-methylnaphthalene as the sole source of carbon, and reference to the PAH-degrading capacity was obtained from the literature for all the identified genera (Hedlund *et al.*, 2001, Vanbroekhoven *et al.*, 2004, Ma *et al.*, 2006, Poonthrigpun *et al.*, 2006, Rodrigues *et al.*, 2009), *PAH-RHDα* gene sequences were detected in only 2 of the isolates.

**Table II-2:** Analysis of the 16S rRNA gene sequences from the isolated strains, and their tentative assignment to different taxonomic categories

| Isolate code | Sequence accession no.[a] | BOX group[b] | Closest phylogenetic relative | | |
|---|---|---|---|---|---|
| | | | BLAST-N identity[c] | % | Accession no.[d] |
| 1 | GU935753 | 1 | *Serratia* sp. | 99 | EF111121.1 |
| 2 | GU935754 | 4 | Uncultured *Klebsiella* sp. | 95 | EU344923.1 |
| 5 | GU935755 | 2 | *Acinetobacter johnsonii* | 98 | FJ263917.1 |
| 6 | GU935756 | 1 | *Serratia* sp. | 97 | EU109729.1 |
| 7 | GU935757 | 1 | *Klebsiella* sp. | 93 | DQ923489.1 |
| 10 | GU935758 | 1 | *Pseudomonas* sp. | 96 | FJ424813.1 |
| 12 | GU935759 | 1 | Uncultured *Pseudomonas* sp. | 96 | EU705005.1 |
| 13 | GU935760 | 1 | *Pseudomonas* sp. | 98 | FN429930.1 |
| 14 | GU935761 | 1 | *Pseudomonas* sp. | 97 | AB088548.1 |
| 15 | GU935762 | 1 | *Pseudomonas* sp. | 94 | DQ839561.1 |
| 16 | GU935763 | 2 | *Klebsiella terrigena* | 95 | AF129442.1 |
| 19 | GU935764 | 1 | *Serratia proteamaculans* | 97 | AY559499.1 |
| 20 | GU935765 | 1 | *Klebsiella* sp. | 97 | EU545402.1 |
| 21 | GU935766 | 1 | *Acinetobacter johnsonii* | 98 | AM184278.1 |
| 22 | GU935767 | 1 | *Rhizobium* sp. | 99 | EF599760.1 |
| 23 | GU935768 | 1 | Uncultured *Klebsiella* sp. | 97 | EU344923.1 |
| 24 | GU935769 | 1 | *Pseudomonas* sp. | 97 | FJ789687.1 |
| 25 | GU935770 | 1 | Uncultured *bacterium* | 99 | GQ069755.1 |
| 26 | GU935771 | 1 | *Serratia* sp. | 96 | EF111121.1 |
| 27 | GU935772 | 1 | Uncultured *Pseudomonas* sp. | 98 | DQ295987.1 |
| 28 | GU935773 | 3 | *Klebsiella* sp. | 95 | DQ229100.1 |
| 31 | GU935774 | 2 | Uncultured *Klebsiella* sp. | 97 | EF679185.1 |
| 32 | GU935775 | 1 | Uncultured *Pseudomonas* sp. | 95 | DQ295987.1 |
| 36 | GU935776 | 4 | *Klebsiella ornithinolytica* | 100 | AF129441.1 |
| 37 | GU935777 | 2 | *Pseudomonas* sp. | 97 | FN429930.1 |
| 40 | GU935778 | 1 | *Pseudomonas* sp. | 99 | AB506040.1 |
| 41 | GU935779 | 1 | *Rhizobium* sp. | 98 | EU741078.1 |
| 42 | GU935780 | 2 | *Klebsiella* sp. | 97 | EU888474.1 |
| 44 | GU935781 | 1 | *Vibrio proteolyticus* | 93 | DQ995521.1 |

[a] GenBank sequence accession numbers of the respective isolate
[b] Number of genotypes clustered in the same BOX group
[c] Blast-N max identity classification
[d] GenBank sequence accession number of most closely related bacterial sequence(s)

A positive PCR amplification product was obtained only for isolates #10 (*Pseudomonas sp.*) and #25, an unknown member of the family *Enterobacteriaceae*. However, the lack of amplification does not exclude the presence of dioxygenases in the other isolates; instead, this result may indicate the presence of novel genes encoding PAH-degrading enzymes in these hydrocarbonoclastic organisms. Moreover, because we did not monitor the complete cleavage of the benzene rings in this study, it cannot be excluded that the isolated strains oxidize only the methyl group of 2-methylnaphtalene. It is possible that the *ndo*-negative isolates use the methyl group as a source of carbon and energy without cleaving the benzene ring. Phylogenetic analysis, by BLAST, of our *PAH-RHDα* gene sequences and their closest relatives showed that both *PAH-RHDα* genes clustered together with the archetypal *nahAc* gene carried by the plasmid pNAH20 (Yen *et al.*, 1982, Eeva *et al.*, 2000, Sota *et al.*, 2006) (Figure II-2). As identical *PAH-RHDα* genes with high homology to those in naphthalene-degrading plasmids occur in different bacterial families, it is reasonable to speculate that horizontal gene transfer may also play a role in the occurrence and spreading of hydrocarbonoclastic capacity in the SML. Horizontal exchange of genes encoding the degradation of xenobiotic compounds is a common phenomenon in biofilms (Singh *et al.*, 2006). A preponderance of biofilm growing cells in the SML could favor ecological processes that occur in communities with cells in close proximity, such as gene transfer and quorum sensing (Cunliffe *et al.*, 2009b).

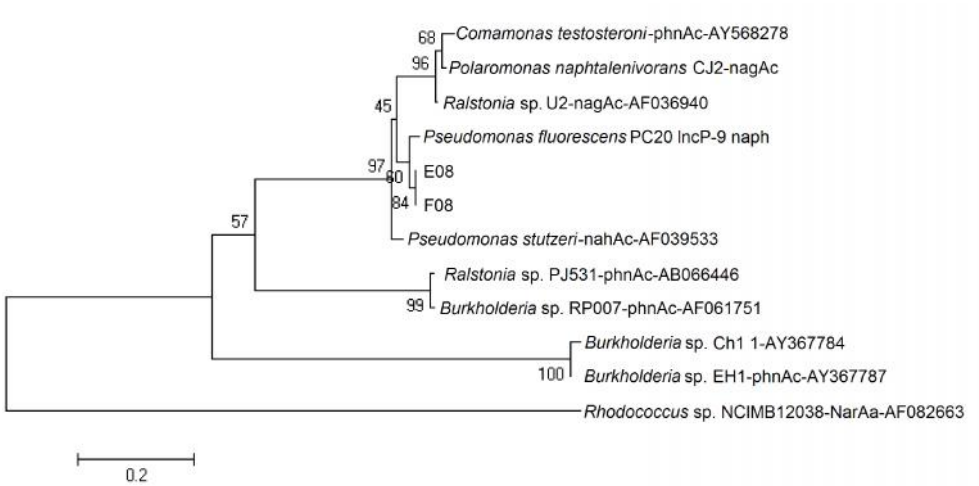


**Figure II-2:** Phylogenetic relationships between dioxygenase-encoding genes amplified from the isolated strains (E08 was obtained from clone 25, F08 from clone 10). The tree was constructed by using the neighbor-joining method and bootstrapping analysis (1000 repetitions). The numbers on the branches indicate percentages of bootstrap values. The scale bar represents the percentage of amino acid divergence.

## The effect of sampling site on the community structure of the bacterioneuston

### *Whole-community and Pseudomonas structural diversity*

Because most microorganisms are refractory to cultivation, culture-independent methods are fundamental in the characterization of the structure of microbial communities in the environment (Amann *et al.*, 1995). DGGE was used to assess the structural diversity of the overall bacterial community. The banding pattern analysis of the PCR-amplified 16S rRNA gene showed a large number of equally abundant bands in bacterioneuston communities from all the sampling sites (Figure II-3). The Shannon diversity index revealed no significant differences between sites (Table II-3). This analysis suggests that the dominant bacterial communities are very similar in the SML of different sampling sites. Earlier studies, using DGGE, found that the dominant bacterioneuston communities were similar in 2 different areas of the Blyth estuary (Cunliffe *et al.*, 2008). Recently, this author also reported that bacterioneuston communities from 2 different areas, close to the Hawaiian Island of Oahu, were more similar to each other than to their subjacent bacterioplankton communities (Cunliffe *et al.*, 2009a).

Due to the preponderance of *Pseudomonas* among the bacteria isolated from the PAH-enrichment culture, and because of the recognized ability of the organisms of this genus to degrade PAHs, we paid particular attention to the diversity of a *Pseudomonas* genetic marker in the SML at all sites. The *Pseudomonas*-specific *gacA* gene was used as a genetic marker for this genus. This gene influences the production of several secondary metabolites in *Pseudomonas spp.*, and it can be used as a complementary genetic marker for detecting bacteria of this genus in environmental samples (De Souza *et al.*, 2003).

Interestingly, contrary to what was observed for the bacterial fingerprints, analysis of the *Pseudomonas* community showed a significant (Anova, $P < 0.05$) increase in diversity (Table II-3) from the outer (Stn S1) to the middle (Stn S3) and inner (Stn S5) sections of the estuary, following the concentrations of PAH and organic matter. This pattern may explain the high abundance of *Pseudomonas* strains recovered from the SML-PAH enrichments obtained in the area with higher levels of lighter (low-molecular-weight) PAHs. As previously mentioned, members of this genus are known for their ability to metabolize light PAH compounds and other monoaromatic hydrocarbons. The *gacA* DGGE pattern of the isolated strains, and the *gacA* profile of whole bacterioneuston, showed a common band with the mobility exhibited by

isolate #27 (Figure II-4). This band is present in the bacterioneuston from Stns S1, S2 and S3, but is absent in samples from Stns S4 and S5, at the inner section of the estuary. Interestingly, isolate #27 showed a high homology with an uncultured *Pseudomonas* which has been linked to the transformation of organic compounds in coastal seawater (Xiaozhen *et al.*, 2007). These results suggest that the distribution of *Pseudomonas* populations may also be related to the quality and composition of lipophilic organic compounds in the SML.

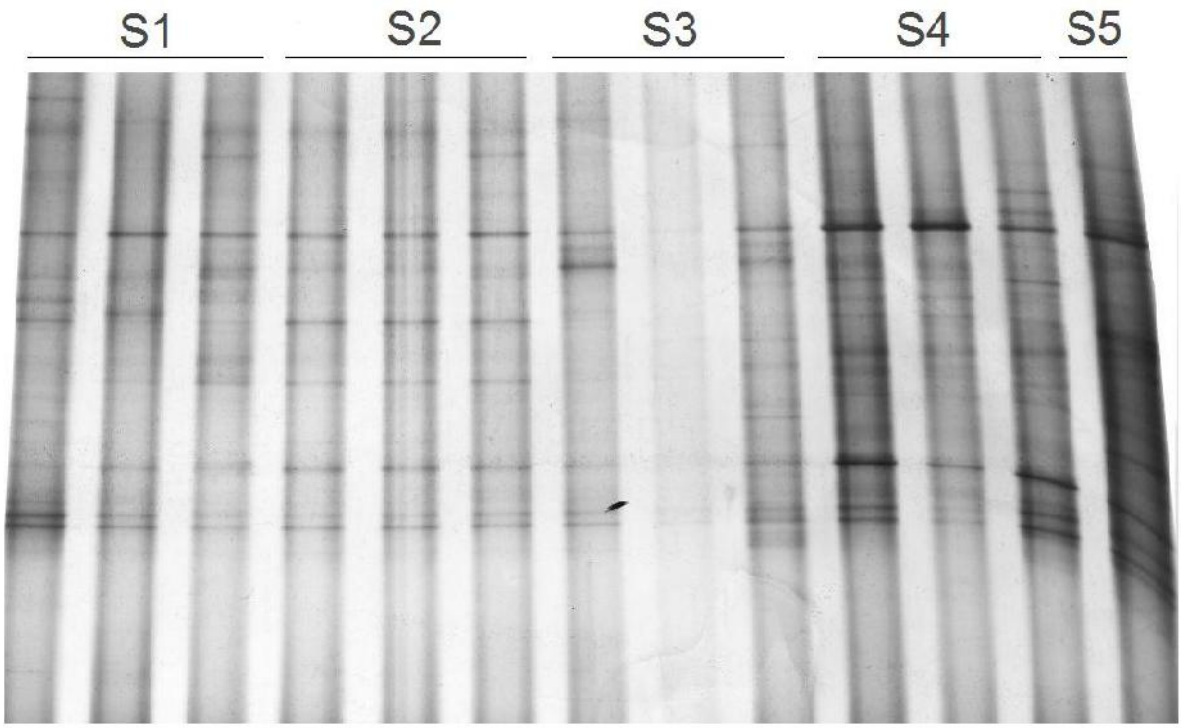


**Figure II-3:** Denaturing-gradient gel electrophoresis fingerprints of 16S rRNA gene fragments amplified from bacterioneuston obtained from the surface microlayer at Stns S1, S2, S3, S4 and S5.

**Table II-3:** Mean and standard deviation of the Shannon diversity indices calculated from denaturing-gradient gel electrophoresis (DGGE) profiles of bacterial 16S rRNA and the *Pseudomonas*-specific *gacA* gene in bacterioneuston from the surface microlayer (SML) at Stns S1, S2, S3, S4 and S5

| DGGE profile | Shannon diversity indices at site | | | | |
|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | S5 |
| *Pseudomonas-specific gacA* | 0.9±0.2 | 1.3±0.5 | 2.0±0.2 | 2.0±0.2 | 2.2±0.2 |
| *Bacterial* 16S rRNA | 2.36±0.29 | 2.24±0.24 | 2.44±0.29 | 2.22±0.37 | 2.58 |

*It was only possible to obtain one replicate of *Bacterial* 16S rRNA in site S5.

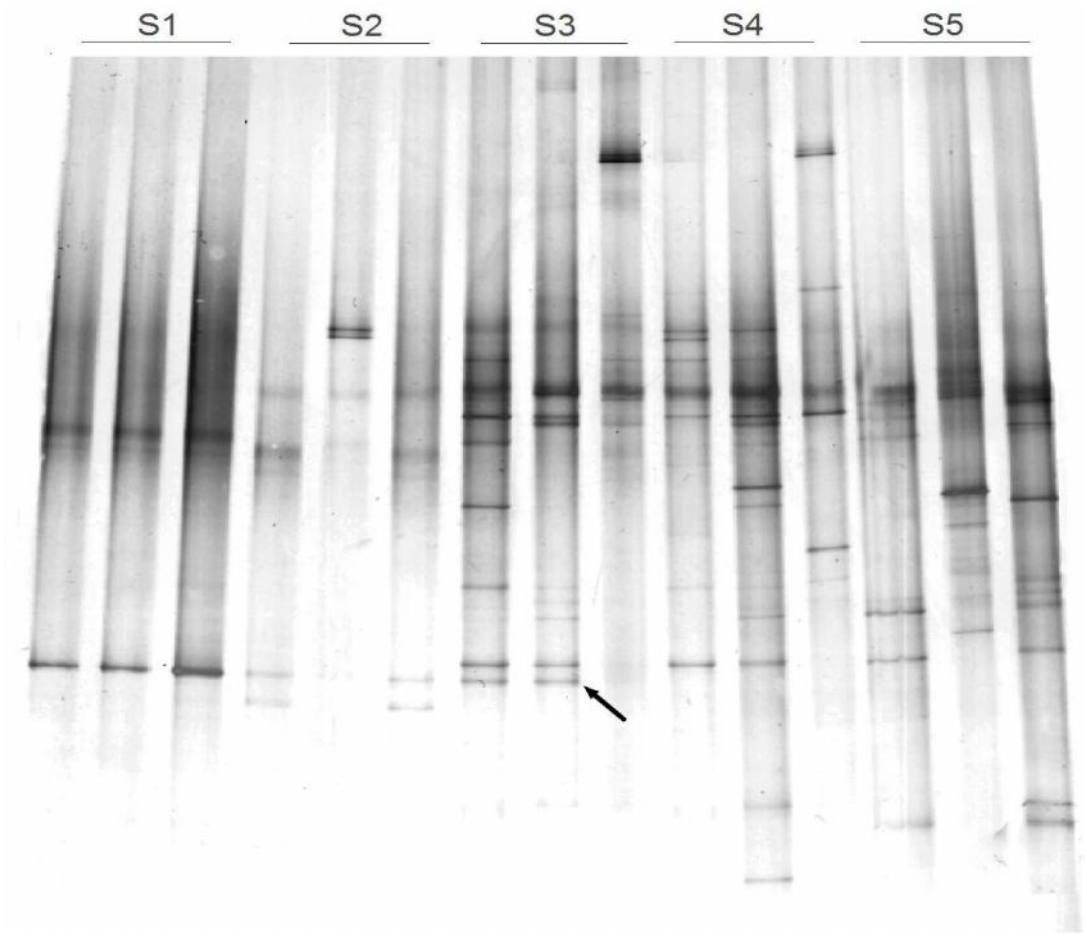


**Figure II-4:** Denaturing-gradient gel electrophoresis (DGGE) fingerprints of *Pseudomonas-specific gacA* gene fragments amplified from bacterioneuston in the surface microlayer collected at Stns S1, S2, S3, S4 and S5. The position of the arrow corresponds to the melting behaviour of fragments corresponding to isolate #27, classified as an uncultured *Pseudomonas sp.*

**Microbial abundance overall and in the *Gammaproteobacteria***

FISH was applied in this study to assess the spatial variability in the total abundance of microorganisms and also for quantifying members of the domain *Bacteria* and the *Gammaproteobacteria* in the SML (Amann *et al.*, 1990, Manz *et al.*, 1992, Karner *et al.*, 1997, Daims *et al.*, 1999, Pernthaler *et al.*, 2001). The overall abundance of microorganisms estimated from DAPI counts ranged from $2.3 \pm 0.2 \times 10^6$ cells $ml^{-1}$ at Stn S1, to $5.0 \pm 0.9 \times 10^6$ cells $ml^{-1}$ at Stn S5, defining a significant (Anova, $P < 0.05$) gradient of enrichment from the outer to the inner sections of the estuary (Figure II-5). The total cell number is within the range ($4.9 \times 10^5$ cells $ml^{-1}$ to $6.2 \times 10^6$ cells $ml^{-1}$) observed in the Ria de Vigo, Spain, estuary (Zdanowski *et al.*, 1997). The pattern of variation that we detected corresponds, in general, to the structure of an estuary in which bacterial abundance and phytoplankton biomass are maximal at the intermediate sections (Wright *et al.*, 1983, Fuks *et al.*, 1991, Cunha *et al.*, 2003). This distribution is probably related to the quality (lability) of the available organic matter along the estuary. The relative abundance of organisms of the domain *Bacteria* varied between $50.4 \pm 3.6\%$ at Stn S2 and $66.7 \pm 9.2\%$ at Stn S4 (Figure II-5).

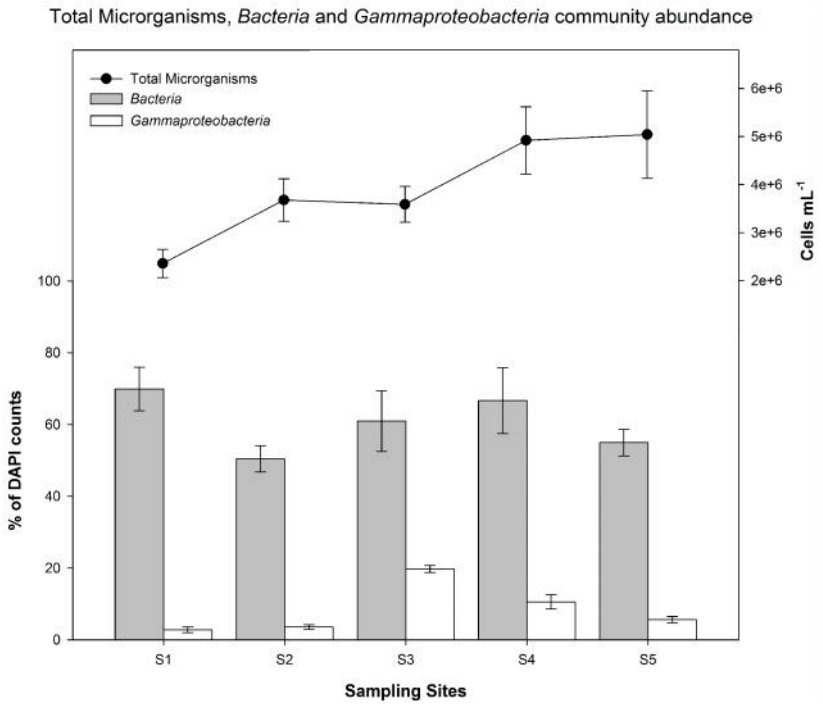


**Figure II-5:** Variations in total prokaryote abundance (as counted by 4 ,6-diamidino-2-phenylindole [DAPI] staining) and relative abundance of *Bacteria* and *Gammproteobacteria* (% of DAPI counts) determined by fluorescence *in situ* hybridization in bacterioneuston in the surface microlayer collected at Stns S1, S2, S3, S4 and S5. (In the scale: 2e+6 = $2 \times 10^6$ etc.)

The proportion of cells hybridized by the *Bacteria* probes is within the 60% average reported for the Delaware estuary (Kirchman *et al.*, 2005). The missing percentage could be related to other groups not covered by this study. Results of a CARD-FISH analysis, revealed that up to 37% of the total cell numbers recovered from the SML in high mountain lakes hybridized with *Archaea*-specific probes (Auguet *et al.*, 2008). The relative abundance of organisms of the domain *Bacteria* did not show a defined trend of spatial variation along the estuary. On the contrary, the highest value for the relative abundance of organisms of the *Gammaproteobacteria* (19.7 ± 1.0%) was found in the SML at Stn S3 (Figure II-5), where naphthalene and phenantrene were detected. *Gammaproteobacteria* were selected as a target for FISH analysis because this group includes many of the PAH-degrading genera, such as *Alcanivorax*, *Cycloclasticus*, *Pseudomonas*, *Oleiphilus*, *Oleispira* and *Thalassolituus (*Watanabe, *2001,* Head *et al., 2006)*. Although the abundance and distribution of microorganisms in marine and estuarine waters is influenced by complex biotic and abiotic interactions (Shiah *et al.*, 1995), the high concentration of low-molecular-weight PAHs, generally easily degraded (Yamada *et al.*, 2003), at sampling Stn S3 can be related to the high relative abundance of *Gammaproteobacteria* at this site. Exposure to hydrocarbons has been shown to decrease bacterial diversity, as a result of selection in favor of PAH-degrading bacteria (Roling *et al.*, 2002, Castle *et al.*, 2006). Nonetheless, other sources of organic carbon might also have influenced the distribution of this group. The preference of *Gammaproteobacteria* for high concentrations of nutrients has been previously reported, and peaks of abundance appeared to be related to particular point sources of nutrients (Bouvier *et al.*, 2002, Henriques *et al.*, 2004).

**Conclusions**

To our knowledge, we are the first to have isolated and characterized hydrocarbonoclastic strains of bacterioneuston from estuarine SML exposed to chronic OH contamination; the most dominant isolates belonged to the genus *Pseudomonas* (members of the *Gammaproteobacteria*). The SML is the first marine habitat to be in contact with hydrophobic OH pollutants after an oil spill accident. The possibility of using hydrocarbonoclastic bacterioneuston isolates in a bioremediation strategy for the rapid degradation of oil slicks should be examined in future studies. The SML has the potential to be a natural 'seed bank' that can be useful for isolating and 'domesticating' novel bacteria for PAH degradation. The ability of the hydrocarbonoclastic bacterioneuston to withstand and degrade toxic

compounds, together with its capacity to survive in this environment, make the isolates recovered in this study interesting candidates for future research on *in situ* SML bioremediation.

The cultivation-independent analyses (FISH counts and *Pseudomonas*-DGGE) agreed with the isolation results. These findings indicate that the abundance of *Gammaproteobacteria* and the diversity of *Pseudomonas* in the bacterioneuston have undergone significant changes in areas contaminated with elevated levels of OH in the Ria de Aveiro. However, the ecological role of the bacterioneuston in the degradation of hydrophobic OH pollutants is still unclear. Future work should focus on more in-depth functional analyses of genes whose expression/repression are most likely related to SML contamination by OH compounds.

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# Chapter III

## CHAPTER III

# Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities

**Francisco J.R.C. Coelho, Rui J.M. Rocha, Ana C.C. Pires, Bruno Ladeiro, José M. Castanheira, Rodrigo Costa, Adelaide Almeida, Ângela Cunha, Ana Isabel Lillebø, Rui Ribeiro, Ruth Pereira, Isabel Lopes, Catarina Marques, Matilde Moreira-Santos, Ricardo Calado, Daniel F.R. Cleary and Newton C.M. Gomes**

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**Abstract:** An experimental life support system (ELSS) was constructed to study the interactive effects of multiple stressors on coastal and estuarine benthic communities, specifically perturbations driven by global climate change and anthropogenic environmental contamination. The ELSS enables the control of salinity, pH, temperature, ultraviolet radiation (UVR), tidal rhythms and exposure to selected contaminants. Unlike most microcosms previously described, the ELSS enables true independent replication (including randomisation). In addition to this, it can be assembled using commercially available materials and equipment, thereby facilitating the replication of identical experimental setups in different geographical locations. The present study aimed the validation of the reproducibility and environmental quality of the system by comparing chemical and biological parameters recorded in our ELSS with those prevalent in the natural environment. Water, sediment microbial community and ragworm (the polychaete *Hediste diversicolor*) samples were obtained from four microcosms after 57 days of operation. In general, average concentrations of dissolved inorganic nutrients ($NO_3^-$; $NH_4^+$ and $PO_4^{3-}$) in the water-column of the ELSS experimental control units were within the range of concentrations recorded in the natural environment. While some shifts in bacterial community composition were observed between *in situ* and ELSS sediment samples, the relative abundance of most metabolically active bacterial taxa appeared to be stable. In addition, ELSS operation did not significantly affect survival, oxidative stress and neurological biomarkers of the model organism *H. diversicolor*. The validation data indicates that this system can be used to assess independent or interactive effects of climate change and environmental contamination on benthic communities. Researchers will be able to simulate the effects of these stressors on processes driven by microbial communities, sediment and seawater chemistry and to evaluate potential consequences to sediment toxicity using model organisms such as *Hediste diversicolor*.

**Keywords:** microcosm, microbial ecology, pyrosequencing, climate change, marine sediments, benthic communities

## Introduction

One of the greatest scientific challenges currently faced by researchers worldwide is to understand how human activities alter ecosystems and how ecosystems will respond to future perturbations. A key question is to what extent changes in ocean temperature, acidity and UV radiation will affect contaminant toxicity and what impact this will have on the marine biota (Doney, 2010, Hader *et al.*, 2011). Although there is an increased awareness of the potential for interactions between climate change and chemical contaminants, we only have a rudimentary understanding of how multiple stressors interact to affect (communities of) organisms (Schiedek *et al.*, 2007, Gao *et al.*, 2012, Passow *et al.*, 2012). Thus, it is important that we gain a mechanistic understanding of how anthropogenic stressors affect coastal and estuarine marine ecosystems. These biomes provide valuable ecosystem services such as fisheries, filtering, detoxification and carbon sequestration. However, they are among the most impacted of global ecosystems (Barbier *et al.*, 2010).

The processes involved in ecosystem response to perturbation often occur over a wide range of temporal and spatial scales with unknown interactions, making it difficult to establish cause-effect relationships in natural systems (Benton *et al.*, 2007). In particular, there is no standardized approach for the evaluation of global change effects on different marine ecosystems. Small scale models, *e.g.* microcosms, can be a useful tool to assess such complex global problems (Benton *et al.*, 2007). Microcosms are simplified ecosystems, designed to simulate natural environments under controlled conditions (Roeselers *et al.*, 2006). These model systems allow the testing of hypotheses and ecological theories on populations or communities with a high degree of experimental control and replication, which would be very difficult to achieve through field observation or *in situ* experimentation (Benton *et al.*, 2007, Jessup *et al.*, 2004). Moreover, microcosms enable researchers to perform experiments with potentially toxic contaminants, which would rarely (if ever) be carried out in the field.

In the present study, we assessed the extent to which microcosm conditions affect the sediment bacterial communities and the endobenthic species *Hediste diversicolor* (Müller, 1776, formerly known as *Nereis diversicolor*). Bacterial communities are fundamental players in all relevant geochemical cycle. However, despite the overwhelming importance of bacterial mediated processes, the potential impact of climate change on the composition and activity of bacterial assemblages is still poorly understood (Reid, 2011). *Hediste diversicolor* is a key species in shallow coastal systems: it is omnivorous, an active predator, and is highly prone to

predation by waders, fish and crabs (Scaps, 2002). This species has been widely used as a model or sentinel organism in the assessment of sub-lethal impact of contaminant in estuaries, both in the field and laboratory microcosms (Moreira *et al.*, 2006).

An important, but often overlooked, prerequisite for performing microcosm experiments is to determine whether the structural and functional properties of the source ecosystem are well represented in the microcosm, and the extent to which the experimental results are biased by the microcosm design and conditions (Leser, 1994). In the present study our main goals were to develop and evaluate an innovative experimental life support system (ELSS) designed to: 1) study the interactive effects of multiple stressors on coastal and estuarine benthic communities, *i.e.*, environmental stressors resulting from climate change and contamination; and 2) enable researchers to carry out complex but well replicated experiments. We also aimed to develop a versatile system that could be assembled in different marine regions across the world using commercially available materials and equipment. The physical, chemical and biological parameters of marine environments can be highly variable at the regional scale. This variability determines, to a given extent, how intensively global climate change affects these environments. Therefore, it is important to develop tools to evaluate the applicability of current models in predicting changes in local ecosystem health and function.

## Methods

### ELSS basic architecture

The basic concept of our ELSS was to provide a versatile framework for microcosm simulation of climate change scenarios in coastal and estuarine environments that could be easily replicated around the world. To achieve this goal, the system was developed using: 1) affordable materials and equipment, which are readily available in local or online stores (Table III-S1 for a list of the main material and equipment employed to assemble the ELSS, as well as their respective suppliers), and 2) a modular construction system that will enable researchers to work over a wide range of configurations, thus allowing them to address specific research questions using statistically robust experimental designs.

The ELSS mimics fundamental aspects that condition biological activity in marine ecosystems, namely photoperiod, light intensity (including photosynthetically active radiation, PAR) and tidal cycles. Additionally, it is possible to control temperature, ultraviolet radiation

(UVR), salinity and pH. The ELSS is composed by two frames of 16 microcosms (32 in total) (glass tanks 25 cm high, 28 cm length and 12.4 cm width, each with a maximum functional water volume of approximately 3 L), which makes it possible to run up to 8 treatments simultaneously, each one with a maximum of 4 independent replicates for each treatment. All replicates can be arranged in a randomized split-plot design (Figure III-1 and Figure S III-1).

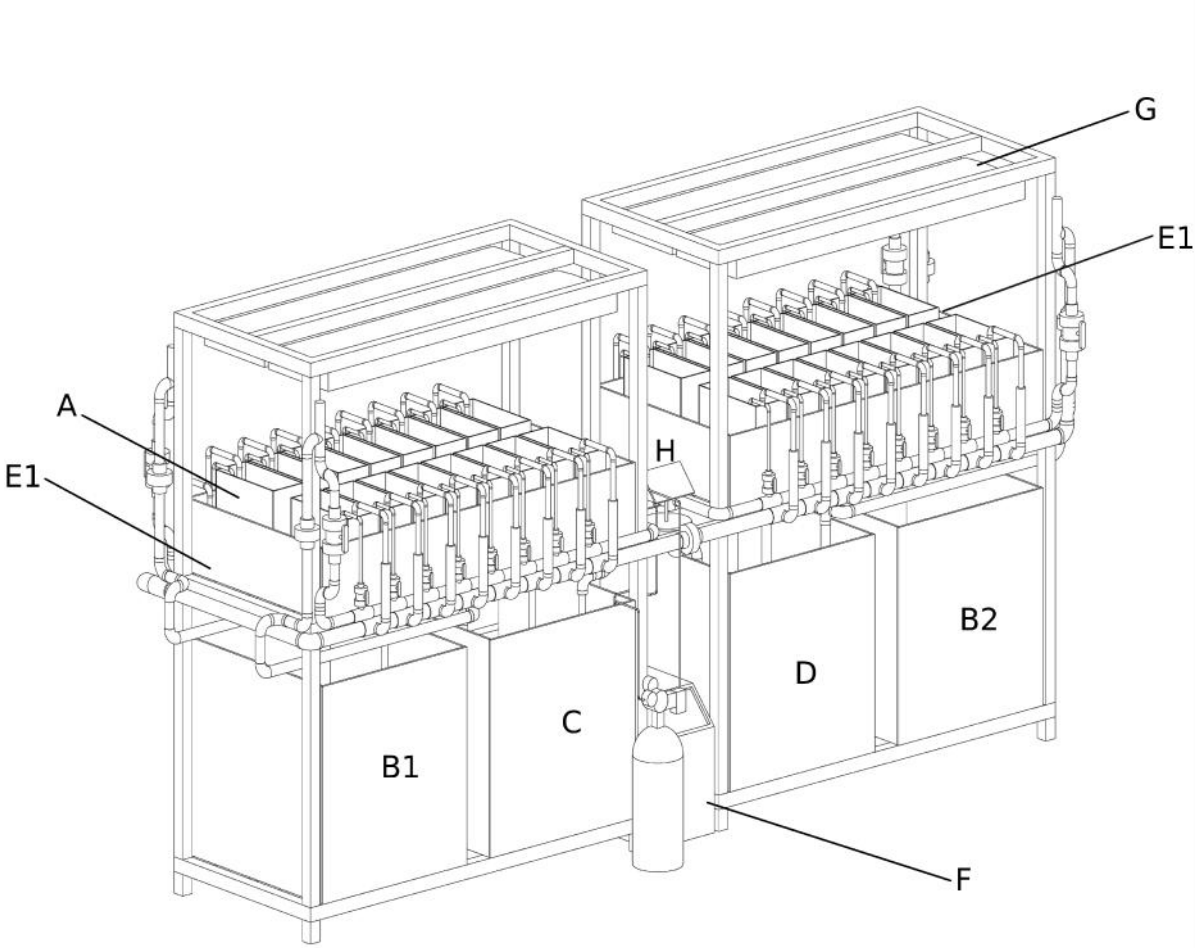


**Figure III-1:** Experimental life support system (ELSS) general scheme. A – independent microcosm; B- saltwater reservoir; C – acidified saltwater reservoir; D – normal pH saltwater reservoir; E – water bath; F – refrigerator; G – lightning system (a vinyl frame can be included to isolate the light from the luminaires); H – pH control system. More detailed schemes of the artificial life support system are given in the supporting information.

*Tidal cycle and water circulation control system*

The ELSS is operated using synthetic saltwater, which is prepared by mixing freshwater, purified by a four stage reverse osmosis unit (Aqua-win RO-6080) with a commercially available salt mixture (Tropic Marin Pro Reef salt – Tropic Marine, Germany) (Figure III-1, B1 and B2). The water for tidal cycles is prepared 24 h before use, in order to allow salt and water to mix. No water recirculation is employed in order to avoid cross contamination between experimental treatments and to avoid chemical artefacts that may be promoted by re-using the same water over time. Thus, the ELSS can be described as a flow-through non-recirculated system operated with synthetic saltwater. Its hydraulic system enables microcosms to be operated under any desired tidal regime (*e.g.*, diurnal, mixed, semi-diurnal). Each tidal regime is controlled as follows: newly prepared synthetic saltwater is pumped (Aquabee UP 3000) into the saltwater reservoirs (Figure III-1, C and D) for pH adjustment (if necessary); the ELSS is equipped with 4 such reservoirs (each with an approximate volume of 230 L). For high tide events, saltwater is pumped using a submersible pump (Aquabee UP 3000) from reservoirs C and D (Figure III-1) through an independent pipe system of polyvinyl chloride (PVC) tubes into each microcosm. The saltwater flow rate is manually controlled by a PVC valve located above each microcosm (Figure S III-1, S III-2 and S III-3). Low tide events are simulated using outflow submersible pumps (Rena flow 400 C), operated with digital timers; each glass tank (the microcosm) is equipped with a single pump positioned inside a PVC cylinder and protected with a mesh screen (to avoid clogging) (Figure S III-4). During low tide events the water is discharged from the microcosms through an outflow pipe, and drained to a collector.

*pH control system*

Water pH can be manipulated by acidifying the water stocked in the tide reservoirs by bubbling $CO_2$ through a diffuser (Figure S III-5) (Gattuso *et al.*, 2009). The diffuser operates with a water pump (Aquabee UP 3000) to maximize $CO_2$ gas mixing in saltwater. $CO_2$ addition is controlled with a feedback system that includes a combination of a pH electrode connected to a controller ($V^2$ control pH controller, Tropical Marine Centre, UK) and a pressure regulator with an integrated solenoid valve ($V^2$ pressure regulator pro, Tropical Marine Centre, UK). The digital display of the controller allows visualization of actual pH in

the saltwater reservoirs and pH monitoring with the pH electrode. The controller opens the solenoid valve whenever pH rises above the set value; $CO_2$ is then injected until water pH returns to the pre-set value. We tested the pH system under the experimental validation parameters to simulate a pH reduction of 0.3 units. The average pH value measured in low tide every two days along a week, (Figure SIII-10), was 7.67± 0.07 (NBS scale).

*Temperature control system*

In order to compensate for potential temperature fluctuations during experimental trials, namely those promoted by the illumination system implemented in our ELSS (see below), about two thirds of the microcosms height was immersed in one of two main water tanks (Figure III-1, E1 and E2). These water bath tanks are perforated at the bottom and connected through a 40 mm PVC pipe with a valve. The connection between water bath tanks ensures that temperature is the same in all microcosms. For conducting experiments with different temperatures the connection valve between the water bath tanks can be closed, allowing the setting of two distinct water temperature treatments. The water in the tanks is continuously pumped by a canister filter pump (SunSun HW-302) through a cooler equipped with a thermostat (Teco TR10) set to the desired temperature. The canister filter pump (composed by mechanical filtration sponges and activated carbon to remove any debris from the freshwater in the bath) operates with a flow rate of 1000 L $h^{-1}$ (Figure S III-6). Four submersible 200 W heaters equipped with thermostats (Rena Cal 200) are placed inside the water bath tanks to modify water temperature to previously set values. The simultaneous operation of the cooling and heating system minimises water temperature fluctuations to ± 0.5ºC.

*Lighting control system*

The ELSS is equipped with 4 ReefSET® programmable luminaire systems for diurnal light cycle and controlled UV simulation. Each luminaire holds 4 UV fluorescent tubes (SolarRaptor, T5/54W, Germany) and 4 full spectra fluorescent tubes (AquaLight, T5/54W/10000K,Germany) disposed alternately under a reflector (Figures S III-7 and S III-8). The luminaire system incorporates a dimming ballast that allows the adjustment of light intensity by varying the voltage supply. ReefSET® proprietary software enables the simulation

of a range of scenarios by varying the percentage of light intensity (see http://www.reefset.com).

**Water chemistry analysis**

Water samples for dissolved inorganic nutrient (nitrate $NO_3^-$, ammonium $NH_4^+$ and *o*-phosphate $PO_4^{3-}$) determination were collected from each microcosm at the beginning of the experiment, after 21 days and at the end of the experiment. Water aliquots were immediately filtered (Whatman GF/C glass-fibre filter) and stored frozen at -20 ºC until analysis. $NO_3^-$ determination followed the 8039 method described in the Hach Spectrophotometer (DR 2000) standard analytical procedures (Hach, USA, DR2000, 44863-00). The determination of $NH_4^+$ and $PO_4^{3-}$ concentrations were carried out following standard spectrophotometric methods described elsewhere (LimnologiskMetodik, 1992). The analytical quality control was ensured by duplicate samples and by the analysis of blanks between samples.
Visual inspection of $NO_3^-$, $NH_4^+$ and $PO_4^{3-}$ histograms revealed significant deviations from normal distributions. The distributions remained significantly deviant after logarithmic and square-root transformation. We, therefore, tested for significant differences in dissolved inorganic nutrient concentration among sampling events using a repeated measures permutational analysis of variance with the adonis() function (Vegan package) in R (http://www.r-project.org/; Accessed 4 May 2012). The script used is provided in the supporting information.

**Biological validation**

*Sediment sampling*

Four sediment cores were collected at the east margin of Mira channel (40°37"N, 8°44"W), one of the main channels of the Ria de Aveiro lagoon (Portugal) in May 2011. The Ria de Aveiro is a shallow mesotidal coastal lagoon connected with the Atlantic Ocean through a single inlet, and characterized by four main channels with several secondary narrow channels, inner basins and extensive intertidal areas (Dias *et al.*, 2001). Several studies have shown that Ria de Aveiro has a moderate level of eutrophication and low overall human influence when compared to other estuarine systems (Ferreira et al., 2003, Lopes et al., 2007).

Plexiglass cores of undisturbed sediment samples (10 cm deep, 27 cm length and 10.6 cm width) were collected and each core transferred directly into individual microcosms (4 in total) of the ELSS (Figure III-1, A) in May 2011. Microcosms containing the sediment were brought back to the laboratory and connected to the ELSS less than 2 h after sampling. The ELSS was operated continuously during 57 days.

*Experimental validation parameters*

The ELSS system was programmed to simulate the specific seasonal characteristics of the coastal lagoon system (Ria de Aveiro) at the sampling site where and when sediment cores were collected. Salinity was adjusted to simulate the conditions recorded at the sampling location and kept constant (32.6 ± 1.5) during the experiment, with synthetic saltwater being prepared as described above in the "tidal cycle and water circulation control system" section. Prior to each high tide, water salinity was checked. All microcosms were exposed to a uniform semi-diurnal tidal regime, experiencing two high tides and two low tides daily. Each tidal cycle took approximately 1 min. The duration of each tidal event minimizes pH and salinity fluctuations between the reservoirs and the microcosms. The minimum and maximum water levels above the sediment surface were approximately 5 cm (low tide) and 10 cm (high tide). During each tidal cycle about 50% of the water volume of each microcosm was exchanged (~1.5 L), thus simulating the water renewal percentage recorded for the central area of the estuarine system recreated in the ELSS (Ria de Aveiro) (Dias *et al.*, 2001).

Water pH was adjusted to 8.0 and water temperature to 19ºC, which corresponds to the average values recorded at the sampling location. The average pH monitored in the system was 7.97± 0.07 (NBS scale). With the luminaires system set to its maximum power, the PAR value measured for the full spectrum fluorescent lamps was 260.50 ± 56.30 μmol photons $m^{-2}$ $s^{-1}$, and the UV-A (320-400 nm) irradiance emitted by the UV lamps was 2875.91 ± 264.62 mW $m^{-2}$ (Lamp spectrum - Figure III-S9, and measurement details can be consulted in supporting information). In order to mimic summer photoperiod and light conditions at Portuguese latitudes during the time of sampling, a 14 hour diurnal light cycle was simulated, with light intensity varying from 50% to 100% of the total fluorescent tube intensity (Table II-S2). Since UV-A radiation is practically unaffected by changes in ozone depletion and plays an important role in biological systems, including photo-repair mechanisms (Bargagli, 2005), a similar amount of UV-A integrated irradiance was maintained constant among microcosms.

This was achieved by activating the UV lamps for four h a day at maximum intensity and filtering the UV-B component with a glass panel in the luminaires.

*Profiling of the active bacterial community*

*RNA extraction, denaturing gradient gel electrophoresis and pyrosequencing*

Four composite samples each corresponding to four sediment cores (~1 cm of top sediment with a 1 cm diameter) were obtained *in situ* (ConBs), and from the microcosm after 21 days (ConIs) and 57 days (ConFs) of operation. Environmental samples were collected at the study site, separated from each other by at least 1 m. Microcosm samples were obtained from four independent microcosms. Approximately 1 g of the aerobic sediment layer was directly transferred into a 2.0 ml screw cap tube containing Lysing Matrix E (FastRNA® Pro Soil-Direct Kit, Qbiogene Inc.,CA) and immediately immersed in liquid nitrogen (Liu *et al.*, 2011). Samples were kept at -80º C until RNA extraction.

Total RNA was isolated from sediment samples using the FastPrep® Instrument (Qbiogene, Inc, CA), for 40 s at a speed setting of 6.0 according to the manufacturer's instructions. Residual DNA was removed using Turbo DNA-free kit (Applied Biosystems, Austin, TX, USA). Total RNA was then converted to single stranded cDNA using random hexamer-primed reverse transcription by applying SuperScript® III Reverse Transcriptase Kit (Invitrogen). The complete removal of DNA was confirmed by PCR. Universal bacterial primers U27 and 1492R were used to amplify *ca.* 1450 bp of the 16S rRNA cDNA (Weisburg *et al.*, 1991). Specific products were detected in cDNA samples after reverse transcription, but not in their corresponding source RNA samples (data not shown).

Denaturing-gradient gel electrophoresis (DGGE) was used to monitor structural variation in bacterial communities in ConBs, ConIs and ConFs. Briefly, a nested PCR approach was used to amplify the 16S rRNA gene sequence from the samples. For the first PCR, the universal bacterial primers U27 and 1492R were used. For the second PCR, 1 µl of the product of the first PCR was used as template with bacterial DGGE primers 968F-GC and 1401R (*ca.* 433 bp) (Nübel *et al.* 1996). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel containing 6 to 10% acrylamide (Rotiphorese) with a gradient of 40 to 58% of denaturants. The run was performed in Tris-acetate-EDTA buffer (0.5 M Tris-Base, Sigma, 0.05 M EDTA, Sigma; 0.1 M $CH_3CO_2Na$, Sigma, pH 8.0) at 60 °C at

a constant voltage of 220 V for 16 h on a DCode vertical electrophoresis apparatus (universal mutation detection system; Bio-Rad). The DGGE gels were silver stained (Heuer *et al.*, 2001). The processing of the DGGE gels was carried out using the Bionumerics software 6.6 (Applied Maths, Kortrijk, Belgium). A barcoded pyrosequencing approach was used for an in depth microbial community analysis of ConBs and ConFs samples. Fragments of the 16S rRNA (cDNA) were sequenced for each sample with primers V3 Forward (5´ - ACTCCTACGGGAGGCAG-3') and V4 Reverse (5´ - TACNVRRGTHTCTAATYC-3') using the 454 Genome Sequencer FLX Titanium (Life Sciences Roche Diagnostics Ltd, West Sussex, UK). Sequences were analysed with QIIME software package following published recommendations (Kuczynski *et al.*, 2011). Details on sequence quality analysis and assignment can be found in supporting information. Sequences can be downloaded from the NCBI Short Read Archive (STUDY SRP013200).

Two square matrices 1. containing the abundance of all OTUs per sample generated with Qiime and 2. containing band 'abundance' based on band intensity and position of the DGGE gel were imported into R. Both were $\log_{10}(x+1)$ transformed and distance matrices constructed using the Bray-Curtis index with the vegdist() function in the vegan package in R (Oksanen *et al.*, 2008). The Bray-Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Legendre *et al.*, 2001, Cleary, 2003, Cleary *et al.*, 2004a, Cleary *et al.*, 2004b, de Voogd *et al.*, 2009). Total rarefied OTU richness per sample was estimated with a self-written function (Gomes *et al.*, 2010). Variation in OTU composition among treatments was tested for significance using the Adonis() function. In the Adonis() analysis, the Bray-Curtis distance matrix of OTU composition was the response variable with treatment as independent variable. The number of permutations was set at 999; all other arguments used the default values set in the function. Variation in the relative abundance of the most abundant higher taxa (three most abundant phyla; three most abundant classes and the six most abundant orders) was tested for significance with an analysis of deviance using the glm() function in R. Since the data was proportional, a glm with the family argument set as binomial was first applied. However, the ratio of residual deviance to residual d.f in the models exceeded 1, so the family was set to "quasibinomial". In the quasibinomial family the dispersion is not fixed at one so it can model over-dispersion.

*Hediste diversicolor stocking in microcosms*

In addition to *H. diversicolor*, other marine invertebrates (the isopod *Cyathura carinata*, and the mudsnail *Hydrobia ulvae*) were introduced to the microcosms to ensure that all microcosms were colonized with meiofauna at minimal density levels. However, for the validation of the ELSS system, only data from *H. diversicolor* was used as this species has been widely used as a model or sentinel organism to assess contaminant impact in the field and laboratory (Moreira *et al.*, 2006)

*Invertebrate collection*

The three invertebrate species were collected in June 2011 at two reference sites located in the Mondego (40º 08' N and 8º 50' W; *C. carinata*) and Mira (*H. diversicolor* and *H. ulvae*; 37°40' N and 8°45' W). More details about the sampling location and procedures can be found in supporting information. *Hediste diversicolor* (7/microcosms), *C. carinata* (4/microcosm) and *H. ulvae* (20/microcosm) were introduced in the microcosm after the 21 day period of stabilization to the microcosms. These organisms were retrieved from the sediment at the end of the experiment, counted and immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Pools of 2 - 3 ragworms from each microcosm were weighed (fresh weight) and homogenised in ice-cold phosphate buffer (50 mM, pH=7.0 with 0.1% Triton X-100) (1:5 m/v). Homogenates were centrifuged at 10,000 *g* for 10 min. at 4ºC and supernatants were divided in aliquots and stored at -80 °C.

*Biochemical parameters*

The total content in protein of each aliquot was determined spectrophotometrically according to Bradford (1976) using bovine □-globulin as standard. Catalase (CAT) activity was measured spectrophotometrically at 240 nm, following Aebi (1984). Superoxide dismutase (SOD) activity was assessed spectrophotometrically at 505 nm, using the RANSOD (RANDOX™) SOD kit and following the procedure described in the manual provided by the supplier. Glutatione *S*-transferase (GST) activity was measured spectrophotometrically at 340 nm following Hadig *et al.* (1974). Acetylcholinesterase (AChE) activity was measured spectrophotometrically at 414 nm, following Ellman *et al.* (1961). Enzyme activities were measured in triplicate and expressed in nmol of hydrolyzed substrate per min per mg of

protein, except for CAT and SOD. CAT was expressed in μmol of hydrolyzed substrate per min per mg of protein and SOD in units per gram of protein.

Lipid peroxidation (LPO) was evaluated through the quantification of by-products of the peroxidation of membrane lipids, like malondialdeyde (MDA), after their reaction with 2-thiobarbituric acid (TBA), following Buege and Aust (1978). The concentration of thiobarbituric acid reactive substances (TBARS: □ = $1.56 \times 10^6$ $M^{-1}cm^{-1}$) was measured spectrophotometrically at 535 nm and expressed in nmols of MDA equivalents per mg of protein).

The average values recorded for each parameter, in the organisms exposed in the microcosm control units, were compared with *in situ* and laboratory values reported in the literature for *H. diversicolor*, collected in the same estuary during the same period of the year (Moreira *et al.*, 2006). The values of AChE, CAT, GST, SOD and LPO activity deviated significantly from normality (Shapiro-Wilk normality test, $P = 0.046$, $P < 0.001$, $P = 0.013$, $P = 0.007$, and $P = 0.015$, respectively). With the exception of GST after logarithmic transformation (Shapiro-Wilk normality test, $P = 0.112$), the deviations remained significant after logarithmic and square-root transformation. GST exhibited homogenous variances between groups (Bartlett test, chi-squared = 1.31, df = 1, $P = 0.251$). Therefore, AChE, CAT, GST, SOD and LPO were tested for significant differences between microcosm and reported values in literature using the Adonis() function and tested GST using a Student's t.test(). In the Adonis() analysis, the Euclidean distance matrix of enzyme activity was the response variable with treatment as independent variable. The number of permutations was set at 999; all other arguments used the default values set in the function.

## Results and discussion

### *Water nutrient validation*

During the experimental period, the concentrations of $NO_3^-$ did not change significantly (repeated measures Adonis, $F_{2,9} = 5.35$, $R^2 = 0.543$, $P = 0.09$). $NO_3^-$ varied from $29.96 \pm 1.10$ μmol $l^{-1}$ at the beginning, to $42.86 \pm 10.1$ μmol $l^{-1}$ after 21 days and $32.38 \pm 1.31$ μmol $l^{-1}$ at the end of the experiment. $NH_4^+$ varied from $0.87 \pm 0.27$ μmol $l^{-1}$ at the beginning, $1.35 \pm 0.74$ μmol $l^{-1}$ after 21 days and $1.42 \pm 0.48$ μmol $l^{-1}$ at the end of the experiment. There was no significant difference in $NH_4^+$ concentration between sampling events (Repeated

measures adonis, $F_{2,9} = 1.29$, $R_2 = 0.223$, $P = 0.27$). $PO_4^{3-}$ varied from 1.07 ± 0.05 μmol $l^{-1}$ at the beginning to 1.12 ± 0.05 μmol $l^{-1}$ after 21 days and 1.07 ± 0.05 μmol $l^{-1}$ at the end of the experiment (repeated measures Adonis analysis: $F_{2,9} = 1.33$, $R_2 = 0.229$, $P = 0.35$) (Figure S III-11). In summary, the environmental range of water parameters (temperature, salinity and pH) and dissolved inorganic nutrients ($NO_3^-$, $NH_4^+$ and $PO_4^{3-}$) measured in our ELSS validation were comparable to those recorded in several Portuguese coastal systems (Table S III-3).

*Biological validation*

*Sediment bacterial diversity*

DGGE fingerprinting was used first as a proxy (Cleary *et al.*, 2012) to assess the effect of the microcosm on bacterial community structure. Ordination analysis of band profiles revealed significant differences (Adonis, $F_{2,9} = 1.58$, $R^2 = 0.260$, $P = 0.007$) in composition among sampling events (Figure S III-12). A 16S RNA cDNA-based barcode pyrosequencing approach provided a deeper characterization of sediment bacterial communities from ConBs and ConFs. Barcode pyrosequencing has been successfully used for in-depth studies of microbial diversity in several ecosystems (Roesch *et al.*, 2007, Gomes *et al.*, 2010, Keijser *et al.*, 2008, Pires *et al.*, 2012). Most sequence-based studies use DNA libraries. However, DNA from dormant cells, "dead" cells or extracellular DNA may bias the results (Urich *et al.*, 2008, Gaidos *et al.*, 2011). RNA cDNA libraries, in contrast, represent more recent activity and allow insight into compositional differences and *in situ* activity of microbial community members in a given setting (Urich *et al.*, 2008). To examine changes in OTU richness occurring during ELSS experiments, rarefaction curves were generated (Figure III-S13) for ConBs and ConFs. Controlling for sample size (n = 1800 sequences), OTU richness varied from 751.75 ± 12.76 in ConBs to 519.64 ± 8.23 in ConFs. Rarefied OTU richness was clearly lower at the end of the experiment. Shifts in bacterial community composition were assessed using PCO ordination of bacterial OTUs (97%) (Figure III-S14). Composition differed significantly (Adonis analysis: $F_{1,6} = 3.89$, $R^2 = 0.039$, $P = 0.025$) between sampling events. Most of the abundant OTUs (large grey circles; ≥ 50 sequences) were shared between both treatments. However, certain dominant populations (OTUs) showed stronger associations to either ConBs or ConFs samples.

Microcosm manipulation affected bacterial composition by reducing the number of rare OTUs and increasing the abundance of specific OTUs. This probably occurred because the microcosm is a more stable and less heterogeneous environment. Reduced disturbance may have allowed competitively superior groups to out-compete and effectively eliminate other species. This phenomenon is in line with the intermediate disturbances hypothesis (IDH) (Connell, 1978). Such an effect can also occur within different habitats in natural marine environments, which are commonly subjected to several levels of heterogeneity and disturbance regimes (Gingold *et al.*, 2010). For example, the presence of bioturbating shrimps in marine sediment habitats has been shown to induce changes in composition of sediment bacterial communities and increase bacterial diversity (Laverock *et al.* (2010).

Bacterial composition analysis showed that 74.03 ± 4.28% of all reads were assigned to the *Proteobacteria* at the beginning of the experiment. This proportion rose to 95.74 ± 1.47% at the end of the experiment. The different classes of this phylum are often abundant in marine sediment (Gomes *et al.*, 2010, Jiang *et al.*, 2009). Within the *Proteobacteria*, the *Gammaproteobacteria* was the most dominant class; their relative abundance ranged from 36.39± 7.08%, at the beginning of the experiment to 47.12± 4.14% at the end. The *Gammaproteobacteria* is the largest *Proteobacteria* group in terms of diversity and includes a wide range of phenotypic and metabolic diversity with recognized importance (Kersters *et al.*, 2006). The relative abundance of the *Deltaproteobacteria* class increased significantly after microcosm manipulation from 18.60 ± 6.04% at the beginning of the experiment to 42.70 ± 5.25 % at the end (Anova, $F_{1,6}$ , P = 0.003). Our results show that this trend was mainly related to an increase in the relative abundance of the *Desulfobacterales* order, which includes anaerobic sulphate reducing bacteria (SRB) (Figure III-2). SRB play an essential role in a variety of processes in anoxic marine sediment including organic matter turnover, pollutant detoxification and the carbon and sulphur cycles (Zhang *et al.*, 2008). SRB use sulphate as an electron acceptor in the degradation of organic compounds resulting in the production of sulphide through the dissimilatory sulphate reduction pathway (Muyzer *et al.*, 2008). Our ELSS was set to simulate the central area of the Ria de Aveiro mesotidal system, where approximately 50% of the water volume is renewed between tides (Dias *et al.*, 2001). However, the original sampling site in particular, is subject to intense water renewal, and being at an intertidal zone, the sediment is exposed to air during low tide. This implies that the microcosms simulated a more anoxic environment than that of the original sampling site, leading to an increased relative abundance of anaerobic *Desulfobacterales*. Introduction of

ragworm to the system might also influence bacterial community structure. Although bioturbation is generally associated with an increase in oxic zones in the sediment, infaunal activity can also augment anaerobic processes such as sulphate reduction (Bertics *et al.*, 2010).

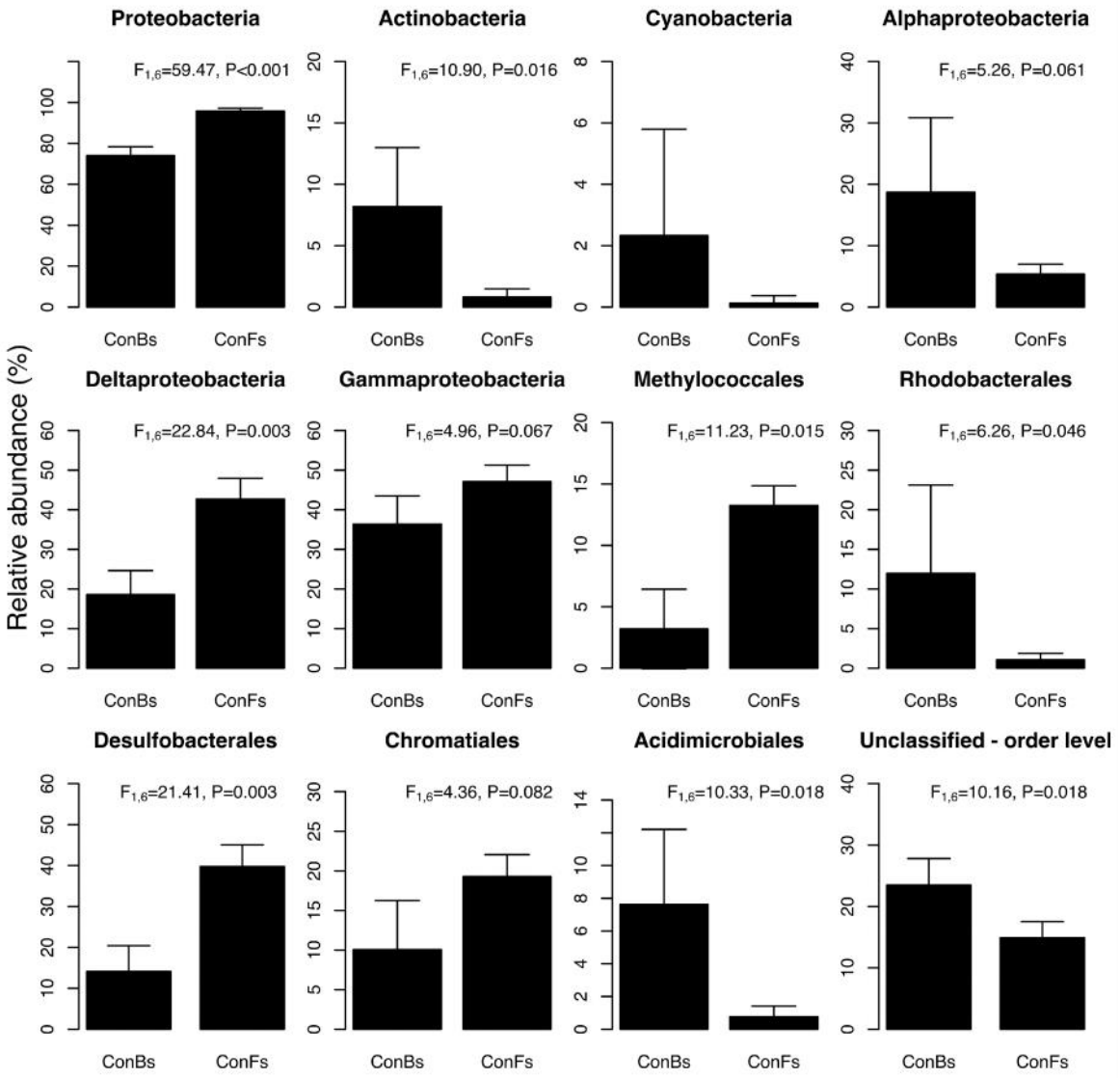


**Figure III-2:** Relative abundance of the most dominant bacterial taxa (three most abundant phylum; three must abundant classes and the six must abundant orders) the sediment collected in the environment (ConBs) and from the sediment retrieved from the microcosm after 57 days of operation (ConFs). Summary from analysis of deviance (glm with 'quasibinomial' family) is indicated above the bars. Note that analyses of deviance was not perform on *Cyanobacteria*, since sequences affiliated with this group were not detected in all samples.

Nevertheless, the simulated conditions did not exclude important functional aerobic groups in sediment biogeochemical processes. The abundance of OTUs belonging to the *Methylococcales* order increased after microcosm manipulation from 3.19 ± 3.23% at the beginning of the experiment to 13.26 ± 1.60% at the end. This order includes several members that oxidize methane under aerobic or microaerobic conditions (Bowman, 2005).

In contrast to the *Deltaproteobacteria,* the abundance of *Alphaproteobacteria* declined from 18.70 ± 12.14% at the beginning of the experiment to 5.36 ± 1.60% at the end. This was mainly related to a reduction in the abundance of the *Rhodobacterales* order, which declined from 11.98 ± 11.13% at the beginning of the experiment to 1.08 ± 0.80% at the end. Within the *Rhodobacterales*, several OTUs belonging to the *Rhodobacteraceae* family were detected. *Rhodobacteraceae* includes the *Roseobacter* clade, one of the major marine groups; 20% of coastal and 15% of mixed-layer ocean bacterioplankton communities consist of members of this clade (Sørensen *et al.*, 2005). Given that members of this group are likely present in water, their higher relative abundance in the sediment samples retrieved from the environment could be due to near-shore seawater infiltration, thus explaining their reduction following the microcosm experiment. Several phylotypes related to the *Roseobacter* clade were found in an endovaporitic microbial mat and the authors attributed this finding to seawater infiltration in the mat (Sørensen *et al.*, 2005). Other less abundant phyla such as *Cyanobacteria* and *Actinobacteria* were also less abundant at the end of the experiment.

The distribution of dominant (≥ 50 sequences) OTUs across all samples is visualized using a heatmap (Figure S III-15). Results from this analysis are in line with the pattern detected in the relative abundance analysis. Several abundant OTUs detected at the beginning of the experiment remained among the most dominant OTUs at the end of the experiment, including several known aerobic and anaerobic groups (*e.g.*, *Gammaproteobacteria* – OTU 2987, OTU 2315, OTU 3254*; Deltaproteobacteria* - *Desulfobacteraceae* – OTU 3129; *Methylococcales* - 1648). As outlined above, several sulphate reducing bacterial OTUs increased their abundance in microcosm samples (*e.g.*, *Desulfobacteraceae* OTUs – 1724; 1325), whereas members of the *Rhodobacterales* order declined in abundance at the end of the experiment (*e.g.*, OTUs - 1569; 675).

*Hediste diversicolor biochemical parameters*

*Hediste diversicolor* has been indicated as an adequate sentinel species for transitional waters due to its wide distribution, sensitivity and its role in the functioning and structure of marine ecosystems (Scaps, 2002, Moreira *et al.*, 2006, Bouraoui *et al.*, 2009, Solé *et al.*, 2009). Ragworms usually move actively around the sediment surface or within their burrow systems looking for food (Kristensen, 2001). Furthermore, these organisms are both sediment eaters (Solé *et al.*, 2009) and suspension feeders (Kristensen, 2001 and references cited), features that increase the chances of exposure to contaminants associated with different sediment components, overlying water and interstitial water.

At the end of the experiment 96.40% of stocked *H. diversicolor* were recovered, indicating that the architecture and the conditions of the system did not compromise the survival of these organisms even during long-lasting exposure. Figure III-3 shows boxplots for values recorded for each biochemical parameter monitored for *H. diversicolor* specimens stocked in the microcosms of our ELSS, as well as ranges of values available in the study of Moreira *et al.* (2006) for this species, collected in the same estuary and in the same period of the year. The comparability with organisms collected at the same period of the year is of particular importance as the influence of seasonal (*e.g.*, water temperature) factors, physiological factors, sexual condition, gametogenesis period and behaviour in antioxidant enzyme levels is well known (Sun *et al.*, 2008). Compared to the previous study of Moreira *et al.* (2006), no significant differences were observed in the activity of AChE (Adonis, $F_{1,7} = 0.89$, $R^2 = 0.113$, $P = 0.380$; $F_{1,17} = 1.75$, $R^2 = 0.094$, $P = 0.174$, comparing with *in situ* and laboratory data from Moreira *et al.*, 2006, respectively), thus, denoting no neurologic adverse effects of microcosm exposure in the polychaete. The activity of CAT and GST were significantly higher in the present study in comparison to results obtained by Moreira et al. (2006) (Adonis for CAT: $F_{1,7} = 6.66$, $R^2 = 0.487$, $P = 0.061$; $F_{1,17} = 16.67$, $R^2 = 0.495$, $P = 0.002$ and Student-t test for GST: $t_{4,5} = 5.6$, $p<0.001$ and $t_{4,15} = 5.6$, $p<0.001$, respectively, when compared with laboratory and *in situ* data for each enzyme). However, the differences registered in GST activity between the present work and the results obtained by Moreira *et al.* (2006) with organisms exposed, under controlled condition, in the laboratory was 22%, which is close to the 20% threshold commonly considered to be within the normal range of activity of the enzyme and to have no biological significance (Olsen *et al.*, 2001). The activity measured for SOD and LPO in *H. diversicolor* exposed in the ELSS was significantly lower than that

reported by the latter authors (Adonis for SOD: $F_{1,7}$ = 339.25, $R_2$ = 0.979, P = 0.008; $F_{1,17}$ = 846.06, $R_2$ = 0.980, P = 0.001 and Adonis for LPO: $F_{1,7}$ = 78.79, $R_2$ = 0.918, P = 0.008; $F_{1,17}$ = 100.04, $R_2$ = 0.855, P = 0.001). These observations indicate that the organisms were not under neurologic or oxidative stress. The apparent depression in SOD activity may also have been the result of the different methods applied to assess this enzyme in both studies.

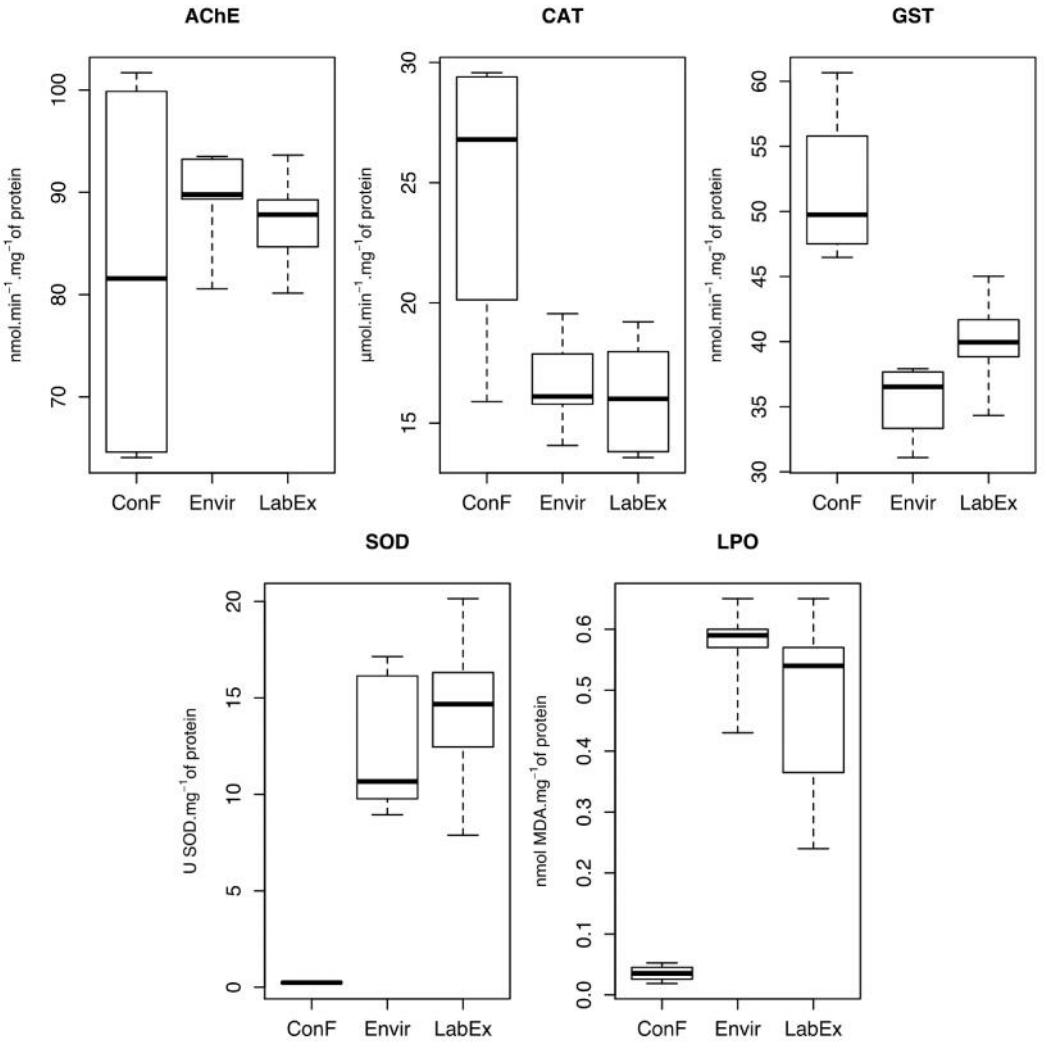


**Figure III-3:** Boxplot of the values recorded for each biochemical parameter measured in *Hediste diversico* after 36 days in the microcosms (ConF) and reported in the literature for *in situ* (Envir) and laboratory valu (LabEx) of the same species collected at the same site and season (Moreira *et al.*, 2006). The boxed ar represents the mean ± quartile and the whiskers extend to the minimum and maximum values.

Temperature and the sediment sulphide content have been pointed out as factors responsible for the induction of SOD activity in *H. diversicolor*, while anoxia seems to have a non significant effect on the activity of this enzyme (Abele-Oeschger *et al.*, 1994, Sun *et al.*, 2008). Thus, the lower temperature to which the ragworms have been exposed in the microcosm, in comparison to the field conditions, may have been responsible for the depression in SOD activity. Temperature effect is also likely to affect the activity of the other tested enzymes, since a similar decreasing trend has been reported for antioxidant enzyme activities in invertebrate species with decreasing temperatures (Khessiba *et al.*, 2005; Sroda and Cossu-Leguille, 2011; Kong *et al.*, 2012). However, the temperatures that produced a significant reduction in the activity of stress biomarkers in the referenced studies were lower than those recorded in the ELSS, and in some cases variation with temperature displayed a sex-dependent tendency (Sroda and Cossu-Leguille, 2011). Similarly, contradictory relationships between temperature and SOD activity in *H. diversicolor* have been reported (Abele-Oeschger *et al.*, 1994, Sun *et al.*, 2008). Although the ELSS appeared to induce anaerobic conditions, a sulphide rich environment and promote the abundance of SRB, our results failed to show oxidative stress induction in ragworms.

**Experimental life support system applications**

In this study we have described the development and validation of an experimental microcosm framework capable of simulating fundamental dynamics of coastal systems. Water inorganic nutrient concentrations remained fairly stable during validation trials, exhibiting comparable values to those recorded in the coastal lagoon. Bacterial community structure validation revealed that the microcosm operational programme introduced a shift that favoured the SRB group in particular. However, these changes do not necessarily imply a strong shift in community functioning, given that the most abundant classes were still detected in samples at the end of the experiment. Nevertheless, if deemed necessary, aerobic conditions can be stimulated by choosing different tidal ranges with different percentages of water renewal. At the end of the validation period, the biomarkers of *H. diversicolor* were within the same magnitude of values reported in the literature for organisms collected in the same estuary, under reference conditions.

The ELSS described here can be easily reproduced and operated under a moderate budget. We estimated that the replication of this system would cost approximately 15000

euros. Assuming 0.098 euros per Kilowatt hour$^{-1}$ (average price of electricity for industry in the European Union for the second semester 2012 - http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=nrg_pc_205&lang=en), a daily operational cost of approximately 4.09 euros was estimated. If necessary, the system architecture is sufficient flexible to allow for the replacement of several subsystems in order to fit specific research needs. Its modular construction enables the setting of a multitude of statistically robust complex experimental designs. By changing the pre-sets of the ELSS values with respect to temperature and pH subsystems, it is possible to simulate several scenarios of global climate change, such as the postulated increase in temperature and reduction in pH in marine environments. If users choose to use natural seawater and operate under a closed recirculated water system, it is important that other parameters related to inorganic C species, such as dissolved inorganic carbon or total alkalinity, are monitored since carbonate chemistry can be affected. Irradiance values can also be manipulated by varying lamp intensity and time of operation. For example, scenarios predicting an increase in UV-B can be simulated by simply removing the glass filter used in our validation trials and by manipulating lamp intensity.

This system can be used to establish cause-effect relationships on the impact of climate change and other anthropogenic stressors on coastal marine benthic communities and processes. For example, the ELSS could be used to study bacterial mediated degradation (*e.g.*, petroleum hydrocarbon degradation) in coastal systems under various climate change scenarios. *Hediste diversicolor* stress indicators could then be applied to assess the toxicity of degradation products. The data thus obtained can provide the basis for our understanding how climate change and pollution may interact to affect coastal marine ecosystems in the future.

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# Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities

## Supporting information

## Methods

### Systems basic architecture

**Table S III-1:** Artificial life support system main components, their manufacturers and suppliers.

| | | |
|---|---|---|
| Reverse osmosis unit | Aqua-win RO-6080 | www.aquawin.com.tw |
| Inflow pump | Aquabee UP 3000 | http://www.aquabee- |
| Outflow pump | Rena flow 400 C | www.planetrenadirect.com |
| Refrigerator | Teco TR10 | www.tecoonline.eu |
| Canister filter pump | SunSun HW-302 | www.sunsun-china.com |
| Luminaire systems | ReefSET® LM DIM 1208 (8 x 54 W) | www.reefset.com |
| Fluorescent tubes | AquaLight, T5/54W/10000K | www.aqualight.de |
| UV Fluorescent tubes | SolarRaptor, T5/54W | www.aqualight.de |
| Submersible 200 W heaters | RenaCal | www.rena.fr |
| pH controller | V² control, TMC | www.tropicalmarinecentre.co.uk |
| Solenoid valve | V² pressure regulator pro, TMC Centre | www.tropicalmarinecentre.co.uk |
| $CO_2$ bottle | V² Cylinder 567g (CGA320 conn.) | www.tropicalmarinecentre.co.uk |
| Synthetic Sea Salt | Tropic Marin Crystal Sea ® | www.meisalt.com |
| PVC valves (Solvent socket) | PVC-U ball valve (Pressure) tap 20 mm | Local store |
| PVC valves (Solvent socket) | PVC-U ball valve (Pressure) tap 32 mm | Local store |
| PVC valves (Solvent socket) | PVC-U ball valve (Pressure) tap 40 mm | Local store |
| Union (Solvent socket) | PVC-U (Pressure) 16 mm | Local store |
| Union (Solvent socket) | PVC-U (Pressure) 20 mm | Local store |
| Union (Solvent socket) | PVC-U (Pressure) 32 mm | Local store |
| Union (Solvent socket) | PVC-U (Pressure) 40 mm | Local store |
| Union (Solvent socket) | PVC-U (Pressure) 50 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 16 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 20 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 25 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 32 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 40 mm | Local store |
| 90º Elbow (Solvent socket) | PVC-U (Pressure) 50 mm | Local store |
| Reducing Bush (Solvent socket) | PVC-U (Pressure) 20 x 16 mm | Local store |
| 90º Tee (Solvent socket) | PVC-U (Pressure) 32 mm | Local store |
| 90º Tee (Solvent socket) | PVC-U (Pressure) 50 mm | Local store |
| 90º Red. Tee (Solvent socket) | PVC-U (Pressure) 40 x 25 mm | Local store |
| 90º Red. Tee (Solvent socket) | PVC-U (Pressure) 32 x 20 mm | Local store |
| Conical Reducer (Solvent socket) | PVC-U (Pressure) 50 x 40 mm | Local store |
| Spigot Connect. (Solvent socket) | PVC-U (Pressure) 20 x 20 mm | Local store |
| PVC Solvent Cement | TANGIT® PVC-U | Local store |
| PVC Pipe | PVC-U (PN 10) 16, 20, 25, 32, 40 and 50 | Local store |
| Microcosm | 5 mm glass, glued with neutral silicone | Local store |
| Saltwater Reservoir | 10 mm glass, glued with neutral silicone | Local store |
| Waterbath tank | 10 mm glass, glued with neutral silicone | Local store |
| Structure | Stainless Steel 118 structure 40 x 2mm | Local store |
| PVC panel | PVC black panels (6mm) | Local store |

## Artificial life support system detail schemes

**Figure S III-1:** Lateral front view of the experimental life support system.

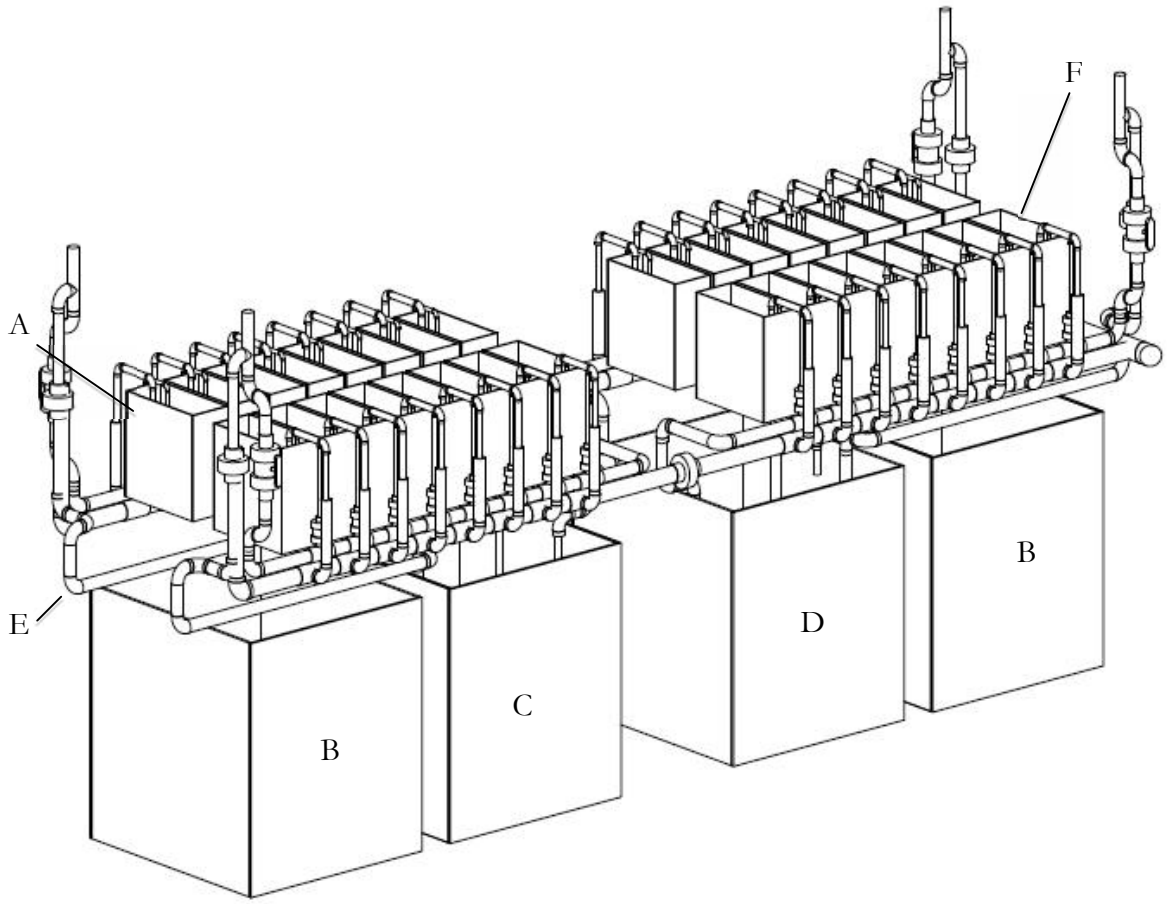


**Figure S III-2:** Water and tide circulation system detail (Front). A – independent microcosm; B- saltwater reservoir; C – acidified saltwater tide reservoir; D – normal pH saltwater tide reservoir; E – inlet pipe (to return the exceeding water to the tide reservoir); F –outlet pipe (to discharge the microcosm waste water after tide circulation).

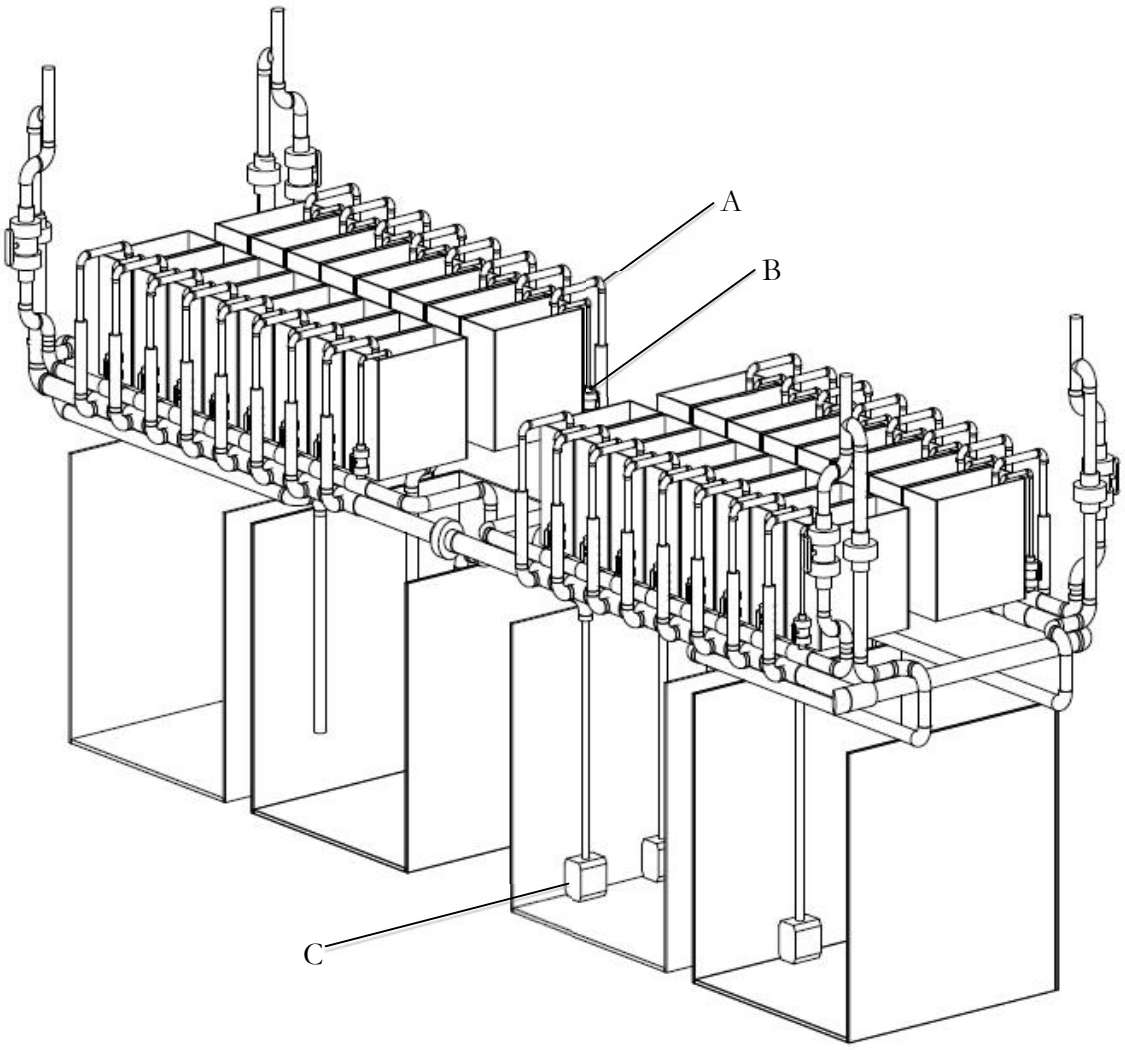


**Figure S III-3:** Water and tide circulation system detail (Back). A – outlet pipe from the microcosm; B – inlet pipe in the microcosm; C – inflow water pump.

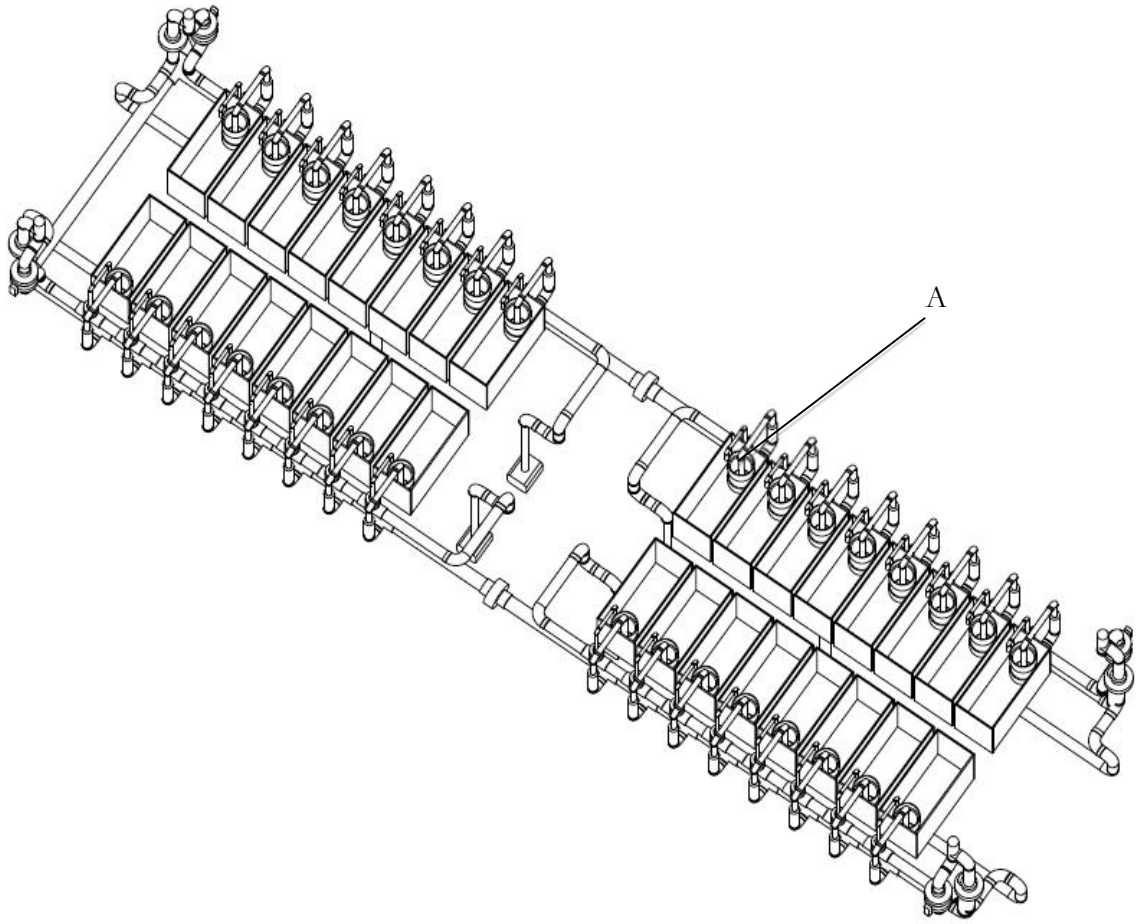


**Figure S III-4:** Water and tide circulation system detail (top view). A - Outflow pump positioned inside a PVC cylinder and protected with a mesh screen.

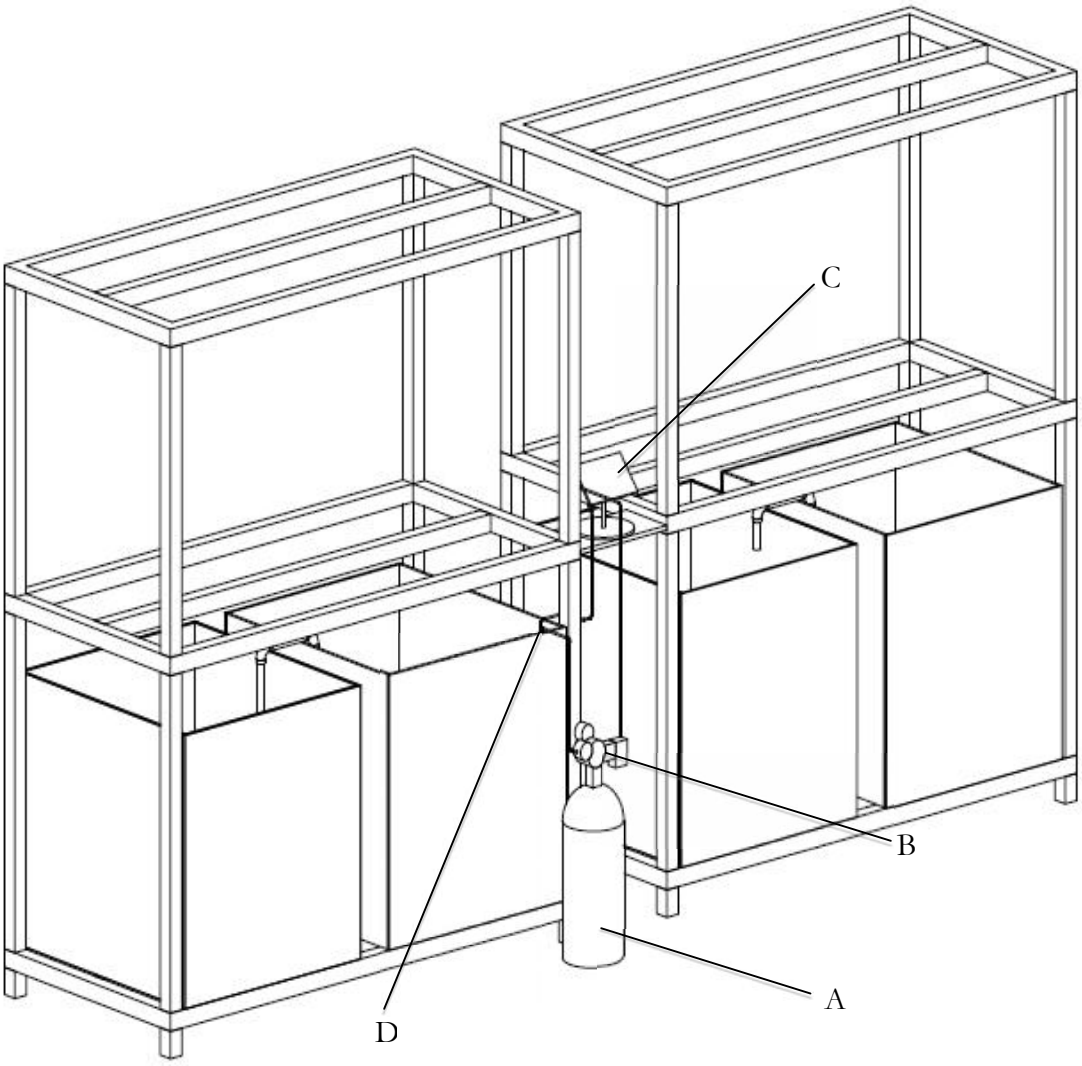


**Figure S III-5:** pH control system detail: A - $CO_2$ bottle; B – solenoid valve; C – pH controller; D – pH electrode probe.

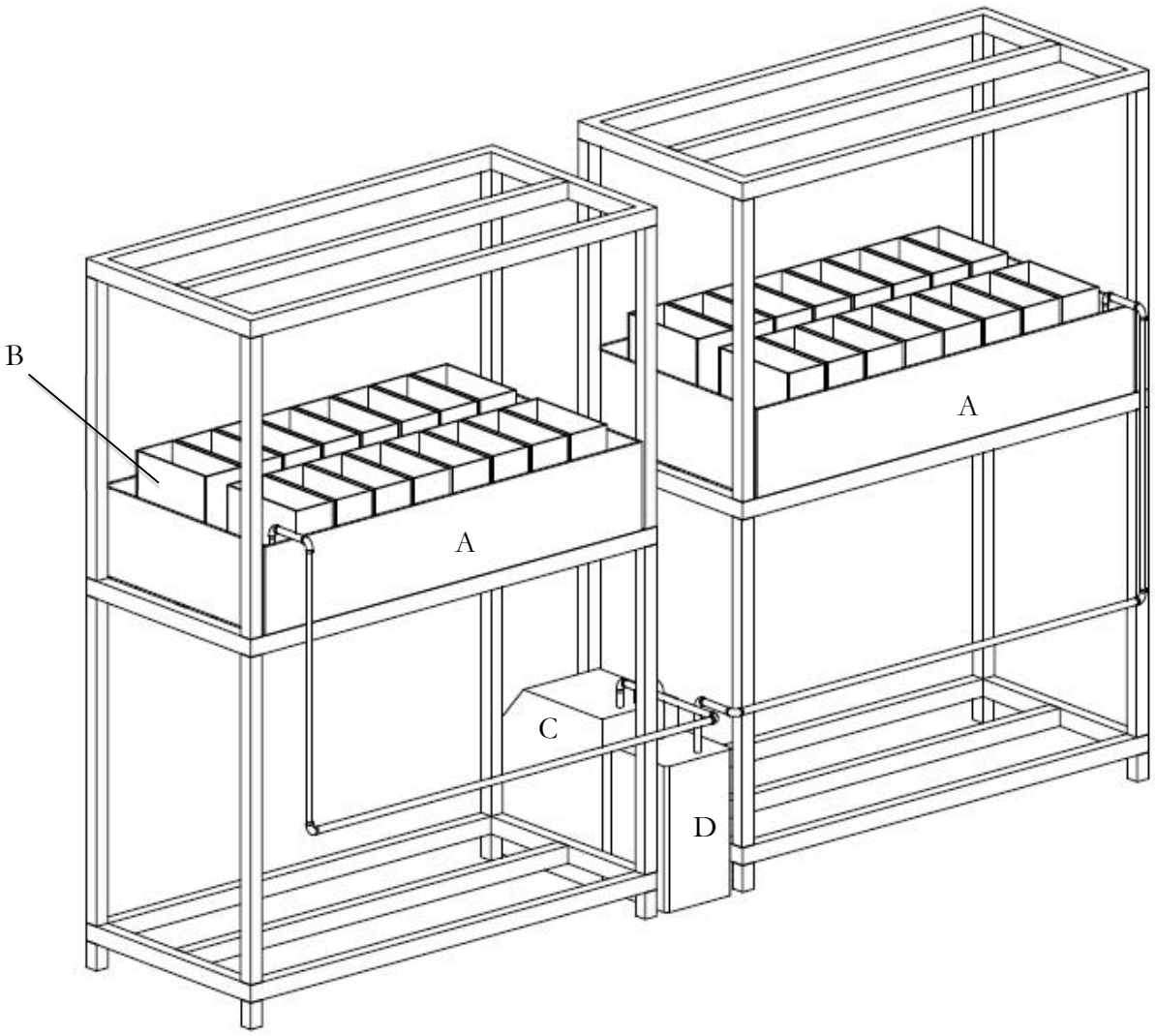


**Figure S III-6:** Water bath detail (from back). A – waterbath (the 2 tanks were drilled in the bottom and connected through a 40 mm PVC pipe); B – individual microcosm; C – refrigerator; D - canister filter pump; E - Stainless Steel 118 structure 40 x 2mm.

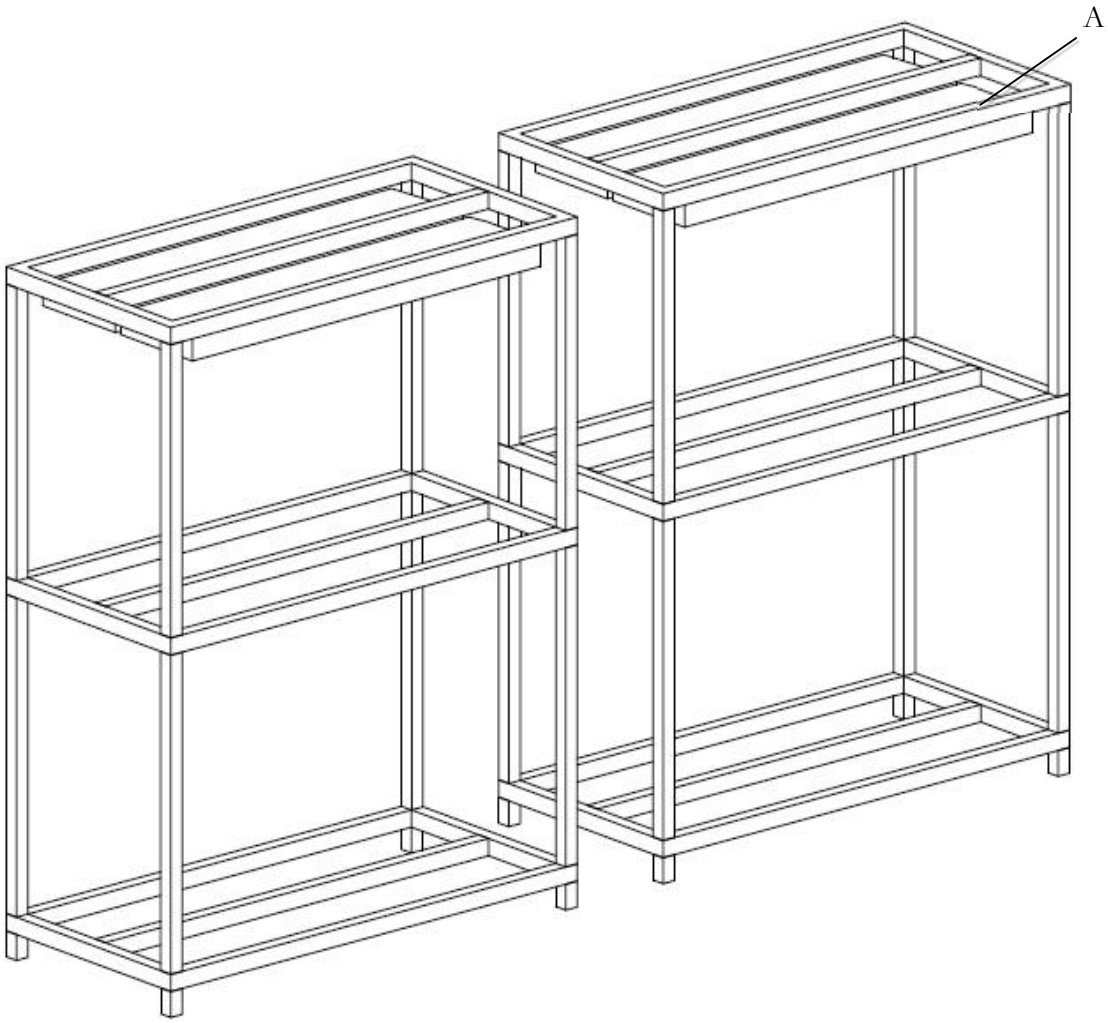


**Figure S III-7:** Lighting system detail: A - luminaire.

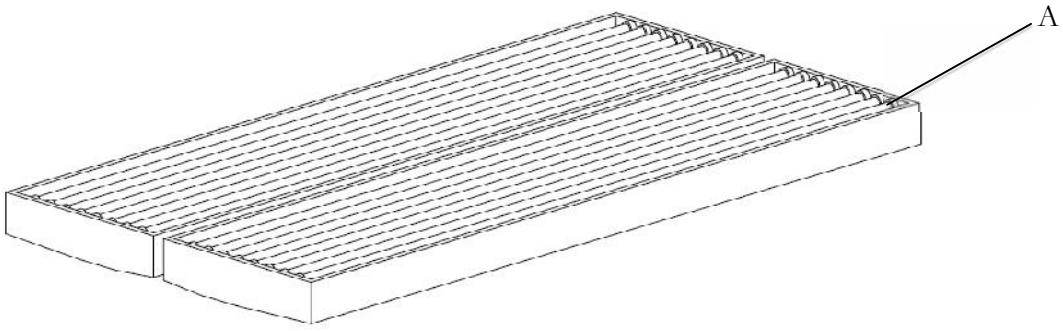


**Figure S III-8:** Luminaire detail: A – daylight and ultraviolet lamps dispose alternately.

**Lighting system**

Photosynthetic Photon Flux Density (μmol photons $m^{-2}$ $s^{-1}$) was measured with a Quantum Meter with a separate sensor (Model MQ-200, Apogee Instruments, Logan, Utah, USA). The spectrum of photosynthetic active radiation was acquired using a USB2000 spectroradiometer, with spectral resolution of 0.38 nm (USB2000-VISNIR, grating #3, Ocean Optics, Duiven, Netherlands) and a 400 μm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics). UV irradiances were accessed with a spectro-radiometer connected to a monochromator that provides information on the spectral irradiance with a resolution of one nanometer. Spectral irradiance was obtained by the BenWin+ Software (Bentham Instruments, Reading, UK) (Ribeiro *et al.*, 2011). Photosynthetic Photon Flux Density, PAR and UV spectra were measured under each luminaire in minimum tide high in the middle and border microcosm.

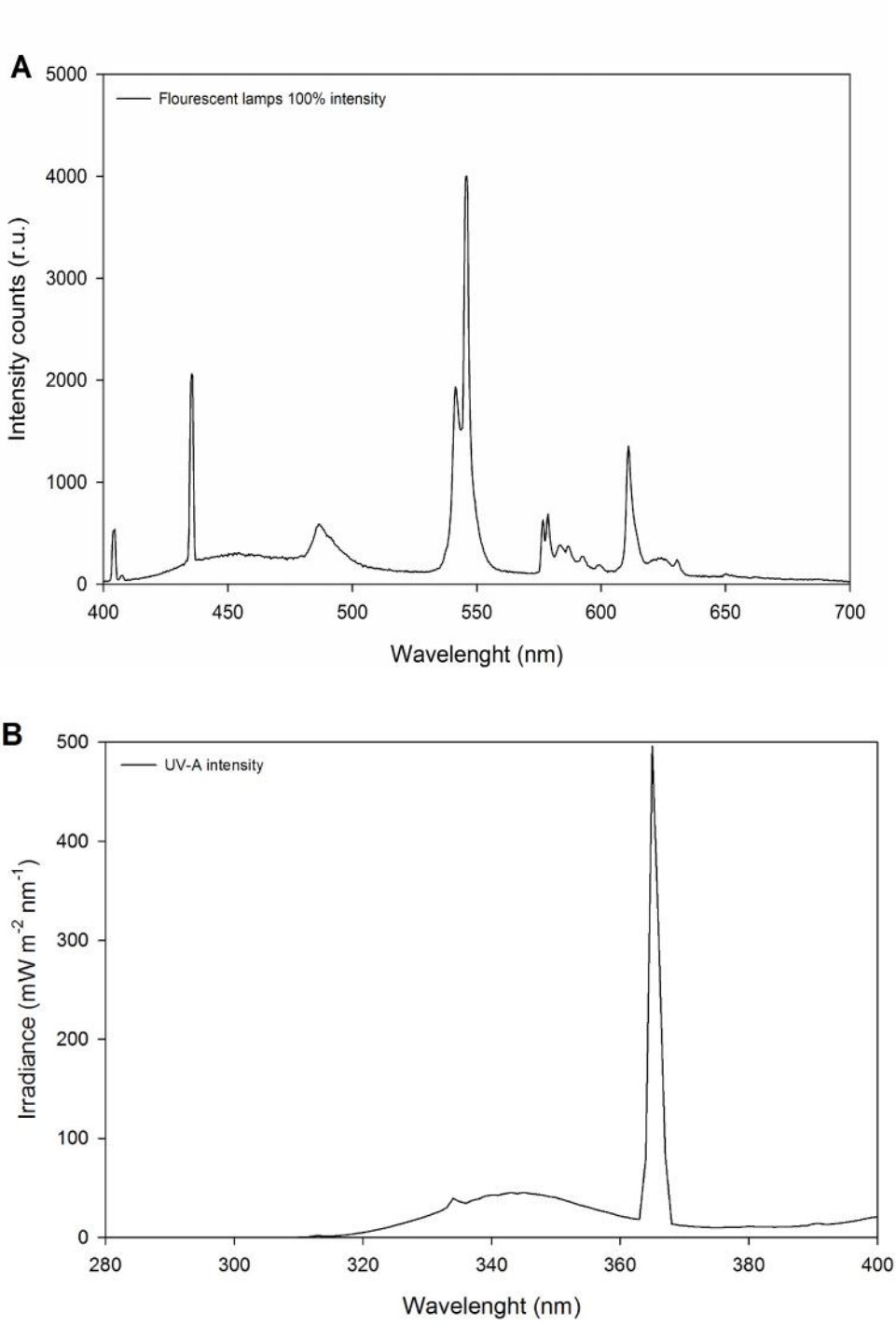


**Figure S III-9:** Experimental light spectra. A - Spectrum of photosynthetically active radiation (PAR: 400–700 nm) of the fluorescent tubes set to 100% intensity. B- Spectral distribution of UV radiation lamps set to 100% intensity and filtered with glass filter.

**Table S III-2:** Diurnal cycles of the PAR and UV integrated radiation intensities expressed as percentages of maxima intensities of the respective fluorescence tubes.

| Time | Intensity level of fluorescence tubes (% of lamp intensity) | |
|---|---|---|
| | Fluorescent tubes | UV lamps |
| 1:00 | 0 | 0 |
| 2:00 | 0 | 0 |
| 3:00 | 0 | 0 |
| 4:00 | 0 | 0 |
| 5:00 | 0 | 0 |
| 6:00 | 50 | 0 |
| 7:00 | 60 | 0 |
| 8:00 | 70 | 0 |
| 9:00 | 80 | 0 |
| 10:00 | 90 | 0 |
| 11:00 | 100 | 100 |
| 12:00 | 100 | 100 |
| 13:00 | 100 | 100 |
| 14:00 | 100 | 100 |
| 15:00 | 90 | 0 |
| 16:00 | 80 | 0 |
| 17:00 | 70 | 0 |
| 18:00 | 60 | 0 |
| 19:00 | 50 | 0 |
| 20:00 | 0 | 0 |
| 21:00 | 0 | 0 |
| 22:00 | 0 | 0 |
| 23:00 | 0 | 0 |
| 24:00 | 0 | 0 |

**pH control system**

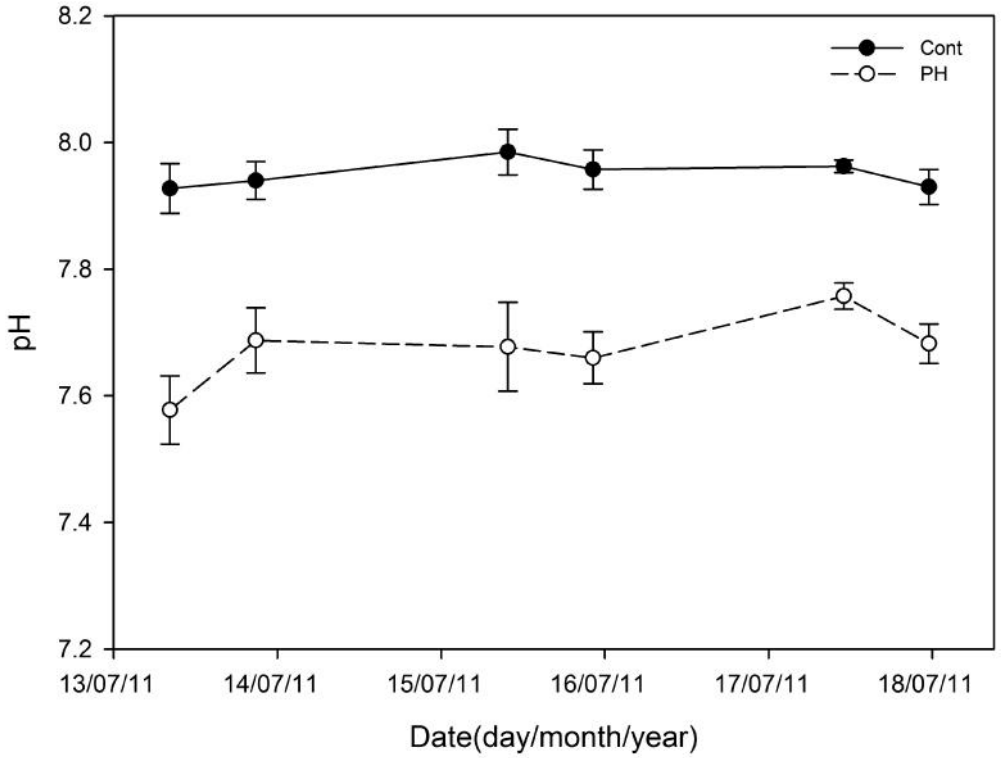


**Figure S III-10:** Average pH measured during one week every two days in control and reduced pH treatments. For each treatment the pH was measured in four microcosms at the end of each low tide. In order to achieve a pH reduction of 0.3 in each microcosm, the acidified reservoir water was adjusted to a pH value of 7.2.

**Collection of invertebrates and microcosm exposure**

The invertebrate species were collected by hand, at the low tide (*H. diversicolor* and *H. ulvae* at a salinity of 33-35 and water temperature 23°C and *C. carinata* at a salinity of 30 and water temperature 21°C) 21 days after the beginning of the experiment, and transported to the laboratory in plastic containers filled with local water. In the laboratory the organisms were separated from debris and a pool of organisms with a similar body size, for each species, was obtained. From this pool, 30 organisms, for *H. diversicolor* and *H. ulvae*, were randomly chosen for the evaluation of their biomass, as dry weight. For dry weight estimations, organisms were left in filtered (10 μm) local water for 24 h, time after which they were dried at 60°C for 72 h. The dry weight was then estimated to the nearest 0.01 g (Kern ALS-A, Kern and Sohn, Balingen, Germany). Mean (± standard deviation) biomass of organisms to be used was 15 (±4.7) mg for *H. diversicolor* and 0.90 (±4.7) mg for *C. carinata*. For *H. ulvae* (n = 30), organisms

with a shell length and width of 5.28 (±0.35) mm and 2.61 (±0.20) mm, respectively, were used. Organisms to be used in the microcosm were randomly distributed into groups of seven, twenty and four, respectively for *H. diversicolor*, *H. ulvae* and *C. carinata* (corresponding, respectively, to a density of 0.03, 0.09, and 0.02 organisms $cm^2$, thus, avoiding stress due to overloading of the system, as this density is below the densities observed at the Mira estuary and those reported in literature to induce stress due to overloading-approximately 0.8 and 1.8 organisms $cm^2$ (Costa *et al.*, 2001, Barnes, 2001) and maintained in 50 ml polypropylene vials filled with local estuarine water, at 19 to 21ºC under a photoperiod of $16h^L$:$8h^D$. The day the experiment was started, *i.e.*, approximately 24 h after being collected, groups of seven worms, twenty mudsnails and four isopods were placed into four microcosm independent control units in contact with the sediment (see previous material and methods sections).

## Validation of inorganic nutrient concentration in water

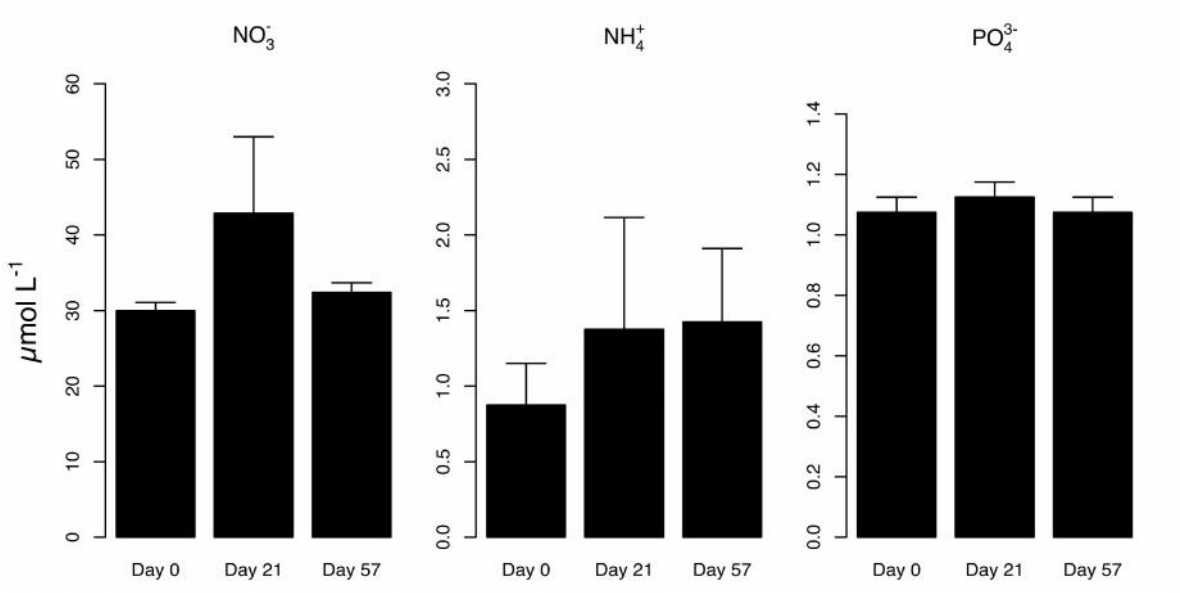


**Figure S III-11:** Concentration of dissolved inorganic nutrients (nitrate $NO_3^-$; ammonium $NH_4^+$ and o-phosphate $PO_4^{3-}$) in water ELSS control at the beginning of the experiment, after 21 days (ConIw) and at the end of the experiment.

**Table S III-3:** The environmental range of water parameters (temperature, salinity and pH), and of dissolved inorganic nutrients (nitrate $NO_3^-$, ammonium $NH_4^+$ and o-phosphate $PO_4^{3-}$) in Portuguese coastal systems and in the ELSS microcosms (control experimental units).

| Estuarine system | Assessment period | Area/stations | Temp (ºC) | Sal. | pH | Nitrate ($NO_3^-$) | Phosphate ($PO_4^{3-}$) | Ammonium ($NH_4^+$) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Douro 41º14'N 08º66'W | Monthly Dec 2002/ Dec 2003 | 10 stations along the estuary (? stations) | 7-25 | 0-35 (≈30) | ----- | 1-499 µmol $L^{-1}$ (10-50* µmol $L^{-1}$) | 0.2-6.8 µmol $L^{-1}$ (0.5-1.0*µmol $L^{-1}$) | 0.3-159 µmol $L^{-1}$ (5-15*µmol $L^{-1}$) | (Azevedo *et al.*, 2008) (Table 3; Fig 3) |
| Ria, Aveiro 40º38'N 08º45'W | June 2001 September 2001 | 2 stations Barra; Costa Nova | 18-21 | 22-34 | ----- | 3-20** µmol $L^{-1}$ | 0.5-1.3 µmol $L^{-1}$ | ---------- | (Lopes *et al.*, 2007) (Table 1) |
| Mondego 40º08'N 08º5'W | Monthly Jan 1999/ Jan2003 | 3 stations South arm | 8-22 | 0-34 | ----- | 1-36 µg$L^{-1}$ | 0.2-3.1 µg$L^{-1}$ | 1-15 µg$L^{-1}$ | (Lillebø *et al.*, 2005) (Table 2) |
| Tejo 38º8'N 09º1'W | Spring Summer Mar 1999/ Nov 2005 | 3 stations | 13-26 | 1-36 | 7.1-9.0 | 0.2-146***µmol $L^{-1}$ | 1.8-9.2 µmol $L^{-1}$ | ---------- | (Gameiro *et al.*, 2007) (Table 4) |
| | July 2001 May 2002 June 2003 | 19 stations along the estuary (4 stations) | 17-25 (17-21) | 0-35 (≈30) | ----- | 10-60 µmol $L^{-1}$ (10-25* µmol $L^{-1}$) | 1-4 µmol $L^{-1}$ (1-3* µmol $L^{-1}$) | ?-60 µmol $L^{-1}$ (3-15* µmol $L^{-1}$) | (Brogueira *et al.*, 2007) (Fig 2) |
| Sado 38º27'N 08º43'W | May 1996 September 1997 | 14 stations along the estuary (6 stations) | ----- | 0-35 (≈30) | ----- | ?-50* µmol $L^{-1}$ (≤10* µmol $L^{-1}$) | 1-4* µmol $L^{-1}$ (0.5-1.5* µmol $L^{-1}$) | 2-8* µmol $L^{-1}$ (1-3* µmol $L^{-1}$) | (Cabeçadas *et al.*, 1999) |
| Ria, Formosa 37ºN 08ºW | Monthly June1987/ May1988 | 22 stations along the coastal lagoon | ----- | ----- | ----- | 10-50* µmol $L^{-1}$ | 0.4-1.1* µmol $L^{-1}$ | 2-10* µmol $L^{-1}$ | (Gameiro *et al.*, 2007) |
| | June 2001 July2002 | 3 stations | 15-25 | 36-37 | ----- | 0.4-4.1 µmol $L^{-1}$ | 0.1-0.5 µmol $L^{-1}$ | ≤1.2 µmol $L^{-1}$ | (Loureiro *et al.*, 2006) |
| Guadiana 37º11'N 07º25'W | Apr May Jun Jul Sep 1997 | 10 stations | ----- | 0-35* | ----- | 0.?-75* µmol $L^{-1}$ | 0.?-10* µmol $L^{-1}$ | 0.?-10* µmol $L^{-1}$ | (Rocha *et al.*, 2002) |
| ELSS microcosmos | May Jun Jul 2012 | 4 control replicates | 19.9 ± 1.7 | 32.6±1.5 | 7.969 ±0.069 | 29-53 µmol $L^{-1}$ | 1.1-1.2 µmol $L^{-1}$ | 0.6-2.3 µmol $L^{-1}$ | This study |

*Values inferred from charts; **($NO_x$); ***DIN

**Profilling of the active bacterial community**

**16S rRNA sequence analysis**

Only sequences containing exact matches to primer sequences and barcode tags were used for further analysis. Raw sequencing reads were quality trimmed according to published recommendations (Kuczynski *et al.*, 2011) and the QIIME software package (http://qiime.sourceforge.net/; Accessed 8 October 2012) was applied to analyze the results of the runs. The 'pick_otus.py' function in QIIME with the usearch quality filter and the default sequence similarity threshold of 0.97 was used to assign sequences to operational taxonomic units (OTUs). The usearch quality filter is a pipeline script developed using USEARCH to perform filtering of noisy sequences, chimera check and OTU picking (http://qiime.org/tutorials/usearch_quality_filter.html; Accessed 8 October 2012). Chimera filter was performed *de novo* using UCHIME and against a reference database. UCHIME has better sensitivity than the previously most sensitive database method ChimeraSlayer and is more than 1000 times faster (Edgar *et al.*, 2011).

A representative OTU set was chosen with the 'pick_rep_set.py' function using the 'most abundant' method. Sequences belonging to the representative set were classified taxonomically with the 'assign_taxonomy.py' function in QIIME with the Naive Bayesian rRNA RDP Classifier method and 80% minimum confidence score. Chimeric sequences, plant organelles or sequences not classified as *Bacteria* (*e.g.*, *Archaea*) were removed prior to statistical analysis. The 'make otu_table.py' function in QIIME was used to generate a square matrix containing the abundance of all OTU's per sample that was used in subsequent analysis.

After quality screening with QIIME pipeline a total of 26252 sequences, corresponding to 3658 OTU´s, were identified in sediment control samples from the environment and from the microcosm. Per sample, sequences ranged from 1819 in Con2B to 4830 in Con3F, corresponding to 534 and 1305 OTU´s, respectively.

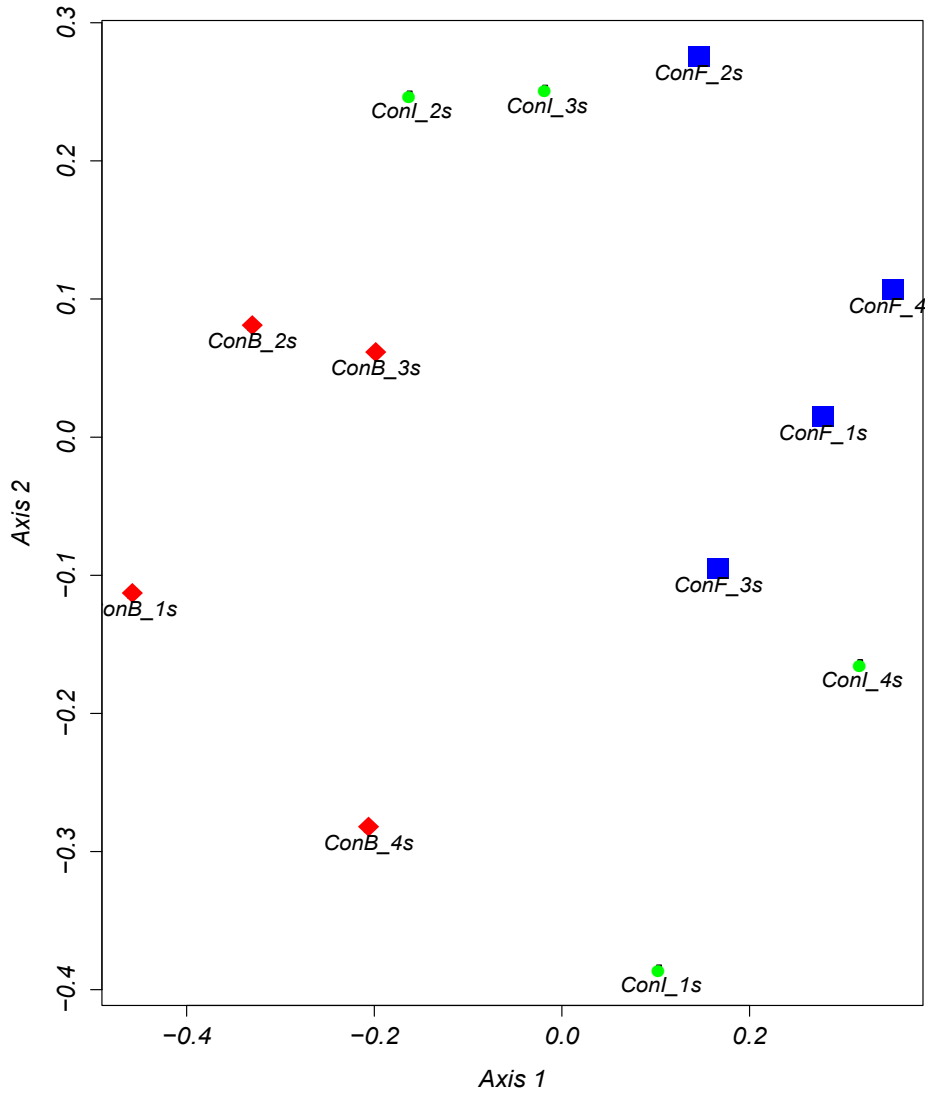


**Figure S III-12:** Principal coordinates analysis of denaturing-gradient gel electrophoresis fingerprints of 16S rRNA gene fragments amplified at time 0 (ConBs), 21 days (ConIs) and 57 days (ConFs)

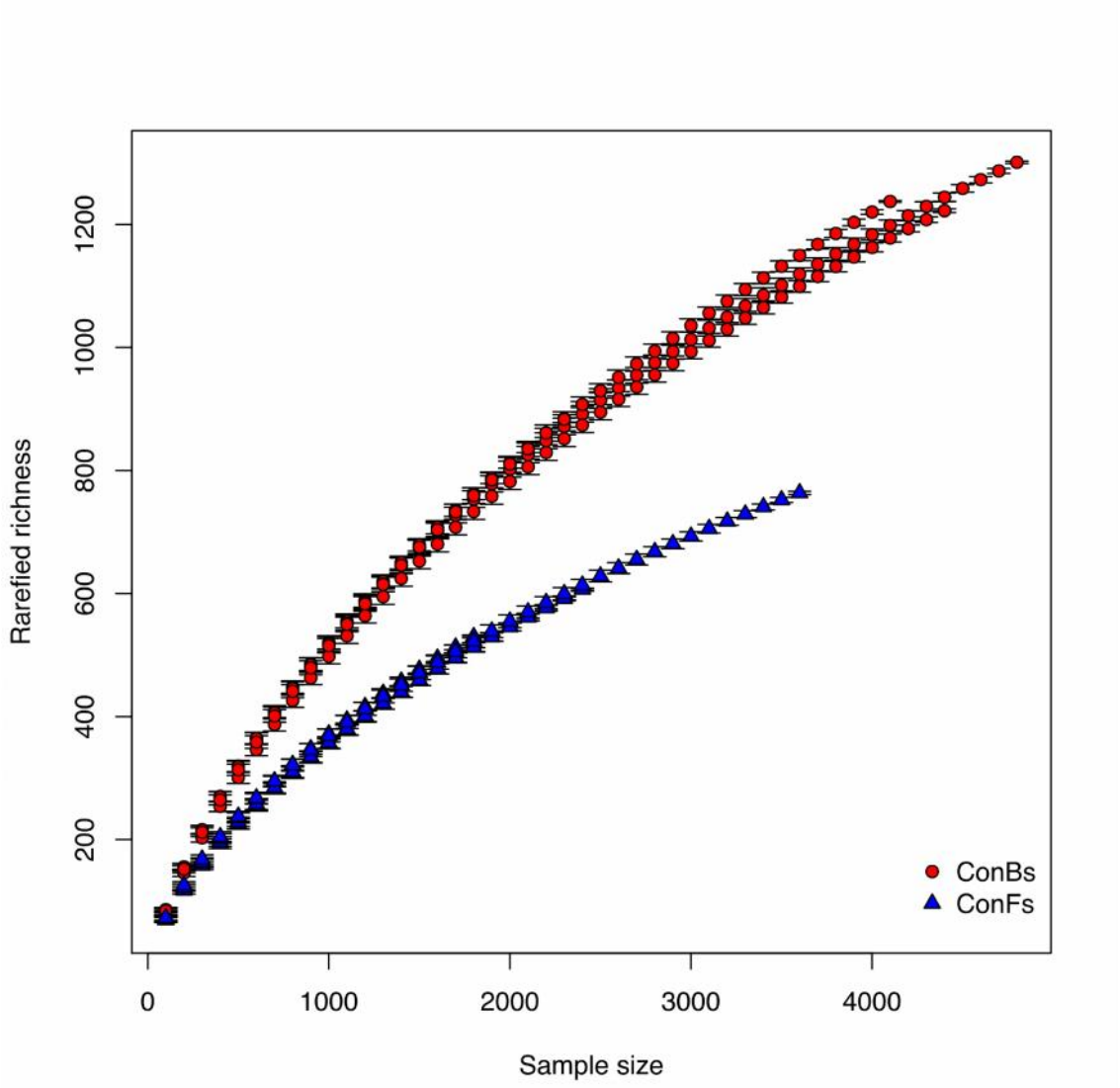


**Figure S III-13:** Rarefied OTU richness as a function of the number of sequences from ConBs (environment) and ConFs (microcosm) samples.

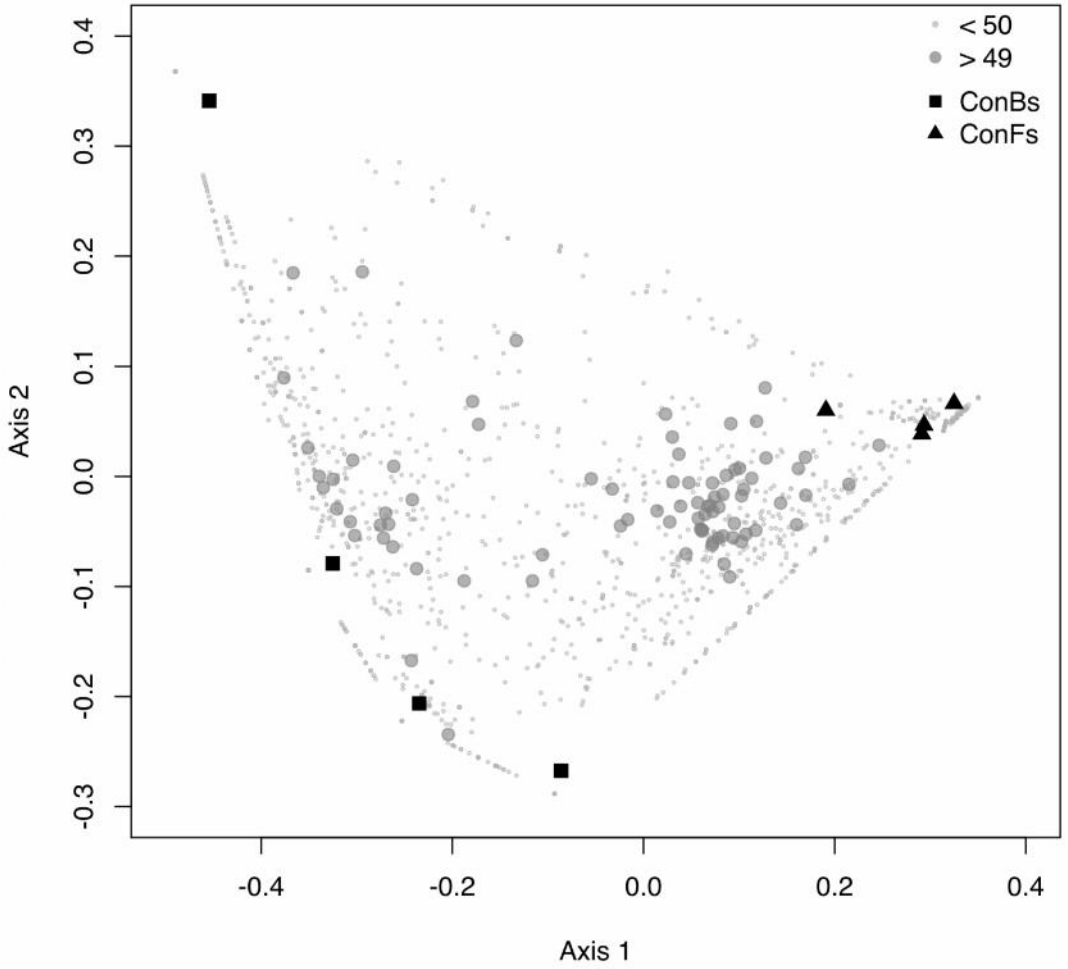


**Figure S III-14:** Principal coordinates analysis of operational taxonomic unit (OTU) composition. The first two axes of a PCO ordination based on a matrix of OTU composition of sediment control samples from the environment (ConBs) and from the microcosm after 57 days operating (ConFs).

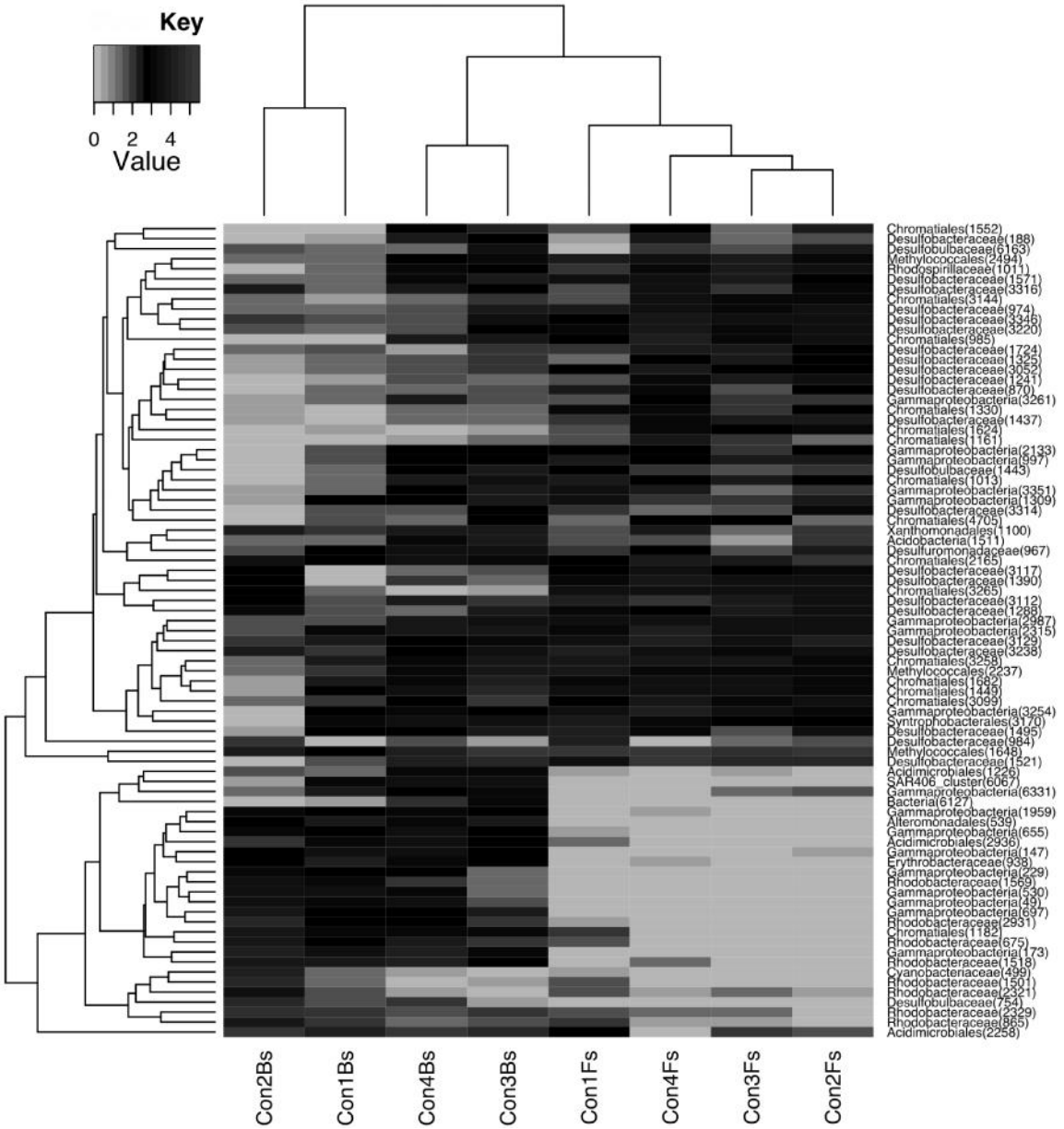


**Figure S III-15:** Heatmap showing the abundance of dominant 16S rRNA cDNA sequence reads (≥ 50 sequences). The heatmap was generated using the function heatmap2() in the R package gplots (http://www.cran.r-project.org/). The OTUs were log-transformed and clustered according to their occurrence by UPGMA hierarchical clustering. The code of each OTU is indicated in brackets.

**Multivariated Repeated Measurements with Adonis ()**

#This script will analyse multiple time effect on repeated inorganic nutrient concentration measures. Script details and rational can be found on theBioBucket website (http://thebiobucket.blogspot.pt/2011/04/repeat-measure-adonis-lately-i-had-to.html#more). Briefly, repeated measures on one sample are not independent events, so permutations across samples were not performed. The script allows temporal correlation within samples. The order of time points is changed from 1,2,3 to 3, 1, 2 or 2,3,1. Each nutrient was analysed separately.

```
#Import data
nutrients.df<-read.table("nutrients.matrix.txt",header=TRUE,as.is=TRUE)

#choose data
site<-as.factor(nutrient.matrix[,1])
time<-as.ordered(nutrient.matrix[,2])
NO3<-as.matrix(nutrient.matrix[,3])

#(btw, using dist() defaults to euclidean distance)

print(fit <- adonis(dist(NO3) ~ time, permutations=1))

#set number of permutations

B<-1999

# setting up frame which will be populated by random r2 values:
pop <- rep(NA, B + 1)
# the first entry will be the true r2:
pop[1] <- fit$aov.tab[1, 5]

#set up a "permControl" object:
# turn off mirroring as time should only flow in one direction
```

```
ctrl <- permControl(strata = site, within = Within(type = "series", mirror = FALSE))
# Number of observations:
nobs <- nrow(NO3)

#check permutation (...rows represent the sample id):
#within in each repeated sample (= sites) timepoints are shuffled,

shuffle(nobs, control = ctrl)

#loop:
# in adonis(...) permutations = 1,

set.seed(123)
for(i in 2:(B+1)){
  idx <- shuffle(nobs, control = ctrl)
  fit.rand <- adonis(dist(NO3) ~ time[idx], permutations = 1)
  pop[i] <- fit.rand$aov.tab[1, 5]}
#p value
print(pval <- sum(pop >= pop[1]) / (B + 1))

#aov.tab

str(fit$aov.tab)
```

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# Chapter IV

# Ocean acidification and oil contamination have a synergistic effect on microbial community composition

**Francisco J.R.C. Coelho, Daniel F.R. Cleary, Rui J.M. Rocha, Ana C.C. Pires, José Castanheira, Artur M. S. Silva, Mário M. Q. Simões, Vanessa Oliveira, Ana Lillebo, Adelaide Almeida, Ângela Cunha, Rodrigo Costa, Ricardo Calado, and Newton C. M. Gomes**

**Abstract:** There is near unanimous consensus that climate change has the potential to alter marine ecosystems. However, researchers are still far from understanding the extent and consequences (if any) of climate change interactions with other anthropogenic stressors. Due to its complex nature, the effects of climate change (*e.g.* ocean acidification) and other anthropogenic stressors have mainly been accessed independently. A possible way of understanding these interactions is through experimentation under well-controlled conditions. With this work we demonstrate that the combination of reduced water pH and oil contamination can alter the structure of the active bacterial communities of shallow estuarine sediment. Anaerobic sulphate reducing bacteria drastically reduced their relative abundance under oil contamination and pH values projected for the end of this century. 16S RNA (cDNA) based predicted metagenome suggested that several oil contaminant degradation pathways could be affected in these conditions. Taken together, these results indicate that in future ocean reduced pH conditions the impact of an oil spill can be substantially different than under current pH levels.

**Keywords**; microbial ecology; pyrosequencing; climate change

## Introduction

Marine environments are exposed to a range of factors that are fundamentally altering the chemistry of the ocean at unprecedented rates (Doney 2010). Since the beginning of the industrial revolution, human activities, particularly the burning of fossil fuels, have increased atmospheric carbon dioxide ($CO_2$) from approximately 280 ppmv (parts per million volume) (Indermühle *et al.,* 1999) to nearly 394 ppmv in 2012 (NOAA Earth System Research Laboratory, 2012).

Between one-quarter to a one-third of the excess $CO_2$ has been absorbed by the oceans, reducing seawater pH and inducing modifications in fundamental chemical balances between inorganic carbon species (Sabine *et al.,* 2004, Richard *et al.,* 2009). This process, known as ocean acidification, is often referred to as "the other $CO_2$ problem" (Doney *et al.,*

2009). The magnitude of this process is such that modelled predictions on the disruption of the ocean carbonate chemistry have no parallel in the past 300 million years of earth´s geological record (Hönisch *et al.,* 2012).

The implementation of the Montreal protocol has successfully reduced ozone-depleting substances (Mckenzie *et al.,* 2011). However, increasing concentrations of greenhouse gases are expected to interact with ozone and alter its spatial distribution and exchanges between the stratosphere and troposphere, with potential effects on UVR levels that reach the earth (Bais *et al.,* 2011). Furthermore, warming, acidification and changes in ocean stratification patterns are also expected to increase the penetration of UVR in the water column and enhance organism exposure to UV-B in forthcoming years (UNEP 2010).

In addition to the effects on the global climate system, human activities such as fossil fuel combustion, fertilizer use, and industrial activity have been seriously and adversely affecting coastal and open-ocean environments for decades, as they cause a continuous influx of pollutants (including oil hydrocarbons, pesticides and heavy metals) (Doney 2010). Oil hydrocarbons (OH) are probably one of the most widespread pollutants in oceans worldwide. The Deepwater Horizon tragic accident alone released an estimated volume of $4.4\times10^6 \pm 20\%$ barrels of oil ($7.0\times10^5$ $m^3$) into the ocean (Crone and Tolstoy 2010).

Both ocean acidification and UVR have the potential to interact with other anthropogenic stressors such as oil hydrocarbons leading to changes in marine ecosystems (Fabry *et al.,* 2008, Hader *et al.,* 2011). However, we still lack a fundamental knowledge on how these stressors will interact, compromising our ability to predict sudden contamination impacts in future ecosystems. Understanding the impacts of these interactions on microbial communities and microbial mediated biogeochemical processes is fundamental to determine how ecosystems will react. Microbial communities and their activities drive important ecosystem functions. For example, the ability of bacteria to use OH as a carbon and energy source greatly influences the environmental detoxification of an oil spill (Head *et al.,* 2006). We hypothesized that the impact of an oil spill in marine ecosystems causes a significant shift on microbial communities under increased UV-B and reduced water pH. We tested this hypothesis by characterizing the independent and interactive effect of these stressors on estuarine sediment microbial communities using an experimental life support system (ELSS). This system allows simulating coastal and estuarine ecosystems and has been previously validated to simulate climate change scenarios on benthic communities (Coelho *et al.,* in press).

## Methods

*Aromatic and aliphatic hydrocarbon analysis*

Aliphatic and aromatic hydrocarbons were determined using a soxhlet extraction followed by a GC-MS analysis (Yan *et al.*, 2009). Approximately 5 g of sediment were collected from oil contaminated treatments (OnOi, UVOi, pHOi and UVTot) and from control treatments (Cont). The sediment was homogenized and freeze-dried before extraction. The sediment was spiked with 25 μl (2 μg/ml) deuterated recovery surrogate standards (Supelco) containing naphthalene-$d_8$, acenapthlene-$d_{10}$, phenanthrene-$d_{10}$, chrysene-$d_{12}$ and perylene-$d_{12}$ and soxhlet extracted with 150 ml dicloromethane for 24 h naphthalene-$d_8$, acenapthlene-$d_{10}$, phenanthrene-$d_{10}$, chrysene-$d_{12}$ and perylene-$d_{12}$ and soxhlet extracted with 150 ml dicloromethane for 24 h (Yan *et al.* 2009). The first fraction, corresponding to aliphatic hydrocarbons, was eluted with 15 ml of n-hexane. The second fraction, containing PAH compounds, was collected by eluting 30 ml of n-hexane/dichloromethane (1:1, v:v). The two fractions were combined for analysis. The determination of the two hydrocarbons fraction was performed on a Network GC system 6890/5973 (Agilent Technologies, USA) mass spectrometer with a 30 m, 0.25 mm, 0.25 μm film thickness VF-5MS fused silica column in the selective ion mode (SIM) for aromatic hydrocarbons or in scanning mode for aliphatic hydrocarbons. More details can be found in hydrocarbon analysis section in supporting information.

*Active bacterial community profiling*

Four composite samples consisting of four sediment cores (~1 cm of top sediment with a 1 cm diameter) were collected in the microcosms after 57 days of operation. Approximately 1 g of the sediment layer was directly transferred into a 2.0 ml screw cap tube containing Lysing Matrix E (FastRNA® Pro Soil-Direct Kit, Qbiogene Inc.,CA) and immediately immerse in liquid nitrogen (Liu *et al.* 2011). Samples were kept at -80 ºC until the RNA extraction.

Total RNA was isolated from sediment samples using the FastPrep® Instrument (Qbiogene, Inc, CA), for 40 s at a speed setting of 6.0 according to the manufacturer's instructions. Residual DNA was removed using Turbo DNA-free kit (Applied Biosystems,

Austin, TX, USA). Total RNA was then converted to single stranded cDNA using random hexamer-primed reverse transcription by applying SuperScript® III Reverse Transcriptase Kit (Invitrogen). The complete removal of DNA was confirmed by PCR. Universal bacterial primers U27 and 1492R were used to amplify *ca.* 1450 bp of the 16S rRNA gene (Weisburg et al., 1991). Specific products were detected in cDNA samples after reverse transcription, but not in their corresponding source RNA samples (data not shown).

A barcoded pyrosequencing approach was used for microbial community analysis. Fragments of the 16S ribosomal RNA (rRNA) gene were sequenced for each sample with primers V3 Forward (5'-ACTCCTACGGGAGGCAG-3') and V4 Reverse (5'-TACNVRRGTHTCTAATYC-3') using the 454 Genome Sequencer FLX Titanium (Life Sciences Roche Diagnostics Ltd, West Sussex, UK). Only sequences containing exact matches to primer sequences and barcode tags were used for further analysis.

*Sequence Analysis*

Raw sequencing reads were quality trimmed according to published recommendations (Kuczynski *et al.* 2011) and the QIIME software package (http://qiime.org; Accessed 4 May 2012) was applied to analyze the results of the runs. Sequences can be downloaded from the NCBI ShortRead Archive, accession numbers (study accession number - SRP021163). The 'pick_otus.py' function in QIIME with the uclust method and the default sequence similarity threshold of 0.97 was used to assign sequences to operational taxonomic units (OTUs). A representative OTUs set was chosen with the 'pick_rep_set.py' function using the 'most abundant' method. Sequences belonging to the representative set were classified taxonomically with the 'assign_taxonomy.py' function in QIIME with the Naive Bayesian rRNA RDP Classifier method and 80% minimum confidence score. Chimeric sequences (identified using the parallel identify chimeric 'seqs.py' function in QIIME), plant organelles or sequences not classified as *Bacteria* (*e.g.*, *Archaea*) were removed prior to statistical analysis. The 'make otu_table.py' function in QIIME was used to generate a square matrix containing the abundance of all OTUs per sample that was used in subsequent analysis.

Total rarefied OTUs richness per sample was estimated with a self-written function (Gomes et al., 2010) in R (http://www.r-project.org/; Accessed 6 January 2013). Rarefied OTUs richness was selected for a minimum sampling size of 1800 sequences. Shapiro-Wilk

test() was used to test for normality and Bartlett test() was used to test the assumption of homogenous variance. Analysis of variance (Anova) was then used to test if rarefied richness differed significantly between treatments. Variation in OTUs composition among treatments was tested for significance using the adonis() function in vegan (Oksanen *et al.*, 2012). In the Adonis analysis, the Bray-Curtis distance matrix of OTUs composition was the response variable with treatment as independent variable. The number of permutations was set at 999. The argument strata was included to allow randomizations only within each luminaire (Oksanen *et al.* 2012). Variation in composition among treatments was assessed with Principal Coordinates Analysis (PCO), using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was log10 (x+1) transformed and used to produce a distance matrix based on the Bray-Curtis distance with the vegdist() function in vegan.

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved Sates (PICRUST-http://picrust.github.com/picrust/Accessed 24 January 2013) software package to predict the metagenome functional content from the 16S rRNA dataset. The 'pick_reference_otus_through_otu_table.py' was used to pick OTU´s against a reference collection with OTU´s assigned at a 97% similarity. The OTU table obtained was then normalize dividing each OTU by the know/predicted 16S copy number with 'normalize_by_copy_number.py' function. The 'predict_metagenomes.py' function was used to create a square matrix, containing normalized OTU abundance multiply by each predicted functional trait abundance per sample, that was used in subsequent analysis.

Variation in OTUs and KO relative abundances was analyzed with a mixed-model approach, treating oil, UV-B and reduced pH as fixed effects and the luminaires as the random effect. Since we wanted to examine the interaction between oil, UV-B and pH we included an interaction term. The mixed-model was fitted using glmer function in the lme4 package with family argument set to binomial (Bates *et al.*, 2011). Overdispersion was accounted for by including individual level variability as a random effect (Bates *et al.*, 2011). The significance on the main effects and interaction terms was tested using the z -statistics. With this approach pseudo-replication was accounted for (*i.e.* oil contamination, reduced water pH and UV-B treatment split within four luminaires).

**Results and discussion**

*Sample Collection and Experimental Manipulation*

A fully factorial experiment was designed using 32 independent microcosms with three treatments: UV-B radiation, water pH and OH contamination. Each treatment had two levels: UV-B (with and without), pH (normal and reduced) and Oil (with and without). The treatments were replicated into four random independent microcosms and arranged in a randomized split-plot design with UV-B as the whole plot and pH and oil at the sub-plot levels. The design enabled us to distinguish 8 distinct groups. These groups included 1. Cont (no UV-B, normal pH and no oil), 2. OnpH (no UV-B, reduced pH, no oil), 3. OnUV (UV-B, normal pH and no oil), 4. UVpH (UV-B, reduced pH, no oil), 5. OnOi (no UV-B, normal pH, oil), 6. UVOi (UV-B, normal pH, oil), 7. pHOi (no UV-B, reduced pH, oil) and finally 8. UVTot (UV-B, reduced pH, oil). The experiment was divided into two phases. In the first phase, the sediment was stabilized during 22 days. In this phase pH was gradually reduced in reduced pH treatments while the UV-B system operated with the same intensity during all the experiment. The second phase started on day 22, with the simulation of an oil spill by pouring oil into oil contaminated treatments. This phase lasted 35 days, until we visually confirmed the degradation of the most part of the oil from the water surface. Polycyclic aromatic and aliphatic hydrocarbon analysis from sediment collected from oil contaminated and control treatments did not reveal significant differences (Figure S IV-1) and S IV-2), further suggesting that most of the oil hydrocarbons were already degraded at this time.

The microcosms were subjected to a uniform semi-diurnal tidal regime, experiencing two high tides and two low tides daily. Water pH was manipulated by bubbling $CO_2$ into water (Gattuso and Lavigne 2009) reservoirs prior addition into the microcosms. The acidified water was supplied with a pH value of 7.2 to the microcosms in each high tide. The sediment carbonate chemistry impacted the water pH that tended to increase along each tide. Nonetheless, the minimum pH reduction in relation to control microcosms was 0.3, which falls within the 0.3-0.4 pH reduction modeled for global sea surface for the year 2100 (Caldeira *et al.*, 2003). The average pH was 7.64±0.11 in acidified microcosms (measured at the end of each low tide, before the addition of new acidified water) and 7.95 ±0.09 in the control microcosms (Figure S IV-3).

A constant UV-B irradiance of 1426.36±93.32 $mWm^{-2}$, during 4 h per day around noontime, was used in microcosms with UV-B. This value is similar to the typical DNA-damaging UV dose observed in Lisbon in June. Since UV-A radiation is practically

unaffected by changes in ozone depletion and plays an important role in photo-repair mechanisms (Bargagli 2005), a similar amount of UV-A integrated irradiance of 2875.91±264.62 $mWm^{-2}$ was supplemented to the microcosms.

*Active bacterial community*

The active bacterial community from each treatment was characterized by using a barcoded pyrosequencing approach of the bacterial 16S rRNA (cDNA) gene. Sequences were assign to operational taxonomic units (OTUs) based on their sequence similarity with a cut off value of 97% (Roesch *et al.,* 2007). To examine changes in OTUs richness, rarefaction curves were generated for all treatments (Figure S IV-5). Controlling for sampling size (n= 1800 sequences) rarefied OTUs richness varied from 490±68.52 for OnOi, to 557±48.68 for OnUV (Figure S IV-3). No significant differences in OTUs richness were detected between treatments (Anova, $F_{7,24}$ = 1.29, $R^2$ = 0.868, P = 0.54). On the contrary, there was a significant variation in OTUs composition between treatments (Adonis analysis: $F_{7,31}$ = 1.563, $R^2$ =0.313, P<0.001). A principal coordinate analysis (PCO) using the Bray-Curtis distance of OTUs composition was applied to visualize the dissimilarities between the different treatments (Figure IV-1).

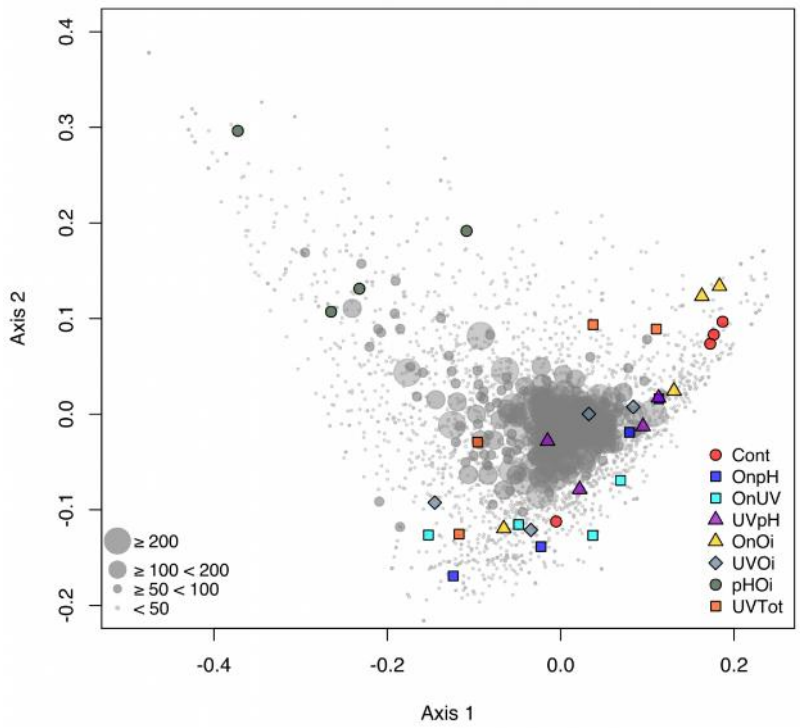


**Figure IV-1:** Principal coordinates analysis of operational taxonomic unit (OTU) composition. The first two axes are shown based on a matrix of OTU composition. **Cont** - control with no treatment; **OnpH** - reduced pH; **OnUV** - exposed to UV-B; **UVpH** - reduced pH and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and reduced pH; **UVTot** - contaminated with oil and exposed to UV-B and reduced pH.

The primary and secondary axis of variation was between the Cont group and the samples retrieved from the pHOi group. UVTot group also exhibited a variation from other groups, although less pronounced than pHOi. Using the RDP classifier, with a confidence threshold of 80%, 128313 out of 129282 (99.25%) qualified sequences were assigned to known phyla. *Proteobacteria* was the most represented phylum varying from 95.92±1.35% of total identified OTUs in OnOi to 88.55±5.20% in pHOi group. We therefore, tested the interactive effect of oil, pH and UV-B on the relative abundance of the three most representative *Proteobacteria* groups classified at order level (Figure IV-2). Together, these four groups varied from 76.12±9.91% in pHOi to 87.21±2.39% in Cont (The relative abundance of all *Proteobacteria* orders is available in supportive information Figure S IV-8). Within these four orders the most notable effects in relative abundance was detected in *Desulfobacterales*. We detected a significant interactive effect between oil and reduced pH treatment in the relative abundance of *Desulfobacterales* (Wald Z test, z = -3.046, P = 0.002). Indeed, in line with the PCO analysis, the most drastic effect was a decrease in the relative abundance of *Desulfobacterales* in microcosms with oil under reduced pH. In oil contaminated microcosms the relative abundance of *Desulfobacterales* decreased from 50.71±6.96%, under normal water pH and without UV-B, to 19.56±7.36%, in microcosms with reduced pH without UV-B. On the other hand, no significant effect of oil (Wald Z test, z = 1.386, P = 0.166), UV-B (Wald Z test, z = -1.186, P = 0.235) or pH (Wald Z test, z = -1.408, P = 0.159) alone was detected, thus suggesting a synergistic interaction between oil and reduced water pH (Figure IV-2).

Such a drastic reduction in active *Desulfobacterales* members could have consequences for the degradation of organic pollutants in contaminated marine sediments under reduced water pH without UV-B. *Desulfobacterales* order includes diverse anaerobic sulphate-reducing bacteria. These bacteria are characterized by their ability to use sulphate as terminal electron acceptor in the oxidation of $H_2$ and organic compounds. This directly links them to the cycling of carbon and sulphur and to the degradation of organic contaminants, including oil hydrocarbons, in a variety of habitats (Muyzer and Stams 2008, Zhou *et al.,* 2011). The observed decrease thus suggests that after oil contamination and under reduced pH the degradation of organic contaminants can be substantially affected. Interestingly, although no significant differences were detected in hydrocarbon concentration between treatments with oil, the highest values for several aliphatic (*e.g.* 1-decene and dodecene) and aromatic hydrocarbons (*e.g.* acenaphtylene, acenaphthene and fluorene) were detected in combined oil and reduced pH treatment (Figure S IV-1 and S IV-2). However, these values showed high

standard deviation hampering the detection of significant differences in the amount of hydrocarbon removal between different groups.

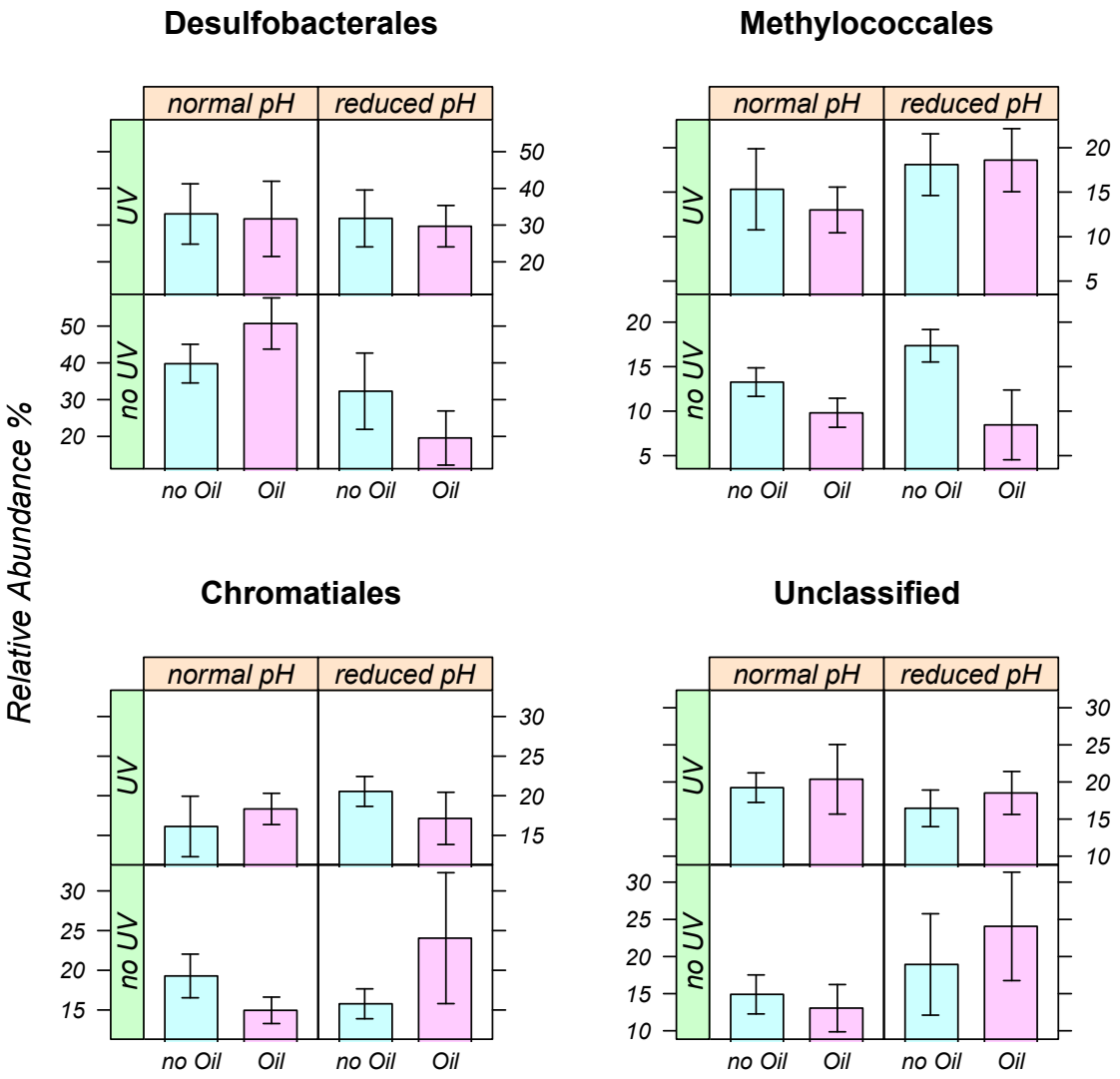


**Figure IV-2:** Relative abundance of OTUs classified at order level under the independent and combined effects of reduced pH, UV-B exposure and oil contamination. Sequences reads (from 454 Genome Sequencer FLX Titanium) were assigned to OTUs and classified at order level with QIIME software (http://qiime.org). The three most abundant orders are shown.

A significant interactive effect on the relative abundance of *Desulfobacterales* was also detected between oil, UV-B and reduced pH treatment (Wald Z test, z = -3.046, P = 0.002). However, in this case, the relative abundance of *Desulfobacterales* was 29.70±0.75, similar to 31.83±0.79 registered in oil contaminated sediments under normal pH with UV-B. Probably, in this case, oil hydrocarbon photochemical modification due to the presence of UV-B masked the interaction between oil and pH detected without UV-B.

A significant interaction was also detected in oil, UV-B and reduced pH treatment in the relative abundance of *Methylococcales* (Wald Z test, z = 2.156, P = 0.031). However, contrary to what was observed in *Desulfobacterales*, the relative abundance of *Methylococcales* was the highest in the microcosm with oil, UV-B and reduced pH. In oil contaminated microcosms, the relative abundance of this order increase from 9.81±1.63%, under normal pH and without UV-B, to 18.59±3.54%, under reduced pH and in the presence of UV-B. Functionally, order *Methylococcales* includes aerobic bacteria capable of oxidizing and utilizing methane (methanotrophic) and other $C_1$ compounds (methylotrophic) as source of carbon and energy (Bowman 2005). Methanotrophic bacteria respond rapidly to increase methane concentrations, and it has been suggested that they may act as an important biofilter that limits the release of $CH_4$ from the ocean to the atmosphere (Kessler *et al.*, 2011). This sudden increase can be caused by the combined presence of UV-B and pH. The presence of UV-B can induce the formation of C1 compounds through photochemical degradation of oil hydrocarbons whereas pH has been shown to exert a strong influence on other methylotrophic bacteria, in ocean acidification mesocosms experiments (Roy *et al.,* 2012). Indeed, an independent effect of pH (Wald Z test, z = 1.672, P = 0.094) and oil (Wald Z test, z = -1.846, P = 0.064) was detected in order *Methylococcales*.

*Predicted metagenome analysis*

Here we used the PICRUSt software (http://picrust.github.com/picrust/) to predict metagenome functional contents based on the Kyoto encyclopedia of genes and genomes (KEGG) classification to identify potential bacterial traits in our samples. Given the drastic decrease in *Desulfobacterales* the focus was specifically set on the relative abundance of KEGG orthologs (KO) assigned to the category "xenobiotics and degradation". The independent and interactive effect of oil, pH and UV-B on the relative abundance of the six most representative pathways was analysed (Figure IV-3). The relative abundance of all KEGG categories and of all xenobiotics and degradation pathways is available in supportive information Figures S IV-6 and S IV-7. The predicted metagenome for these categories was consistent with taxon responses. A significant interaction between oil and reduced pH in benzoate degradation (Wald Z test, z = -3.14, P = 0.002), caprolactam degradation (Wald Z test, z = -3.40, P < 0.002) and ethylbenzene degradation (Wald Z test, z = -4.53, P < 0.001)

pathways was found. The relative abundance of KO assigned to these pathways decreased with oil contamination under reduced pH conditions in the absence of UV-B.

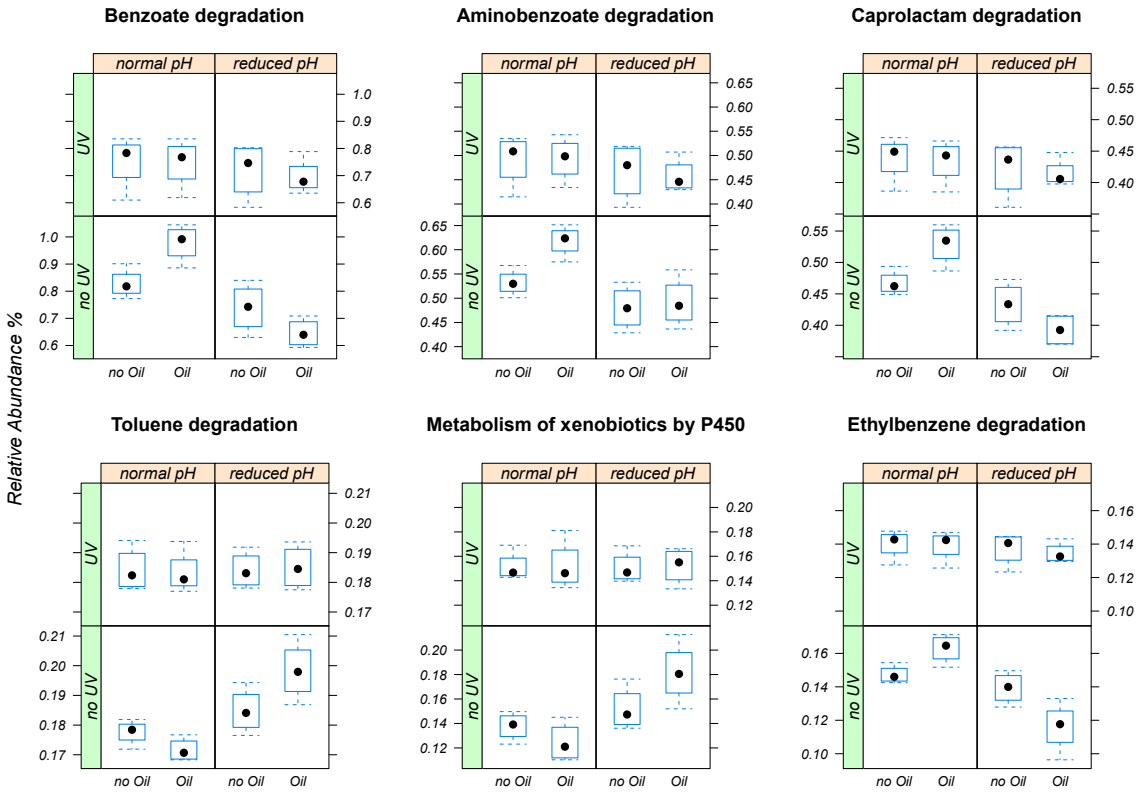


**Figure IV-3:** Relative abundance of the predicted genes assigned to KEGG metabolism category for xenobiotics biodegradation and metabolism under the independent and combined effects of reduced pH, UV-B exposure and oil contamination. Metagenome functional content was predicted from 16S rRNA sequence dataset against a set of reference metagenomes with PICRUSt software (http://picrust.github.com/picrust/). The five most abundant, plus PAH, degradation pathways are shown.

These results suggest that in these conditions, the pathways responsible for degrading these compounds are underrepresented in relation to the other treatments. However, metagenome prediction also revealed functional implications that were not clear from the community analysis. An interaction between reduced pH and oil in toluene degradation (Wald Z test, $z = 3.2$, $P = 0.001$) and metabolism of xenobiotics by cytochrome P450 (Wald the Z test, $z = 3$, $P < 0.0027$) with a consequent increase in the relative abundance of KO orthologs was observed. Importantly, these results reinforced the hypothesis that the observed changes under combined effect of oil and pH can have potential functional implications on contaminant degradation pathways.

## Conclusions

Impacts of ocean acidification and reduction of water pH can be exacerbated in coastal and estuarine waters due to increased eutrophication and the consequent development of hypoxic and anoxic zones (Howarth *et al.,* 2011). The major effect detected in our multifactorial experiment was a reduction of *Desulfobacterales* order due to a synergistic interaction between oil contaminated sediments and reduced water pH. Considering the role that sulphate-reducing bacteria have in anaerobic OH degradation, such a drastic reduction can alter the detoxification capacity of marine sediment, increasing the toxicity of impacted ecosystems. Interestingly, probably due to photochemistry modifications of OH, a moderate level of UV-B appears to attenuate these effects. Therefore, it is likely that the magnitude of these interactions and their effects will depend on the level of UV-B that penetrates the impacted ecosystem. Oxidation of organic compounds coupled to the reduction of sulphate to sulfite may account for more than 50% of organic matter mineralization in the sea bed (Jorgensen 1982). Given that acidification will not be limited to surface waters and will also occur in the deep ocean (Orr *et al.,* 2005) with low or absent ultraviolet radiation, the degradation of OH in these environments can be considerably affected with widespread consequences to higher trophic levels. Considering the implications of the observed effects to microbial communities we suggest that future studies focus on the mechanisms that underlie the synergistic interactions between ocean acidification and oil contamination.

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# Ocean acidification and oil contamination have a synergistic effect on microbial community composition
# Supporting information

## Hydrocarbon analysis

Aliphatic and aromatic hydrocarbons were determined using a Soxhlet extraction followed by a GC-MS analysis. Approximately 5 g of homogenized and freeze-dried sediment were spiked with 25 μl (2 μg/ml) deuterated recovery surrogate standards (Supelco) containing naphthalene-$d_8$, acenapthlene-$d_{10}$, phenanthrene-$d_{10}$, chrysene-$d_{12}$ and perylene-$d_{12}$ and soxhlet extracted with 150 ml dicloromethane for 24 h (Yan *et al.*, 2009). The extract was concentrated by a rotator evaporator up to a volume of about 2-3 ml, and change solvent into 10 ml n-hexane which further reduce to approximately 1-2 ml. A 2:1 alumina/silica gel column with anhydrous sodium sulfate overlaying the alumina (remove small quantities of water) was used to fractioned the extract. The first fraction, corresponding to aliphatic hydrocarbons, was eluted with 15 ml of n-hexane. The second fraction, containing PAH compounds, were collected by eluting 30 ml of n-hexane/dichloromethane (1:1, v:v). The two fractions are combined for analysis. Sample extract was reduced to 1 ml and further to 0.2 ml with a gentle purified nitrogen stream. A known quantity (2 mg/ml) of the internal standard, hexamethylbenzene, was added prior to GC-MS. The determination of two hydrocarbons fraction was performed on a GC analyses were carried using an Agilent Network GC system, namely an Agilent 6890 gas chromatograph equipped with a mass selective detector (MSD 5973) with electron impact ionization (EI) operating at 70 eV (Agilent Technologies). A VF-5MS fused silica column (30 m x 0.25 mm i.d and 0.25 μm film thickness) was used for analytes separation. The GC–MS conditions for sample analysis were as follows: the injection was performed manual in the splitless mode, at transfer-line and injector temperature maintained at 300 ºC, and ion source at 230 ºC. Helium was used as carrier gas at a flow of 1.3 ml $min^{-1}$. Initial oven temperature was 60ºC, then ramped to 200 ºC at 10 ºC $min^{-1}$, followed by another ramp step to 300 ºC at a rate of 5º C $min^{-1}$, and held for 8 min. The electron impact ionization mode conditions were at ion energy 70 eV. The mass scanning ranged between m/z 20 and m/z 500.

Before sample analysis, a standard mixture and a procedural blank were run in sequence to check for contamination, peak identification and quantification. The

concentration of the 16 US EPA priority PAHs was done by using an internal standard mixture (Supelco) peaks area method. To quantify the aliphatic hydrocarbons fraction two internal standards were used: undecane (n-$C_{11}H_{24}$) and tetracosane (n-$C_{24}H_{50}$). Deutorated PAH surrogate standard were added to sediment samples to monitor the procedures of sample extraction, cleanup and analysis. The surrogate recoveries added to sediment samples were 72 ± 15% for naphthalene-$d_8$ (n = 20), 73 ± 15% for acenaphthene-$d_{10}$ (n = 20), 89 ± 20% for phenanthrene-$d_{10}$ (n = 20), 86 ± 18% for chrysene-$d_{12}$ (n = 20) and 86 ± 18% for perylene-$d_{10}$ (n = 20).

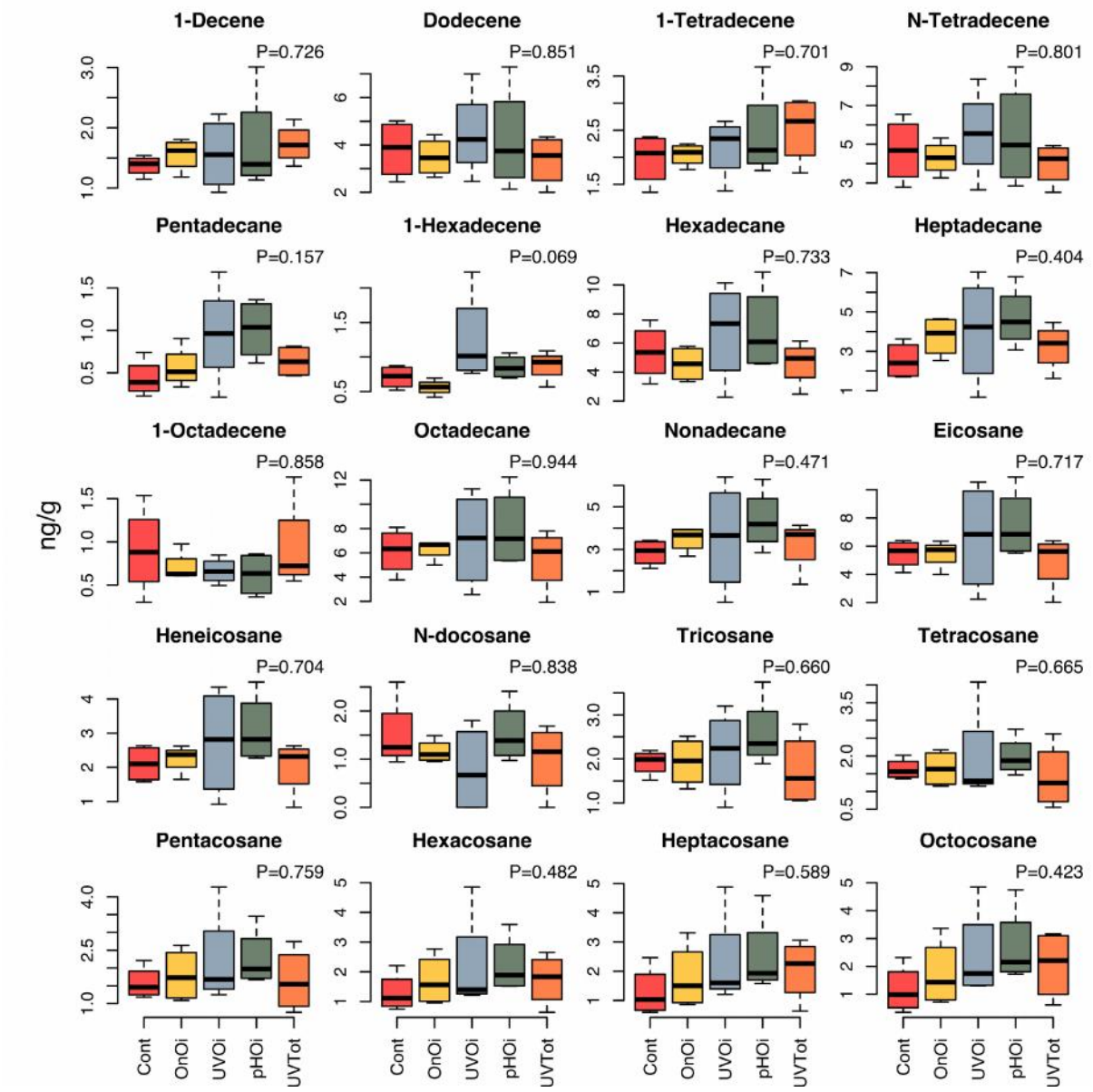


**Figure S IV-1:** Aliphatic hydrocarbon concentration in sediments from **Cont** - control with no treatment; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified. Summary from analysis of deviance (Kruskal-Wallis test) is indicated in the upper right corner of each graph.

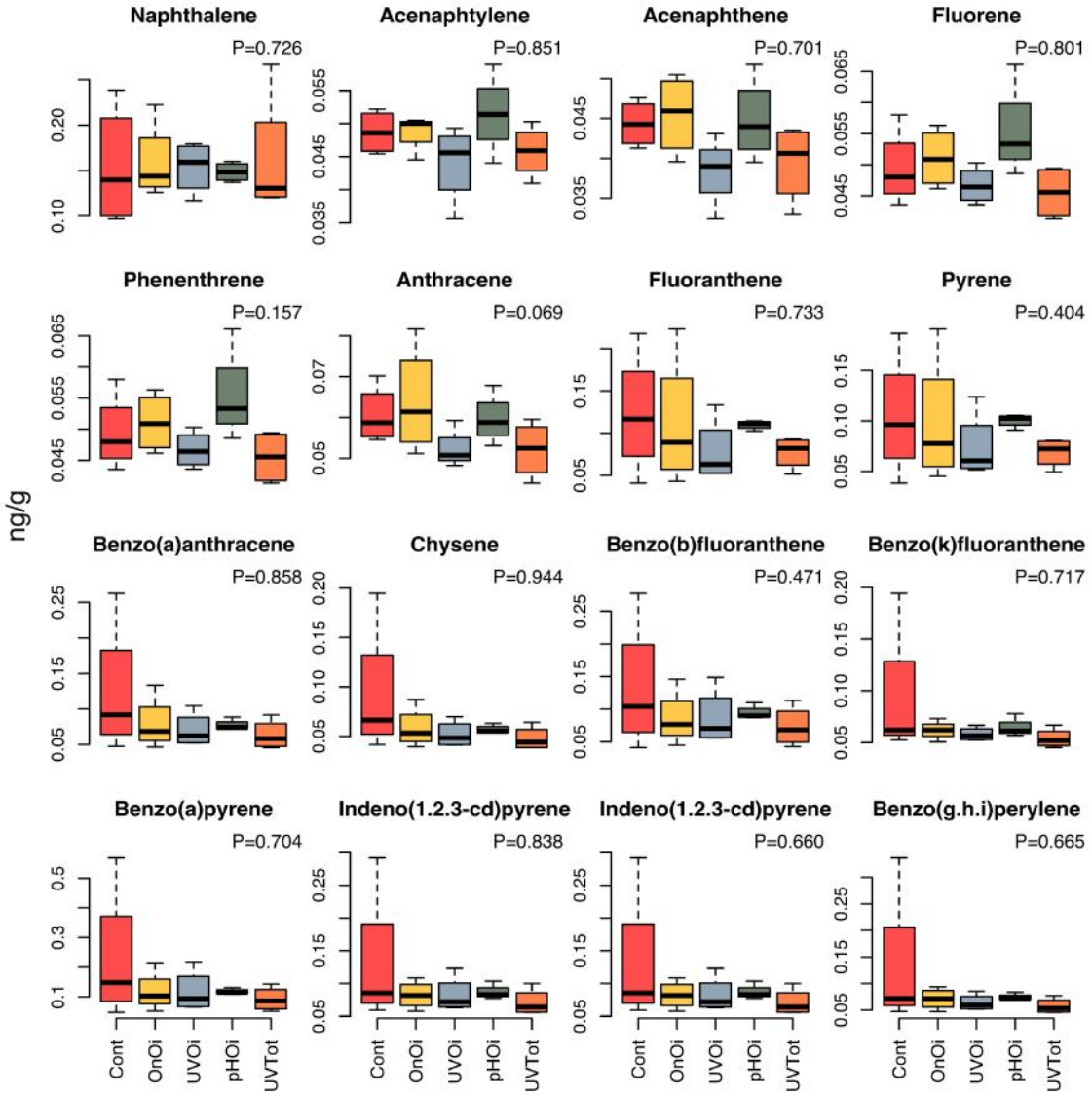


**Figure S IV-2:** Polycyclic aromatic hydrocarbon concentration in sediments from **Cont** - control with no treatment; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified. Summary from analysis of deviance (Kruskal-Wallis test) is indicated in the upper right corner of each

**Water acidification**

The pH was manipulated by bubbling $CO_2$ into the water. The $CO_2$ addition was controlled with a feedback system that included a combination of pH electrode with a controller ($V^2$ control pH controller, Tropical Marine Centre, UK) and a pressure regulator with a integrated solenoid valve (V2 pressure regulator pro, Tropical Marine Centre, UK). The controller allowed the visual representation of the actual pH. The acidified reservoir thank was monitored with a pH electrode and the controller opened the valve when the pH was lower than the set value. $CO_2$ was then delivered until return of the pH to the pre-set level. The acidified water was supplied to the microcosms in each high tide. In the stabilization period, the pH in the acidified reservoir thank was gradually decreased 0.19 units every two days, until a pH of 7.2. The microcosms were then stabilized for 10 more days until the oil contamination. The pH was monitored in the acidified and control microcosm with a calibrated pH meter (Orion Star portable meter, Thermo Fisher Scientific Inc, USA) (Figure S IV-3) before each low tide. The average pH in the control microcosms was 7.95 $\pm$0.07 and in the acidified microcosms was 7.67$\pm$0.07.

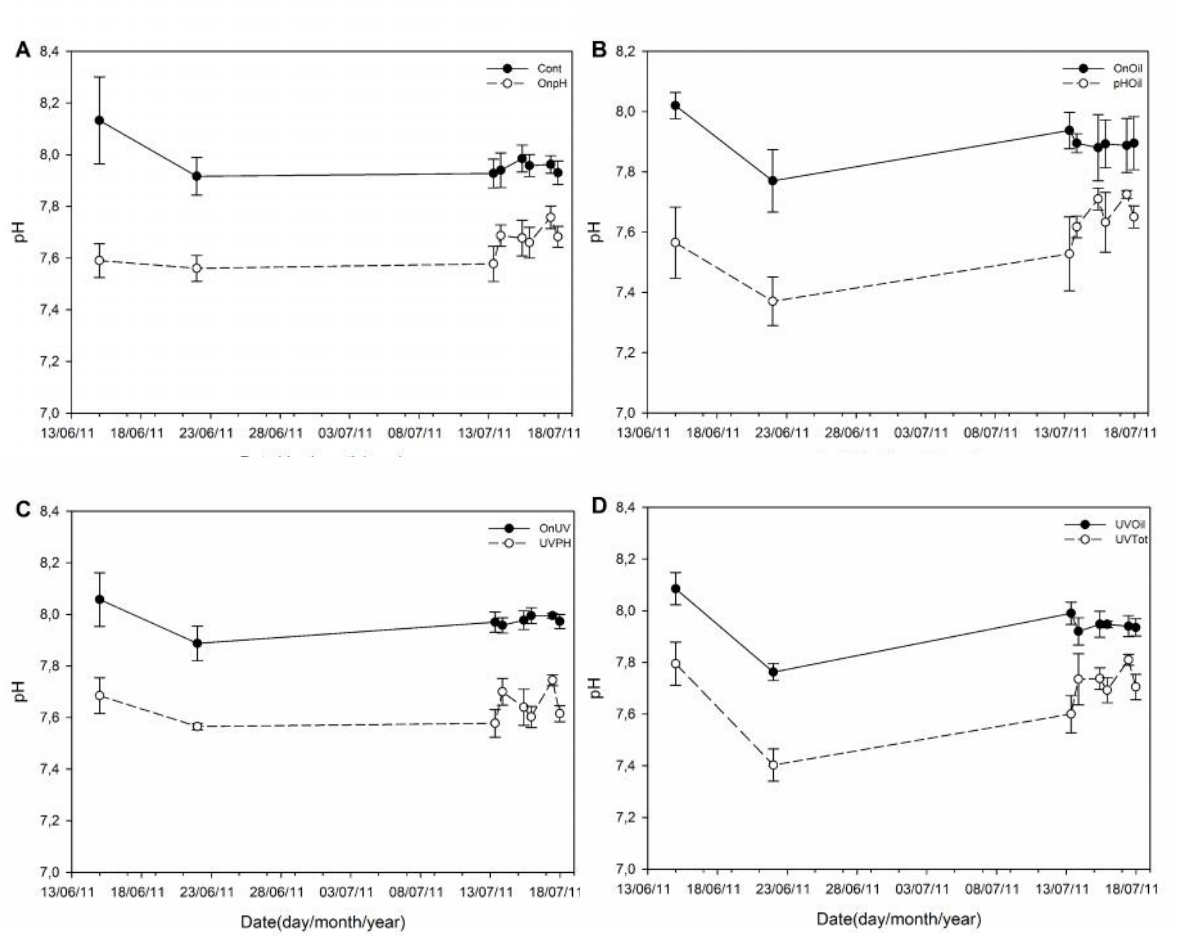


**Figure S IV-3:** Average pH variations in the different microcosms after the stabilization period. In the last week pH was monitored every other day right before the low tide. **Cont** – control; **OnpH** – Low pH; **OnOi** –oil contaminated; **pHOi** – Low pH oil contaminated; **OnUV** – UV-B; **UVpH** – UV-B with low pH; **UVOI** – UV-B oil contaminated; **UVTot** - contaminated with oil and exposed to UV-B and water acidified.

**Lighting system**

The artificial life support system was equipped with 4 custom-made ReefSET® programmable luminaire systems for diurnal light cycle and controlled UV-B simulation. Each luminaire holds 4 UV-B fluorescent tubes (SolarRaptor, T5/54W, Germany) and 4 full spectrum fluorescent tubes (AquaLight, T5/54W/10000K, Germany) disposed alternately under a reflector. The luminaire system incorporates dimming ballast that allows the adjustment of light intensity by varying the voltage supply. The ReefSET® proprietary software enables the simulation of several scenarios by varying the percentage of light intensity (http://www.reefset.com). Because during summer months (JJA) the day length varies between ~13 and ~15 h at Portuguese latitudes, a 14 h diurnal light cycle was simulated with light intensity varying from 50% to 100% of the total fluorescent tube intensity (Table S IV-1). With the lamps set to 100% the photosynthetic active radiation (PAR: 400-700 nm) was 260.5±56.3 μmol photons $m^{-2}$ $s^{-1}$ (PAR spectrum - Figure S IV-1.A). Photosynthetic Photon Flux Density (μmol photons $m^{-2}$ $s^{-1}$) was measured with a Quantum Meter with a separate sensor (Model MQ-200, Apogee Instruments, Logan, Utah, USA). The spectrum of photosynthetic active radiation was acquired using a USB2000 spectroradiometer, with spectral resolution of 0.38 nm (USB2000-VISNIR, grating #3, Ocean Optics, Duiven, Netherlands) and a 400 μm diameter optic fiber (QP400-2-VIS/NIR-BX, Ocean Optics).

Two UV-B conditions were implemented in the microcosm ensemble: microcosms irradiated with UV-B and microcosms were the UV-B was screened out. In the microcosms with UV-B, a constant UV-B irradiance of 1426.36±93.32 mW $m^{-2}$, during 4 h per day around noontime, was used. This irradiance corresponds to 50% of the maximum intensity of the UV-B lamps (Table S IV-1). UV-B and UV-A spectra are represented in Figure S IV-1.C. The values of the UV-B irradiance and time exposure were chosen considering the action spectrum for generalized DNA-damage shown in Figure S IV-1.B, which was parametrized by Bernard and Seckmeyer (1997). To find the adequate values, we proceeded as follows: First, the effective DNA-damage UV intensity of the lamps was calculated multiplying the spectrum of Figure S IV-1.C by the weighting function in 1.B and integrating over all spectral range. Next, the typical DNA-damage UV dose observed at Lisbon, in June, was calculated using the observed DNA-damage UV doses available at the Web page of TEMIS (Tropospheric Emission Monitoring Internet Service at http://www.temis.nl/).

TEMIS is part of the Data User Programme (DUP) of the European Space Agency (ESA). Finally, the irradiation duration, in order to have the microcosms exposed to a DNA-damage UV dose similar to the observed one, was calculated dividing the typical DNA-damage UV dose, observed at Lisbon, by the effective DNA-damage UV intensity of the lamps.

To have microcosms without UV-B radiation and maintain UV-A radiation we used a glass filter. The spectrum of the UV filtered radiation is also shown in Figure S IV-1.C. Comparing Figures S IV-1.B and S IV-1.C, it may be seen that the glass filter removed practically all UV radiation with DNA-Damage effect, allowing comparable irradiances at the longer wavelengths of the UV-A, except for the line centered at 365 nm. All the treatments received a similar amount of integrated UV-A irradiance of 2875.91±264.62 mW $m^{-2}$. UV-A and UV-B irradiances were accessed with a spectro-radiometer, place at the minimum tide level (35 cm from the lamps), connected to a monochromator that provides information on the spectral irradiance with a resolution of one nanometer. Spectral irradiance was obtained by the BenWin+ Software (Bentham Instruments, Reading, UK) (Ribeiro *et al.*, 2011). Photosynthetic Photon Flux Density, PAR and UV spectra were measured under each luminaire in minimum tide high in the middle and border microcosms.

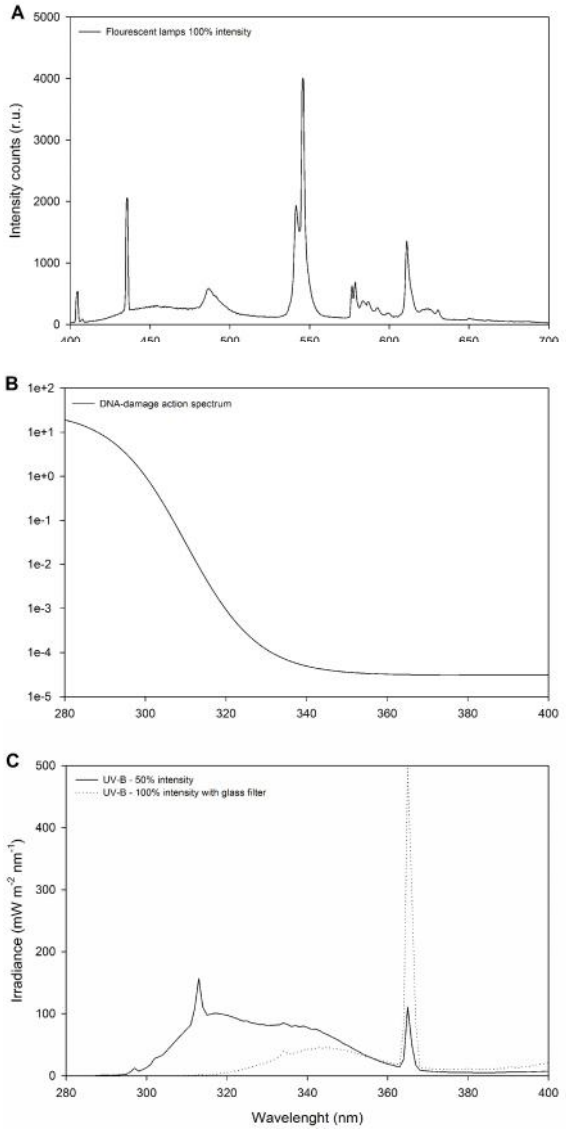


**Figure S IV-4:** Experimental light spectra. A - Spectrum of photosynthetically active radiation (PAR: 400–700 nm) of the fluorescent tubes set to 100% intensity. B- action spectrum for generalized DNA-damage as parametrized by Bernard and Seckmeyer (1997). C - Spectral distribution of UV-B lamps set to 50% intensity and spectral distribution with UV-B lamps set to 100% and with glass filter.

**Table S IV-1:** Full spectrum fluorescence tubes and UV-B fluorescence tubes intensity along the diurnal cycle. UV-B radiation in control microcosms was filtered with a glass panel.

| Time | Intensity level of fluorescence tubes (% of total lamp intensity) | | |
|---|---|---|---|
| | Fluorescent tubes | UV-B lamps – increase UV-B treatments | UV-B lamps – control microcosm |
| 1:00 | 0 | 0 | 0 |
| 2:00 | 0 | 0 | 0 |
| 3:00 | 0 | 0 | 0 |
| 4:00 | 0 | 0 | 0 |
| 5:00 | 0 | 0 | 0 |
| 6:00 | 50 | 0 | 0 |
| 7:00 | 60 | 0 | 0 |
| 8:00 | 70 | 0 | 0 |
| 9:00 | 80 | 0 | 0 |
| 10:00 | 90 | 0 | 0 |
| 11:00 | 100 | 50 | 100 |
| 12:00 | 100 | 50 | 100 |
| 13:00 | 100 | 50 | 100 |
| 14:00 | 100 | 50 | 100 |
| 15:00 | 90 | 0 | 0 |
| 16:00 | 80 | 0 | 0 |
| 17:00 | 70 | 0 | 0 |
| 18:00 | 60 | 0 | 0 |
| 19:00 | 50 | 0 | 0 |
| 20:00 | 0 | 0 | 0 |
| 21:00 | 0 | 0 | 0 |
| 22:00 | 0 | 0 | 0 |
| 23:00 | 0 | 0 | 0 |
| 24:00 | 0 | 0 | 0 |

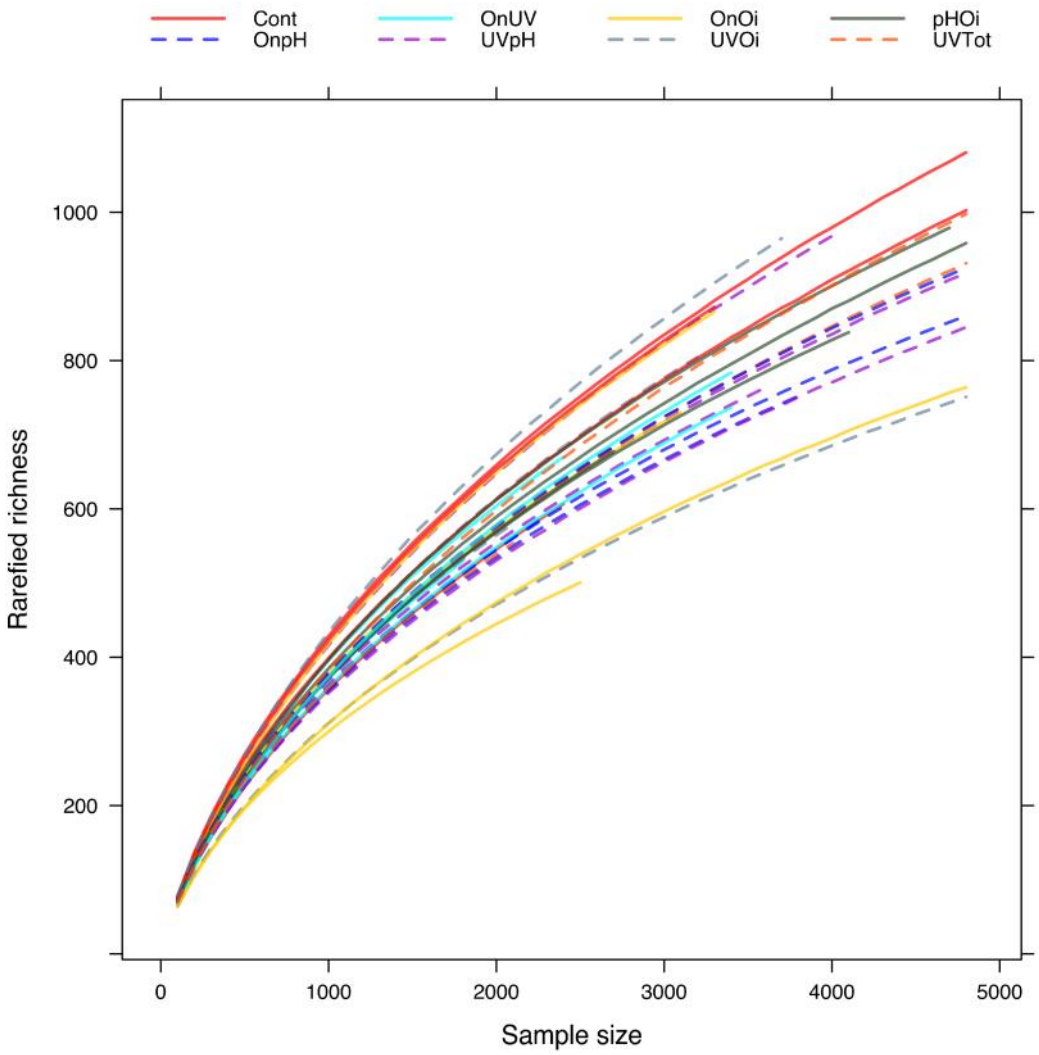


**Figure S IV-5:** Rarefied OTU richness as a function of the number of sequences **Cont** - control with no treatment; **OnpH** - water acidified; **OnUV** - exposed to UV-B; **UVpH** - water acidified and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified.

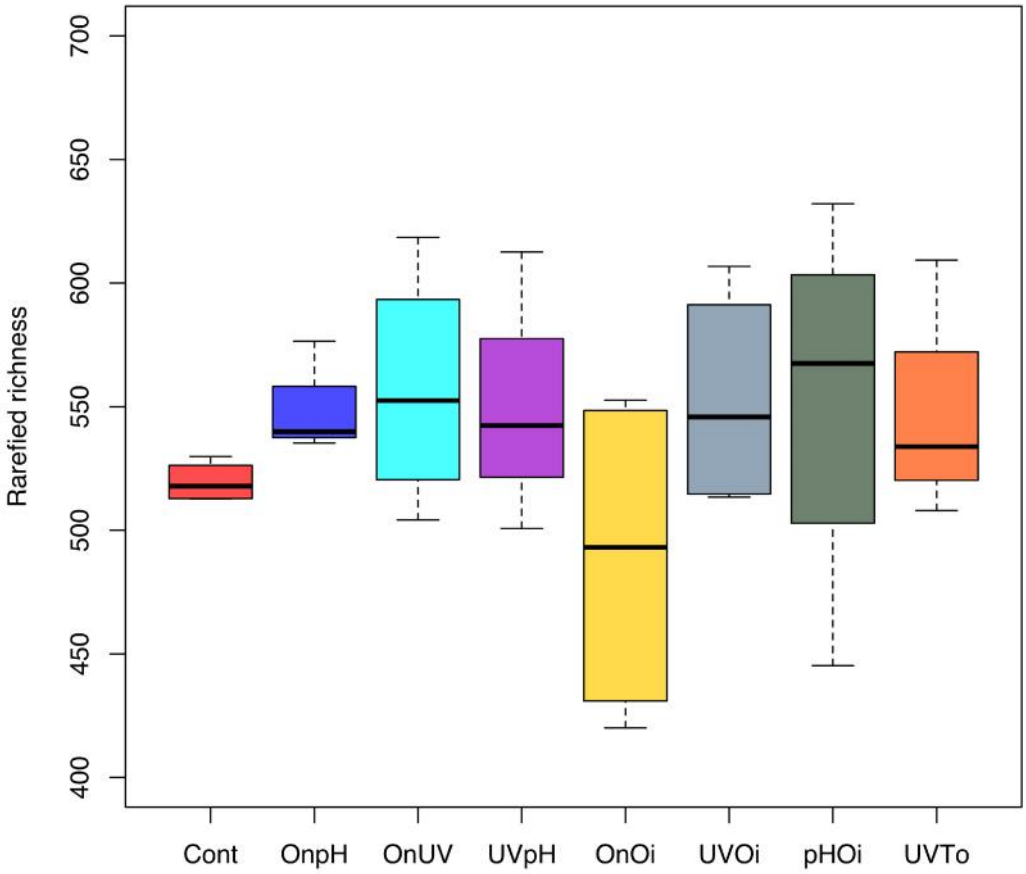


**Figure S IV-6:** Boxplot of rarefied OTU richness controlled for 1800 sequences. The boxed area represents the mean±quartile and the whiskers extend to the minimum and maximum values. **Cont** - control with no treatment; **OnpH** - water acidified; **OnUV** - exposed to UV-B; **UVpH** - water acidified and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified.

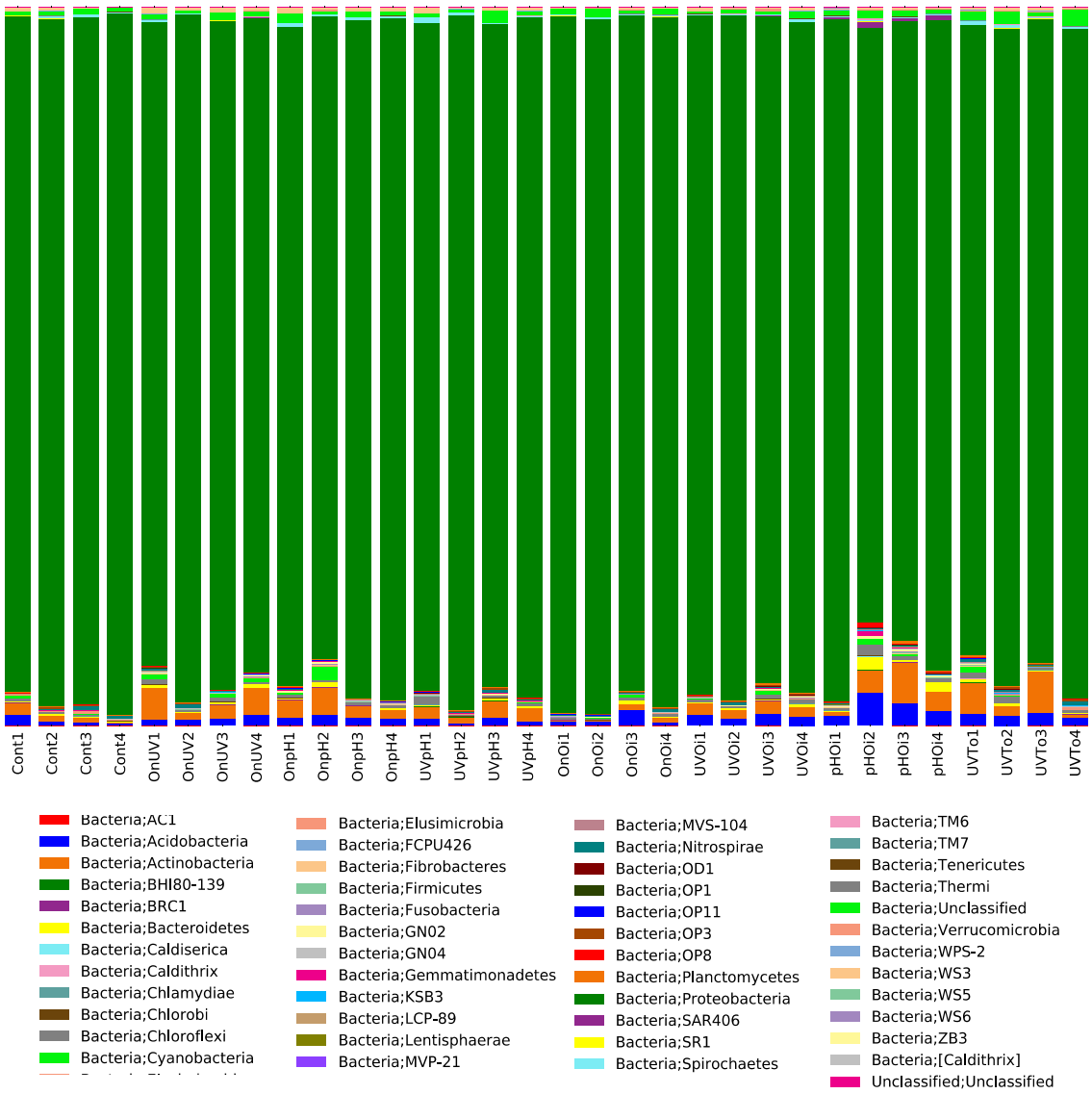


Figure **S IV-7:** Stacking bar plots of the relative abundance of OTU´s classified at phylum level in microcosm sediment treatments. **Cont** - control with no treatment; **OnpH** - water acidified; **OnUV** - exposed to UV-B; **UVpH** - water acidified and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified.

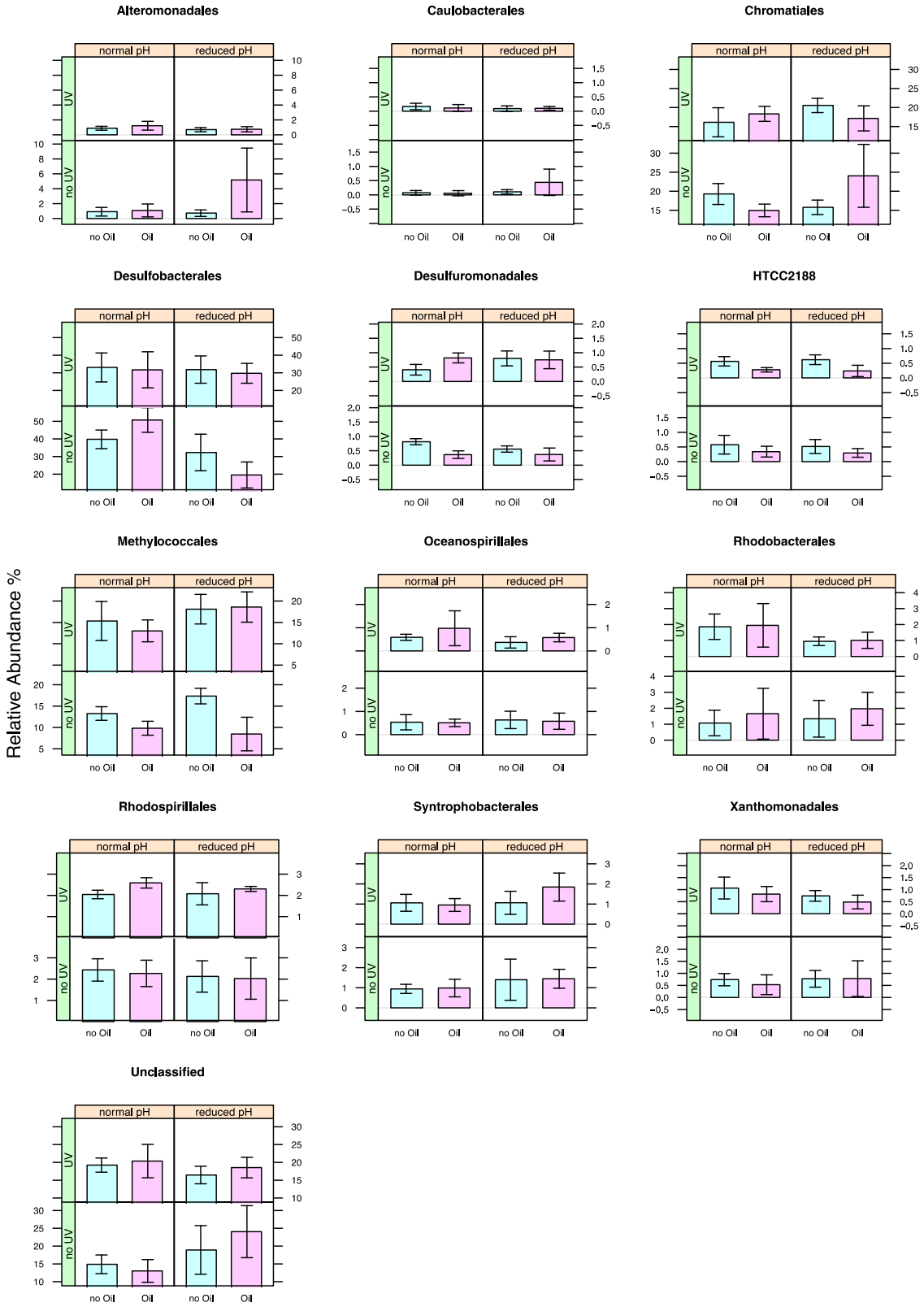


**Figure S IV-8:** Influence of UV-B, pH and Oil on the relative abundance of the most dominant *Proteobacteria* orders (> 100 OTUs).

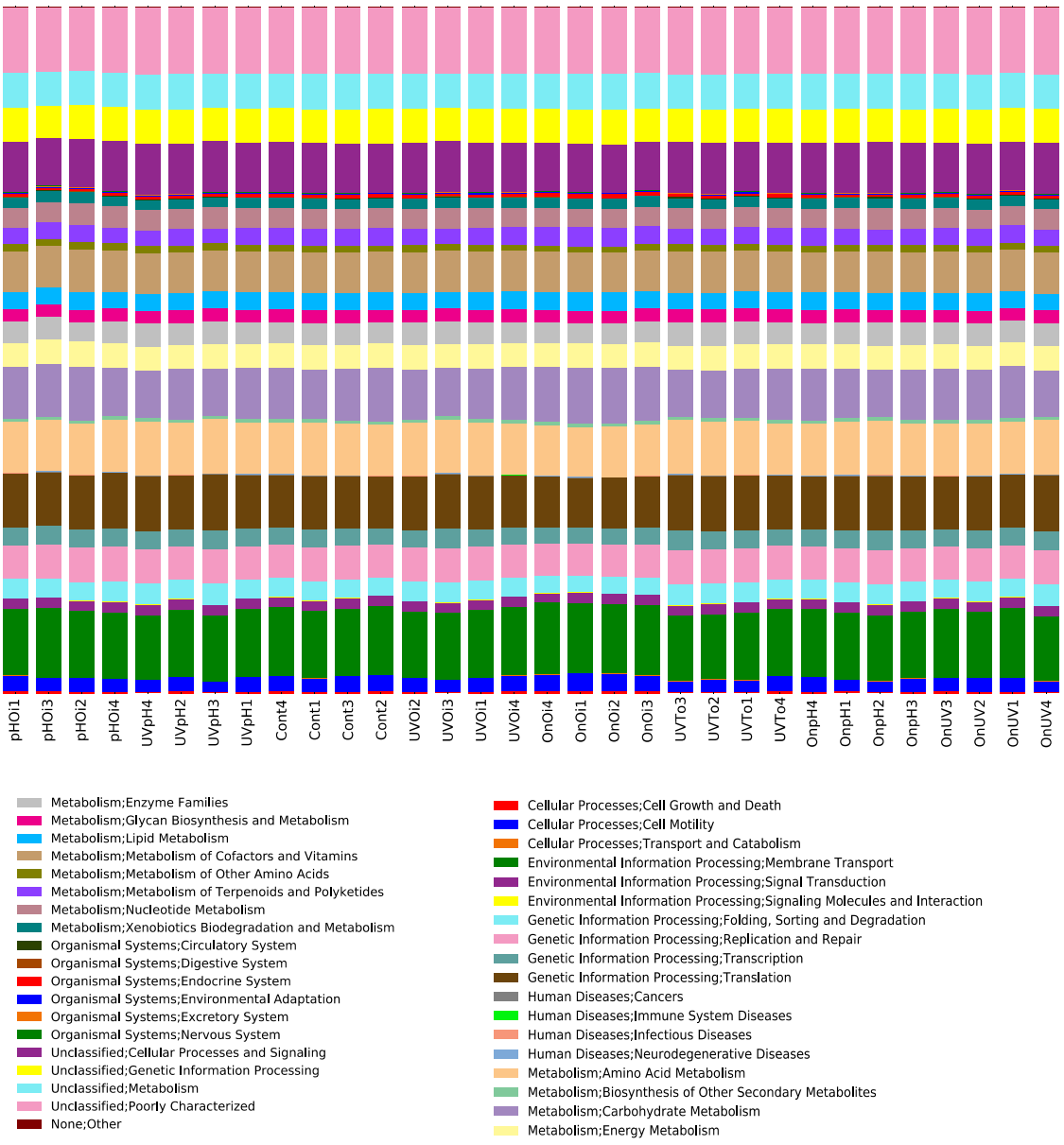


**Figure S IV-9:** Stacking bar plots of the relative abundance of predicted genes assigned to KEGG categories. Predicted metagenome functional content was obtained by analyzing 16S rRNA cDNA sequences with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States software (PICRUSTs). **Cont** - control with no treatment; **OnpH** - water acidified; **OnUV** - exposed to UV-B; **UVpH** - water acidified and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and water acidified; **UVTot** - contaminated with oil and exposed to UV-B and water acidified.

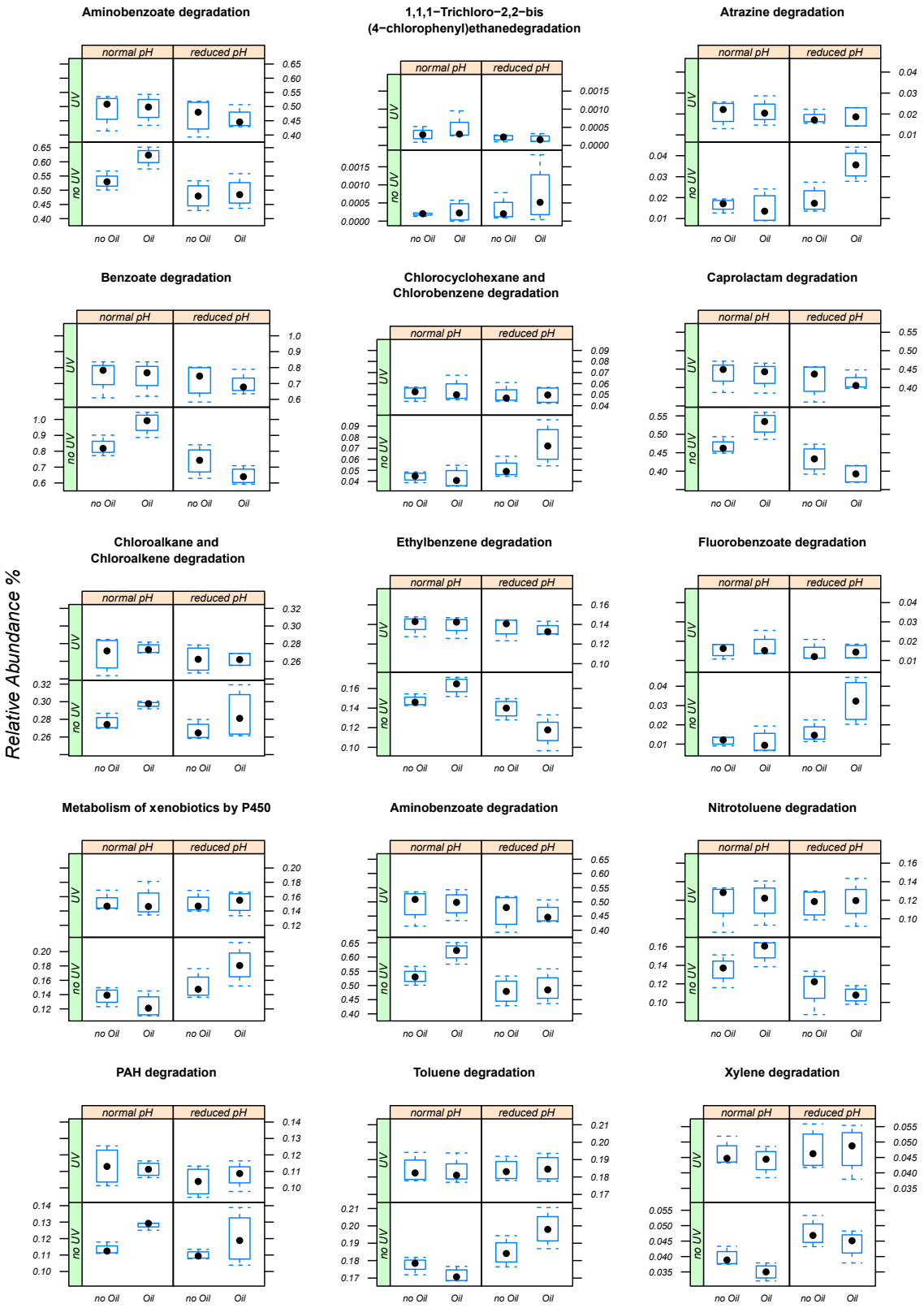


**Figure S IV-10:** Influence of UV-B, pH and Oil on the relative abundance of predicted genes assigned to KEGG category "xenobiotics biodegradation and metabolism".

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# Chapter V

# CHAPTER V
# Discussion and conclusions

## Experimental design and methodological considerations

### From field work to microcosm experiments

This work describes bacteria interactions with oil hydrocarbons (OH) in two distinct environments and under future climate change scenarios. The SML was initially chosen as a model environment due to: a) the enrichment of a wide range of pollutants, including OH, that can be found in this particular compartment of the water column (Wurl *et al.*, 2004); and b) the lack of existing studies that specifically focused on the SML hydrocarbonoclastic bacterial communities. The mesotidal costal lagoon Ria de Aveiro was chosen to perform this study since due to an intense commercial shipping activity it is subject to a continuous efflux of OH, making it suitable to study the responses of bacterioneuston community structure to OH. The collecting points were selected in order to reflect a gradient of oil pollution, extending from the outer section of the estuary to the inner section of canal de Ílhavo. However, this selection is not absent of criticism since the collecting points are also subject to other sources of organic contamination rather than oil, making difficult to access the independent effect of OH pollution.

The difficulty in isolating experimental variables and establishing cause-effect relationships in field studies is a problem not only in microbial ecology studies but also in ecology in general (Jessup *et al.*, 2004, Benton *et al.*, 2007). This justified the development of an experimental life support system that allows high control microcosm experiments designed to study the interactions of bacterial communities and OH under climate change scenarios. The advantages and disadvantages of using small-scale experiments and the development of the microcosm framework are described in chapters I and III, respectively. The logical step after the exploratory study on the hydrocarbonoclastic bacterioneuston community would have been to test several climate change scenarios in the SML. However, simulating the formation of a stable bacterioneuston community in the ELSS with a minimum of realism appeared as a complex task. Even under environmental conditions, the establishment and structure of a complex SML bacterial community is still a poorly understood phenomenon (Cunliffe *et al.*, 2011) that can be affected by several factors such as strong water currents (Santos *et al.*, 2011).

Nonetheless, the simulation of the SML with this system is possible in principle, although additional system optimization efforts would be required. Therefore, the experimental design focused on the microbial community of sediments retrieve from the shallow coastal lagoon Ria de Aveiro.

Shallow estuarine areas provide crucial ecosystem functions. However, the consequences of climate changes and anthropogenic contaminants, such as OH, in these areas remain largely unknown. Since key ecosystem functions in shallow-water sediments are mainly controlled by microorganisms, it is important to understand the effects of these multiple stressors on these communities (Alsterberg *et al.*, 2011).

**Considerations on climate change and oil spill microcosms experiment**

Some important guidelines were followed when programming the experiment: a) the experiment should simulate global change scenarios within the limits established by available models; b) the sediment should be stabilized in the microcosms before the oil introduction; c) the experiment should operate during a reasonable time; d) the microcosms should simulate a shallow environment; e) sediment should be collected intact in the field, in order to preserve its aerobic (~1 cm of top sediment) and anaerobic layers (~10 cm of top sediment); f) sediment sampling within the microcosm should be keep to minimum to avoid microcosm perturbation during the experiment. The acidification of the synthetic water was accomplished by bubbling $CO_2$ into the water. This method is described as an efficient way of manipulating carbonate chemistry in seawater (Gattuso *et al.*, 2009). The lowest average pH was 7.65±0.67 in the acidified microcosms and 7.97±0.07 in the control microcosms, representing a pH decrease of approximately 0.3. This value falls within 0.3-0.4 pH reduction modeled for the ocean surface by the end of this century (Caldeira *et al.*, 2003).

Two limitations of UV-B simulation were taken into consideration. First, the spectral irradiance emitted by UV lamps does not match the solar irradiance. UV lamps emit more short-wave and less long-wave UV-B than the sun (Xu *et al.*, 2010). Since the biological and chemical effects of UVR are wavelength dependent (Rundel, 1983, Santos *et al.*, 2013) a description of wavelength dependency is necessary to simulate increasing UVR scenarios with some degree of realism. Thus, a biological spectral weighting function that describes the effectiveness of the lamp wavelength to produce biological responses is needed to calculate the effective biological UV-B (Andreasson *et al.*, 2006). Second, unlike the effect of increasing

atmospheric $CO_2$ on ocean pH, there is no available information on how the amount of ultraviolet radiation that reaches the water surface will vary in the next century. Although interactions between climate change and stratospheric ozone have the potential to impact future levels of ambient UVR (Thomas *et al.*, 2012, UNEP, 2012, UNEP, 2010), developing models that simulate and can predict future UVR levels requires a broad range of inputs, making this a complex task (Thomas *et al.*, 2012).

In order to address the first limitation, the values of the UV-B irradiance and time of exposure where considered using the action spectrum for generalized DNA-damage parameterized by Bernard and Seckmeyer (1997). In the absence of available models of future UV-B, it was chosen to implement two conditions in the system: microcosms irradiated with UV-B and microcosms where the UV-B was screened out. The typical DNA-damage UV dose observed at Lisbon, in June, was implemented in microcosms irradiated with UV-B. A similar amount of UV-A integrated irradiance was also supplemented to all microcosms (including non UV-B treated) since UV-A radiation is practically unaffected by changes in ozone depletion and plays an important role in photo-repair mechanisms (Bargagli, 2005). Complete description of the system and procedures can be found in chapters III and IV.

The experimental life support system operated during 21 days before the addition of oil in order to allow microbial communities to stabilize under microcosm conditions. During this period, microcosms were gradually acidified until they reached the target pH. The microcosms were kept at the experimental irradiance from day 1. After the oil spill the system operated during 36 days more, in a total of 57 days. The duration of the experiment was established taking into consideration the previous knowledge on microbial dynamics when exposed to oil contamination and the necessary logistics to maintain the experiment. It is well established that microbial community structure can change significantly when exposed to oil hydrocarbon, favoring groups that can use oil hydrocarbons as carbon and energy source (Gomes *et al.*, 2005, Yakimov *et al.*, 2005). Therefore, possible interactive effects of oil, UVR and ocean acidification on microbial community structure could be masked by initial changes due to oil exposure. It is plausible to speculate that potential interactions would only emerge after an initial period. On the other hand, although the ELSS can be set to function by only two operators working in shifts, its functioning requires considerable amounts of water and synthetic salt. The ELSS consumed approximately 96 l of water and 3.3 kg of salt per day of operation. Thus a compromise between these two important factors had to be made and the system operated during 57 days.

Water renewal between tides at the original site from where the sediment was collected is almost 100%. However, since one of the experiment guidelines was to simulate a shallow environment, the microcosms were programmed to simulate a 50% water renewal between tides, similar to that estimated for the central area of the estuary (Dias *et al.*, 2001). Therefore, the sediments in the microcosms were exposed to more anoxic conditions than in their natural environment. These conditions introduced a changing in the original microbial community, favoring anaerobic groups. The relative abundance of *Desulfobacterales* which mainly includes anaerobic bacteria, clearly increased under microcosm conditions. This was not the only change in the microbial community. Although the dominant groups present in the environment were also detected in the microcosms, the microcosm conditions caused a decrease in rare groups. It can be argued that the microcosms failed to fully reproduce the original community detected in the environment. However, the goal of the experiment was not to reproduce with detail the original communities but rather to sustain a microbial community that preserved some of their original structural and functional characteristics. Importantly, water inorganic analysis and stress indicators of the model organisms *Hediste diversicolor* suggested that the changes in the microbial community structure did not affect the microcosm artificial ecosystem.

**Microbial communities and oil hydrocarbon degradation in estuarine environments – main conclusions and unanswered questions**

In this study, *Pseudomonas* emerged as a representative genus within the hydrocarbon degrading bacteria in the SML. The ability of Pseudomonads to degrade OH and their role in bioengineered remediation strategies is not new (Menn *et al.*, 2008). However, this was the first time that hydrocarbon-degrading isolates were obtained from the SML. As referred, the SML is the first water compartment to be in contact with OH pollutants after major oil spills. Therefore, *Pseudomonas* isolates adapted to this layer can be ideal targets for engineer or promote (through biostimulation strategies) during oil spills. Two important aspects arise from this work. The first refers to the pattern of variation along the oil gradient observed in *Pseudomonas* DGGE profile. Due to the role of *Pseudomonas* in organic matter degradation these patterns can imply a potential functional change that should be explored in future studies. A metatranscriptomic approach could be used to characterize the expression of genes related to organic matter and OH degradation in this layer. The second aspect is the evidence of

horizontal gene transference between different bacterial isolates. Horizontal gene transfer is a rapid mechanism that microorganisms use to share OH degrading genes when facing a sudden input of OH (American Academy of Microbiology, 2011). Future work should focus on this process that can be determinant in the capacity of bacterial communities to degrade oil in contaminated environments.

The microcosm experiment demonstrated that the impact of an oil spill in the active bacterial communities capable of degrading oil will be substantially different in future ocean pH levels. *Desulfobacterales* order relative abundance decreased drastically after the oil spill simulation under reduced ocean pH when compared to normal pH. Moreover, a significant change in predicted genes associated with several OH degradation pathways was also detected, confirming that the observed changes in *Desulfobacterale*s will probably have functional consequences in OH degradation. Anaerobic degradation of oil hydrocarbons by microorganisms in marine sediments reduces toxic hydrocarbons (Rothermich *et al.*, 2002). Therefore, a drastic reduction in *Desulfobacterales* could significantly change the capacity of coastal and estuarine systems to degrade OH with widespread consequences in higher trophic levels. Importantly, the pH reduction simulated in this study is probably the best-case scenario for coastal and estuarine environments. Increased eutrophication in these areas will exacerbate the effect of ocean acidification due to the development of hypoxic and anoxic zones (Howarth *et al.*, 2011). Interestingly, a moderate level of UV-B appears to attenuate these effects probably because of photochemistry modifications of OH. Therefore, it is likely that the magnitude of these interactions and their effects will depend on the level of UV-B that penetrates the impacted ecosystem. Future work must be done to unravel the mechanisms that underlie the associated changes to clarify the drastic decrease of *Desulfobacterales* and the toxicity of oil degradation products under these conditions. Other observed shifts in the structure of microbial communities should also be the focus of future research. A significant increase of *Methylococcales* in oil contaminated sediment under reduced pH and UV-B was also registered. Methanotrophic bacteria may act as an important biofilter that limits the release of $CH_4$ to the atmosphere, and therefore the ecological relevance of the observed trends should also be accessed in future studies.

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# Appendix

# Interactive effects of global climate change and pollution on marine microbes: the way ahead

**Francisco J.R.C. Coelho , Ana L. Santos, Joana Coimbra, Adelaide Almeida, Ângela Cunha, Daniel F.R. Cleary, Ricardo Calado and Newton C. M. Gomes***

**Ecology and Evolution**

**Abstract:** Global climate change has the potential to seriously and adversely affect marine ecosystem functioning. Numerous experimental and modelling studies have demonstrated how predicted ocean acidification and increased ultraviolet radiation (UVR) can affect marine microbes. However, researchers have largely ignored interactions between ocean acidification, increased ultraviolet radiation and anthropogenic pollutants in marine environments. Such interactions can alter chemical speciation and the bioavailability of several organic and inorganic pollutants with potentially deleterious effects, such as modifying microbial mediated detoxification processes. Microbes mediate major biogeochemical cycles, providing fundamental ecosystems services such as environmental detoxification and recovery. It is, therefore, important to understand how predicted changes to oceanic pH, UVR and temperature will affect microbial pollutant detoxification processes in marine ecosystems. The intrinsic characteristics of microbes, such as their short generation time, small size and functional role in biogeochemical cycles combined with recent advances in molecular techniques (*e.g.*, metagenomics and metatranscriptomics) make microbes excellent models to evaluate the consequences of various climate change scenarios on detoxification processes in marine ecosystems. In this review, we highlight the importance of microbial microcosm experiments, coupled with high-resolution molecular biology techniques, to provide a critical experimental framework to start understanding how climate change, anthropogenic pollution and microbiological interactions may affect marine ecosystems in the future.

## Introduction

Anthropogenic concentrations of carbon dioxide ($CO_2$) have increased from approximately 280 ppm (parts per million) in preindustrial times (Indermühle *et al.*, 1999) to nearly 394 ppm in 2012 (NOAA Earth System Research Laboratory, 2012). Levels of $CO_2$ in the atmosphere now exceed limits considered natural for most animals and plants (Ehleringer *et al.*, 2002). The best known postulated consequence of an increasing atmospheric $CO_2$ concentration is global warming, which may, among other things, lead to sea level changes, promote ocean stratification, and alter the sea-ice extent and patterns of ocean circulation (Doney *et al.*, 2012). In addition to the above, increased atmospheric $CO_2$ will also lead to a net air-to-sea flux of $CO_2$, thereby reducing seawater pH and modifying the chemical balance

among inorganic carbon species. This process, known as ocean acidification, is often referred to as "the other $CO_2$ problem" (Henderson, 2006). In contrast to other climate change scenarios, ocean acidification is a direct consequence of increased atmospheric $CO_2$ and does not depend on uncertainties related to other climate change predictions (Doney *et al.*, 2009).

Although international treaties have been effective in reducing atmospheric concentrations of ozone depleting substances, increased greenhouse gas concentrations have the potential to affect the spatial distribution of ozone and its exchange between the stratosphere and the troposphere; this, in turn, will influence ultraviolet radiation (UVR) levels reaching the Earth's surface (UNEP, 2010, UNEP, 2012). Higher UVR levels have also been shown to disrupt aquatic food webs and reduce the biological sinking capacity of aquatic environments for atmospheric $CO_2$ (Hader *et al.*, 2007, Fabry *et al.*, 2008).

In addition to the effects of anthropogenic activities on global climate change, fossil fuel combustion, fertilizer use, and industrial activity have adversely affected coastal and open-ocean environments for decades, providing a continuous influx of pollutants [including oil hydrocarbons (OH), pesticides and heavy metals] into these ecosystems (Doney, 2010). With respect to OH, natural seepage alone introduces about $6\times10^5$ metric tons $year^{-1}$ of crude-oil to oceans, representing c. 47% of crude oil entering the marine environment. The remaining 53%, results from anthropogenic activities (accidental oil spills, transport activities, refining, storage, and others) (Kvenvolden *et al.*, 2003). Among scientists, there is a growing awareness that ocean acidification and increased UVR have the potential to alter contaminant transfer in aquatic food webs, and modify the structure of aquatic trophic webs and that the biomagnification of contaminants may lead to increased toxicity in marine ecosystems (Pelletier *et al.*, 2006, Fabry *et al.*, 2008).

Microbial communities play a central role in the global recycling of pollutants. For example, the oil-catabolic versatility of microbes, particularly bacteria, ensures that oceans are not completely covered with an oil film (Head *et al.*, 2006). Despite the importance of microbes in the process of global recycling of anthropogenic pollutants, the potential interactions of ocean acidification, UVR, anthropogenic pollutants and marine microbial communities have been largely ignored. Little is known about how ocean acidification and increased UVR can interact with anthropogenic pollutants will affect microbial communities and biogeochemical cycling.

Moreover, although ocean acidification and increased UVR have the potential to affect microbial assemblages (Riebesell *et al.*, 2007, Santos *et al.*, 2012b, Liu *et al.*, 2010), very little is known about the effects on microbial mediated pollutant detoxification and how this will impact pollutant pathways (Figure A-1).

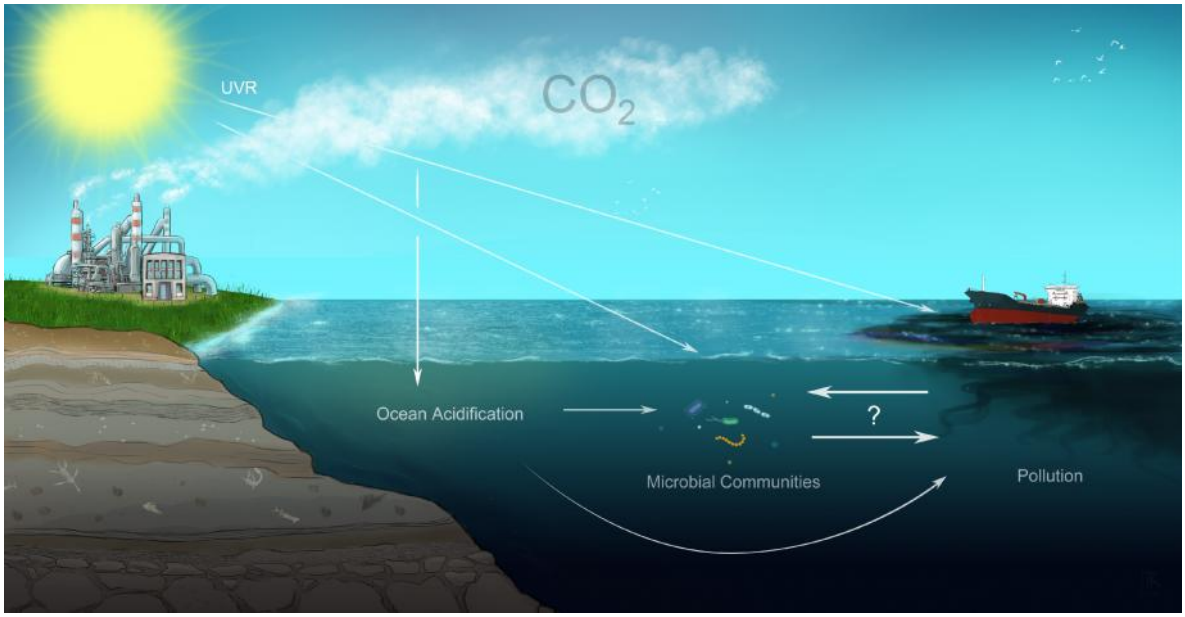


**Figure A-1**: Interactions between ultraviolet radiation, ocean acidification, anthropogenic pollution and microbial communities. Climate change has the potential to influence pollutant toxicity by acting directly on pollutant chemistry or indirectly by affecting microbial mediated detoxification.

The aim of this review is to present the recent advances in our understanding of the consequences of interactions between ocean acidification, increased UVR, anthropogenic pollutants and marine microbial communities. Recent technological advances in molecular microbiology as a means to improving our ability to study potential interactive effects are also discussed.

## Ocean acidification and UVR interactions with marine microbial communities – what we know so far

If $CO_2$ emissions continue unabated, oceanic pH will decline 0.3 to 0.4 units by the end of this century, and up to 0.7 units in 2300 (Caldeira *et al.*, 2003). When $CO_2$ dissolves in seawater, carbonic acid ($H_2CO_3$) is formed and quickly dissociates into hydrogen ($H^+$) and

bicarbonate ($HCO_3^-$) ions. A hydrogen ion can then react with a carbonate ion ($CO_3^{-2}$) to form bicarbonate. This process leads to increased partial pressure ($pCO_2$), increased concentrations of $H_2CO_3$, $HCO^-_3$, and $H^+$, and reduced concentrations of $CO_3^{-2}$ (Fabry *et al.*, 2008). These changes in carbonate chemistry have serious implications for marine organisms that depend on minerals such as calcite and aragonite to produce shells and skeletons (*e.g.*, corals, mollusks, echinoderms and crustaceans). Indeed, the available data suggests that calcification rates will be affected under future $pCO_2$ scenarios (Fabry *et al.*, 2008).

A key question is how microbial communities and microbial mediated biogeochemical processes will be affected by ocean acidification. Joint *et al.* (2011) recently argued that given that microbial assemblages have always experienced variable pH conditions, the appropriate null hypothesis to be tested is that "there will be no catastrophic changes in marine biogeochemical processes driven by phytoplankton, bacteria and archaea". In response to this article, Liu *et al.* (2010), performed a meta-analysis of published data and suggested that changes in microbial structure and function are possible. Both authors provide valid arguments to a complex issue that we have only just started to understand. So far, existing studies suggest that microbial mediated processes such as carbon and nitrogen cycles may be affected. For example, Riebesell *et al.* (2007) showed that a phytoplankton community responded to higher $CO_2$ concentrations (three times the present $pCO_2$ conditions) in seawater by an up to 39% increase in net primary production. Increased $pCO_2$ may also impact the nitrogen cycle. The filamentous cyanobacterium *Trichodesmium*, a major contributor of new nitrogen in oligotrophic oceans, has been shown to increase carbon and nitrogen fixation rates by 35 to 100% at $pCO_2$ levels predicted for 2100 (Hutchins *et al.*, 2007). In addition to nitrogen fixation, other components of the nitrogen cycle may also be altered by ocean acidification. Nitrification can be affected by pH driven changes in the availability of ammonia ($NH_3$). Beman *et al.* (2010) suggest that a reduction in nitrification rates of 3-44% can occur within a few decades. With respect to bacterial communities there is little information and the existing studies are less clear. Most of the studies regarding bacteria under ocean acidification scenarios have been performed in large pelagic mesocosm systems that study the effect of carbonate chemistry modifications through the food web. These experiments are capable of realistic simulations where indirect effects from interactions with phytoplankton can be studied. Experiments such as these have demonstrated that bacterial abundance and activity can vary due to phytoplankton shifts under high $pCO_2$ (Grossart *et al.*, 2003, Allgaier *et al.*, 2008). Regarding community structure, large mesocosms and small-scale approaches have

revealed contrasting effects. In large pelagic mesocosms dominant bacterial community shifts were not related to $pCO_2$ (Roy *et al.*, 2012), whereas in small microcosm systems, pH levels predicted for the year 2100 had a significant impact on bacterial structure (Krause *et al.*, 2012).

It is clear that we still have much to learn about microbial dynamics under elevated $pCO_2$ levels, particularly with respect to the underlying mechanisms that trigger some of the observed trends. Furthermore, the impact of ocean acidification on microbial function needs to be addressed with more focus on local or regional conditions, since the magnitude of carbonate changes will vary across regions. For example, anthropogenic stressors exacerbate ocean acidification through the development of hypoxic and anoxic zones due to increased eutrophication in coastal and estuarine areas. Low oxygen waters are more acidic than ocean waters. In a model saline estuary the development of hypoxia is enough to reduce pH levels by more than 0.5 units (Howarth *et al.*, 2011).

**Effects of UVR in marine microbial communities**

Researchers have studied the effects of UVR for some decades. An important impetus for studying UVR was the concern for the ozone layer, which had been adversely affected by chlorofluorocarbons. Following implementation of the Montreal protocol that placed restrictions on ozone-depleting substances, ozone levels in the atmosphere are no longer declining (McKenzie *et al.*, 2011). However, recovery of the stratospheric ozone layer to 1980's levels is not likely to occur in the next decades (Weatherhead *et al.*, 2006). In fact, the area of the Antarctica ozone hole reached a maximum in 2006 (Nasa, 2009) and in 2011 a record destruction of the ozone layer over the Arctic was reported (Manney *et al.*, 2011). Therefore, changes in UV radiation levels in the future will depend on changes in various atmospheric factors, besides total ozone, including clouds, aerosols, as well as surface reflectivity (or albedo), in some locations. Other factors, including tropospheric gaseous pollutants and stratospheric temperature, may also play a role (WMO, 2010). Due to the complexity of factors influencing changes in UV radiation levels reaching the Earth's surface, future trends in UV radiation levels are uncertain and contrasting predictions exist. For example, while some prediction models indicate that by the 2090's mean erythemal UV levels will drop by up to 12% worldwide compared to values recorded in 1980 (Bais *et al.*, 2011), other models indicate that UV-B levels will increased in the Northern Hemisphere in response to reductions in the amount of aerosols and clouds (Hegglin *et al.*, 2009, Watanabe *et al.*, 2011).

It is well known that the amount of UVR that reaches the earth's surface has important consequences for aquatic ecosystems. UVR is the most photochemically reactive waveband of incident solar radiation and can have genotoxic, cytotoxic and ontogenetic effects on aquatic organisms (Bancroft *et al.*, 2007). It is commonly divided into three wavelength ranges: UV-A (320-400 nm), UV-B (290-320 nm), and UV-C (<290 nm). DNA absorbs only weakly at longer UV wavelengths (Jones *et al.*, 1987). Thus, the biological effects of UV-A are usually considered to be indirect, resulting from intracellular generation of reactive oxygen species (ROS), oxidative damage to lipids, proteins and DNA (Pattison *et al.*, 2006). UV-C wavelengths are generally not deemed to be environmentally relevant, given that they are almost completely screened out of the atmosphere by oxygen and ozone. UV-B is the highest energy wavelength of solar radiation that reaches the Earth's surface and the UV wavelength mostly affected by shifts in the ozone layer (Andersen *et al.*, 2002). UV-B radiation can cause damage to nearly all biomolecules by direct absorption or indirectly as a result of enhanced formation of ROS (Vincent *et al.*, 2000).

Environmental effects of UVR radiation are generally attenuated by protective strategies displayed by living organisms, such as avoidance, photochemical quenching and repair. The overall stress imposed by UVR exposure thus reflects a balance between damage, repair and the energetic costs of protection, while it may also affect energy consumption and the biochemical composition of cellular material, resulting in lower survival and growth rates (Vincent *et al.*, 2000). UVR represents an important stressor for bacteria in aquatic ecosystems, as their simple haploid genomes provide little or no functional redundancy (Garcia-Pichel, 1994). In general, exposure to UV-B reduces extracellular enzymatic activities (Herndl *et al.*, 1993, Santos *et al.*, 2012), oxygen consumption (Joux *et al.*, 2009) and leucine and thymidine incorporation (Sommaruga *et al.*, 1997, Santos *et al.*, 2012). Different bacterial groups have also been shown to vary in their sensitivity to UVR and the potential to repair UVR induced damage (Fernández Zenoff *et al.*, 2006, Santos *et al.*, 2012). *Gammaproteobacteria* have been identified as the most UV-resistant group in several aquatic environments (Santos *et al.*, 2012, Alonso-Sáez *et al.*, 2006, Ordoñez *et al.*, 2009). Field studies have also identified the *Bacteroidetes* group as UV resistant (Alonso-Sáez *et al.*, 2006, Fernández Zenoff *et al.*, 2006). The *Alphaproteobacteria* group, on the other hand, has been reported to be UV-sensitive (Alonso-Sáez *et al.*, 2006). Among *Alphaproteobacteria*, the SAR11 cluster, which is potentially the most abundant and ubiquitous clade of heterotrophic marine bacteria in the oceans (Morris *et al.*, 2002a) were found to be particularly sensitive to solar UVR (Alonso-Sáez *et al.*, 2006, Ruiz-

González *et al.*, 2012). High UVR sensitivity of SAR11 was attributed to the high A+T content (69%) reported for the genome of the representative member of this group, *Pelagibacter ubique* (Giovannoni *et al.*, 2005b). The UV sensitivity of the SAR11 group is also supported by recent observations of the disappearance of sequences affiliated to *Pelagibacter* in natural Patagonian bacterioplankton communities following an 8-day exposure to PAR, PAR+UV-A and PAR+UV-A+UV-B (Manrique *et al.*, 2012). Other studies, however, indicate stimulation of SAR11 activity by light, potentially associated with the presence of proteorhodopsins (Giovannoni *et al.*, 2005a, Lami *et al.*, 2009, Mary *et al.*, 2008). Further studies are necessary to elucidate how environmental factors, particularly UVR, affect SAR11 diversity and activity.

The differential sensitivity to UVR exhibited by the most abundant bacterial groups present in the bacterioplankton is of paramount importance for the biogeochemical impact of enhanced UVR on ecosystems. The rationale for this assumption is the contrasting activity displayed by different groups of bacteria on the utilization of DOM (Cottrell *et al.*, 2000). For example, UVR-sensitive *Alphaproteobacteria* populations seem to be responsible for a large part of low-molecular-weight DOM uptake, while the more UVR-resistant *Bacteroidetes*, tend specialize in high-molecular-weight DOM uptake (Cottrell *et al.*, 2000, Alonso-Sáez *et al.*, 2006). Therefore, changes in bacterial community structure triggered by increased UV-B levels may promote dramatic shifts in DOM pathways (Morris *et al.*, 2002b).

**Synergistic effect of UVR, ocean acidification and anthropogenic pollutants**

*Will interactions between UVR, ocean acidification and anthropogenic pollutants affect marine microbes?*

Despite the fact that several studies have shown that environmental, physical, and chemical parameters directly affect the toxicity of anthropogenic pollutants, the interactive effects of UVR and ocean acidification on the chemistry of these pollutants and their effects on marine microbial communities have received very little attention. Increased UVR levels and changes to ocean pH will certainly affect the chemistry of several natural compounds and environmental pollutants, thus altering the way that they will interact with marine organisms. Polycyclic aromatic hydrocarbons (PAH), one of the most common compounds associated to OH pollution, are ideal examples of photoactive contaminants that are strongly absorbed in the UV-A and UV-B spectral regions. It is known that PAH toxicity to marine organisms may increase with exposure to UVR. This increase is largely regulated by two processes: namely,

photosensitization and photooxidation reactions. Both of these processes have the potential to release phototoxic aromatic hydrocarbons into the environment, which are more toxic than their parent compounds (Krylov *et al.*, 1997).

Reduced oceanic pH has the potential to affect the adsorption of metals by organic particles. Generally, organic particles are negatively charged and, as pH declines, surface sites become less available to adsorb positive ions like metals (Millero *et al.*, 2009). This aspect is particularly important due to the fact that more than 99% of the total concentration of most metals in seawater corresponds to organic complexes (Millero *et al.*, 2009). Small deviations in the concentration of elements such as Cu and Cd can have a serious effect on the health of marine organisms (Millero *et al.*, 2009). Organic materials though are often nonhomogeneous and of unknown structure. It is essential that we gain a better understanding of metal speciation in organic complexes (Millero *et al.*, 2009, Doney *et al.*, 2009).

A key question is whether there is any evidence of an interaction between UVR and ocean acidification, on one hand and anthropogenic pollutants on the other. The answer is yes, although several details are missing and require further research. Photoenhanced toxicity of PAH due to UVR exposure has already been observed in a variety of organisms. On crab larvae no significant effect of PAH and UVR exposure was detected on larval crab mortality after independent exposure but the combined effect of both, resulted in up to 100% mortality, (Peachey, 2005). This phenomenon has also been verified in isolated bacterial strains (McConkey *et al.*, 1997), although only a few studies have addressed the effect of UVR photo-modified pollutants in complex microbial assemblages (Pelletier *et al.*, 2006, Petersen *et al.*, 2008). In a microcosm experiment designed to study the effects of increased UV-B in the presence of the water soluble fraction of crude oil, an increase in mortality was observed in the phytoplankton community exposed to UV-B. In this scenario, the toxic effects on phytoplankton led to a release of carbon and other nutrients that stimulated bacterial growth (Pelletier *et al.*, 2006). A similar effect was reported in sediment microcosms (Petersen *et al.*, where algal 14C-incorporation and chlorophyll a content both declined in sediments exposed to UV-light and pyrene. At the same time, oxygen consumption and the release of N and P increased, suggesting an increase in bacterial activity (Petersen *et al.*, 2008).

It has been suggested that altered water $CO_2$ chemistry in combination with other environmental stressors may modify the responses of organisms, and even ecosystems, to these stressors in ways that differ substantially from the action of only a single stressor (Fabry *et al.* 2008). Indeed, ocean acidification in combination with elevated nutrient inputs can

accelerate the expansion of filamentous turfs at the expense of calcifying algae in a synergistic response 34% greater than the sum of their individual effects (Russell *et al.*, 2009). Recently, it was reported that DNA damage in amphipods was 2.7 times higher in metal contaminated sediment under an increase $pCO_2$ (750 µatm) scenario (Roberts *et al.*, 2012). However, nothing is known about possible changes in the response of marine microbes to anthropogenic pollutants under increased $pCO_2$ scenarios.

Generally, very little is known about the potential interactions (antagonistic, additive, or synergistic) between different pollutants. Although it lies outside the scope of this review, this topic is important given that marine ecosystems are exposed to a myriad of novel chemical substances that can react in unexpected ways (Crain *et al.*, 2008).

*Microbial mediated detoxification*

An important question to be addressed is whether the effects of ocean acidification and UVR will affect microbial communities in such a way that it may alter microbial mediated detoxification of anthropogenic pollutants. In addition to directly measurable physiological effects on marine microbes, UVR and ocean acidification can also indirectly affect the toxicity of anthropogenic pollutants by inducing shifts in microbial community structure; they can also alter microbial mediated detoxification processes. For example, the bioavailability of inorganic nutrients required for bacterial growth, such as nitrogen and phosphorus, is a key factor in successful ecosystem detoxification of PAH (Atlas *et al.*, 1972). Anything that alters the nitrogen cycle, such as ocean acidification has the potential to alter microbial mediated PAH detoxification processes and consequently PAH toxicity.

A critical potential effect of ocean acidification is an alteration of metal bioavailability. Metals interact with microbes in various ways, and are involved in virtually all aspects of microbial growth and metabolism (Gadd, 2010). Changes in iron chemistry are particularly important, given that iron is a limiting nutrient for marine phytoplankton in large oceanic regions (Sunda *et al.*, 2010). The $pCO_2$ values predicted for the year 2100 would reduce iron uptake by diatoms and coccolithophores by 10 to 20% (Shi *et al.*, 2010). The reduction in iron availability is believed to be related to pH induced binding of iron to organic ligands, thus reducing biologically available Fe(III) (Shi *et al.*, 2010; Sunda, 2010). The net effect of ocean acidification on iron chemistry is still unclear. For example, although lower pH also increases iron binding to organic ligands, the solubility of Fe(III) increases with water acidification in

surface ocean waters (Figure A-2) (Millero *et al.*, 2009). Changes in iron bioavailability due to ocean acidification have, therefore, potentially harmful effects (Shi *et al.*, 2010). Iron is also an important factor in the detoxification of hydrocarbons, by influencing the activity of enzymes that catalyze the oxidative breakdown of PAH (Dinkla *et al.*, 2001, Santos *et al.*, 2008). Monooxygenase and dioxygenase enzymes, essential in most microbial PAH degradation pathways, require a metal cofactor which is often iron (Bugg, 2003). The activity of several key enzymes, including toluene monooxygenase, in the degradation of the aromatic hydrocarbon toluene by *Pseudomonas putida*, was found to be reduced under iron limiting conditions (Dinkla *et al.*, 2001). The complexity of biological, chemical and environmental interactions in natural environments restricts our ability to establish cause-effect relationships. Understanding the interactive effects of climate change and anthropogenic pollutants on microbial communities is a complex task. It requires the study of a multitude of chemical and biological pathways, which may only be experimentally addressed in detail under controlled conditions.

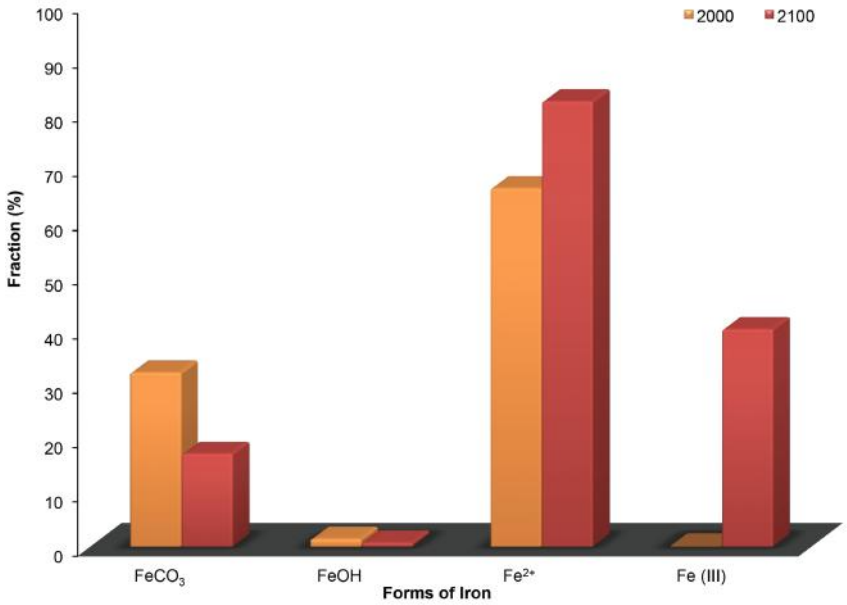


**Figure A-2:** One hundred years scenario of forms of iron in surface ocean waters considering the 0.4 pH units decrease modelled by Caldeira and Wickett (2003), at 25ºC and salinity of 35. Adapted from Millero *et al.* (2009). Since coastal and estuarine areas are very different between them, modeling on speciation of metals in these areas is needed for a more complete and accurate scenarios.

## Microcosm coupled with molecular biology technologies as an experimental framework

It is important that we obtain a mechanistic understanding of the effects of ocean acidification and UVR on pollutant toxicity and degradation. Pollution events, however, occur over a wide range of spatial and temporal scales making it difficult to gauge cause-effect relationships. This difficulty is enhanced when studying additional levels of complexity such as interactive effects with different stressors. In such cases, small-scale models, such as micro- and mesocosms, can be useful tools.

Micro- and mesocosms are simplified systems, constructed to mimic natural environments under controlled conditions (Roeselers *et al.*, 2006). Both simplified systems are powerful tools that have facilitated the study of several ecological processes, including research on predator-prey coevolution, ecosystem level-selection, resource competition and adaptive radiation (Jessup *et al.*, 2004). Both systems have advantages and disadvantages (Table A-1). The major advantage of these setups is that they enable a high degree of experimental control and replication. The level of control provided is virtually impossible to obtain through standard field surveys (Benton *et al.*, 2007). In addition to this, micro- and mesocosms enable researchers to experiment with highly toxic substances that would not be possible *in situ*. While there is some concern that these models are too small, both spatially and temporally, to be useful, the goal of these experiments is not to fully reproduce nature in a laboratory model system, but rather to simplify complex ecosystems so that essential dynamics can be captured (Jessup *et al.*, 2004). The distinction between micro- and mesocosms is somewhat arbitrary. Mesocosms tend to be outdoor and larger in size, increasing biological and spatial complexity (Petchey *et al.*, 2002), but diminishing experimental control and reducing replicability. In contrast, small microcosm setups allow a degree of experimental control and replication that is difficult to achieve with larger outdoor mesocosms.

**Table A-1:** Main advantages and disadvantages of micro- and mesocosm experiments

| | Environment | Microcosm | Mesocosm |
|---|---|---|---|
| Ease of replication | + | +++ | ++ |
| Precise control over environmental parameters | + | +++ | ++ |
| Treatments under investigation can be highly controlled | + | +++ | ++ |
| Space and temporal scale | +++ | + | ++ |
| Multitrophic interactions | +++ | ++ | +++ |
| Functional ecosystem mimicry | +++ | ++ | ++ |
| Circumvent over-simplification | +++ | + | ++ |

\+ - Limited

++ - Moderate

+++ - Full control

Of course, the size and design of these experiments will always depend on the research question. Research on potential interactions between climate change and anthropogenic pollutants can greatly benefit from the experimental control of small microcosm systems. Many field studies only provide correlative evidence of certain phenomena. Microcosm experiments can help to elucidate whether there is an actual mechanistic effect. Additionally, small-scale experiments with microbes can overcome microcosm scale related limitations associated with studying larger organisms. Due to the small size and short generation times of microbes, it is possible to simulate complex temporal and spatial scales within microcosms (Jessup *et al.*, 2004). Climate change interactions with anthropogenic pollutants could be tested over several generations. For example, Collins and Bell (2006) simulated evolutionary responses of *Chlamydomonas* populations exposed to increasing concentrations of $CO_2$ using microcosm experiments. Nevertheless, temporal and spatial scales should be considered with care when performing experiments with microbes. Enclosure within small experimental containers can induce shifts in microbial communities known as the "bottle effect" (Ferguson *et al.*, 1984). It is important to monitor microbial communities and determine the extent results are biased by microcosm enclosure.

Currently, one of the key gaps in our understanding of how climate change may affect microbial communities is the lack of microcosm systems designed to simulate predicted scenarios in marine environments. For example, the effect of carbonate chemistry manipulation on microbial communities has mainly been assessed in larger mesocosms. Reliable microcosm systems designed to mimic fundamental dynamics of marine environments and capable of simulating climate change scenarios are needed. However, developing microcosm systems capable of simulating climate change scenarios such as increase UVR or ocean acidification is not a trivial task. For example, the spectral irradiance emitted by UVR lamps does not match natural solar irradiance. UVR lamps emit more short-wave and less long-wave UVR than the sun (Xu *et al.*, 2010). A possible solution is to calibrate the lamps with a biological spectral weighting function that describes the effectiveness of lamp wavelength to produce biological responses (Andreasson *et al.*, 2006). However, this experimental setup is complex for long term outdoor microcosm experiments since UVR lamps must be continuously calibrated in order to account for daily and seasonal light variation. If the experimental setup does not involve wavelength isolation, microcosms can be directly exposed to sunlight. Recently, Gao *et al.* (2012) exposed microcosms directly to several levels of solar radiation to simulate the synergistic effects of light exposure and increased $pCO_2$ in phytoplankton at different depths. Likewise, there are several methods that simulate future changes in seawater chemistry. $CO_2$ bubbling, addition of high-$CO_2$ seawater and combined addition of acid and $HCO^{3-}$ are the three approaches that most closely mimic future scenarios of shifts in seawater chemistry (Gattuso *et al.*, 2009). Probably, the easiest to implement in microcosms is $CO_2$ bubbling with pH stats systems. In these systems, pH is monitored continuously and a controller valve increases or reduces the addition of $CO_2$ when pH deviates from a set value (Gattuso *et al.*, 2009).

As referred to above, the use of microcosms to address fundamental ecological questions is not new. However, this approach combined with recent advances in microbe characterization technologies can provide an important framework to start unravelling how climate change and pollution may interact to affect several levels of biological organization (Figure A-3).

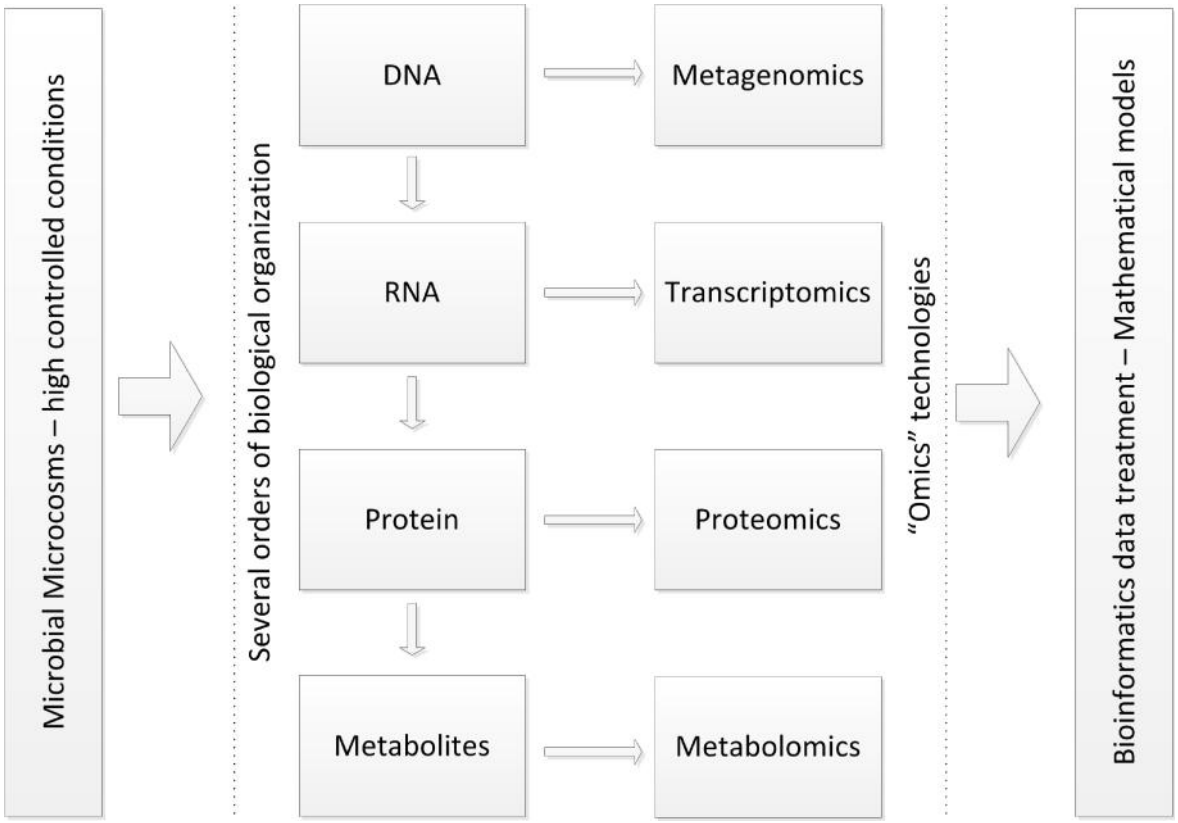


**Figure A-3:** Microbial microcosm couple with "omics" technologies can provide an excellent tool to gain mechanistic insights into climate change and anthropogenic pollution interactive effects at several levels of biological organization.

The recent development of molecular technologies has enabled scientists to assess the structure and function of microbes in a range of different environments including soil, sediment, water and within animal and plant hosts. The development of high-throughput DNA sequencing technologies has been a milestones in the field of metagenomics and metatranscriptomics. Metagenomic analysis, however, only provides structural and putative functional information of the microbes under study. In order to restrict focus to the active (as opposed to dormant, Urich *et al.* 2008) members of microbial communities and the genes expressed, several protocols were developed to sequence actively transcribed RNA and messenger RNA (mRNA) (also known as metatranscriptomics). Metatranscriptomic analysis can facilitate the study of microbial responses to rapid environmental change (*e.g.* an oil spill), thereby linking structural shifts to community function (Mason *et al.*, 2012). In parallel, advances in efficient chromatographic separation coupled with mass spectrophotometry-based approaches have enabled high-throughput protein identification. The study of the entire set of proteins (proteome) produced by a given microbial community in a particular environment

has led to a new field known as metaproteomics. Together with metagenomics and metatranscriptomics, metaproteomics can facilitate the study of cellular responses to changing environmental conditions. Metabolite profiling in complex biological samples is also an emerging field. This new approach, known as metabolomics, involves the quantitative and qualitative analysis of the complete set of metabolites present in a sample, providing additional information on metabolic and physiological potential.

While these technologies have greatly improved our ability to acquire data, they have also created new challenges. The current rapid development of "omic" technologies is unique in that it actually exceeds the rate of chip performance evolution in the computing industry, also known as Moore's law (Gilbert *et al.*, 2012). However, our ability to integrate the large amount of "omic" data is maturing rapidly. For example, the construction of co-occurrence and correlation networks from presence-absence or abundance data, in a process know as network inference, is being increasingly used to predict microbial interactions (Faust *et al.*, 2012). Recently, Larsen *et al.* (2012) used an artificial neural network to develop a model that predicts the abundance of microbial taxa as a function of environmental conditions and biological interactions. This method can be seen as a first step in the application of bioclimatic modeling to predict microbial community and environmental interactions under future global change scenarios.

Microcosm simulations must be developed in line with data acquisition from the field and modeling. Microbial observatories that generate long-term data series from different habitats and across several gradients (*e.g.*, polluted *versus* non-polluted areas or areas of volcanic activity where $CO_2$ gas is released into the water creating a natural gradient of pH levels (Hall-Spencer *et al.*, 2008) can also provided valuable information on potential interactive effects. Furthermore, data series such as these can be used to confirm or refute hypotheses formulated from microcosm experiments.

**Concluding remarks**

Understanding the full extent of interactive effects of global climate change and pollutants on microbes is a complex task, which entails the study of a multitude of interactions. In addition to this, the information about these interactions is scarce and studies in this field are still in their infancy. Further studies are needed to evaluate how the effects of oceanic pH and UV radiation (UVR) will affect microbial detoxification processes in marine

ecosystems. The experimental data gather so far allow us to predict that independent and interactive effects of UVR and ocean acidification will probably affect microbial community structure and function. Given the importance of microbial mediated processes, there is a potential for the disruption of key ecosystem services. At present, there are some major technical challenges that still need to be met with respect to reliable and replicable integrated approaches to simulate predicted climate change scenarios and evaluate how they will affect the toxicity of pollutants and the functioning of microbial communities. This endeavour demands statistically robust experiments under controlled conditions, where biological and non-biological markers of environmental function can be accurately identified and quantified. Microcosm experiments paired with new "omics" technologies, along with field surveys can provide an excellent framework to ascertain the effect of anthropogenic pollutant toxicity and microbial function under different climate change scenarios. It is important that we start to identify interactions resulting from global climate change and anthropogenic pollution in order to mitigate known and novel environmental threats.

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