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Planctomycetes cell viability studies: perspectives of toxicity assessment using zeta potential

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Centro Interdisciplinar de Investigação Marinha e Ambiental

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Carlos Eduardo de Bento Flores

Dissertação de Mestrado apresentada à
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Mestrado em Biologia Celular e Molecular
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Dissertação para a candidatura ao grau de Mestre em Biologia Celular e Molecular submetida à Faculdade de Ciências da Universidade do Porto.

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"Pedras no caminho?

Guardo todas, um dia vou construir um castelo…"

Fernando Pessoa

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Resumo

Atualmente, a qualidade da água tem uma enorme importância, devido ao forte impacte das práticas industriais e agrícolas nos ambientes aquáticos e consequentemente na biologia dos seres vivos. Isto é particularmente crítico para os microorganismos unicelulares, que estão completa e diretamente expostos aos poluentes. Até um certo nível, os microrganismos conseguem tolerar a exposição aos poluentes, mantendo a viabilidade celular. Devido à sua fácil manipulação e elevada sensibilidade, as bactérias têm sido amplamente usadas como biosensores para a avaliação da qualidade de águas. Planctomycetes é um filo de bactérias peculiares e ubíquas, normalmente encontradas em diversos habitats aquáticos e em associação a algas, esponjas e outros organismos. Testes de toxicidade envolvendo planctomycetes foram apenas recentemente reportados. O potencial zeta é o potencial elétrico específico de uma partícula (viva ou não) quando está em solução aquosa. Embora um vasto número de estudos tenha sido realizado com bactérias e medições de potencial zeta, nenhum relacionou diretamente este parâmetro com a viabilidade celular. Esta relação poderá ser relevante para ensaios de toxicidade, devido à alteração das cargas na dupla camada externa das células (onde se mede o potencial zeta) quando as bactérias são expostas a uma concentração tóxica de um poluente.

Neste sentido, o principal objetivo deste estudo é a realização de um *screening* da viabilidade celular de diferentes bactérias (focando em particular o peculiar grupo dos planctomycetes) e complementar a análise com medições de potencial zeta. Para a realização deste objetivo seis planctomycetes, *Rhodopirellula baltica* e cinco estirpes isoladas de macroalgas marinhas (UC9, UC17, MsF5, LF2, Sm4), foram estudados após exposição a poluentes. Adicionalmente, duas bactérias normalmente usadas em bioensaios de toxicidade, *Pseudomonas putida* e *Escherichia coli*, e uma bactéria patogénica para peixes de água salgada, *Vibrio anguillarum*, foram estudadas para comparação. O crescimento das diferentes estirpes foi caracterizado. A indução da morte celular por micro-ondas e fervura foi também realizada de modo a definir a melhor forma de matar as células para posteriores estudos de potencial zeta. O efeito dos poluentes (metais pesados, nitritos, nitratos, amónia, fosfatos, detergentes, fungicidas, fenol, hidrazina, azida sódica e substâncias ativas de fármacos) em diferentes bactérias foi avaliado através da análise da viabilidade celular e medições de potencial zeta. Para a análise da viabilidade celular, cada cultura líquida bacteriana em fase exponencial foi exposta a várias concentrações dos diversos poluentes. Após exposição, as bactérias foram cultivadas, usando o método da gota em placa.

Na generalidade, as estirpes exibiram uma grande variação na sensibilidade aos poluentes, pela ordem: *V. anguillarum* > planctomycetes > *P. putida* > *E. coli*. *E. coli* foi resistente a todos os poluentes, com a exceção do fenol e da azida sódica. De todos os poluentes testados, o cobre, o Ridomil®, a hidrazina e o fenol foram os mais tóxicos. Os resultados obtidos demonstraram que os planctomycetes tiveram uma elevada sensibilidade e um comportamento de dose-resposta a alguns poluentes, nomeadamente aos metais pesados. Os planctomycetes resistiram a concentrações extremamente elevadas de nitratos, nitritos e amónia. Foram também as únicas bactérias sensíveis ao Previcur N®. A azida sódica reduziu a viabilidade celular em *E. coli*, *P. putida* e *V. anguillarum*, mas não nos planctomycetes. Contudo, este composto afetou a respiração celular dos planctomycetes, embora com menor influência do que nas outras bactérias estudadas. A análise *in silico* de alguns alvos proteicos da ação dos poluentes corroborou a discussão dos resultados obtidos. Observou-se tanto o aumento como a diminuição dos valores do potencial zeta em células estacionárias e mortas, comparando com as células em fase exponencial. Após exposição ao crómio, *E. coli* pareceu comportar-se como uma partícula inerte, não mostrando uma relação entre potencial zeta e viabilidade celular. Em oposição, essa relação foi observada na estirpe LF2, quando exposta a metais pesados, mas não em relação à exposição com outros poluentes. A concentração celular, ao contrário do fotoperíodo, influenciou o potencial zeta. Estudos de microscopia eletrónica de transmissão mostraram modificações morfológicas na estirpe UC17 após exposição ao arsénio, tais como: descondensação do DNA e o aumento do número de zonas eletro-transparentes.

Concluindo, os nossos resultados revelaram uma resposta bacteriana diversificada em relação aos poluentes a que as bactérias foram expostas, o que deverá afetar a estrutura das comunidades microbianas em ecossistemas sob pressão, e fornecer novas informações sobre a ecofisiologia dos planctomycetes. Para além disso, os nossos resultados sugerem que os planctomycetes poderão ser considerados potenciais candidatos para bioensaios de toxicidade. Particularmente a estirpe LF2, que parece ser um bom biosensor, com base no potencial zeta, para a análise de águas contaminadas com metais pesados. Este trabalho proporciona orientações para futuros estudos em biotecnologia e microbiologia celular, também como novos horizontes para o estudo de um dos grupos bacterianos menos caracterizados, Planctomycetes.

Palavras-chave: bioensaios, planctomycetes, poluição, potencial zeta, toxicidade, viabilidade celular.

Abstract

Nowadays, water quality is of major relevance due to industrial and agricultural activities, which have a strong impact in aquatic environments and consequently on the biology of the organisms. This issue is particularly critical for unicellular microorganisms which are completely and directly exposed to pollutants. Up to a certain level, microorganisms can tolerate pollutants, maintaining cell viability. Due to their easy manipulation and high sensitivity, bacteria have been widely used as biosensors in order to assess water quality. Planctomycetes is a particular ubiquitous bacterial phylum, commonly found in a wide range of aquatic habitats and in association with algae, sponges and other organisms. Toxicity tests involving planctomycetes were only recently reported. Zeta potential is the specific electrical potential of a particle (living or not), when it is in an aqueous solution. Although a wide range of studies have been performed with bacteria and zeta potential measurements, none of them related directly this parameter with cell viability. This relation might be relevant for toxicity assays, due to the alteration of charges in the cells' external Double Layer (where the zeta potential is measured) when bacteria are exposed to a toxic pollutant concentration.

In this sense, the main goal of this study was to do a general screening of the cell viability of different bacteria (with focus in the peculiar planctomycetes group) after exposure to pollutants and complement this analysis with zeta potential measurements. To achieve this goal, the growth of six different planctomycetes, *Rhodopirellula baltica* and five strains isolated from marine macroalgae (UC9, UC17, MsF5, LF2, Sm4), was assessed after pollutants exposure. In addition, two common bacteria used in toxicity bioassays, *Pseudomonas putida* and *Escherichia coli*, and one pathogen of marine fishes, *Vibrio anguillarum*, were studied for comparative purposes. The growth of the different strains was characterized. Induction of cell death by microwaves or heat bath was also performed in order to define the best way to kill the cells for further studies of zeta potential. The effect of pollutants (heavy metals, nitrites, nitrates, ammonium, phosphates, detergents, fungicides, phenol, hydrazine, sodium azide, and active pharmaceutical ingredients) in the different bacteria was assessed by a cell viability screening and zeta potential measurements. For cell viability assessment, each liquid culture of microorganism in exponential phase was exposed to several pollutant concentrations. After exposure, bacteria were cultivated using the drop plate method.

In general, the strains exhibited a great range of sensitivity to pollutants in the following order: *V. anguillarum* > planctomycetes > *P. putida* > *E. coli*. *E. coli* showed

resistance to all pollutants tested, with the exception of phenol and sodium azide. Of all the pollutants tested, copper, Ridomil®, hydrazine and phenol were the most toxic. Results obtained revealed that planctomycetes had a high sensitivity and dose-response behavior to some pollutants, namely heavy metals. Planctomycetes resisted to extremely high concentrations of nitrate, nitrite and ammonium but they were the only bacteria sensitive to Previcur N®. Sodium azide reduced cell viability in *E. coli*, *P. putida* and *V. anguillarum*, but not in planctomycetes. However, this compound affected planctomycetes cell respiration, although with slighter effect than in other bacteria. *In silico* analysis of some protein targets of the pollutants action supported the discussion of the obtained results. Both increasing and decreasing of zeta potential values were observed in stationary and dead cells, in comparison to exponential cells. After chromium exposure, *E. coli* seemed to behave as an inert particle, not showing a relation between cell viability and zeta potential. A relation between these two parameters was evident in strain LF2 when exposed to heavy metals, but not to other pollutants. Cell concentration, but not photoperiod, influenced zeta potential. Transmission electron microscopy studies showed morphological alterations in the strain UC17 after exposure to arsenic, such as DNA decondensation and increasing of electron transparent areas.

In conclusion, our results evidenced a diverse response of bacteria towards pollutants, which may influence the structuring of microbial communities in ecosystems under stress, as well as, provided new information about the ecophysiology of planctomycetes. Furthermore, our results suggested that planctomycetes could be considered potential candidates for toxicity bioassays. In particular strain LF2, which seems a good biosensor to assess water contaminated with heavy metals, based on zeta potential. This work provide guidelines for future biotechnological, cell and molecular microbiology studies, as well as, new horizons in the study of one of the most still uncharacterized bacterial group, Planctomycetes.

Keywords: bioassays, cell viability, planctomycetes, pollution, toxicity, zeta potential.

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Abbreviations

®	registered trademark
μ	specific growth rate
μL	microlitre
μM	micromolar
μm	micrometer
100x	one hundred times
16S rRNA	16S ribosomal ribonucleic Acid
aa	aminoacids
AChE	acetylcholinesterase
ALAD	d-aminolevulinic acid dehydratase
APIs	active pharmaceutical ingredients
As	arsenic
ATPase	adenosinetriphosphatase
ATSDR	Agency for Toxic Substances and Disease Registry
AU	arbitrary units
BD	Becton Dickinson and Company
CAT	catalase
Cd	cadmium
CECT	Spanish Type Culture Collection (Colección Española de Cultivos Tipo)
Cr	chromium
Cu	copper
DDT	dichlorodiphenyltrichloroethane
DL	double layer
DNA	deoxyribonucleic acid
e.g.	*exempli gratia*
ELS	electrophoretic light scattering
EPA	United States Environmental Protection Agency
EPSs	exopolysaccharides
EROD	ethoxyresorufin-O-deethylase
ESIC	electrostatic interaction chromatography
et al.	*et alii*
ETC	electron transport chain
Exp.	exponential

g	gram
g	mean double (generation) time
GPx	glutathione peroxidase
GSH	glutathione
GST	glutathione S-transferase
h	hours
i.e.	*id est*
ICM	intracytoplasmic membrane
IUPAC	International Union of Pure and Applied Chemistry
k	mean growth rate constant
L	liter
LA	Luria Agar
LB	Luria Broth
LPO	lipid peroxidation
LPS	lipopolysaccharide
LUCA	last universal common ancestor
M	molar
M13 medium	M13 Verrucomicrobium medium
MFO	mixed-function oxidase system
mg	milligram
min	minutes
mL	milliliter
MLSA	multi-locus sequence analysis
mM	millimolar
Mn	manganese
Mo	molybdenum
MTs	metallothioneins
mV	millivolt
NA	Nutrient Agar
NB	Nutrient Broth
Ni	nickel
nm	nanometer
nmol	nanomole
NPi	National Pollutant Inventory
OC	oxygen consumption
ºC	degree Celsius

OD	optical density
ORFs	open reading frames
PAHs	polycyclic aromatic hydrocarbons
PATRIC	Pathosystems Resource Integration Center
PEPCK	phosphoenolpyruvate carboxykinase
PHO	phosphate regulon
POPs	persistent organic pollutants
PVC	Planctomycetes-Verrucomicrobia-Chlamydiae
R^2	correlation coefficient
RNA	ribonucleic acid
rpm	rotations per minute
rRna	ribosomal ribonucleic acid
s	seconds
S/m	siemens per meter
SD	standard deviation
SOD	superoxide dismutase
spp.	species
Sta.	stationary
TBARS	thiobarbituric acid reactive substances
TEM	transmission electron microscopy
TM	trademark
UNESCO	UN Educational, Scientific and Cultural Organization
w/v	weight per volume
WHO	World's Health Organization
YETI	yeast environmental toxicity indicator
YTV	tyrosine-threonine-valine domain protein
Zn	zinc
ζ	zeta

1. Introduction

1.1. The impact of water pollution

As a fundamental resource for Earth sustainability, water has an essential role in the equilibrium of the ecosystems. Men's existence is closely related to the use of water and according to WHO (World's Health Organization), water quality is an important issue for human life, dignity and development. Whereas in the past this issue did not receive the deserved attention, nowadays, water pollution has reached a primary relevance both in developed and in some developing countries (WHO 2003, 2012), mainly because of the increasing scarcity of potable water resources. Although contaminants can enter in ground and surface waters from both natural and anthropogenic sources, the main contribution for water contamination comes from anthropogenic activities, mostly due to the intensive exploitation of the water resources and the enhancing of the water spoiling. Leading the causes of water exploitation and pollution are industry and agriculture (*Annex1*). The sudden and quick development of these two activities has been contributing to severe water exposure to diverse toxicants.

There are two major origins of water pollution: point and non-point sources (Letson 1992). Point sources are confined and easily identifiable sources where pollutants are or may be discharged, such as: pipes, conduits, containers, vessels or discrete fissures. The starting places for these discharges are commonly sewage treatment plants, industrial (e.g. oil and gas extraction, mining, manufacturing) and government facilities (e.g. military bases), animal feedlots or ships. In contrast, non-point source pollution comes from many and diffuse sources, resulting in runoffs which carry the natural and human-made pollutants, *i.e.*, although the contaminants have a point source initially, the long-range transport ability of the contaminant and its potential multiple origins make the original source difficult to define exactly. Non-point pollution comprises frequently: i) runoffs from agriculture rich in fertilizers and pesticides, or effluents from urban places, which carry oils, grease and toxic chemicals; ii) sediments from eroding streambanks or improperly managed construction sites, crop and forest lands; iii) salts from irrigation practices and acid drainage from abandoned mines; iv) wastes from livestock, pets and faulty septic systems containing bacteria and nutrients;

v) atmospheric deposition and hydromodification. Actually, in the majority of countries, the pollution from non-point sources is considered the most important cause of water pollution. According to EPA (United States Environmental Protection Agency) reports the effect of “nonpoint source pollutants” on waters varies frequently and may not always be fully assessed (EPA 2007).

1.1.2. The Pollutants

A diversity of pollutants from chemical, physical, radioactive or pathogenic microbial nature can be added to water bodies. These pollutants can be a threat for living-organisms, directly, such as POPs (persistent organic pollutants) used as pesticides (e.g. DDT and toxaphene); or indirectly, causing changes in water conditions that allows harmful reactions or activities for the ecosystem, such as eutrophication (Hogan 2010).

Even after water treatment, some pollutants remain in waters (Westerhoff 2005). In addition, some treatments such as the chlorination process, one of the most used water preservative methods to inhibit bacterial contamination, can originate genotoxic compounds in the water (Park et al. 2000).

Many lists of “priority pollutants” have been made in order to ensure water quality, varying according to the country and the agency responsible for monitoring water quality (ATSDR 2011; Directive 2008/105/EC; EPA 2007; NPi 2007). The main source of these pollutants found in natural waters is quite distinct and comprises frequently more than one kind of pollutants. For instance, heavy metals are frequently found in industrial wastes. On the other hand, phosphates, nitrates, nitrites, ammonia and pesticides are mainly associated to fertilizers and other products commonly used in agricultural activities. Other kind of common pollutants are compounds with antimicrobial, antifungal and/or antiviral activity which are used as antiseptics and disinfectants, such as phenol and detergent by-products. Furthermore, pollutants such as hormones and active pharmaceutical ingredients (APIs) are emerging on the environment, due to the exponential consumption of cosmetics, pharmaceuticals and other health care products. Despite their chemical diversity, their ecological impact remains largely unknown and only recently they have been considered as deserving priority attention (Sanderson et al. 2004).

Water contaminants have a variety of mechanisms of action in cells, which may include: i) inhibition of enzymes; ii) binding to ion channels and regulatory proteins; iii) changes in gene expression; iv) generation of reactive oxygen species; v) limitation of cell growth; vi) destruction of cell envelope (namely, cell wall and cell membrane).

Some of the compounds (e.g. APIs) have also an unpredictable biological activity, in certain conditions (Daughton and Ternes 1999). Additionally, many of the aforementioned pollutants are also carcinogenic or potentially carcinogenic (e.g. hydrazine, heavy metals) (WHO 1987).

1.2. The assessment of pollution in waters

Various studies have assessed the water quality of water bodies exposed to point and non-point sources. However, the absence of a universal and cost-effective method, able to cover a wide range of toxicants, makes the correct analysis of the majority of water supplies difficult or even impossible. The importance of this issue is even greater when the countries need to comply with National or European water quality regulations, such as the Water Framework Directive (Hering et al. 2010). Currently, major efforts have been made to develop new methods that are easy to use, sustainable and economically feasible.

Two main approaches are used to evaluate and monitor water quality: physic-chemical analyses and biological approaches (bioassays). The former consists in determining the presence and concentration of hazardous substances in water, using analytical methods. Apart from the accuracy and sensitivity of these methods, they are typically time-consuming and their high selectivity only provides the detection of targeted compounds (single or a small group of pollutants). Furthermore, many different pollutants may co-exist in water and sometimes the toxicity lies in the mixture of pollutants rather than in each compound alone. On the other hand, bioassays can surpass these disadvantages and complement physic-chemical studies, providing crucial information. Indeed, there are more and more studies using both approaches and giving to the biomonitoring a vital relevance for water quality assessment (*see for review*: Besse et al. 2012; Rodriguez-Mozaz et al. 2007).

1.2.1. Bioassays

Since the discovery of the first biological method, bioassays have been increasingly employed in ecotoxicology. The main purpose of these methods is to detect changes in water quality and the presence (or not) of environmental pollution by detection of changes in health status, physiological features or behavioural responses from biological material such as: antibody reactions, enzymes, cells, tissues, whole organisms or even populations. In addition, some of these methods can also have an

important role in the determination of organism communities' abundance (Gall et al. 2012). The increasing utility of bioassays in water environments is mainly due to: i) their ability to detect chemical material that chemical analyses cannot detect because of methodological limitations (van der Schalie et al. 2001); ii) the response of aquatic organisms, which is more sensitive and reliable; iii) the better assessment in case of mixed pollution and in samples with a complex chemical nature, without a prior knowledge of the pollutant physic-chemical properties; and iv) the frequent low cost associated to the application of this kind of approaches (Batzias and Siontorou 2006). In addition, these methods can give important information about the mechanisms of action of the pollutants and their toxic effects on living organisms (Jastrzębska and Buszewski 1999; Ravera 2001).

These methods may be divided into two main groups: the biosensors / biomarkers and the bioindicators / biomonitors. Even though both concepts seem to be confused in some studies, they have several differences. The main advantage of bioindication is to provide continuous and real-time information about water quality, using bioindicators to characterize environmental situations (Namieśnik and Wardencki 2000; Radecki and Radecka 1995). Thus, the biological indicators are chosen according to several features, like: a sedentary life; wide distribution; simple identification and sampling; high accumulating ability of pollutants (Buszewski et al. 2002; Ravera 2001). Many biological methods have been developed using evolutionary higher organisms such as plants, fishes, bivalves and crustaceans, to monitor water quality (*Annex 2*). However, most of them are used mainly to study cell biomarkers from specific tissues.

In contrast, biosensors or biomarkers are designations used when the contaminant's detection is performed at a cellular, biochemical or molecular level. In this context, the main biological material used can be nucleic acids, enzymes, antibodies, hormone receptors or microorganisms (Table 1). Especially in aquatic ecosystems, the employment of biosensors can simplify, at a reduced cost, the biological monitoring (Dutka and Bitton 1989; Eltzov and Marks 2011). The use of biosensing implies the pollutant recognition by a biological element, which is in intimate contact with a physical transducer (Figure 1). The transducer converts the biological recognition event into a measurable signal. In fact, its high sensitivity enables a toxicant determination at a trace and ultratrace level (Jastrzębska and Buszewski 1999). Different types of signal transduction might be the basis of biosensor classification in distinct groups (e.g. optical, electrochemical and thermal) (Lei et al. 2006).

Table 1. The main biosensors used in environmental monitoring. Table based on Allan et al. 2006 and Lei et al. 2006.

Biosensor base	Principle	Advantages	Disadvantages
Enzymes	- Enzymatic reactions are accompanied by the production or consumption of CO_2, NH_3,H_2O_2, H^+ or O_2. - The substrates activate or inhibit enzyme or other protein activity.	- Ability, in some cases, to modify catalytic properties or substrate specificity by genetic engineering. - Various transducers easily detect and correlate this species to the substrates.	- The main limitation is the lack of specificity in differentiating among compounds of similar classes.
Immunosensors	- High selective pollutant extraction and/or quantification based on antigen/antibody interactions.	- More versatile than enzyme. - More selective and specific. Affinity is significantly higher than for other biomolecules. - Can be created in order to bind to a wide range of compounds.	- They have to be developed and characterized for each compound. - Cross-reactivity with analogues and metabolites. - False positives.
DNA	- A single-stranded oligonucleotide probe is immobilized as a recognition material. - Interactions of an immobilized double-stranded DNA with low molecular weight pollutants.	-Base-pairing interactions between complementary sequences are both specific and robust.	- Molecular instability and expensive techniques
Whole-cell	- Recognition elements by measuring the general metabolic / physiological status of the cells.	- Many enzymes and co-factors that co-exist in the cells give them the ability to consume and hence detect a large number of chemicals.* - Can be easily manipulated and adapted to consume and degrade new substrates.	- *This may compromise their selectivity. - Low reproducibility.

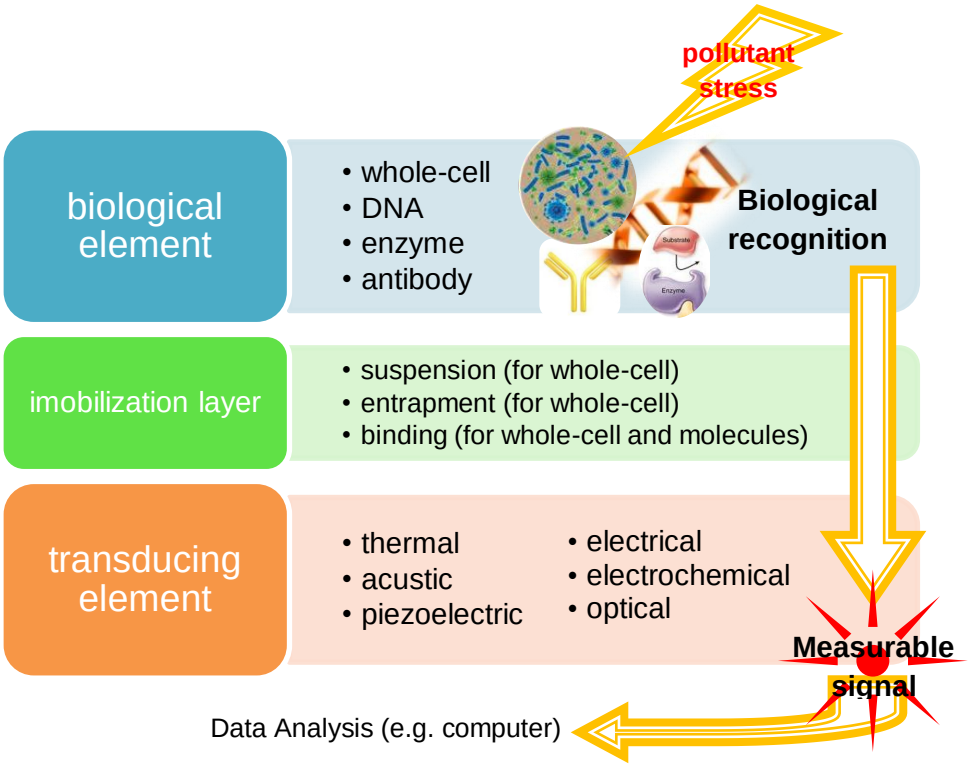


Fig.1 – General structure of a biosensor and schematic representation of the signal production by biosensors.

1.2.1.1. Microbial bioassays

Bacteria and other unicellular organisms are in direct contact with the environment, which is in constant physic-chemical alteration. Therefore, during biological evolution these organisms developed complex mechanisms of defense and adaptation in order to survive. Due to their easy manipulation and high biotechnological application, microbial biosensors have been widely used for water quality assessment (Eltzov and Marks 2011). There are many examples of whole-cell biosensors: cell lines of mammals and plants, microalgae, filamentous microfungi, yeasts and several species of bacteria. Some of the most common tests to assess water quality are: the ones based on *Chlorella vulgaris* (an unicellular green alga, widespread in fresh waters) (Cronin et al. 2004); the Spirotox test (Nałęcz-jawecki 2005), based on *Spirotostomum ambiguum* (a protozoan ciliate present in clean rivers and lakes); the yeast environmental toxicity indicator – YETI (using *Saccharomyces cerevisiae*) (Knight et al. 2004) and the Microtox®, which consists in measuring the natural luminescence of *Aliivibrio fischeri* (Komlos et al. 2010).

Moreover, a wide range of studies based on bacteria have been performed according to different techniques. Among the most common bacterial biosensors, there are bioluminescent bacteria (Charrier et al. 2011), nitrifying bacteria (Woznica et al.

2013), sulphur-oxidizing bacteria (Oh et al. 2011) and bacterial communities from activated sludge (Jordan et al. 2010). Although the advantages brought by these and others bacterial biosensors, very few techniques have been commercialized.

1.3. The relevance to study Planctomycetes

More and more, there is a pressing need to extend the microbiology horizons beyond the classical models (such as *E. coli* or *Saccharomyces* spp.), in order to provide better information about cell biology (Fuerst 2011). Planctomycetes are crucial pieces for this understanding, due to their peculiar morphology and physiology, associated to their evolutionary importance.

1.3.1. Morphology and Physiology

Two of the most notorious features of planctomycetes morphology are their permanently condensed DNA and their particular cell wall. The absence of peptidoglycan in the proteinaceous cell walls of the planctomycetes facilitates the obtainment of pure cultures by the use of antibacterial agents in media (e.g. vancomycin and beta-lactams) (Cayrou et al. 2010). Another distinctive feature is the internal compartmentalization defined by single bilayer membranes (the intracytoplasmic membrane - ICM) or double membranes with two bilayers, first reported by Lindsay et al. (1997). This compartmentalization allows the division of the planctomycetes cell plan in two major regions: paryphoplasm and pirellulosome (Lindsay et al. 1997, 2001) (Figure 2, a). Additionally, it also enables the formation of a membrane-bound nucleoid in *Gematta obscuriglobus* (Fuerst 1991) and other compartments as an organelle surrounded by a single bilayer membrane, named anammoxosome, found only in anaerobic ammonium oxidation (anammox) planctomycetes (van Niftrik et al. 2004). This distinctive group of planctomycetes displays an important role in nitrogen cycle and nitrogen-rich-wastewater remediation (Kartal et al. 2010; Kuenen 2008), it was also estimated that they are responsible for generating 50 % of the nitrogen in atmosphere (Jetten 2008).

This has been the accepted cell plan in the last decade. However, a new concept for the planctomycetes cellular envelope is gaining support based on genetic and structural evidences (Lage et al. 2013; Santarella-Mellwig et al. 2013; Speth et al. 2012). Regarding these data, the planctomycetes possess an outer membrane equivalent to the one of Gram negative bacteria and refutes the presence of the

intracytoplasmic membrane (Figure 2, b) (*for Gram negative membrane structure see Fig. 5b, 1.4.2. Cellular organisms and zeta potential*).

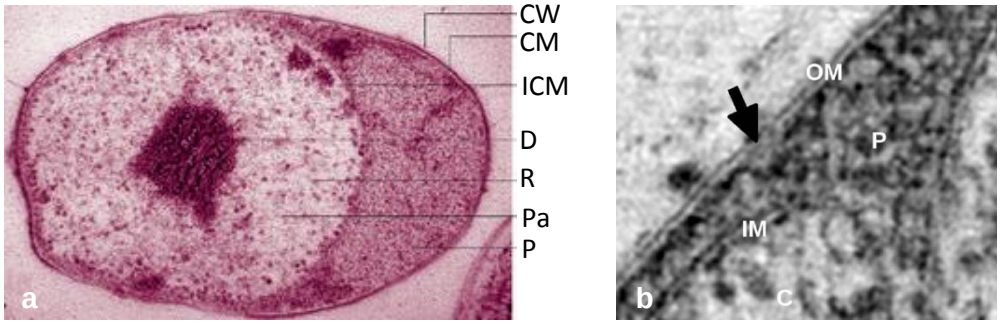


Fig.2 – The general cell plan of planctomycetes (a). CW, cell wall; CM, cytoplasmic membrane; ICM, intracytoplasmic membrane; D, condensed DNA; R, ribosomes; Pa, paryphoplasm; P, pirellulosome (*Adapted from* Thomson et al. 2003). The new concept for the planctomycetes cell envelope (b). OM, outer membrane; P, periplasm; IM, inner membrane (= CM); C, cytoplasm (*Adapted from* Santarella-Mellwig et al. 2013).

The majority of the proteins detected in several planctomycetes' cell walls showed an aminoacid composition rich in proline and cysteine, which are involved in cell wall stabilization (Giovannoni et al. 1987; König et al. 1984; Liesack et al. 1986). For instance, in *Rhodopirellula baltica* it was detected by proteomic analysis a tyrosine-threonine-valine domain protein (YTV) with many cysteine and proline residues (Hieu et al. 2008). Furthermore, some of the previous studies confirmed that this aminoacid composition conferred resistance to boiling in 10 % sodium dodecyl sulphate by planctomycetes.

The budding reproduction is other typical characteristic of these microorganisms. Only anammox planctomycetes, which is considered a divergent marine order (Rachel 2009), and the classis Phycisphaerae (Fukunaga et al. 2009) uses binary fission.

Also unusual in bacteria, it is the ability of some of these organisms to produce sterols (it was suggested they might regulate the membrane fluidity) (Pearson et al. 2003) and the presence of encoding genes for C_1 transfer enzymes. Interestingly, this feature does not have a correlation with the production or metabolization of the C_1 compounds, but it was proposed that could be related with adaptations to subtoxic conditions in the ocean (Chistoserdova et al. 2003; Woebken et al. 2007).

Almost all plactomycetes are heterotrophic aerobes, with the exception of the anammox group, which is anaerobic and chemoautotrophic (Kuenen 2008). The life style of planctomycetes is quite diverse. Their life cycle can involve swarmer (mobile) and sessile cells (Grade et al. 2005). They can also live either as free-living cells or in aggregates (Fuchsman et al. 2012). In this way, the diversity of morphotypes (Fuerst

1995) and the particular physiology allowed these organisms to colonize a wide range of habitats.

1.3.2. Ubiquity

Although frequently present in low abundance, planctomycetes are found throughout the different aquatic and terrestrial environments such as: marine waters (Fuchsman et al. 2012), freshwater (Bondoso et al. 2011), rizosphere (Sheng et al. 2012), polluted environments (Chouari et al. 2003), soil (Buckley et al. 2006) and many others. Their environmental ubiquity is strongly supported by analysis of *16S rRNA*-based gene clone libraries derived from environmental microbial communities (Bowen et al. 2012; Buckley et al. 2006; Smith et al. 2012; Torres-Cortés et al. 2012).

In marine particles these microorganisms reach high quantities: in intertidal marine sediments there are 10^8 cells per mL (Musat et al. 2006); in ocean detritus drifting through the water, planctomycetes are the main components forming “marine snow” (Delong et al. 1993); in association with blooms of marine diatoms (Morris et al. 2006). The remarkable ubiquity of these microorganisms is extended even to extreme environments: arid habitats (e.g. Atacama Desert) (Drees et al. 2006), extreme saline habitats (Bernhard et al. 2012), acidic habitats (Ivanova and Dedysh 2012; Urbieta et al. 2012), Antarctic lakes (Tang et al. 2013), thermophilic habitats at temperatures higher than 55 ^oC (Giovannoni et al. 1987), 75 ^oC (in oil reservoirs with water production) (Li et al. 2010), or 85 ^oC (in deep-sea hydrothermal vents) (Byrne et al. 2009).

In addition, it was detected a wide and different association of planctomycetes to other organisms, such as: other bacteria (Castenholz 1985), macroalgae (Lage and Bondoso 2011), sponges (White et al. 2012), coral (Webster and Bourne 2007), gut of termites (Kohler et al. 2008) and crustaceans (Chaiyapechara et al. 2012; Fuerst et al. 1997). For example, the genus *Rhodopirellula* dominates the surface microbial community of *Laminaria hyperborean*. This organism is crucial for coastal productivity around the world and the bacterial symbiosis has certainly an important role in nitrogen and carbon turnover (Bengtsson and Ovreas 2010).

1.3.3. Evolutionary relevance

Initially, based on oligonucleotide catalogues, Planctomycetes were classified as a group of *Eubacteria* (Woese 1987), in opposition to its previous classification as fungi. More recent analysis using concatened protein-coding genes and genomes showed that these microorganisms form a distinct phylum of bacteria (Jun et al. 2010; Strous et al. 2006). Indeed, phylogenetic studies, both with rRNA sequences (Brochier and Philippe 2002), or with whole proteomes (Jun et al. 2010), suggested that planctomycetes are the deepest branch among all phyla of the *Bacteria* domain.

Nowadays, diverse studies have been clustering planctomycetes with other bacterial phyla, forming the Planctomycetes-Verrucomicrobia-Chlamydiae (PVC) superphylum (Figure 3). The members of PVC group display several features in common, as: internal compartmentalization (Fieseler et al. 2004; Lee et al. 2009); the presence of some genes for peptidoglycan biosynthesis pathway, but the absence of peptidoglycan in their cell walls; the ability to synthesize sterols; the DNA permanently condensed. The PVC includes soil and marine bacteria, symbionts, species from anoxic habitats and even human pathogens (Glockner et al. 2010; Wagner and Horn 2006). The morphologic and physiologic approximation of some PVC members to eukaryotes is also evident, in particular in relation to planctomytcetes.

Different models were made to explain the evolutionary mechanisms of planctomycetes: planctomycetes as precursors of eukaryotes; the retention of certain features of a proto-eukaryote LUCA (Forterre and Gribaldo 2010) or a convergent re-evolution of a eukaryote-like plan.

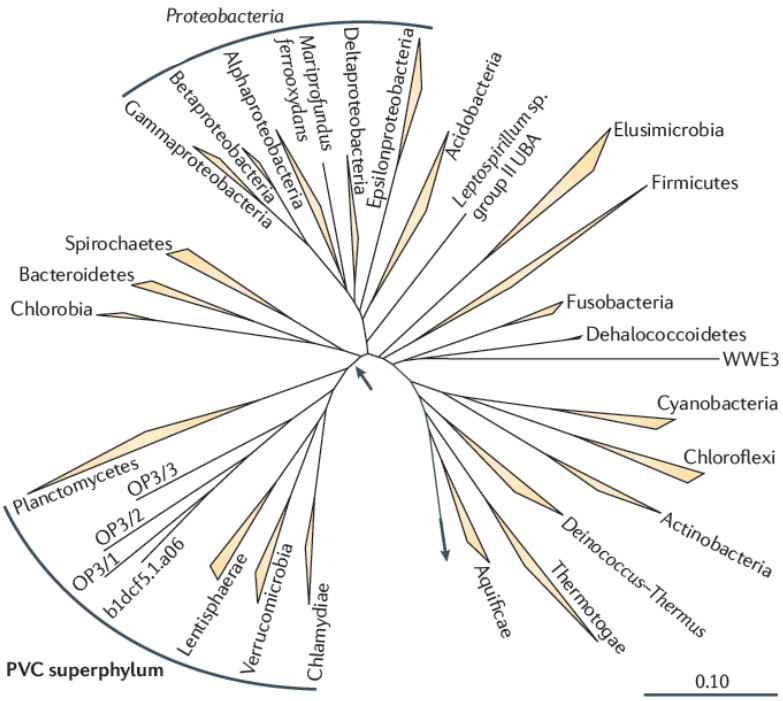


Fig.3 – Phylogenetic tree based on 23S ribosomal RNA gene, representing the relationship of the PVC superphylum (arrow) to other bacterial phyla. The arrow next to Aquificae indicates the outgroup (the domains *Archaea* and *Eukarya*). The scale bar represents 0.1 substitutions per nucleotide position. (*Adapted from* Fuerst and Sagulenko 2011).

The lack of detailed genetic systems (mainly in non-annamox planctomycetes) is an important barrier in the study of planctomycetes, although there is a quite large collection of genomic information and the evidence of some molecular signatures (Gupta et al. 2012).

Currently, only the genome of *Rhodopirellula baltica* was described in detail (Glockner et al. 2012) and only approximately 32 % of the *open reading frames* (ORFs) have a predicted function. Also in genus *Rhodopirellula*, a large number of isolates, mainly from European seas, have recently been analyzed by multi-locus sequencing (MLSA) (Winkelmann et al. 2010). Furthermore, significant progresses in the genetics of the morphogenesis, cell division and signal transduction of planctomycetes are ongoing (Jogler et al. 2012; Speth et al. 2012; van Niftrik et al. 2009).

1.4. The Zeta Potential

Zeta (ζ) potential is the electrical potential of a particle in an aqueous solution at the slipping plane, which is the interface between the electrical double layer of the particle and the bulk liquid. It can also be synonymous of electrokinetic potential in colloidal systems, according to IUPAC (International Union of Pure and Applied Chemistry) (IUPAC 1997).

1.4.1. The Double Layer (DL)

In the exact moment when a particle (such as a gas bubble, a liquid droplet, an object or a cell) is placed into a liquid, on the surface of the respective particle appears a structure formed by two parallel layers – the double electrical layer (Figure 4).

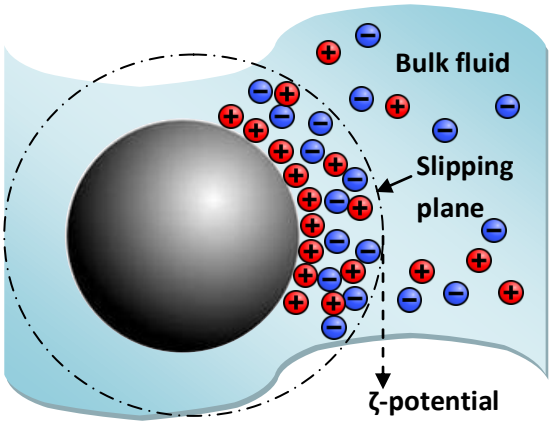


Fig.4 – Schematic representation of the double layer (DL) formation on a negatively-charged particle and the interface in contact to the remaining fluid, where zeta potential is measured. Another DL can be formed, depending on the particle nature.

The structure of the first layer (the surface charge) consists only in entities with a single charge (either negative or positive), which is opposite to the particle's native charge. In contrast, the second layer (the mobile/diffuse layer) is loosely associated to the particle and has a specific composition for each particle, due to the distribution of ions and counter ions. In this layer the ions can freely move in the fluid under electric activity.

Zeta potential and the surface charge can be strongly related, but they can also have opposite charges (Kirby 2010). However, it is the zeta potential of each particle that controls interactions to other particles or compounds in the solution, and not the surface charge of the particle (Kirby 2010). For instance, particles with high zeta potential (either positive or negative), when in contact, will repeal each other.

1.4.2. Estimation of zeta potential

In general, zeta potential is used as a physical indicator to measure the charge stability of a dispersed system. This parameter indicates the attraction or repulsion degree between the adjacent charged particles in a liquid (Lyklema 1995).

Moreover, the charge at the slipping plane will be very sensitive to the concentration and the type of ions in the solution. In this way, factors like pH, ionic strength and concentration of specific ions of the dispersion media have a major influence in the measurements (Baik and Lee 2010; Wnek and Davies 1977).

1.4.2.1. Methodological approaches

Although still impossible to measure directly the zeta potential, it can be calculated using theoretical models, or experimentally determined through the electrophoretic mobility – by electrokinetic or electroacoustic phenomena.

The electrokinetic phenomena (employed in the present work) consists in the application of an electric field that causes the migration of particles in solution toward an electrode which has an opposite charge (anode or cathode), acquiring a velocity that is correlated to a zeta potential value (Bazant and Squires 2004; Delgado et al. 2007). The velocity of the particles is measured using a laser beam, according to the Laser Doppler Velocimetry technique (Avidor 1974; Matthews and O'Connor 1978; Stoltz et al. 1984) and their mobility is converted into a zeta potential value by inputting

the dispersant viscosity through the Smoluchowski (Sze et al. 2003) or the Huckel (Kirby and Hasselbrink 2004) theories.

On the other hand, the electroacoustic phenomena measures the dynamic electrophoretic mobility, which is similar to electrophoretic mobility previously described, but at higher frequency (Dukhin and Goetz 2004).

Nowadays, there are some instruments available to estimate the zeta potential with high sensitivity and avoiding possible contaminations. One of the most common is Zetasizer nano® from Malvern (used in this work).

1.4.2.2. Applications

Zeta potential has a wide range of applications (Marsalek 2012). Two of the most important are: i) to test the effect of compounds (e.g. additives) in a dispersion system in order to improve the formulation stability and increase the shelf life of products (Malhotra and Coupland 2004); ii) to assess the impact of some changes (e.g. hydrolysis or gas ingress) during the food packing or storage (Aresta et al. 2013; Freitas and Müller 1998). Among the different applications of zeta potential, its use as a predicting tool in bioassays for water toxicity assessment has never been referred.

1.4.3. Cellular organisms and zeta potential

As any particle, the physical stability of cellular living organisms can also be correlated with zeta potential values. Different studies have been made with a diversity of organisms or cell lineages, including: fungi (Liu et al. 2012) and yeasts (Jenkins et al. 2012), algae (Oukarroum et al. 2012), plant cells (Judy et al. 2012), animal cells (e.g. epithelial and cancer cells) (Dombu et al. 2012; Rosenholm et al. 2010) and bacteria (Tariq et al. 2012).

Being related to surface charges, the analysis of zeta potential has even more importance in the study of unicellular organisms, such as bacteria, since they are directly exposed to abiotic pressures. Consequently their cell surface plays a crucial role in the maintenance of homeostasis and the cell shape, turgor support, growth, division, interaction with immunological factors or other organisms, adhesion, exchange of compounds and protoplast protection from chemical and physical insults.

In addition, the majority of bacteria is in intimate contact with water, either by living in aquatic environments or by possessing a hydrated surface to facilitate the nutrient transport and waste expulsion (Beveridge and Graham 1991). Therefore, there

are several molecules (phosphates, for example) in the cell envelope, which allow the maintenance of a surface hidrophilicity. This is essential for optimal cell function and confers a net negative electrostatic surface charge to the bacterial cells growing at physiological pH, and it depends on the composition of the bacterial cell envelope (Bayer and Sloyer 1990; Beveridge 1988) (Figure 5).

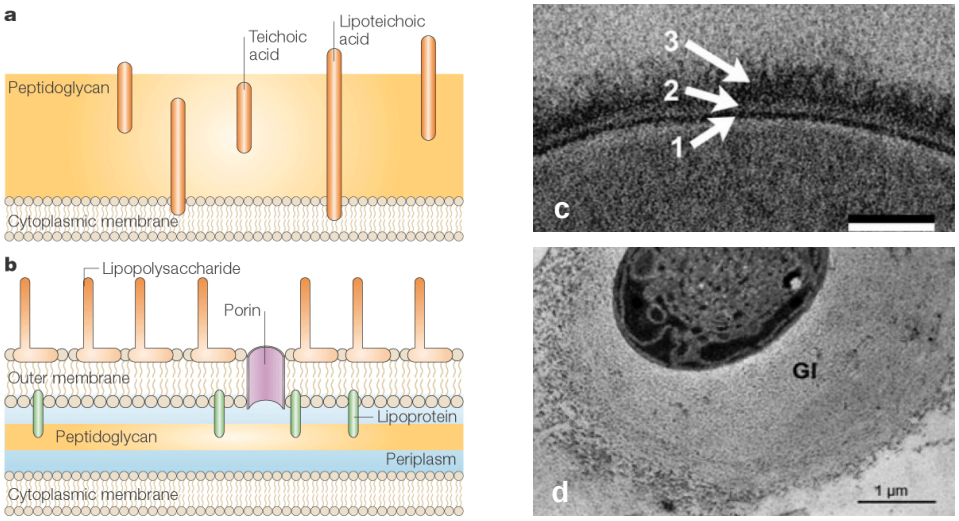


Fig.5 – Cell wall structure of Gram positive (a) and Gram negative (b) bacteria. (*Adapted from* Cabeen and Jacobs-Wagner 2005). External surface layers of *Bacillus subtilis* (Gram positive) (c). The barr represents 50 nm. 1, peptidoglycan; 2, Slime layer and 3, Capsule (*Adapted from* Matias and Beveridge 2005). External glicocalyx (Gl) of *Aquisphaera giovannonii*, a planctomycete (d) (*Adapted from* Lage et al. 2013).

In Gram positive bacteria, the presence of phosphoryl and carboxylate groups in the teichoic acids (Figure 5, a), which are placed in the peptidoglycan cell wall, are mainly responsible for the surface cell electronegativity. On the other hand, in Gram negative bacteria, the peptidoglycan is not directly exposed to the extracellular environment, and the surface electronegativity is mainly due to carboxylate groups of the lipopolysaccharides (LPS) located in the outer membrane, as well as the presence of proteins (e.g. porins), which contributes to the surface net charge distribution (Faraudo et al. 2010) (Figure 5, b). Externally to the cell wall, other surface layers and compounds as exopolysaccharides (EPSs) are also responsible in influencing the cell surface charge (Figure 5, c-d).

A large portion of metabolic energy is used to synthesize and maintain all these macromolecular components in order to provide a better anionic or cationic distribution in outer cell surface region, which is responsible for the interactions between the cell and other charged surfaces.

Similarly to other small particles, the electrostatic charge of bacterial cells cannot be measured directly, consequently it is necessary to use an indirect approach. Earlier, several methods have been developed to assess the surface charge of bacteria, among the most usual techniques in bacteriology there are: microelectrophoresis (Brinton and Lauffer 1959; Moyer 1936), electrostatic interaction chromatography (ESIC) (Wood 1980), aqueous two-phase partitioning (Stendahl et al. 1977), isoelectric equilibrium analysis (Sherbet et al. 1972) and electrophoretic light scattering (ELS) (Lytle et al. 1999). In this study we used the ELS method for zeta potential measurement from electrophoretic mobility, and the Smoluchowski equation for zeta potential calculation. Briefly, this is a very rapid and easy method for estimating zeta potential values based on the determination of the frequency shifts of a laser light caused by the scattered light from the particles (Figure 6).

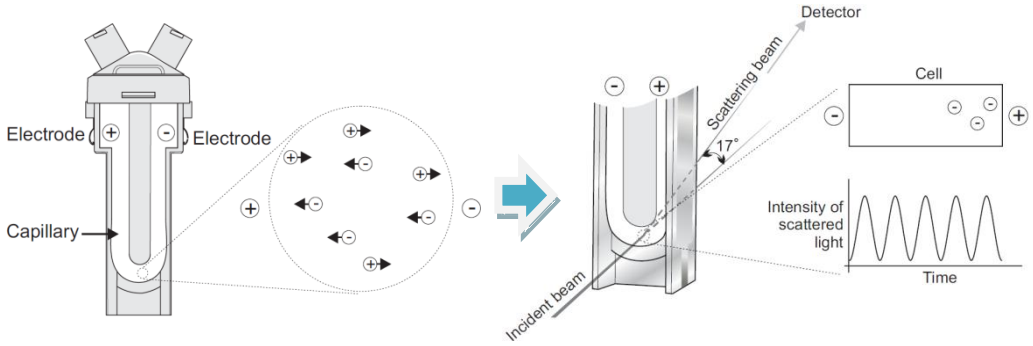


Fig.6 – Illustration of the ELS method in a "dip cell" (which has two electrodes) (*Adapted from* Malvern 2004).

The method consists in providing two types of motions in the particles' suspension. The first motion is random (a Brownian motion, with the electric field off), which is responsible for the cell's movement into and out a light beam. This motion scatters light, which is detected by a photodetector. After this, the electric field is turned on and the particles migrate towards a positive electrode (in case of bacteria cell). Due to this motion, the magnitude and direction of this particle is given by a drift velocity vector, which can be determined in an electric field by measuring the frequency shift of the frequency spectrum. Finally, in the case of most of the bacteria (and even viruses), their size allows the use of the Helmholtz-von Smoluchowski equation for the zeta potential calculation from particle's electrophoretic mobility (Hiemenz and Rajagopalan 1997; Malvern 2004).

Initially, ELS was used by Ware and Flygare in 1971. Since then, this method has been proved useful as a tool for a variety of applications in the study of bacterial physiology, such as bacterial adhesion (Jones et al.1997), solubilisation of minerals

(Blake et al. 1994), cell permeability (Magia et al. 1995), study of surface cell proteins (e.g. porins) (Swanson et al. 1997), resistance to antimicrobial molecules (Bengoechea et al. 1998), capsulation and susceptibility to phagocytosis (Watt et al. 2003), relationship between cations and cell surface (Bundeleva et al. 2011), extracellular polymers (Tsuneda et al. 2003) and quorum sensing (Eboigbodin et al. 2006).

Moreover, although some studies associate growth medium conditions, starvation, death (Soni et al. 2008) and growth phases (Hayashi et al. 2003) with zeta potential values, none correlated changes in zeta potential with alteration of cell viability.

1.4.4. Citotoxicity and zeta potential

Chemical changes interfere with the net charges of bacterial cell surfaces, inducing stress and ultimately loss of cell viability. Different studies have already linked different heavy metals exposure (Collins and Stotzky 1992; Schott and Young 1973), or antimicrobial molecules (Morris et al. 1995) with alteration of cell surface charge.

However, up to a certain level, a living cell has the ability to rebalance the ions in and out of the cell surface in order to maintain cell viability, avoiding toxicity effects and consequently cell death (Brandys et al. 1999). In contrast, when a bacterial cell is no longer viable, unbalances occur and the bacterium becomes unable to compensate these net charges alterations.

In a suspension, the alteration of cell stability and viability will affect first the outer layer of cell's DL, due to the loss of capacity to keep the charges under equilibrium. Accordingly, zeta potential, which is measured in DL interface, might indicate the alteration of cell integrity in a dispersion system, due to toxic exposure, for example.

A previous study (Lage et al. 2012) using planctomycetes members from the *Pirellula-Rhodopirellula-Blastopirellula* clade (one of the main targets in planctomycetes' research) demonstrated differences in zeta potential values between the different planctomycetes strains and after exposure of *Rhodopirellula* strain LF2 to different heavy metal concentrations.

2. Aims

The main goal of this work was to assess the cell viability of different bacteria after exposure to various pollutants. A wide range of toxicants were tested in eleven bacterial strains, including several planctomycetes, in order to compare the bacterial growth responses.

We intended to provide new insights about planctomycetes' physiology, as well as to verify the utility of planctomycetes as bioindicators of polluted waters. Due to their ubiquity and thus, their adaptation ability to a wide range of environments, planctomycetes might be useful biological tools to be used in pollution assessment.

In addition, pollution effect in selected strains was also evaluated based on zeta potential. This instrumental tool may provide an innovator and fast bioassay to identify contamination in water. For the implementation of the biosensor we aimed to establish a relationship between the zeta potential and the bacterial cell viability.

Furthermore, *in silico* analysis of the genes related to toxicity aspects was performed in order to provide useful guidelines for further molecular studies.

3. Material and Methods

3.1. Bacterial strains and culture conditions

Planctomycetes strains LF2, UC9, UC17, Sm4, MsF5 were isolated from the biofilm community on the marine macroalgae surface (Lage and Bondoso 2011), and are part of the LEMUP (Laboratory of Microbial Ecophysiology of University of Porto, Porto, Portugal) microbial collection. Strain *Rhodopirellula baltica* was isolated from the Baltic sea (Schlesner 1994). *Escherichia coli* was isolated from a water sample of the Rio Febros - Avintes, Portugal (Cabral and Marques 2006). *Pseudomonas putida* NB3L was isolated from a marine sponge (Pimenta 2010) and *Vibrio anguillarum* from a sea bass's kidney. It was also included in the study the strain *Aliivibrio fischeri* CECT 524 (Spanish collection).

Planctomycetes were cultivated on modified M13 medium (Lage and Bondoso 2011). *E. coli* was cultivated in Luria Broth (LB) / Luria Agar (LA) (10 g/L Tryptone, Cultimed; 5 g/L Bacto™ Yeast Extract, BD; 1 g/L D-glucose, Merckr; 5 g/L sodium chloride, Merck; 1.6 % agar Bacteriological American Type, Cultimed). *A. fischeri*, *P. putida* and *V. anguillarum* were cultivated in Nutrient Broth (NB) / Nutrient Agar (NA) (5 g/L Bacto™ Peptone, BD; 3 g/L Yeast extract; 1 g/L D-glucose; 1.6 % agar Bacteriological American Type) made with natural sea water.

The purity of all the liquid cultures used in the following assays was assessed by optical microscopy (Leica DM750) at 100x (oil immersion).

3.2. Evaluation of culture growth

The assessment of culture growth was monitored spectrophotometrically (Genesys™ 10 Series, Thermo Spectronic), measuring the optical density at 600 nm (OD_{600nm}) from liquid cultures initiated with a ratio (culture:medium) of 1:10 for planctomcycetes and 0.1:10 for the other microorganisms. The cell concentration was measured every 30 minutes for *P. putida* and *A. fischeri*, every 20 minutes for *V. anguillarum* and *E. coli*, and every hour for Planctomycetes (during 32 hours), until the stationary growth phase. The liquid cultures were incubated at 37 ºC for *E. coli* and 26 ºC for the other bacteria, on the adequate medium. For *P. putida* the measurements were made on NB (with sea water) and M13 media. In the case of *P. putida*, *E. coli*, *V.*

anguillarum and *A. fischeri*, the final growth curve estimated was a result of the mean of three growth curves.

Growth curves, doubling times, tendency lines and correlation coefficients were estimated using Microsoft Office Excel 2007 for Windows. The mean doubling (generation) time (*g*) for each curve was calculated according to the equation 1 and the growth rate with the equation 2, both in exponential growing phase:

$$g = \frac{0.693}{\mu}, \textbf{ Equation 1} \quad ; \quad k = \frac{1}{g}, \textbf{ Equation 2}$$

Where μ represents specific growth rate and was determined directly from the logarithmic plot of the growth data, according to the tendency line. The mean growth rate constant (*k*) represents the rate of growth during the exponential phase and is expressed as the number of generations per unit time. The correspondent doubling time for each bacteria was calculated as an average of the three estimated g, with the associated standard deviation.

3.3. Cell death experiments

Experiments to induce cell death were performed using 4 mL of liquid culture in exponential phase for each microorganism. Two different methods were used to induce death: i) microwaves and ii) water bath. In the former method, liquid cultures were exposed to 100, 300, 450 and 700 watts for 30, 60 or 90 seconds. The second method was performed by the exposure of liquid cultures to 60, 75 and 95 ºC for a period of 1 to 30 minutes for planctomycetes and 2, 5 or 10 minutes for other bacteria. Controls were made without exposure.

Cell death was confirmed by the drop plate method on the adequate medium, *i.e.* three drops of 10 µl from the liquid culture (after exposure) were placed on the adequate media for each strain. The plates were incubated at 37 ºC for *E. coli* and 26 ºC for other microorganisms. The microbial growth was checked after 24 hours for *E. coli*, *A. fischeri*, *V. anguillarum* and *P. putida* or 3 days for planctomycetes, using magnifying glasses (Leica GZ4). Images of the cultures were recorded using the GenoPlex system (VWR). Different growth levels were represented in a range of 0 to 4 (Figure 7), where 4 represents the maximum growth level (same as control) and 0 represents the absence of growth. The level 0.5 was applied when only one or two colonies were formed, and after a longer incubation period.

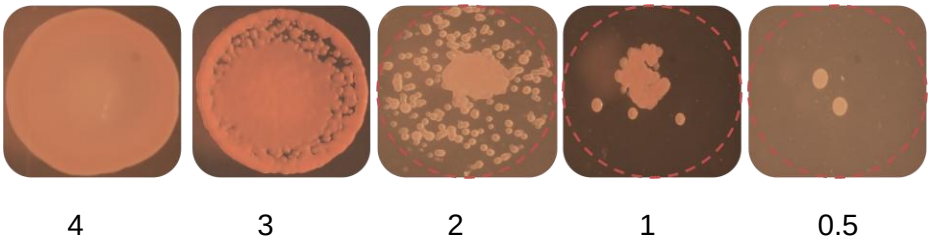


Fig.7 – Bacterial cultures, each drop represents a different growth level (0.5-4). The dashed lines correspond to the area where the drop was placed.

3.4. Toxicity assays

3.4.1. Chemicals

A wide range of pollutants was tested such as: zinc (as $ZnCl_2$, Merck), chromium (as $KCr(SO_4)_2 \cdot 12H_2O$, Merck), copper (as $CuCl_2 \cdot H_2O$, Merck), arsenic (as KH_2AsO_4, Sigma), nickel (as $NiCl_2 \cdot 6H_2O$,Sigma-Aldrich), cadmium (as $CdCl_2 \cdot 2 \cdot 1/2H_2O$, Sigma-Aldrich), nitritie (as $NaNO_2$, Merck), nitrate (as $NaNO_3$, Merck), ammonia (as NH_4Cl, M&B), phosphates ($NaH_2PO_4 \cdot H_2O$, Merck), sodium azide (NaN_3, Riedel-de-Haen), phenol (C_6H_6O, Sigma-Aldrich), hydrazine (as $(NH_2)_2H_2SO_4$, Sigma-Aldrich), Previcur N® (propamocarb hydrochloride, Bayer CropScience), Ridomil Gold SL® (metalaxil-M, Syngenta Agro) IGEPAL CA-630 (Sigma-Aldrich), a common dish detergent, a common hand cleanser, diclofenac (Sigma-Aldrich), acetaminophen (Sigma-Aldrich) and caffeine (Sigma-Aldrich).

3.4.2. Cell viability assays

Stock solutions of the pollutants were prepared in Milli-Q water for heavy metals (5866 µM), ammonium (1.87 M), nitrate (3.53 M), nitrite (1.45 M), Ridomil® (1 %), Previcur N® (10 %), phosphates (0.83 M), hydrazine (15.40 mM), sodium azide (1.54 M), phenol (1.06 M), diclofenac (31.4 mM), acetaminophen (66.2 mM) and caffeine (51.5 mM). For the detergents (IGEPAL CA-630, dish detergent and hand cleanser), the original formulations were used as stock solutions. The exposure of the microorganisms to the pollutants was performed in liquid cultures in the exponential growing phase. One millilitre of culture was harvested by centrifugation at 13 400 rpm during 60 seconds (MiniSpin®, Eppendorf) and the cells resuspended in one millilitre of each pollutant concentration tested. The same range of concentrations for each

pollutant was used for all the bacteria. The exposure times were 30 and 60 minutes. After pollutant exposure, the suspension was centrifuged and resuspended in 1 mL of Milli-Q water. Afterwards, the cells were cultivated on the adequate medium by means of the drop plate method (as described in section *3.3. Cell death experiments*).

3.5. Zeta potential measurements

Zeta potential was measured using a Zetasizer Nano ZS® equipped with a universal dip cell (Malvern Instruments Ltda.). All experiments were performed at 25 ºC and the values obtained were the average of three measurements. In addition, conductivity and standard deviation of the measurements were also registered. The respective OD_{600nm} for exponential or stationary growth stage were monitored for each bacteria.

For determination of dead cells' zeta potential, the cells were killed by heat bath at 95 ºC during 5 minutes. To evaluate the effect of cell concentration in zeta potential alteration, cultures of LF2, *E. coli* and *P. putida* were diluted in the adequate medium for each strain and the OD monitored. The assessment of the photoperiod influence was performed in LF2 cultures, with three different experiments: i) the culture growth in the light; ii) the culture growth in the darkness; iii) the first 2 days of growth in the darkness (until reaching the exponential phase) with posterior transition to the light. The incubation temperature in the three experiments was 26 ºC.

Measurements of the cells exposed to pollutants were performed after being harvested one millilitre of LF2 culture (13 400 rpm during 60 s) and resuspended it in one millilitre of aqueous pollutant solution (in Milli-Q water). The range of pollutant concentrations was the same as in the one used in cell viability assays. In the case of mixed heavy metal exposure, the cells were exposed to a concentration of 586.6 µM of each metal (in a binary mixture).

In all the zeta potential measurements aforementioned, cell viability was monitored using the drop plate method.

3.6. Oxygen consumption measurements

Respirometric assays with *E. coli*, *P. putida*, *R. baltica* and strain LF2 were performed at Institue for Mollecular and Cell Biology (IBMC). The oxygen consumption was measured with a Clark-type oxygen electrode (Oxygraph System, Hansatech). Temperature was controlled with a water-bath at 37 ºC for *E. coli* and 26 ºC for *P.*

putida, *R. baltica* and strain LF2. Culture samples of *E. coli*, *P. putida*, *R. baltica* and strain LF2 were harvested and resuspended in the adequate growth medium up to a approximate final OD_{600nm} of 0.500 AU for *E. coli*, and 0.200 AU for the other bacteria. One millilitre of culture was placed in the oxygraphic chamber. When a constant slope of the oxygen plot was reached, the culture was exposed to 0.77 M sodium azide in order to determine the effect of this pollutant on respiratory activity. Each experiment was performed three times with independent cultures.

3.7. Ultrastructural study

Only strain UC17 was used for transmission electron microscopy (TEM) studies, conducted at Institute of Biomedical Sciences Abel Salazar (ICBAS). Bacterial cells were harvested from exponential growing phase liquid cultures in M13 medium. Two control experiments were performed: i) with the cells resuspended in Milli-Q water; ii) with the cells resuspended in M13 medium. For the arsenic treatment, the cells were resuspended in 59 µM of arsenic solution. In all the three experiments the exposure time was 16 hours. The cells were fixed for 2 hours in 2.5 % (w/v) glutaraldehyde in marine buffer at pH 7.0 (Watson et al. 1986), and post-fixed in 1 % (v/v) osmium tetroxide for 4 hours and in 1 % uranyl acetate for 1 hour. Cells were dehydrated through a graded ethanol series (50 %; 70 %; 90 %; 100 %) and transferred to propylene oxide. The specimens were subsequently embedded in gradient concentrations of propylene oxide:Epon resin (2:1; 1:1; 1:2; 100 % resin). After polymerization, ultrathin sections were obtained. The sections were then collected in copper grids and stained for 10 minutes in 1 % (v/v) uranyl acetate and 10 minutes in Reynolds lead citrate. Sections were examined in a JEOL 100CXII transmission electron microscope.

3.8. *In silico* analysis

The study of the genomes was performed using the database PATRIC (Pathosystems Resource Integration Center). The analysis was made with the genomes available in the database: 23 for Planctomycetes, 610 for *E. coli*, 17 for *P. putida* and 3 for *V. anguillarum*. We used the *Protein Family Sorter* tool to restrict the research to specific protein families (FIGfams): the demethylases (for planctomycetes); the phosphoenolpyruvate carboxykinase; the cytochromes aa3, bd and c. This tool allowed to examine the distribution of the proteins across different genomes.

4. Results and Discussion

4.1. Evaluation of culture growth

Figure 8 shows the growth curves of the planctomycetes strains, and Table 2 provides the doubling times and the growth rate for each strain. In the conditions used, the strains reached the stationary phase in an approximate OD_{600nm} range of 0.230 AU - 0.300 AU for *R. baltica*, LF2 and MsF5; and 0.300 AU - 0.400 AU for UC17, Sm4 and UC9.

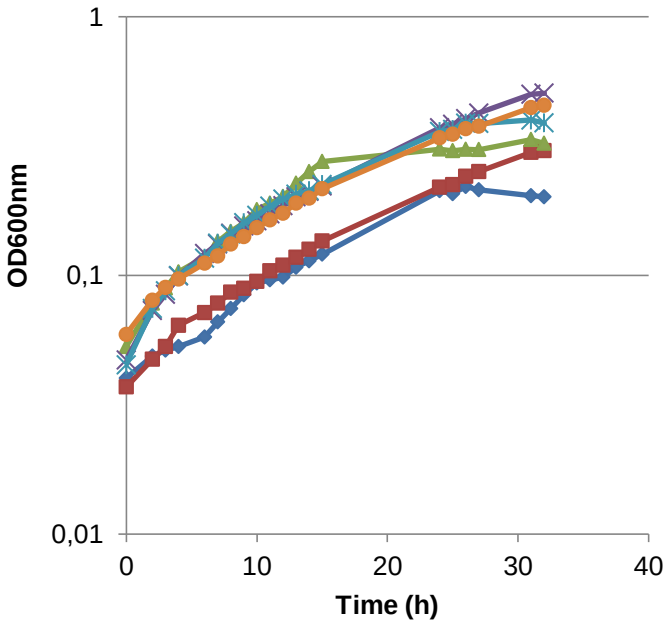


Fig.8 – Growth curve of strains *R. baltica* (♦), LF2 (■), Sm4 (▲), UC9 (×), UC17 (✳) and MsF5 (●) in M13 medium.

Table 2. Parameters analyzed from planctomycetes growth curves.

Bacteria	Doubling time (*g*)	Growth rate (*k*) (generations/h)	Correlation (R^2)*
R. baltica	12 h 12 min	0.08	0.999
LF2	13 h 18 min	0.08	0.999
Sm4	14 h 52 min	0.07	0.998
UC9	15 h 21 min	0.06	0.996
UC17	14 h 40 min	0.07	0.995
MsF5	15 h 42 min	0.06	0.996

* Correlation coefficient, which corresponds to the degree of correlation between the tendency line and the points used for the estimation of the doubling time in the exponential phase.

Regarding the other bacteria tested, their growth curves displayed in Figure 9 showed that they have a faster growth in comparison to planctomycetes strains. *E. coli* is the fastest dividing species, followed by *V. anguillarum*, *A. fischeri* and finally *P. putida* (Table 3).

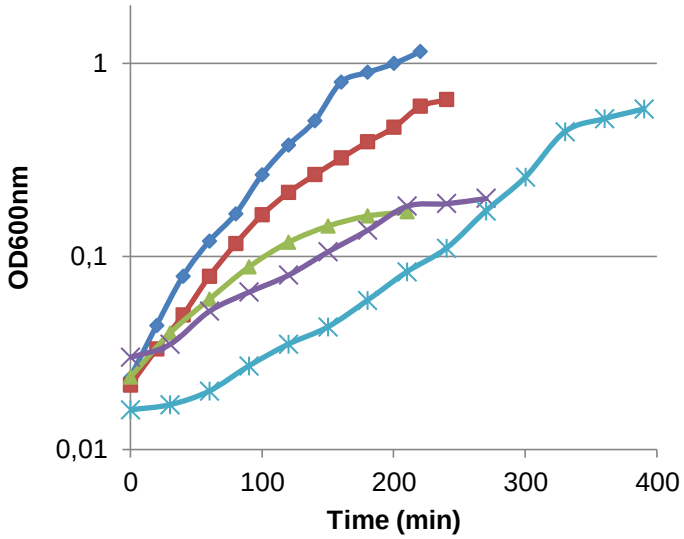


Fig.9 – Growth curve of *E. coli* (♦) in LB medium, *V. anguillarum* (■) and *A. fischeri* (▲) in NB medium, *P. putida* in M13 medium (×) and in NB medium (✳). Each curve represents the mean of 3 growth estimations.

Furthermore, medium NB seemed to be more adequate than the M13 for *P. putida* growth, because of the smaller doubling time observed.

Table 3. Parameters analyzed from the growth curves of *E. coli*, *V. anguillarum*, *A. fischeri* and *P. putida* in the respective medium.

Bacteria	**Doubling time (*g*) ± SD**	**Growth rate (*k*) (generations/h)**	**Correlation (R^2) ± SD***
E. coli	58 min ± 14 min	1.03	0.991 ± 0.004
V. anguillarum	1 h 21 min ± 4 min	0.73	0.994 ± 0.003
A. fischeri	1 h 50 min ± 18 min	0.55	0.978 ± 0.018
P. putida (NB)	2 h 36 min ± 07 min	0.32	0.992 ± 0.007
P. putida (M13)	3 h 8 min ± 41 min	0.38	0.995 ± 0.003

* Correlation coefficient, which corresponds to the degree of correlation between the tendency line and the points used for the estimation of the doubling time in the exponential phase.

In all the growth curves obtained, the end of the exponential phase and the beginning of the stationary phase was observed. This characterization allowed us to select bacteria in the respective growing phase for the following experiments.

4.2. Cell death experiments

In order to find out the best way to kill the bacteria for specific zeta potential analyses, the cell death was assayed by means of microwaves or heat bath exposure. This kind of assays has never been performed with planctomycetes.

For the exposure time assayed, all bacteria maintained the cell viability at 100 watts, with the exception of *A. fischeri* that lost cell viability after 60 seconds of exposure (Table 4). At 300 watts cell viability of all the bacteria was highly affected and among planctomycetes, only three strains could maintain their cell viability (LF2, Sm4 and UC9). Strain LF2 was the most resistant planctomycete to microwaves. Furthermore, among all the bacteria tested, it was the only one that survived after 60 seconds of exposure to 300 watts. This strain was also the only planctomycete that was able to grow at 450 watts, similarly to *E. coli* and *V. anguillarum*. All bacteria died after 90 seconds of exposure to 300 or 450 watts, and in all exposure times at 700 watts.

Table 4. Growth levels of different strains after exposure to 100, 300, 450 and 700 watts, during 30, 60 or 90 seconds in the respective medium.

	Microwaves (watts)											
	100			300			450			700		
Time (s)	30	60	90	30	60	90	30	60	90	30	60	90
R. baltica	4	4	4	0	0	0	0	0	0	0	0	0
LF2	4	4	4	1	1	0	0.5	0	0	0	0	0
Sm4	4	4	4	2	0	0	0	0	0	0	0	0
UC9	4	4	4	2	0	0	0	0	0	0	0	0
UC17	4	4	4	0	0	0	0	0	0	0	0	0
MsF5	4	4	4	0	0	0	0	0	0	0	0	0
E. coli	4	4	4	1	0	0	1	1	0	0	0	0
P. putida	4	4	4	3	0	0	0	0	0	0	0	0
A. fischeri	4	0	0	0.5	0	0	0	0	0	0	0	0
V. anguillarum	4	4	4	2	0	0	1	0	0	0	0	0

Regarding the heat bath experiment, the most resistant bacteria were *E. coli*, which maintained the cell viability at 75 ºC after 2 minutes, *V. anguillarum*, able to grow even after 5 minutes of exposure to 75 ºC (Table 5), *R. baltica* and strains UC17 and MsF5 (Table 6). *A. fischeri* was the most sensitive to temperature and did not grow even at the lowest temperature tested (60 ºC). All the strains died at 75 ºC after 10 minutes of exposure and at 95 ºC.

Table 5. Growth levels of *E. coli*, *P. putida*, *A. fischeri* and *V. anguillarum* after exposure to 60, 75 and 95 ºC, during 2, 5 and 10 minutes in the respective medium.

	Water Bath (ºC)								
	60			75			95		
Time (min)	2	5	10	2	5	10	2	5	10
E. coli	4	3	3	4	0	0	0	0	0
P. putida	3	1	0	0	0	0	0	0	0
A. fischeri	0	0	0	0	0	0	0	0	0
V. anguillarum	3	2	1	2	2	0	0	0	0

The high tolerance demonstrated by *E. coli* and *V. anguillarum* may be explained by the fact that these bacteria are commonly enterobacteria. *E. coli* inhabits normally the gut of warm blooded mammals or birds (Gordon and Cowling 2003), and *V. anguillarum* is a common enteropathogen of various organs of fishes (*see for review* Frans et al. 2011).

Table 6. Growth levels of the different planctomycetes strains after exposure to 60, 75 and 95 ºC, from 1 to 30 minutes in medium M13.

	Water Bath (ºC)																				
	60							75							95						
Time (min)	1	2	5	10	15	20	30	1	2	5	10	15	20	30	1	2	5	10	15	20	30
R. baltica	4	2	.5	0	0	0	0	1	.5	0	0	0	0	0	0	0	0	0	0	0	0
LF2	4	4	.5	0	0	0	0	.5	0	0	0	0	0	0	0	0	0	0	0	0	0
Sm4	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UC9	4	4	2	1	.5	0	0	.5	0	0	0	0	0	0	0	0	0	0	0	0	0
UC17	4	3	1	0	0	0	0	1	.5	0	0	0	0	0	0	0	0	0	0	0	0
MsF5	4	3	2	.5	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Although the presence of planctomycetes living at high temperatures has been detected (Byrne et al. 2009; Giovannoni et al. 1987; Li et al. 2010), studies of temperature tolerance in mesophilic planctomycetes have never been reported. Being these strains naturally mesophiles, it is notorious their tolerance to some tested temperatures. This feature may be related to their particular morphologic features, such as their proteinaceous cell wall with abundant cysteine amino acids (Liesack et al. 1986). Despite the strong disfavor of this aminoacid in thermophilic proteins, it contains motifs that can be fulfilled by other motifs at high temperatures, transforming the aminoacid to confer thermotolerance (Lau et al. 1999; Rosato et al. 2002; Schneider et al. 2002).

Even though microwaves is a faster killing method, due to the great loss of culture volume associated to this strategy, we preferred to use the heat bath at 95 ºC for further zeta potential measurements with dead cultures.

4.3. Cell viability assays

Being the drop plate method used for the screening of cell viability, all the results obtained were compared with control drops (in Milli-Q water), which corresponds to level 4 in the growth range used (0-4). In general, results showed different growth levels for different bacteria, pollutants and their concentrations, suggesting a diverse bacterial sensitivity (summarized in *Annex 3*). As *A. fischeri* was not able to grow after incubation in Milli-Q water, this species was not included in these assays.

Apart from phenol and sodium azide, no other pollutant affected the growth of *E. coli*. This strain, having been isolated from a contaminated river (Cabral and Marques 2006), is probably very well adapted to polluted environments. Some analyses made by these authors led to the conclusion that this river had high levels of ammonia and that *E. coli* strains isolated had the ability to perform nitrate reduction. Moreover, phenomena of resistance acquisition by *E. coli* have been already described in a wide range of compounds, such as heavy metals (Geslin et al. 2001), detergents and biocides (Gaze et al. 2011), or mixed compounds (Nakajima et al. 1995). This resistance capacity is mainly due to the high plasticity of *E. coli* genome either in virulent or commensal strains (Moriel et al. 2012; Tenaillon et al. 2010; van Elsas et al. 2010), and to the relatively low pollutant concentrations in the contaminated habitats, which cause sub-lethal pressures in the *E. coli* strains as described by some of the previous studies.

In contrast, *V. anguillarum* was, in general, the most sensitive bacterium, which may be due to its lifestyle as an endoparasite of fishes. It normally inhabits inside fishes' organs, where it can be protected from external stresses.

Heavy Metals

Cell viability differences between planctomycetes and the other bacteria tested are shown in Figure 10 (a-f). In general, the concentration 59 µM did not affect the growth of the bacteria tested.

In most cases, a dose-response effect was evident in planctomycetes. Although mechanisms of metal resistance or toxicity were not yet described in planctomycetes, the rich protein nature of their cell wall and the glycoproteins in their fimbriae exhibit a wide range of metal ligand groups, providing a diversity of interactions. These groups include carbonyl groups, sufhydryl groups and polysaccharide complexing sites (Hughes and Poole 1989; Rajkumar et al. 2010).

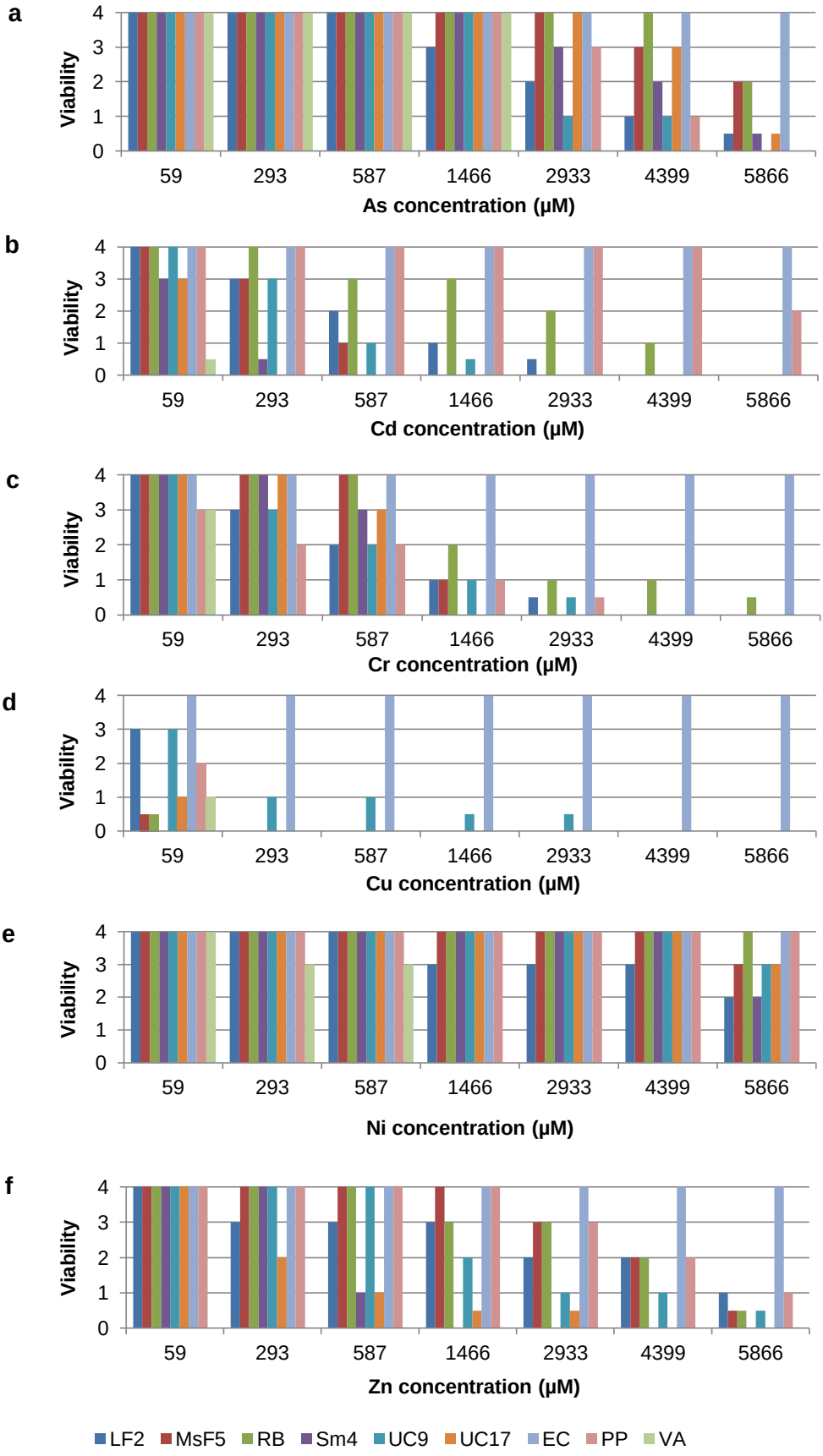


Fig.10 - Bacterial cell viability after 30 minutes of exposure to different heavy metal concentrations: arsenic (a), cadmium (b), chromium (c), copper (d), nickel (e) and zinc (f). RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Regarding planctomycetes cell wall, one of the most important features is certainly the high availability of cysteine amino acids (Liesack et al. 1986) and the YTV domains (Hieu et al. 2008), which have many sulfhydryl groups. In bacteria, heavy metals bind to these groups inactivating proteins.

The sensitivity order showed by planctomycetes to the different metals was: Ni < As < Zn < Cr < Cd < Cu, being *R. baltica* the most resistant strain. Nickel was the only metal that never caused the complete loss of cell viability. Unexpectedly, zinc showed a stronger toxic effect than arsenate, in particular for strain Sm4 and UC17. In fact, other studies suggested that the effects of zinc in microbial communities, as well as in other aquatic living organisms, are underestimated (Paulsson et al. 2000). Furthermore, the relatively low toxicity of arsenic, in the form of arsenate (As^{5+}) can be explained by the lower toxic potential of this form when compared to other arsenic compounds (e.g. As^{3+}) (ATSDR 2007). Copper, which is a widely known biocide (Borkow and Gabbay 2005), was the most toxic metal in this study. Furthermore, high toxicity of copper in other microorganisms was already reported and several associated mechanisms were proposed (Boivin et al. 2005; Ore et al. 2010; Santo et al. 2011; Warnes et al. 2012). Kungolos et al. (2009) observed that copper induced a high toxic effect on different tested species (including the bacteria *A. fischeri*) at concentration levels lower than 0.1 mg/L, which are lower than the ones tested in this study.

In general, *P. putida* was more resistant to metals than the majority of the planctomycetes. The ability observed in *P. putida* to tolerate heavy metals (with the exception of copper) agrees with previous genomic studies which showed that the genome of these bacteria encodes several mechanisms for heavy metals tolerance, maintaining homeostasis. These include two systems for arsenic, one for chromate, four to six systems for divalent cations, two systems for monovalent cations, one metallothionein for metal(loid) binding and four ABC transporters for the uptake of essential Zn, Mn, Mo and Ni (Cánovas et al. 2003; Hu and Zhao 2006).

In the case of *E. coli*, due to its high genomic plasticity (as previously mentioned), several mechanisms of resistance, tolerance and co-tolerance have been reported (Abskharon et al. 2010; Bouzat and Hoostal 2013; Cohen et al. 1991; Su et al. 2011).

Inorganic Nitrogen Compounds

Nitrates, nitrites and ammonium did not cause the complete loss of cell viability in planctomycetes (Fig. 11, a-c). Similarly to *E. coli*, the planctomycetes strains maintained their cell viability when exposed to these compounds and only the strains

LF2 and UC17 were slightly affected by ammonium at the highest concentration (1.87 M) after 30 minutes. On the other hand, *P. putida* and *V. anguillarum* were affected by the three compounds: *P. putida* growth was totally inhibited at 1.77 M nitrates (after 60 min of exposure) and *V. anguillarum* cells were not viable at 1.87 M ammonium. Ammonium toxicity was reported in lower concentration (10 mmol/L), reducing the growth by 65 % of cyanobacteria isolated from freshwater (Dai et al. 2008).

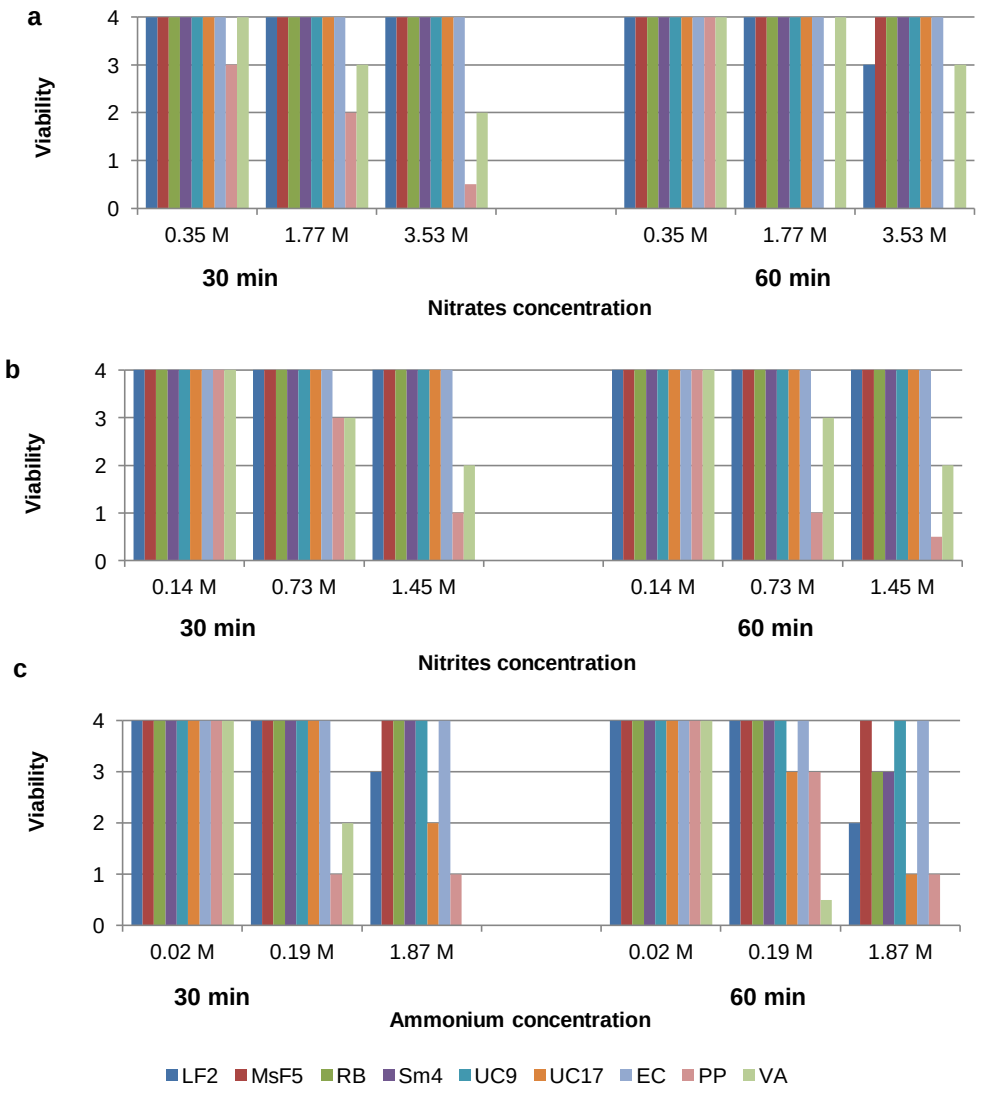


Fig.11 - Bacterial cell viability after 30 or 60 minutes of exposure to different concentrations of inorganic nitrogen compounds: nitrate (a), nitrite (b) and ammonium (c). RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Tolerance to the bacteriostatic action of sodium nitrite in different bacteria was earlier reported (Castellani and Niven 1955). Recently, Fu et al. (2013) induced resistance to nitrite in *E. coli* and *Shewanella oneidensis* at concentration of 5 mM. However, and as far as we know, tolerance to nitrite as tested in this study has never

been reported. In fact, nitrate, nitrite and ammonium, when supplied separately as the only nitrogen source, support the growth of these strains of planctomycetes (unpublished data). Since these planctomycetes were isolated from coastal marine environments, they may be important key players in the inorganic part of the nitrogen cycle in these habitats.

Phosphates

Phosphate is an indispensable nutrient for the formation of nucleic acids and the cell membranes. In this way, the majority of the studies including microorganisms tested the effects of phosphate depletion and not the phosphate toxicity. The main ecological problem of the phosphates in high concentrations is the eutrophication, due to the runoffs with agricultural fertilizers and detergents (Kleinman et al. 2011). But even in high concentrations the microbial community is not affected or in some cases can increase their biomass, as occurred with microalgae (Lehman 1976) and bacteria (Jordaan and Bezuidenhout 2013). Other studies also suggested that the phosphate itself not only does not have a negative effect in the microbial growth (Appenzeller et al. 2001), but also in microbial community structure, ecophysiological index and colony-development index (Sarathchandra et al. 2001).

In the case of *E. coli*, it was early described that when the inorganic phosphate is in excess, the PHO regulon (responsible for phosphorus assimilation) is inactivated and the necessary inorganic phosphate for the cell is taken up by low affinity with inorganic phosphate transporters (Wanner 1993).

In contrast, as observed in Figure 12, phosphates had a strong effect on cell viability of planctomycetes (mainly in *R. baltica*), *P. putida* and *V. anguillarum*. Indeed, at the lowest phosphate concentration (0.08 M) almost all the bacteria were affected and *V. anguillarum* did not grow at all. However, at 0.42 M only *R. baltica* lost completely the cell viability and *E. coli*, MsF5 and Sm4 could tolerate 0.83 M of phosphates even after 60 minutes of exposure.

The strong effect in cell viability may be explained by the use of sodium phosphate. Other studies using phosphate compounds with sodium showed a strong effect in bacterial growth reduction (particularly in Gram negative), which allowed their use to extend the food shelf-life (Dickson et al. 1994; Kim and Marshall 1999). In our work, it was noticed that these compounds can also affect environmental strains. Furthermore, the correlation between the Verrucomicrobia (the closest bacterial group to Planctomycetes) and phosphates has been previously discussed in several studies about dynamics of bacterial community structures (Jordaan and Bezuidenhout 2013; Lindström et al. 2005; Liu et al. 2009). Being Planctomycetes, as well as

Verrucomicrobia, members of the PVC group and sharing various features in common, these bacteria can help to understand the real influence of phosphates among the PVC bacterial cluster.

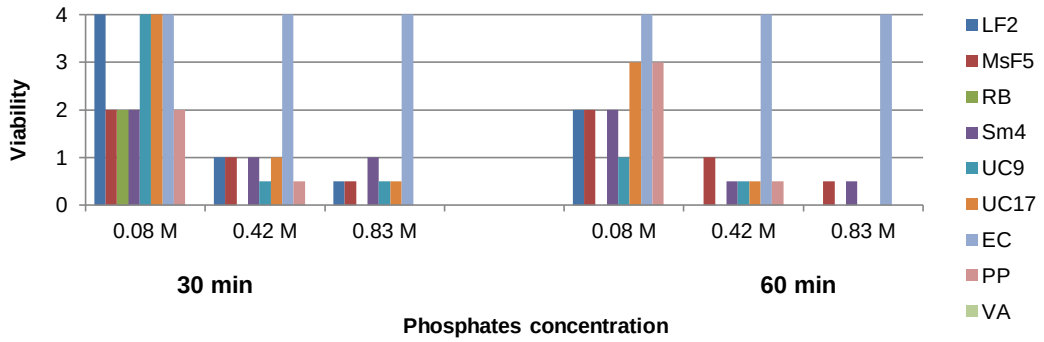


Fig.12 - Bacterial cell viability after 30 or 60 minutes of exposure to different concentrations of phosphates. RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Detergents

The detergents tested had a strong effect on the cell viability of *V. anguillarum* and whenever growth of this species was observed, it was minimal (Figure 13, a-c).

Overall, IGEPAL-C630 was the most toxic detergent, followed by the hand cleanser. However, complete loss of cell viability was only observed at a concentration of 10 % in hand cleanser for strains Sm4 and MsF5, and at 10 % in IGEPAL for MsF5 (in all cases after 60 minutes of exposure). Being surfactants, detergents damage the phospholipidic bilayer of cells, particularly the inner membrane of Gram negative bacteria and promote their self-uptake into the cells. According to Salton (1968) they can penetrate in the cell, react with proteins (causing denaturation) and with lipids (inducing membrane disorganization), and provide the leakage of intracellular low molecular weight material.

However, several resistances to surfactants have been described in Gram negative bacteria, acquired either by chromosomal mutations or plasmid-mediation (Langsrud et al. 2003), and are strongly related to the outer membrane which may prevent their uptake. These resistance mechanisms may be present in the studied bacteria since they are Gram negative. This should also be the case of planctomycetes that recently have been proposed to possess an outer membrane comparable to the Gram negative bacteria (Santarella-Mellwig et al. 2013; Speth et al. 2012).

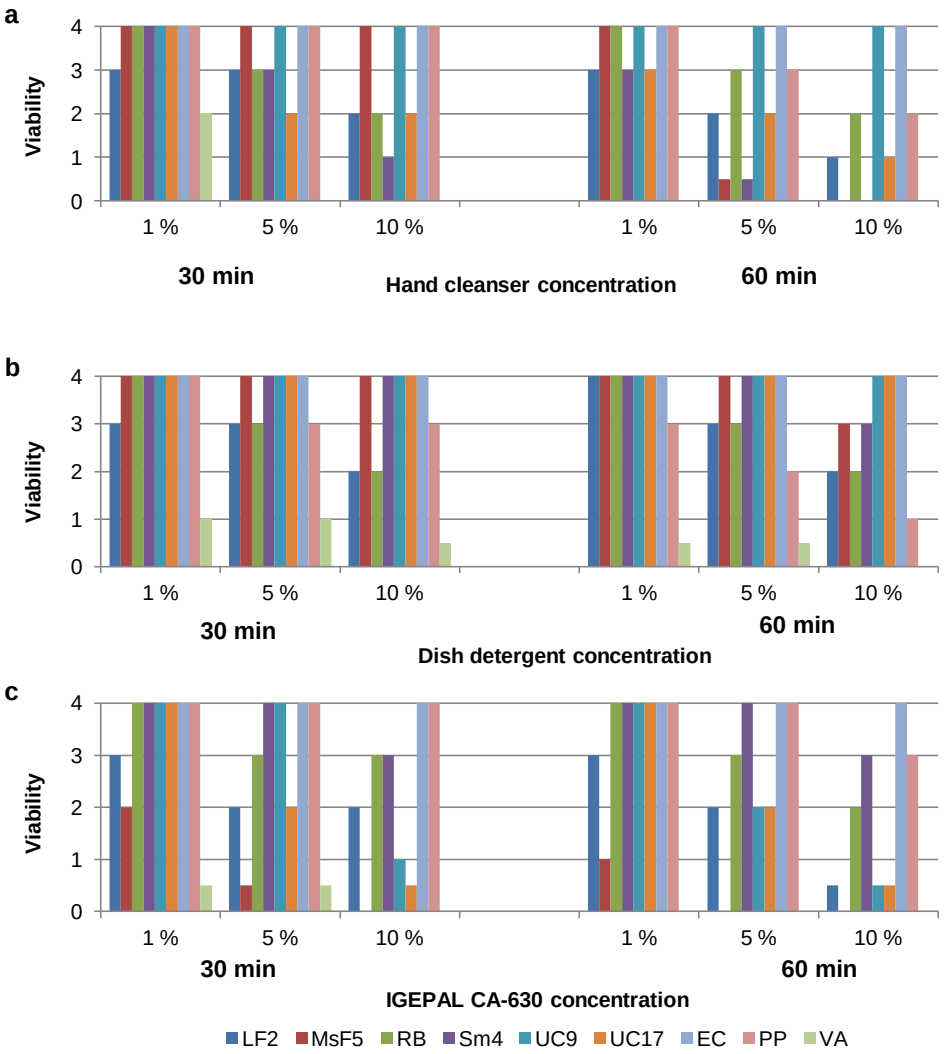


Fig.13 - Bacterial cell viability after 30 or 60 minutes of exposure to different detergents concentrations: hand cleanser (a), dish detergent (b) and IGEPAL CA-630 (c). RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Fungicides

Among the fungicides tested, Ridomil® had a stronger effect than Previcur N® on cell viability of all the bacteria (Figure 14, a-b). Previcur N® only caused the complete loss of cell viability in *R. baltica*, strain LF2 and strain MsF5 (at 10 % of concentration). In contrast, *V. anguillarum* and strain Sm4 lost cell viability when exposed to 0.5 % Ridomil®, and the other planctomycetes at 1 %. Previous studies showed that metalaxyl-M (Ridomil®) was non-toxic for arthropods, vertebrate species (Sukul and Spiteller 2000) and earthworms (Mosleh et al. 2003). On the other hand, tests performed with lower trophic level organisms such as, *A. fischeri*, algae and *Daphnia magna* showed a considerable toxic effect of metalaxyl-M (Kungolos et al. 2009). Considering that concentrations of 1.3 mg/L were found by Graves et al. (2004)

in storm water runoffs, our findings also suggest that metalaxyl-M can cause an environmental risk for non-target organisms.

Whereas Ridomil® inhibits fungal growth and reproduction by imparing the rRNA synthesis (affecting the RNA polymerase activity and consequently the synthesis of nucleic acids), Previcur N® affects the permeability of fungi cells binding to sterols in the membranes and interfering with the biosynthesis of fatty acids and phospholipids within the cell. Planctomycetes contain, in their membranes, lipids like palmitic, oleic and palmitoleic lipids, which are typical of eukaryotes (Kerger et al. 1988). Additionally, *Gemmata obscuriglobus*, a member of the Planctomycetes phylum, produces sterols through an abbreviated pathway which is not common in other bacteria (Pearson et al. 2003). These particular features of planctomycetes may explain their higher sensitivity to Previcur N® in comparision to the other strains tested.

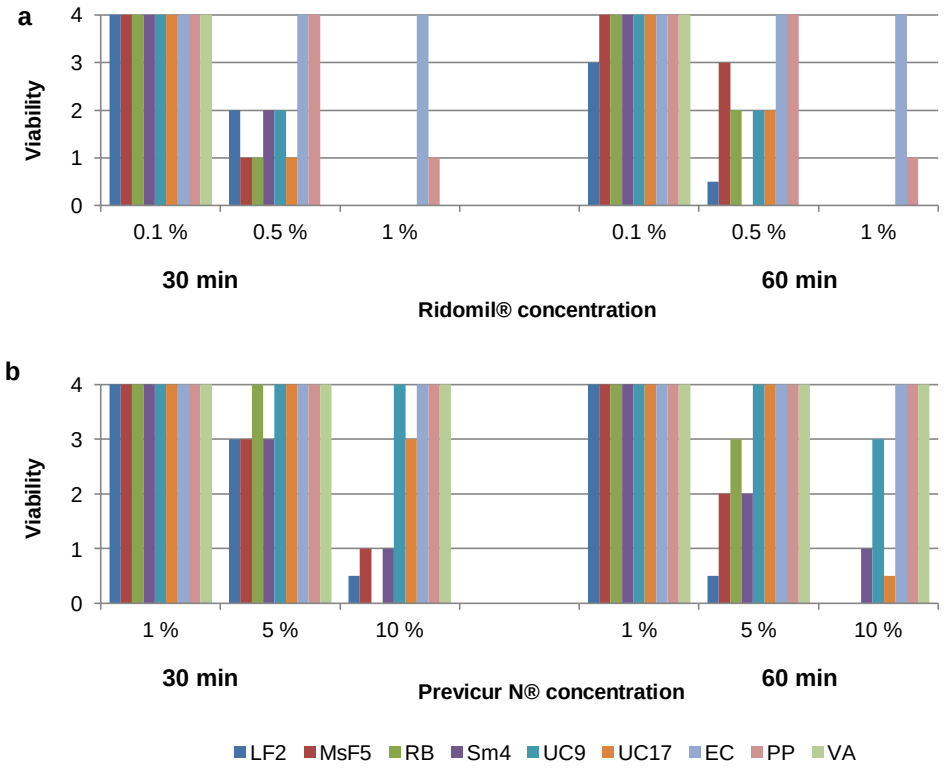


Fig.14 - Bacterial cell viability after 30 or 60 minutes of exposure to different fungicides concentrations: Ridomil® (a) and Previcur N® (b). RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Active pharmaceutical ingredients

The real impact of these ingredients in aquatic living organisms is still unclear and the emergence of APIs is increasing in natural waters (Sanderson et al. 2004). Among the APIs assayed (Figure 15, a-c), diclofenac was the only one that affected the growth of the planctomycetes causing the complete loss of cell viability at 31.4 mM concentration, with the exception of *R. baltica*. Furthermore, it had also a strong effect

on the cell viability of *P. putida* and *V. anguillarum*. The effect of diclofenac in all these bacteria is in agreement with what was previously reported for other microorganisms, such as *A. fischeri* (Yu et al. 2013).

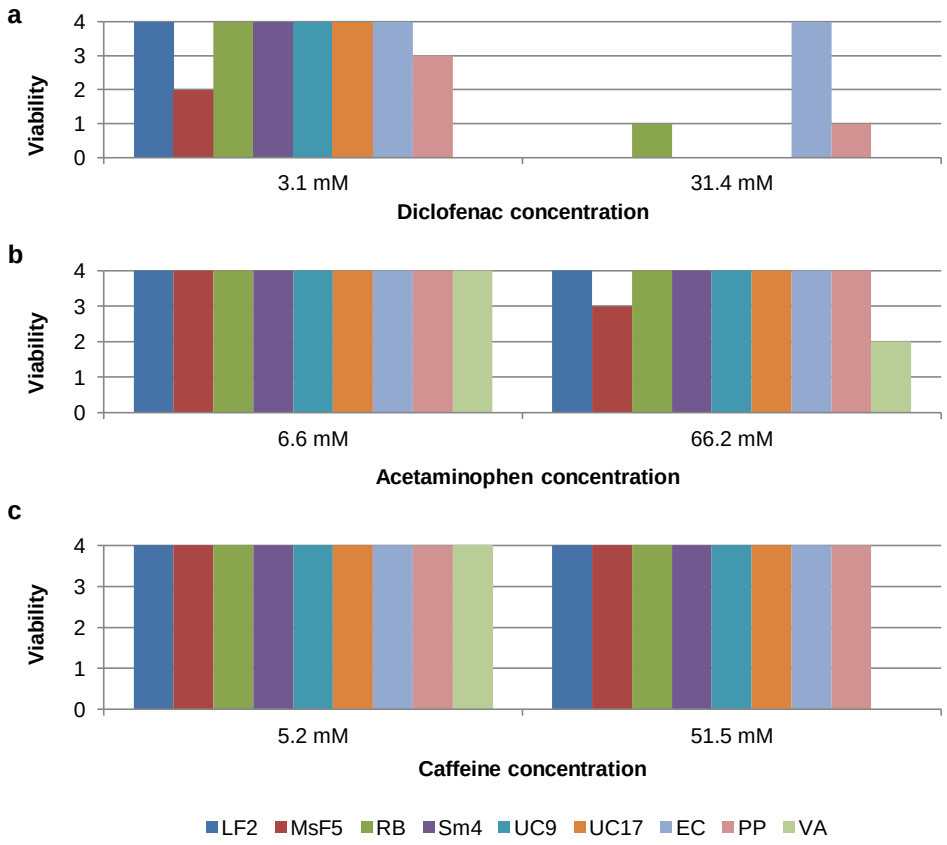


Fig.15 - Bacterial cell viability after 30 minutes of exposure to different APIs concentrations: diclofenac (a), acetaminophen (b) and caffeine (c). RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Caffeine only affected *V. anguillarum* at 51.5 mM concentration. These results disagree with previous ones that indicated the inhibitory effect of caffeine at lower concentrations, mainly in *Pseudomonas* sp., but also in *E. coli* (Dash et al. 2008; Ramanavičienė et al. 2003). However, our results support others that referred the ability of *Pseudomonas* sp. and *E. coli* to tolerate caffeine, mainly due to the N-demethylase activity (Gibson et al. 2012; Summers et al. 2012). Up to now, only one demethylase is referred in genomic microbial databases for planctomycetes (*Gemmata obscuriglobus*) (Table 7).

Table 7. Demethylases present in the genome of *G. obscuriglobus*. The only genome that showed the presence of demethylases (out of 23 analysed using the PATRIC database).

Protein	Protein ID / FIGfam assignment	RefSeq Locus Tag	Length (aa)	Bacteria	Accession number
GbcA Glycine betaine demethylase subunit A	ZP_02735543.1 / FIG00006297	GobsU_01010 0027291	365	*Gemmata obscuriglobus* UQM 2246	NZ_ABGO01 000210

Acetaminophen only slightly affected the cell viability of MsF5 and *V. anguillarum*.

The impact of these APIs in ecosystems has only recently been assessed. The few studies available showed that they can have antibacterial activity as previously described and interfere with the bacteria biofilm communities (Lawrence et al. 2012). However, several bacteria may start adapting to these conditions and changing their genomic information.

Other compounds

Phenol inhibited the growth of all bacterial strains at 0.11 M, just after 30 minutes of exposure (Figure 16). In *V. anguillarum* the growth inhibition also occurred at 0.01 M of phenol after 60 minutes of exposure.

Widely used as disinfectant, phenol acts in the cytoplasmic membrane of Gram negative bacteria, it can enter into the cells by a hydrophobic pathway and then promote a progressive leakage of the intracellular constituents (McDonnell and Russell 2001). Pulvertaft and Lumb (1948) showed that, at lower concentrations (0.032 %) than the ones tested in the present work, phenols agents rapidly lysed growing cultures of *E. coli*, staphylococci and streptococci without involvement of autolytic enzymes. Other studies revealed that phenol can act at the separation point of dividing cells, being the offspring cells more affected (Srivastava and Thompson 1965, 1966).

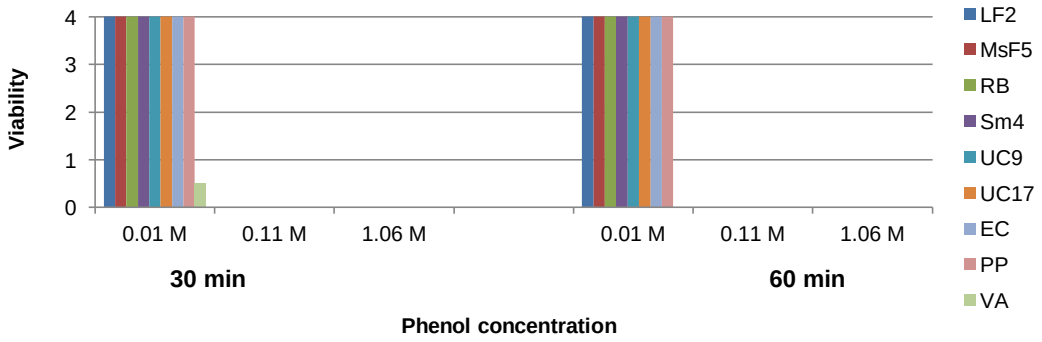


Fig.16 - Bacterial cell viability after 30 or 60 minutes of exposure to phenol. RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Hydrazine at 0.15 mM strongly reduced the cell viability of strains LF2, Sm4 and *P. putida* after 30 minutes of exposure, and also of *R. baltica* and strain UC9 after 60 min (Figure 17). At this concentration *V. anguillarum* did not grow at all. Exposure to 1.54 mM hydrazine for 60 minutes caused the complete loss of cell viability in all the bacteria tested, with the exception of *E. coli*. Apart from the anammox bacteria, resistance to hydrazine, as detected in *E. coli*, is a rare phenomenon in bacteria. This

compound affects the gluconeogenesis of the cells, blocking the conversion of oxaloacetic to phosphoenolpyruvic acids through inhibition of phosphoenolpyruvate carboxykinase (PEPCK) (Prajongtat et al. 2013). *In silico* analysis showed the presence of this enzyme in different planctomycetes genera and in the other bacteria tested (Table 8). In addition, the high toxicity of this compound may be due to the potential induction of DNA damage and consequent inactivation, early well-described in mammalian cells (Bradley et al. 1979; Damjanov et al. 1973; Petzold and Swenberg, 1978) and in salmonella (McCann et al. 1975; Purchase et al. 1978; Sugimura et al. 1976).

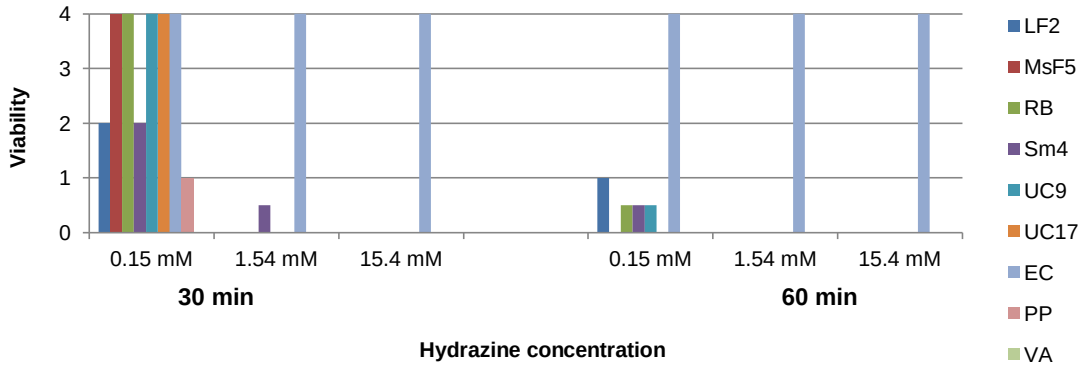


Fig.17 – Bacterial cell viability after 30 or 60 minutes of exposure to hydrazine. RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

Sodium azide affected *E. coli*, *P. putida* and *V. anguillarum* but not planctomycetes (Figure 18). In this way, although planctomycetes are being considered as Gram negative-like bacteria, cell viability results were not in agreement. The growth of *P. putida* was inhibited at 0.77 M and the growth of *V. anguillarum* was inhibited at 1.54 M only after 60 minutes of exposure. Sodium azide is widely used as a respiration inhibitor, interfering with the electron transport chain (ETC) of many different organisms. It is commonly used in media for some Gram positive bacteria, because it is a well-known biocide against Gram negative (Audicana et al. 1995) and few Gram positive bacteria (Das et al. 2005). In bacterial cells, the main targets of sodium azide are the cytochromes of the ETC (Li and Palmer 1993; Little et al. 1996) and ATPases (Daniel 1976; Noumi et al. 1987). Indeed, the effect of sodium azide in *E. coli* cells decreased when mutants altered the protein SecA, which has ATPase activity (Oliver et al. 1990).

Table 8. List of the 13 planctomycetes phosphoenolpyruvate carboxykinase among the 23 genomes analyzed and description of the enzyme found among the other bacteria considered in this study.

Protein / FIGfam assignment	Protein ID	RefSeq Locus Tag	Length (aa)	Pathways involved	Bacteria	Accession number
Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49) / FIG00000834	GAB64027.1	KSU1_D0718	550	Citrate cycle, Pyruvate metabolism, Glycolysis / Gluconeogenesis	planctomycete KSU-1	BAFH01000004
	-	-	525		*Schlesneria paludicola* DSM 18645	AHZR01000030
	YP_003630284.1	Plim_2259	529		*Planctomyces limnophilus* DSM 3776	NC_014148
	YP_003368857.1	Psta_0306	564		*Pirellula staleyi* DSM 6068	NC_013720
	YP_003370088.1	Psta_1553	532		*Pirellula staleyi* DSM 6068	NC_013720
	YP_003370820.1	Psta_2290	546		*Pirellula staleyi* DSM 6068	NC_013720
	YP_004169301.1	Isop_3735	525		*Isosphaera pallida* ATCC 43644	NC_014957
	-	-	508		*Zavarzinella formosa* DSM 19928	AIAB01000143
	ZP_01093904.1	DSM3645_04445	525		*Blastopirellula marina* DSM 3645	NZ_CH672377
	YP_004269567.1	Plabr_1936	532		*Planctomyces brasiliensis* DSM 5305	NC_015174
	ZP_01857289.1	PM8797T_01749	532		*Planctomyces maris* DSM 8797	NZ_ABCE01000048
	EMI17586.1	RMSM_05499	529		*Rhodopirellula maiorica* SM1	ANOG01000778
	EHO67550.1	SinacDRAFT_2770	537		*Singulisphaera acidiphila* DSM 18658	AGRX01000107
Phosphoenolpyruvate carboxykinase [GTP] (EC 4.1.1.32) / FIG00001643	-	-	610		*Gemmata obscuriglobus* UQM 2246	NZ_ABGO01000113

FIGfam assignment	Protein	Length (aa)	Bacteria	Genomes
FIG00000834	Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	540 ± 47.4	*E. coli*	600 (out of 610)
		513 ± 152	*P. putida*	17 (out of 17)
		542	*V. anguillarum*	3 (out of 3)

Furthermore, the genotoxicity of sodium azide in *E. coli* (at 5 mg/ml) (Mamber et al. 1983) and in several *Salmonella typhimurium* strains (de Flora et al. 1984) was earlier described. This genotoxicity is due to the conversion of sodium azide into the mutagenic compound azidoalanine by an exclusive bacterial enzyme – O-acetylserine(thio)lyase.

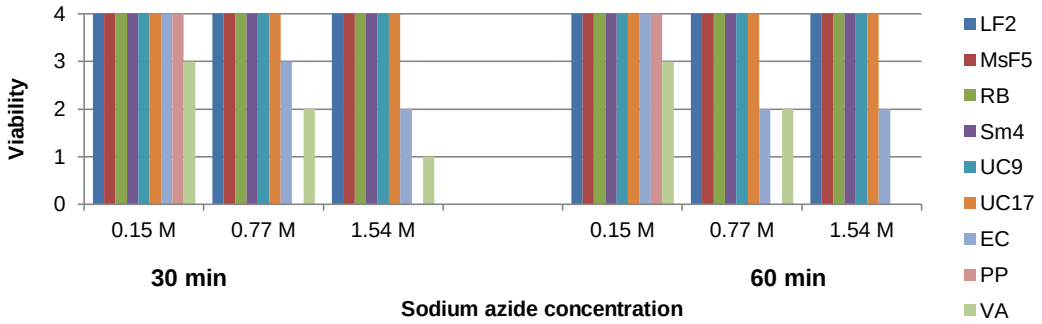


Fig.18 - Bacterial cell viability after 30 or 60 minutes of exposure to sodium azide. RB, *R. baltica*; EC, *E. coli*; PP, *P. putida*; VA, *V. anguillarum*.

4.4. Oxygen consumption measurements

The unexpected resistance to sodium azide displayed by the planctomycetes strains led us to analyze the cell respiration in the studied bacteria. *E. coli* possessed the highest oxygen consumption (OC) (-62.77 ± 2.78 nmol/ml/min), followed by *P. putida* (-41.65 ± 1.03 nmol/ml/min). Planctomycetes presented a much lower oxygen consumption (-11.83 ± 3.33 and -19.13 ± 4.21 nmol/ml/min of, respectively, strain LF2 and *R. baltica*). These differences in the oxygen consumption (Table 9) are in accordance with the differences observed in growth rate of the bacteria studied.

Table 9. Differences in oxygen consumption after introducing sodium azide in liquid cultures of strain LF2, *R. baltica, E. coli* and *P. putida*.

Bacteria	NOC* ± SD (nmol/ml/min)	AOC# ± SD (nmol/ml/min)	% decrease
LF2	-11.83 ± 3.33	-4.32 ± 0.05	63.48 %
R. baltica	-19.13 ± 4.21	-5.14 ± 2.63	73.13 %
E. coli	-62.77 ± 2.78	-10.92 ± 6.35	79.42 %
P. putida	-41.65 ± 1.03	-1.43 ± 0.16	96.57 %

*Normal oxygen consumption (without azide).

#Oxygen consumption after exposure to 0.77 M azide.

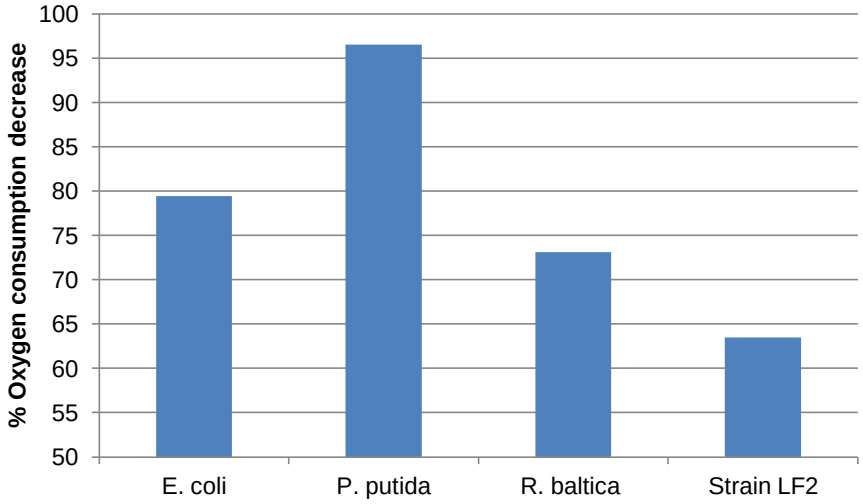


Fig.19 - Decrease in oxygen consumption induced by 0. 77 M sodium azide in liquid cultures of *E. coli*, *P. putida*, *R. baltica* and the strain LF2.

Even though cell viability of planctomycetes was not affected by sodium azide, their cell respiration was affected but lesser than *E. coli* and *P. putida*. Strain LF2 was the least affected strain in the presence of sodium azide (about 63 % OC decrease), followed by *R. baltica* (about 73 % OC decrease). *P. putida* was the most affected bacteria showing approximately 97 % OC decrease (Figure 19).

The bacterial cell respiration is high flexible and may involve various cytochromes (Richardson 2000). *In silico* analysis revealed that several *P. putida* strains not only have cytochrome bd (very common among the bacteria), but also cytochrome c oxidase-related proteins (*Annex 4*), which may explain the stronger effect of sodium azide. Cytochrome c is part of the mitochondrial ETC and is also the one of the main targets of sodium azide in eukaryotic cells (Bennett et al. 1996). Many aerobic bacteria have also cytochrome c proteins participating in nitrogen metabolism and cell respiration (*see for reviews:* Ferguson-Miller and Babcock 1996; Michel et al. 1998), and some studies showed the influence of sodium azide in these proteins (Harrenga and Michel 1999). *In silico* data also showed the presence of cytochrome c proteins in *V. anguillarum* strains, which may explain its high sensitivity in cell viability assays. All the cytochrome c proteins found in *V. anguillarum* are also present in several *P. putida* strains. No cytochrome c involved in cell respiration was found in *E. coli* strains, which is in agreement with its cell respiration involving the cytochromes aa3, bo and bd (Puustinen et al. 1991). No cytochromes bo or aa3 were found in planctomycetes.

The respiratory mechanisms in planctomycetes are not well established yet. Up to now, among the 23 planctomycetes genomes analysed, 25 cytochromes were found being involved or potentially involved in cell respiration: 18 proteins belonging to the

group of cytochrome c, 5 to cyctochrome bd and 2 probable proteins related to the cytochrome cbb3-type (*Annex 5*). Among the cytochromes c proteins in these organisms, some are present also in *P. putida* and *V. anguillarum*, but other ones seem to be particular of planctomycetes.

4.5. Zeta potential measurements

4.5.1. Living and Dead Cultures

The measurements of the zeta potential in living cells showed important differences among the strains tested. Bacterial zeta potential is distributed in the following order: LF2 < UC9 ≈ MsF5 < *R. baltica* ≈ Sm4 < UC17 ≈ *E. coli* < *A. fischeri* < *P. putida* ≈ *V. anguillarum*, from the most negative zeta potential to the less negative (Table 10). Lage et al. (2012) also reported very negative zeta potential values of some planctomycetes strains and suggested a potential relation between the zeta potential values of these strains and the presence of a high number of fimbrae in their morphology. In fact, other studies used zeta potential to distinguish strains with different morphology, such as the presence or absence of LPS (Soon et al. 2011) or capsule (Bayer and Sloyer 1990).

In this study, planctomycetes presented more negative zeta potential values than Gram negative bacteria, mainly during exponential phase. This is particularly the case of strain LF2, which can present zeta potential values between -40 and -55 mV in exponential phase. Furthermore, we detected in stationary growing cultures, a slight decrease of the negative charges (less negative zeta potentials) in LF2, UC9, MsF5, *R. baltica*, *E. coli*, *P. putida*, in contrast to a slight increase in UC17, Sm4, *A. fischeri* and *V. anguillarum*. Previous studies already showed that some strains of Gram negative bacteria varied their zeta potential values according to the growth phase (Soon et al. 2011), but others maintained their zeta potential independently of the growth phase (Tashiro et al. 2010).

Table 10. Determination of zeta potential in living bacterial cultures at exponential (Exp.) and stationary (Sta.) growth phases. Each zeta potential value represents a mean of three measurements.

Bacteria	Growth phase	OD_{600nm}	Zeta Potential (mV)	SD (mV)	Conductivity (S/m)
LF2	Sta.	0.339	-34.1	0.28	1.09
			-33.0	1.77	1.44
	Exp.	0.196	-48.2	2.95	0.94
			-45.5	0.92	0.59
			-47.0	0.70	0.86

Table 10 (continued)

UC9	Sta.	0.390	-31.7	0.99	0.96
			-31.3	1.06	0.83
	Exp.	0.197	-34.9	0.07	0.79
			-34.1	0.42	0.80
			-38.4	0.14	0.91
MsF5	Sta.	0.295	-35.1	0.85	0.85
			-32.0	0.35	0.90
	Exp.	0.220	-35.9	1.13	1.79
			-36.5	0.07	1.52
			-38.3	0.56	0.90
R. baltica	Sta.	0.242	-32.6	0.07	0.90
			-33.0	0.66	0.90
	Exp.	0.164	-35.6	0.14	0.93
			-36.0	0.40	1.20
UC17	Sta.	0.344	-35.1	0.14	0.79
			-37.5	0.28	0.63
	Exp.	0.224	-29.6	0.14	0.60
			-31.1	0.14	0.64
Sm4	Sta.	0.317	-34.7	0.42	0.86
			-37.1	0.42	0.75
	Exp.	0.275	-33.7	0.21	1.12
			-35.1	0.28	1.08
E. coli	Sta.	1.180	-30.0	0.70	0.20
	Exp.	0.500	-37.1	0.30	0.15
P. putida	Sta.	1.100	-19.5	0.78	0.86
	Exp.	0.234	-24.5	1.63	0.59
			-25.2	0.00	0.53
A. fischeri	Sta.	0.145	-28.6	0.85	0.89
			-32.5	0.28	0.41
	Exp.	0.062	-27.6	0.28	0.84
			-28.5	0.92	0.90
V. anguillarum	Sta.	1.325	-27.7	0.71	0.25
	Exp.	0.260	-24.9	1.57	1.35

Similarly to what happened with the culture growth cycle, the zeta potential of dead cells could either decrease or increase (Table 11). However, in this case, the strains that increased their negative charges were UC9, Sm4 and *P. putida*. These small differences in dead cells may be due to the release of charged proteins and other compounds during cell's death. In other studies it was also detected less (but never more) negative zeta potentials (Martinez et al. 2008; Soni et al. 2008).

Table 11. Zeta potential determination in dead cultures. The death was induced at 95 ºC during the exponential growth phase. Living cells were in exponential phase.

	Zeta Potential (mV)		SD (mV)		Conductivity (S/m)	
Bacteria	**Living**	**Dead**	**Living**	**Dead**	**Living**	**Dead**
LF2	-46.9	-34.6	1.52	0.42	0.80	0.94
UC9	-35.8	-36.9	0.21	0.28	0.83	1.11
MsF5	-36.9	-34.0	0.59	0.70	1.40	1.39
R. baltica	-35.8	-32.7	0.27	0.85	1.07	1.25
UC17	-30.4	-29.1	0.14	0.36	0.62	1.24
Sm4	-34.4	-37.0	0.25	0.50	1.10	1.04
E. coli	-37.1	-35.7	0.30	0.40	0.15	0.11
P. putida	-24.5	-26.0	1.60	0.79	0.56	1.18
A. fischeri	-28.1	-26.6	0.86	0.76	0.87	1.21
V. anguillarum	-24.9	-22.6	1.57	0.32	1.35	0.62

Furthermore, the effect of the cell concentration decrease was evident in the decrease of zeta potential values, both in exponential and in stationary phase (Table 12). In several experiments using different bacteria and growth phases the culture dilution led to an increasing of the negative charges. These results are in opposition to the microelectrophoretic study made by Bayer and Sloyer (1990), which described minimal alterations in electrophoretic mobility of *E. coli*, changing cell density. The influence of cell concentration in the increasing of the negative charges is also a factor that contributes to planctomycetes lower zeta potential values, compared to other bacteria in the same growth phase. Therefore, as planctomycetes have fewer generations per time unit in exponential phase than the other bacteria, their zeta potential is more negative (*see 3.1. Evaluation of growth curves*).

Table 12. Effect of cell concentration in zeta potential of *E. coli*, *P. putida* and LF2 cultures.

Strain	Growth phase	OD_{600nm}	Dillution	Zeta Potential (mV)	SD (mV)	Conductivity (S/m)
LF2	Exp.	0.203	100 %	-42.0	2.95	0.94
			75 %	-47.0	2.86	0.96
			50 %	-51.1	3.29	0.50
			50 %	-51.0	0.09	0.40
P. putida	Exp.	0.234	100 %	-24.5	1.63	0.59
			30 %	-27.4	0.28	0.28
			30 %	-26.3	0.28	0.26
E. coli	Sta.	1.180	100 %	-30.0	0.70	0.20
			50 %	-31.3	0.28	0.09
			50 %	-31.7	0.14	0.13
			20 %	-33.5	0.64	0.05
			20 %	-33.9	0.28	0.07

The potential influence of the photoperiod was also assayed in the strain LF2 (Figure 20). In all the conditions tested (bacteria growing in light, in darkness, or only during the first 2 days in darkness) the zeta potential values fluctuated between [-45 mV; -55 mV] during the day. Therefore, all values fall into the normal range considered in this study for the zeta potential values of LF2.

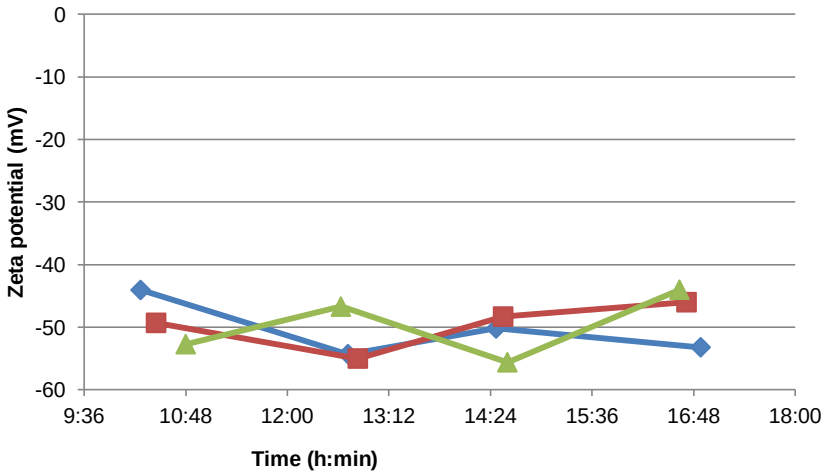


Fig.20 - Evaluation of the photoperiod influence in zeta potential values of LF2 cells in exponential phase: culture growing in light (♦), darkness (▲) or the firts two days in darkness (■)

4.5.2. Exposure to Pollutants

The study of zeta potential after pollutant exposure was conducted mainly with the strain LF2. Although this strain exhibited cell viability variation similar to other planctomycetes, its zeta potential values were more negative than the values of other strains, allowing greater variations of this parameter. The cell viability assessment was performed for all the zeta potential measurements and the results were consistent with the ones obtained previously (*Annex 3*).

Measurements of zeta potential were performed aftber exposure of exponential cultures of LF2 to many kinds of pollutants (Table 13). No relevant zeta potential alterations were observed in the majority of the cases. However, in the exposure to azide, phosphates and detergents, an increasing of the negative charges was detected. This event was also observed in previous studies that related it with absorption and/or degradation of certain compounds, PAHs for example (Rodrigues et al. 2005). There might be a potential relation between cell viability and zeta potential in the case of the inorganic nitrogen compounds and azide, due to the resistance of

planctomycetes to these pollutants (further studies are needed). However, with other pollutants no relation was observed.

Table 13. Influence of several pollutants on zeta potential of strain LF2 and cell viability associated to each measurement.

Bacteria	OD_{600nm}	Conditions	[ ]	Zeta potential (mV)	SD (mV)	Exposure time	Viability
LF2	0.203	Control	-	-43.5	3.57	-	4
		Ammonium	1.87 M	-40.2	1.37	45min	4
		Nitrites	1.45 M	-42.1	1.74	45 min	4
		Nitrates	3.53 M	-38.6	ND*	45 min	4
		Azide	1.54 M	-56.0	1.94	30 min	4
		Previcur®	1 %	-40.9	1.85	30 min	4
			10 %	-40.2	0.46	30 min	0
			10 %	-41.8	0.85	1h 38 min	0
		Phosphates	0.08 M	-51.7	1.25	30 min	3
			0.83 M	-53.9	0.25	30 min	0.5
		Hydrazine	0.15 mM	-47.0	0.20	30 min	1
			0.15 mM	-44.5	0.70	30 min	1
			1.54 mM	-44.0	0.15	30 min	0
	0.180	Control	-	-48.6	3.23	-	4
		Phenol	0.03 M	-43.7	3.41	30 min	2
			0.03 M	-44.5	3.36	30 min	2
		Ridomil®	1 %	-39.9	3.17	30 min	0
		Azide	0.15 M	-63.6	7.40	15 min	4
			0.02 M	-61.6	3.62	15 min	4
			0.02 M	-62.6	4.15	30 min	4
		Dish Det.	1 %	-75.0	12.95	15 min	3
		Hand C.	1 %	-67.5	3.83	15 min	4

* ND, not determined.

Regarding exposure of strain LF2 to heavy metals, a strong increase of zeta potential (decrease in negative charges) was observed with the increasing of metal concentration (Figure 21). In contrast, NaCl did not interfere with the zeta potential.

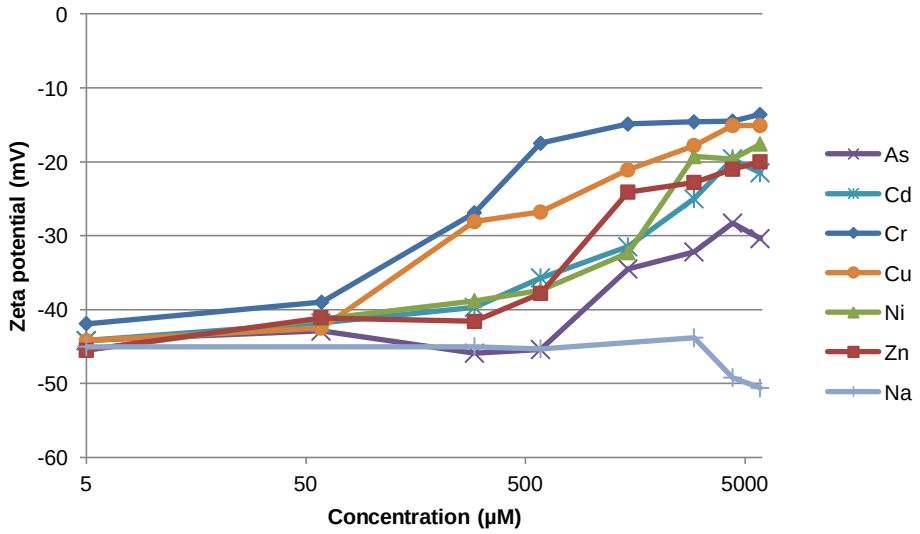


Fig.21 – Effect of heavy metal exposure (As, Cd, Cr, Cu, Ni, Zn) and NaCl in strain LF2 zeta potential.

Furthermore, according to the data obtained after cell viability assessment, a clear relation between the loss of negative charges and the decreasing of cell viability of strain LF2 was evident (Table 14). This finding confirmed previous conclusions made by other authors, suggesting that the surface charge is a function of the viability and nutrient state of cells (Soni et al. 2008). In addition, as strain LF2 was isolated from a marine habitat, this may explain the maintenance of cell viability and the non-reducing effect on its zeta potential after exposure to NaCl.

Table 14. Effect of the increasing of heavy metal concentration or NaCl in zeta potential and cell viability in strain LF2 at OD600nm of 0.202.

Conditions	Concentration (µM)	Zeta potential (mV)	SD (mV)	Conductivity (S/m)	Viability
control	0	-41.9	5.03	0.67	4
	59	-39.0	5.48	0.57	4
	293	-26.9	4.59	0.56	3
	587	-17.5	5.45	0.60	2
Cr	1466	-14.9	6.62	1.25	1
	2933	-14.6	6.58	1.75	0.5
	4399	-14.5	7.83	2.18	0
	5866	-13.6	7.31	2.28	0
control	0	-45.5	6.07	0.62	4
	59	-41.1	6.93	1,03	4
	293	-41.6	5.38	0.46	3
	587	-37.8	6.03	0.76	3
Zn	1466	-24.1	6.35	1.00	3
	2933	-22.8	7.61	1.19	2
	4399	-21.0	7.31	1.36	2
	5866	-20.0	10.30	1.85	1
control	0	-45.5	6.07	0.62	4
	59	-41.3	8.06	0.85	4
	293	-38.8	5.45	0.79	4
	587	-37.4	6.16	1.08	4
Ni	1466	-32.3	7.11	1.04	3
	2933	-19.3	7.53	1.43	2
	4399	-19.6	7.69	1.83	2
	5866	-17.6	8.15	2.01	0.5
control	0	-44.2	5.91	0.59	4
	59	-42.9	7.96	1.02	4
	293	-45.9	6.70	0.79	4
	587	-45.4	5.61	0.67	4
As	1466	-34.5	6.28	0.95	3
	2933	-32.2	6.72	1.06	2
	4399	-28.3	7.51	1.82	1
	5866	-30.4	7.03	1.42	0.5

Table 14 (continued)

control	0	-44.2	5.91	0.59	4
	59	-41.8	6.24	0.65	4
	293	-39.7	6.11	0.49	3
	587	-35.7	8.48	1.27	2
Cd	1466	-31.5	8.38	0.94	1
	2933	-25.0	7.82	1.15	0.5
	4399	-19.6	7.99	1.15	0
	5866	-21.5	7.34	2.07	0
control	0	-44.2	5.91	0.59	4
	59	-42.5	5.31	0.36	3
	293	-28.1	5.79	6.29	0
	587	-26.8	8.56	1.52	0
Cu	1466	-21.1	6.86	0.80	0
	2933	-17.8	7.20	1.23	0
	4399	-15.1	8.80	1.45	0
	5866	-15.1	8.10	1.79	0
control	0	-45.5	6.07	0.62	4
	293	-45.0	6.30	0.83	4
Na	587	-45.3	7.48	0.63	4
	2933	-43.8	8.53	1.47	4
	4399	-49.2	7.01	1.21	4

In comparison with other planctomycetes, LF2 showed a greater shift in zeta potential between the control and the highest metal concentration assayed, providing a strong signal of metal's presence (-28 mV for chromium; -27 mV for nickel and -29 mV for copper) (Figure 22, a-c). However, this difference was not as high as the one observed in *E. coli* (Figure 22, a).

In fact, the influence of heavy metals in the charge alteration of bacteria and yeasts, particularly in the decrease of the negative charges, was reported in many studies. Early studies in 1992 detected the alteration of the electrophoretic mobility in the bacteria *Agrobacterium radiobacter*, *Pseudomonas aeruginosa*, *Bacillus megaterium* and *B. subtilis* in the presence of copper, lead, zinc, mercury, nickel, chromium and cadmium (Collins and Stotzky 1992). Sadowski (2001) described the influence of lead, copper and cadmium in zeta potential modification in the Gram positive *Nocardia* sp. More recently, differences in zeta potential were correlated with the absorption of iron oxide by *Saccharomyces cerevisiae* and *E. coli* (Schwegmann et al. 2010). Additionally, other studies related the zeta potential of different nanometallic particles with their potential toxicity in bacteria (Jiang et al. 2009; Suresh et al. 2010), or in other organisms like *Daphnia* sp. (Griffitt et al. 2008). Some of these studies reported that the zeta potential can work as a tool to understand the toxic mechanism of the metals by the absorption, attachment or repulsion of the particles. In addition, our

results indicate that zeta potential can be used as an indicator of loss of cell viability in planctomycetes. Up to a certain heavy metal concentration, the planctomycetes studied seemed to reach a plateau-like stage, where the reducing effect in zeta potential is slowed down. In strain LF2 this phenomenon happened with all heavy metals tested (Figure 21), and at this phase the bacteria were not able to maintain cell viability. However, this effect was not observed in *E. coli* cells (Figure 22, a), which maintained the cell viability (growth level 4) with the increasing of the negative charges, showing exclusively an effect of charges. Therefore, this effect should be taken into account in toxicity studies performed with *E. coli*.

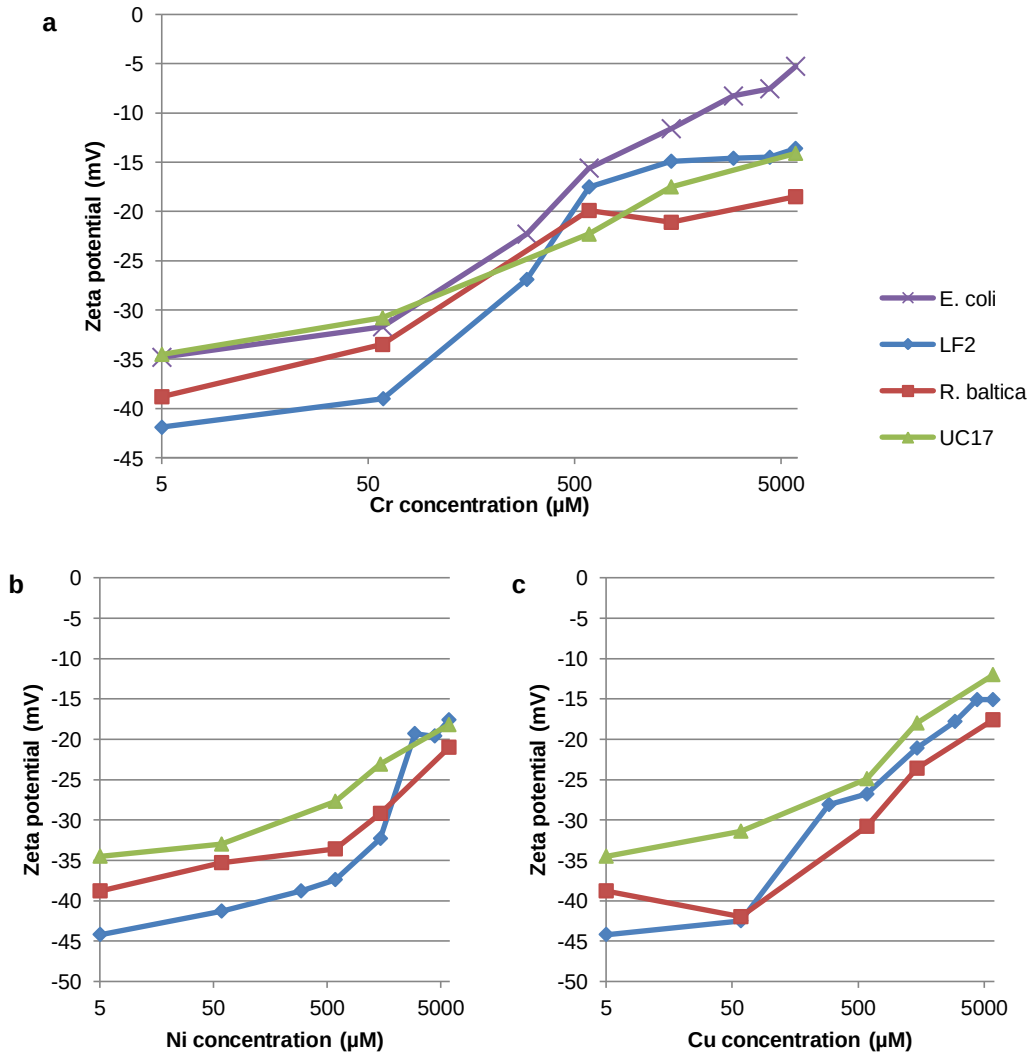


Fig.22 – Comparison of the effect of chromium (a) in strains LF2, UC17 and *R. baltica, E. coli*; nickel (b) and copper (c) in strains LF2, UC17 and *R. baltica*.

In contrast, using the strain LF2 it was possible to detect heavy metals in solution and to distinguish between different toxicity degrees (at least within a certain range), according to the variation of zeta potential. However, between the tested metals there was not a straight relation between the zeta potential values and cell

viability. In this way, it was not possible to identify the most toxic heavy metal based on zeta potential. This virtual application of zeta potential was also explored in other studies, which described its utility as indicator of the bactericidal strength of some antimicrobial compounds (Alves et al. 2010; Chen et al. 2010; Wu et al. 2011).

Moreover, in some cases, the increase of zeta potential values due to the toxic effect of a compound can explain the alteration in the cell adhesion to surfaces in the presence of bactericidal or nutrient-limited conditions (Cowan et al. 1992; Jones et al. 2003; Lewis et al. 1994).

In order to detect some synergistic or antagonistic effects, a preliminary screening of zeta potential and viability alteration was performed with binary mixtures of heavy metals. In general, the viability assays revealed a synergistic effect of the metal mixtures comparing with the previous viability results obtained for each metal (*Annex 3*). As copper caused the cell death either alone or in mixtures, no antagonistic effect between this metal and the other ones was observed. In fact synergistic effects involving copper and other metals are commonly observed in bacteria. Hussein et al. (2004) reported the synergism of Cu and Cr in *Pseudomonas* sp. and Nwuche and Ugoji (2008) described a synergistic effect of Cu and Zn in the reduction of the soil microbial activity. Using 4 different bacteria and 2 yeasts, Collins and Stotzky (1992) detected a synergistic effect of Cu and Ni, which influenced the electrophoretic mobility of the strains. Other studies reported also Cu synergic effects with iron (Bird et al. 2013) and silver (Lin et al. 1996), which had bacteriostatic activity. Additionally, it was possible to relate zeta potential values after heavy metal mixtures exposure to the cell viability results. The less toxic mixtures (As + Cd; As + Ni; As + Ni; Cd + Ni; Cd + Zn and Ni + Zn) were the ones that caused less negative zeta potential values in LF2.

Table 15. Influence of heavy metal mixtures (586.6 µM of each metal) in zeta potential of strain LF2.

Strain	OD_{600nm}	Mixture	Zeta potential (mV)	SD (mV)	Conductivity (S/m)	Viability
LF2	0.202	0 - control	-56.0	6.16	0.361	4
		Cr + As	-20.0	6.49	0.835	0.5
		Cr + Cu	-19.0	5.95	0.959	0
		Cr + Cd	-18.1	6.41	0.734	0.5
		Cr + Ni	-17.3	6.80	0.760	1
		Cr + Zn	-15.6	6.20	0.709	1
		As + Cu	-24.0	7.91	0.765	0
		As + Cd	-36.5	6.61	0.857	2
		As + Ni	-39.3	9.19	0.837	2
		As + Zn	-35.1	7.32	0.849	2
		Cu + Cd	-24.3	6.20	0.831	0
		Cu + Ni	-24.6	7.57	0.800	0
		Cu + Zn	-24.1	6.72	0.772	0
		Cd + Ni	-33.0	7.41	0.758	2
		Cd + Zn	-35.7	7.13	0.897	2
		Ni + Zn	-33.8	8.99	0.841	2

However, zeta potential values after exposure to mixtures were similar to the values obtained after exposure to one of the metals in the mixture – the one that provided a less negative zeta potential when tested individually (Table 14). Therefore, distinction between a toxic single metal and toxic mixtures was not possible exclusively by zeta potential determination. Nevertheless, further research is needed to better understand the real effect of metal mixtures in cellular zeta potential.

4.6. Ultrastructural study

With the microscopy studies performed using the strain UC17 we detected some differences between the two tested controls, with medium M13 (Figure 23, a-b) and Milli-Q water (Figure 23, c-e).

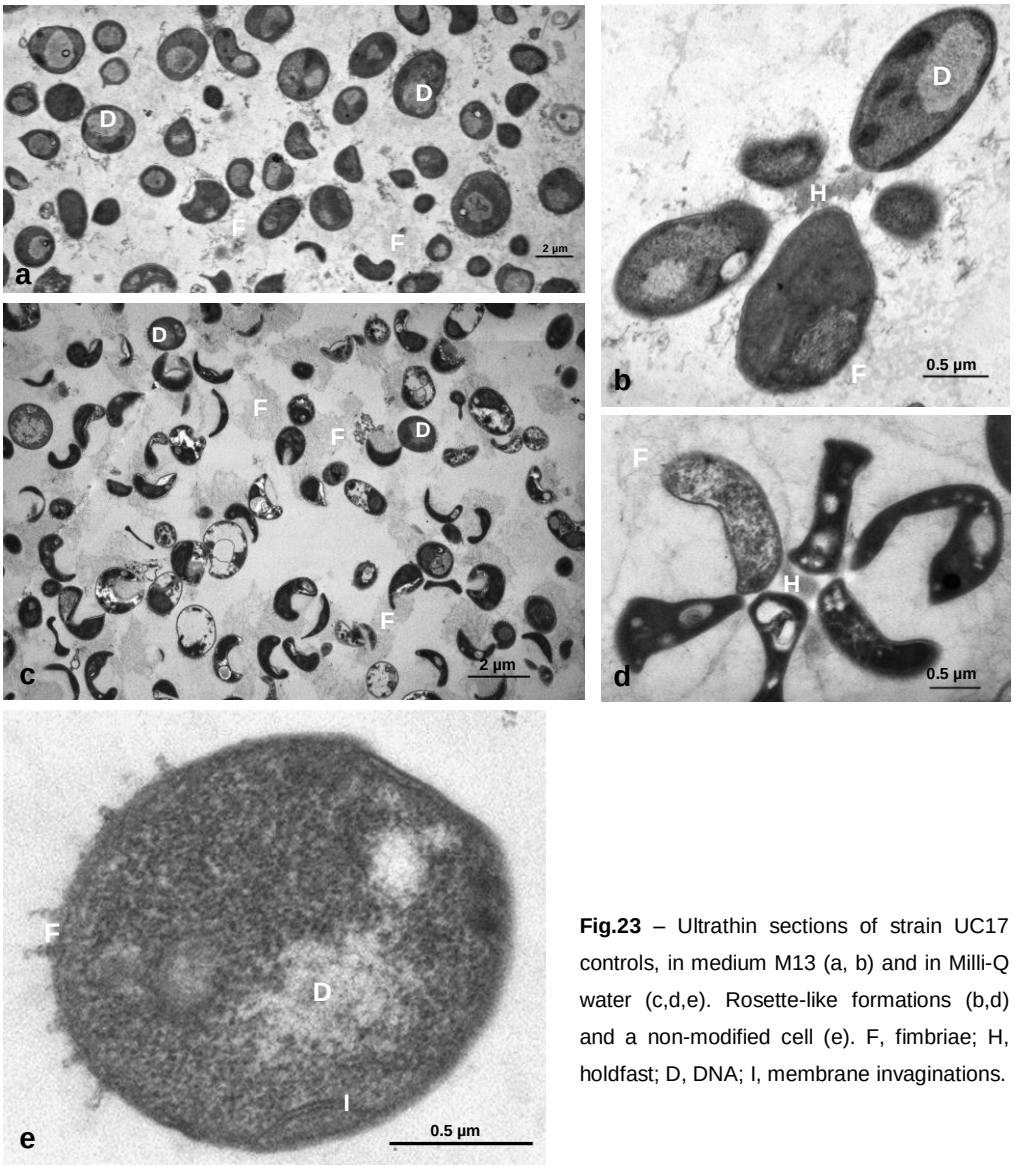


Fig.23 – Ultrathin sections of strain UC17 controls, in medium M13 (a, b) and in Milli-Q water (c,d,e). Rosette-like formations (b,d) and a non-modified cell (e). F, fimbriae; H, holdfast; D, DNA; I, membrane invaginations.

In Milli-Q water, a high number of cells with morphological modifications were observed, namely cell shape changes, which raises problems for further studies (Figure 23, c). Nevertheless, it was verified the high quantity of fimbriae in all the cases, which influences the high negative zeta potential value of this strain.

After arsenic exposure (Figure 24), bacteria revealed high cell disorganization, an increasing in electron transparent areas (with vesicle appearance) (Figure 24, a-c) and DNA decondensation (Figure 24, c).

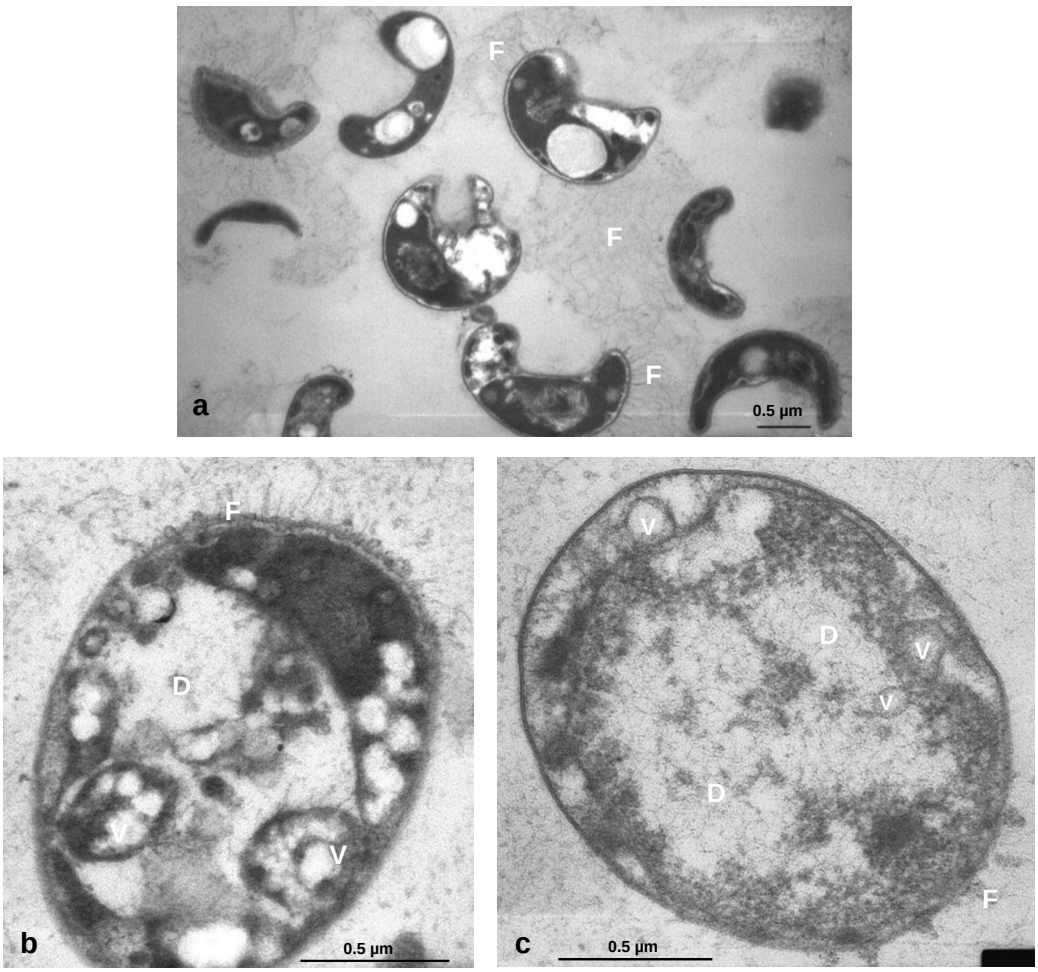


Fig.24 – Ultrathin sections of strain UC17 after 59 µM arsenic treatment during 16 hours (a-c). F, fimbriae; DD, decondensed DNA; V, vesicle-like structures.

According to the cell viability assessment in the TEM experiments, bacteria in Milli-Q water treatment maintained their cell viability while the arsenic-treated bacteria had their cell viability reduced to 0.5 / 1. Therefore, the modifications occurred in the control with Milli-Q water were not sufficient to reduce cell viability of UC17. On the other hand, the reduction of cell viability in the arsenic-treated bacteria can be related to the structural modifications observed.

5. Conclusions

The cell viability studies performed revealed important differences on cell viability of planctomycetes comparing to the other bacteria, which may be relevant for the understanding of the alteration of the microbial community structure in habitats under stress. Furthermore, these different sensitivity responses provided new insights about planctomycetes ecophysiology.

Regarding the pollutants tested, stronger effects were observed in the presence of Ridomil®, phenol, hydrazine and copper. In most cases, *E. coli* was the most resistant bacteria and *V. anguillarum* the most sensitive. The extreme resistance of this *E. coli* strain might be a consequence of the polluted habitat from where it was isolated, allowing the acquisition of resistance.

A potential role in the inorganic nitrogen cycle is envisaged for planctomycetes due to their resistance to extremely high concentrations of inorganic nitrogen compounds.

Although sodium azide did not reduce cell viability of planctomycetes, it affected their cell respiration, but in a slighter way than affected other bacterial species. Further studies are needed to clearly understand the mechanisms of cell respiration in planctomycetes, and the impact of sodium azide in the process.

The measurement of bacterial zeta potential may represent a practical, rapid and easy method to detect heavy metal contamination in water, particurlaly using strain LF2, which has a dose-response behavior and a very negative zeta potential value. Therefore, it was possible to establish a relation between zeta potential and cell viability of these bacteria when exposed to heavy metals. Cell concentration of the cultures and their growth state are factors that must be taken into account when zeta potential measurements are performed.

Thus, a completely new bioassay could be envisaged, with a new biological element and a new transducing element. However, more research is needed to confirm the results obtained, using a large range of concentrations, other planctomycetes and non-planctomycetes strains, as well as, different metals / mixtures.

6. Future Perspectives

In this work we verified that zeta potential is a very promising tool for the assessment waters contaminated with heavy metals. However, some technical limitations make the study of some samples unfeasible. For instance, samples with high conductivity hinder the zeta potential measurement of the cells. Furthermore, in order to compete with the majority of the biosensors already available, it is mandatory that this technique becomes available as a portable device, allowing the assessment *in situ*. Studies with natural waters contaminated with heavy metals, as well as studies comparing this method to commercialized biosensors are needed to completely validate our results.

As a bacterial group with many gaps in their physiological characterization, it is important to continue the research on planctomycetes mechanism of cell respiration, as well as to assess the expression of cytochrome genes and detect the influence of sodium azide *in vitro* (using mutants for example). This compound can also be potentially used in planctomycetes isolation: by pre-washing the sample (e.g. algae) or incorporating azide into the culture media to reduce bacterial contamination as it is done with some specific media for Gram positive bacteria.

Planctomycetes resistance to the inorganic nitrogen compounds may provide important information about their role in the inorganic nitrogen cycle. Additionally, analyses about the potential mechanisms of resistance, or even degradation or bioaccumulation of these compounds might enable planctomycetes to be used in bioremediation of places contaminated with this kind of pollutants.

The presence of particular lipids in planctomycetes associated to the sensitivity to Previcur N®, may lead to new insights about the properties of these lipids and their biotechnological potential.

Our transmission electron microscopy results revealed some particularities in morphology modification. The ultrastructural study of the pollutants effect in planctomycetes should be further extended.

Despite the gaps in the knowledge about the real importance of planctomycetes on the environment and their place in the evolutionary scale, it is certain that these microorganisms have a crucial role in Earth's ecology and might have a high biotechnological potential due to their particular features.

7. References

Abskharon, R.N., Hassan, S.H., Kabir, M.H., Qadir, S.A., El-Rab, S.M.G. and Wang, M.H., 2010. The role of antioxidants enzymes of *E. coli* ASU3, a tolerant strain to heavy metals toxicity, in combating oxidative stress of copper. World J. Microbiol. Biotechnol. 26, 241-247.

Agency for Toxic Substances and Disease Registry (ATSDR), 2007. U.S. Department of Health and Human Services. Public Health Service. Agency for Toxic Substances and Disease Registry Toxicolocigal Profile for Arsenic. August 2007

Agency for Toxic Substances and Disease Registry (ATSDR), 2011. Division of Toxicology and Environmental Medicine. Detailed data table for the 2011 priority list of hazardous substances. That will be the subject of toxicological profiles. From http://www.atsdr.cdc.gov/SPL/.

Alahverdi, M. and Savabieasfahani, M., 2012. Metal Pollution in Seaweed and Related Sediment of the Persian Gulf, Iran. Bull. Environ. Contam. Toxicol. 88, 939-945.

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Roig, B. and Gonzalez, C., 2006. A “toolbox” for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69, 302-322.

Alves, C.S., Melo, M.N., Franquelim, H.G., Ferre, R., Planas, M., Feliu, L., Kowalczyk, W., Andreu, D., Santos, N.C., Fernandes, M.X. and Castanho, M.A., 2010. *Escherichia coli* cell surface perturbation and disruption induced by antimicrobial peptides BP100 and pepR. J. Biol. Chem. 285, 27536-27544.

Appenzeller, B.M.R., Batté, M., Mathieu, L., Block, J.C., Lahoussine, V., Cavard, J. and Gatel, D., 2001. Effect of adding phosphate to drinking water on bacterial growth in slightly and highly corroded pipes. Water Res. 35, 1100-1105.

Aresta, A., Calvano, C.D., Trapani, A., Cellamare, S., Zambonin, C.G. and De Giglio, E., 2013. Development and analytical characterization of vitamin (s)-loaded chitosan nanoparticles for potential food packaging applications. J. Nanopart. Res. 15, 1-12.

Ashworth, J. and Symonds, C., 2013. Simplified Microtox® bioassay for drilling waste disposal on lease soils in Alberta. Can. J. Soil Sci. 93, 109-111.

Audicana, A., Perales, I. and Borrego, J.J., 1995. Modification of kanamycin-esculin-azide agar to improve selectivity in the enumeration of fecal streptococci from water samples. Appl. Environ. Microbiol. 61, 4178-4183.

Avidor, J.M., 1974. Novel instantaneous laser Doppler velocimeter. Appl Opt. 13, 280-285.

Baik, M.H. and Lee, S.Y., 2010. Colloidal stability of bentonite clay considering surface charge properties as a function of pH and ionic strength. J. Ind. Eng. Chem. 16, 837-841.

Batzias, F.A. and Siontorou, C.G., 2006. A knowledge-based approach to environmental biomonitoring. Environ. Monit. Assess. 123, 167-197.

Bayer, M.E. and Sloyer, Jr. J.L., 1990. The electrophoretic mobility of gram-negative and gram-positive bacteria: an electrokinetic analysis. J. Gen. Microbiol. 136, 867–874.

Bazant, M.Z. and Squires, T.M., 2004. Induced-charge electrokinetic phenomena: theory and microfluidic applications. Phys. Rev. Lett. 92, 066101.

Bengoechea, J.A., Lindner, B., Seydel, U., Diaz, R. and Moriyon, I., 1998. *Yersinia pseudotuberculosis* and *Yersinia pestis* are more resistant to bactericidal cationic peptides than *Yersinia enterocolitica*. Microbiology 144, 1509–1515.

Bengtsson, M.M. and Ovreas, L., 2010. Planctomycetes dominate biofilms on surfaces of the kelp *Laminaria hyperborea*. BMC Microbiol. 10, 261.

Ben-Khedher, S., Jebali, J., Kamel, N., Banni, M., Rameh, M., Jrad, A., and Boussetta, H., 2013. Biochemical effects in crabs (*Carcinus maenas*) and contamination levels in the Bizerta Lagoon: an integrated approach in biomonitoring of marine complex pollution. Environ. Sci. Pollut. Res. 20, 2616-2631.

Bennett, M.C., Mlady, G.W., Kwon, Y.H. and Rose, G.M., 1996. Chronic in vivo sodium azide infusion induces selective and stable inhibition of cytochrome c oxidase. J. Neurochem. 66, 2606-2611.

Bernhard, A.E., Marshall, D. and Yiannos, L., 2012. Increased variability of microbial communities in restored salt marshes nearly 30 years after tidal flow restoration. Estuaries Coast. 35, 1049-1059.

Besse, J.P., Geffard, O. and Coquery, M., 2012. Relevance and applicability of active biomonitoring in continental waters under the Water Framework Directive. Trends. Analyt. Chem. 36, 113-127.

Beveridge, T.J. and Graham, L.L., 1991. Surface layers of bacteria. Microbiol. Rev. 55, 684–705.

Beveridge, T.J., 1988. Wall ultrastructure: how little we know. In: Actor, P., Daneo-Moore, L., Higgins, M.L., Salton, M.R.J., Shockman, G.D. (Eds.), Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function. American Society for Microbiology, Washington, DC, USA, pp. 3–20.

Bird, L.J., Coleman, M.L. and Newman, D.K., 2013. Iron and Copper Act Synergistically To Delay Anaerobic Growth of Bacteria. Appl. Environ. Microbiol. 79, 3619-3627.

Blake, II R.C., Shute, E.A. and Howard, G.T., 1994. Solubilization of minerals by bacteria: electrophoretic mobility of *Thiobacillus ferrooxidans* in the presence of iron, pyrite, and sulfur. Appl. Environ. Microbiol. 60, 3349–3357

Boivin, M.E.Y., Massieux, B., Breure, A.M., van den Ende, F.P., Greve, G.D., Rutgers, M. and Admiraal, W., 2005. Effects of copper and temperature on aquatic bacterial communities. Aquat. Toxicol. 71, 345-356.

Bonanno, G., Giudice, R. L. and Pavone, P., 2012. Trace element biomonitoring using mosses in urban areas affected by mud volcanoes around Mt. Etna. The case of the Salinelle, Italy. Environ. Monit. Assess. 184, 5181-5188.

Bondoso, J., Albuquerque, L., Nobre, M.F., Lobo-da-Cunha, A., da Costa, M.S. and Lage, O.M., 2011. *Aquisphaera giovannonii* gen. nov., sp. nov., a planctomycete isolated from a freshwater aquarium. Int. J. Syst. Evol. Microbiol. 61, 2844-2850.

Borkow, G. and Gabbay, J., 2005. Copper as a biocidal tool. Curr. Med. Chem. 12, 2163-2175.

Boufahja, F., Hedfi, A., Essid, N., Aïssa, P., Mahmoudi, E. and Beyrem, H., 2012. An observational study on changes in biometry and generation time of *Odontophora villoti* (Nematoda, Axonolaimidae) related to petroleum pollution in Bizerte bay, Tunisia. Environ. Sci. Pollut. Res. 19, 646-655.

Bouzat, J.L. and Hoostal, M.J., 2013. Evolutionary analysis and lateral gene transfer of two component regulatory systems associated with heavy-metal tolerance in bacteria. J. Mol. Evol. 76, 267-279.

Bowen, J.L., Morrison, H.G., Hobbie, J.E. and Sogin, M.L., 2012. Salt marsh sediment diversity: a test of the variability of the rare biosphere among environmental replicates. ISME J. 6, 2014-2023.

Bradley, M.O., Hsu, I.C. and Harris, C.C., 1979. Relationships between sister chromatid Exchange and mutagenicity, toxicity and DNA damage. Nature (Lond.) 282, 318-320.

Brandys, J., Gawlik, M., Moniczewski, A., Rut-Kowska, A. and Starek, A., 1999. Toxicology, selected aspects. (in Polish) Jagiellonian University Press, Cracow.

Brink, K., Jansen van Vuren, J. and Bornman, R., 2012. The lack of endocrine disrupting effects in catfish (*Clarias gariepinus*) from a DDT sprayed area. Ecotoxicol. Environ. Saf. 79, 256-263.

Brinton, Jr. C.C. and Lauffer, M.A., 1959. The electrophoresis of viruses, bacteria, and cells, and the microscope method of electrophoresis. In: Bier, M. (Ed.), Electrophoresis Theory, and Methods, and Applications. Academic Press Inc, New York, pp. 427–492.

Brochier, C. and Philippe, H., 2002. Phylogeny: a nonhyperthermophilic ancestor for bacteria. Nature 417, 244.

Buckley, D.H., Huangyutitham, V., Nelson, T.A., Rumberger, A. and Thies, J.E., 2006. Diversity of Planctomycetes in soil in relation to soil history and environmental heterogeneity. Appl. Environ. Microbiol. 72, 4522–4531.

Bundeleva, I.A., Shirokova, L.S., Benezeth, P., Pokrovsky, O.S., Kompantseva, E.I. and Balor, S., 2011. Zeta potential of anoxygenic phototrophic bacteria and ca adsorption at the cell surface: possible implications for cell protection from caco3 precipitation in alkaline solutions. J. Colloid Interface Sci. 360, 100–109.

Buszewski, B., Kowalska, J., Pacyna, J., Kot, A. and Namieśnik, J., 2002. Interaction between river catchments and the coastal zone: The EuroCat-Vis-Cat project. Oceanological Stud. 31, 107-116.

Byrne, N., Strous, M., Crépeau, V., Kartal, B., Birrien, J.L., Schmid, M., Lesongeur, F., Schouten, S., Jaeschke, A., Jetten, M., Prieur, D. and Godfroy, A., 2009. Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents. ISME J. 3, 117–123.

Cabeen, M.T. and Jacobs-Wagner, C., 2005. Bacterial cell shape. Nat. Rev. Microbiol. 3, 601-610.

Cabral, J.P. and Marques, C., 2006. Faecal coliform bacteria in Febros river (northwest Portugal): Temporal variation, correlation with water parameters, and species identification. Environ. Monit. Assess. 118, 21-36.

Cánovas, D., Cases, I. and De Lorenzo, V., 2003. Heavy metal tolerance and metal homeostasis in Pseudomonas putida as revealed by complete genome analysis. Environ. Microbiol. 5, 1242-1256.

Castellani, A.G. and Niven Jr, C.F., 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. 3, 154-159.

Castenholz, R.W., 1985. *Heliothrix oregonensis*, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll a. Arch. Microbiol. 142, 164–167.

Cayrou, C., Raoult, D. and Drancourt, M., 2010. Broadspectrum antibiotic resistance of Planctomycetes organisms determined by Etest. J. Antimicrob. Chemother. 65, 2119–2122.

Chaiyapechara, S., Rungrassamee, W., Suriyachay, I., Kuncharin, Y., Klanchui, A., Karoonuthaisiri, N. and Jiravanichpaisal, P., 2012. Bacterial community associated with the intestinal tract of *P. monodon* in commercial farms. Microb. Ecol. 63, 938-953.

Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P. and Thouand, G., 2011. A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor. Anal. Bioanal. Chem. 400, 1061-1070.

Chen, L.C., Kung, S.K., Chen, H.H. and Lin, S.B., 2010. Evaluation of zeta potential difference as an indicator for antibacterial strength of low molecular weight chitosan. Carbohydr. Polym. 82, 913-919.

Chiang, G., Munkittrick, K.R., Urrutia, R., Concha, C., Rivas, M., Diaz-Jaramillo, M. and Barra, R., 2012. Liver ethoxyresorufin-O-deethylase and brain acetylcholinesterase in two freshwater fish species of South America; the effects of seasonal variability on study design for biomonitoring. Ecotoxicol. Environ. Saf. 86, 147–155.

Chistoserdova, L., Jenkins, C., Kalyuzhnaya, M.G., Marx, C.J., Lapidus, A., Vorholt J.A., Staley, J.T. and Lidstrom, M.E., 2004. The enigmatic planctomycetes may hold a key to the origins of methanogenesis and methylotrophy. Mol. Biol. Evol. 21, 1234–1241.

Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J. and Sghir, A., 2003. Molecular evidence for novel planctomycete diversity in a municipal wastewater treatment plant. Appl. Environ. Microbiol. 69, 7354-7363.

Cohen, I., Bitan, R. and Nitzan, Y., 1991. The effect of zinc and cadmium ions on *Escherichia coli* B. Microbios. 68, 157–168.

Collins, Y.E. and Stotzky, G., 1992. Heavy metals alter the electrokinetic properties of bacteria, yeasts, and clay minerals. Appl. Environ. Microbiol. 58, 1592–1600.

Cowan, M.M., Van der Mei, H.C., Stokroos, I. and Busscher, H.J., 1992. Heterogeneity of surfaces of subgingival bacteria as detected by zeta potential measurements. J. Dent. Res. 71, 1803–1806.

Cronin, M.T., Netzeva, T.I., Dearden, J.C., Edwards, R. and Worgan, A.D., 2004. Assessment and modeling of the toxicity of organic chemicals to *Chlorella vulgaris*: development of a novel database. Chem. Res. Toxicol. 17, 545-554.

Dai, G., Deblois, C.P., Liu, S., Juneau, P. and Qiu, B., 2008. Differential sensitivity of five cyanobacterial strains to ammonium toxicity and its inhibitory mechanism on the photosynthesis of rice-field cyanobacterium Ge–Xian–Mi (*Nostoc*). Aquat. Toxicol. 89, 113-121.

Damjanov, I., Cox, R., Sarma, D.S.R. and Farber, E., 1973. Patterns of damage and repair of liver DNA induced by carcinogenic methylating agents in vivo. Cancer Res. 33, 2122-2128.

Daniel, J., 1976. Azide-dependent mutants in *E. coli* K12. Nature 264, 90-92.

Das, A., Silaghi-Dumitrescu, R., Ljungdahl, L.G. and Kurtz, D.M., 2005. Cytochrome bd oxidase, oxidative stress, and dioxygen tolerance of the strictly anaerobic bacterium *Moorella thermoacetica*. J. Bacteriol.187, 2020-2029.

Dash, S.S. and Gummadi, S.N., 2008. Inhibitory effect of caffeine on growth of various bacterial strains. Res. J. Microbiol. 3, 457-465.

Daughton, C.G. and Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change?. Environ. Health Persp. 107, 907–938.

de Flora, S., Zanacchi, P., Camoirano, A., Bennicelli, C. and Badolati, G.C., 1984. Genotoxic activity and potency of 135 compounds in the Ames reversions test and in a bacterial DNA-repair test. Mutat. Res. 133, 161–198.

Definition of electrokinetic potential in "IUPAC. Compendium of Chemical Terminology", 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford, 1997. XML on-line corrected version: http://goldbook.iupac.org (2006) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8.doi:10.1351/goldbook.

Delgado, A.V., Gonzalez-Caballero, F., Hunter, R.J., Koopal, L.K. and Lyklema, J., 2007. Measurement and interpretation of electrokinetic phenomena. J. Colloid Interface Sci. 309, 194-224.

Delong, E.F., Franks, D.G. and Alldredge, A.L., 1993. Phylogenetic diversity of aggregate-attached and free-living marine bacterial assemblages. Limnol. Oceanogr. 38, 924–934.

Di Veroli, A., Selvaggi, R. and Goretti, E., 2012. Chironomid mouthpart deformities as indicator of environmental quality: a case study in Lake Trasimeno (Italy). J. Environ. Monit. 14, 1473-1478.

Dickson, J.S., Cutter, C.G. and Siragusa, G.R., 1994. Antimicrobial effects of trisodium phosphate against bacteria attached to beef tissue. J. Food Prot. 57, 952-955.

Dombu, C., Carpentier, R. and Betbeder, D., 2012. Influence of surface charge and inner composition of nanoparticles on intracellular delivery of proteins in airway epithelial cells. Biomaterials. 33, 9117–9126.

Drees, K.P., Neilson, J.W., Betancourt, J.L., Quade, J., Henderson, D.A., Pryor, B.M. and Maier, R.M., 2006. Bacterial community structure in the hyperarid core of the Atacama Desert, Chile. Appl. Environ. Microbiol. 72, 7902–7908.

Dukhin, A.S. and Goetz, P.J., 2004. Ultrasound for characterizing colloids. In: Somasundaran, P., Markovic, B. (Eds.), Concentrated Dispersions, theory, experiments and applications. American Chemical Society symposium series, Washington, DC, USA pp. 91-120.

Dutka, B.J. and Bitton, G. (Eds.) 1986. Introduction and review of microbial and biochemical toxicity screening procedures. In: Toxicity Testing Using Microorganisms. CRC Press, Boca Raton, Florida

Eboigbodin, K.E., Newton, J.R., Routh, A.F. and Biggs, C.A., 2006. Bacterial quorum sensing and cell surface electrokinetic properties. Appl. Microbiol. Biotechnol. 73, 669–675.

Eltzov, E. and Marks, R.S., 2011. Whole-cell aquatic biosensors. Anal. Bioanal. Chem. 400, 895-913.

European Commission, Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council, Off. J. Eur. Comm. L 348 (2008) 84.

Falugi, C., Aluigi, M.G., Chiantore, M.C., Privitera, D., Ramoino, P., Gatti, M.A., Fabrizib, A., Pinsinoc, A. and Matranga, V., 2012. Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. Mar. Environ. Res. 76, 114-121.

Faraudo, J., Calero, C. and Aguilella-Arzo, M., 2010. Ionic partition and transport in multi-ionic channels: a molecular dynamics simulation study of the OmpF bacterial porin. Biophys. J. 99, 2107-2115.

Ferguson-Miller, S. and Babcock, G.T., 1996. Heme/copper terminal oxidases. Chem. Rev. 96, 2889-2908.

Fieseler, L., Horn, M., Wagner, M. and Hentschel, U., 2004. Discovery of the novel candidate phylum “Poribacteria” in marine sponges. Appl. Environ. Microbiol. 70, 3724–3732.

Forterre, P. and Gribaldo, S., 2010. Bacteria with a eukaryotic touch: a glimpse of ancient evolution? Proc. Natl Acad. Sci. USA 107, 12739–12740.

Foster, W.J., Armynot du Châtelet, E. and Rogerson, M., 2012. Testing benthic foraminiferal distributions as a contemporary quantitative approach to biomonitoring estuarine heavy metal pollution. Mar. Pollut. Bull. 64, 1039-1048.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B. and Rediers, H., 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. J. Fish Dis. 34, 643-661.

Freitas, C. and Müller, R.H., 1998. Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN™) dispersions. Int. J. Pharm. 168, 221-229.

Frias, J.E., Gil, M.N., Esteves, J.L., García Borboroglu, P., Kane, O.J., Smith, J.R. and Boersma, P.D., 2012. Mercury levels in feathers of Magellanic penguins. Mar. Pollut. Bull. 64, 1265-1269.

Fu, H., Chen, H., Wang, J., Zhou, G., Zhang, H., Zhang, L. and Gao, H., 2013. Crp-dependent cytochrome bd oxidase confers nitrite resistance to *Shewanella oneidensis*. Environ. Microbiol. 15, 2198–2212.

Fuchsman, C.A., Staley, J.T., Oakley, B.B., Kirkpatrick, J.B. and Murray, J.W., 2012. Free-living and aggregate-associated Planctomycetes in the Black Sea. FEMS Microbiol. Ecol. 80, 402-416.

Fuerst, J.A. and Sagulenko, E., 2011. Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. Nat. Rev. Microbiol. 9, 403-413.

Fuerst, J.A. and Webb, R.I., 1991. Membrane-bounded nucleoid in the eubacterium *Gemmatata obscuriglobus*. Proc. Natl. Acad. Sci. USA. 88, 8184-8188.

Fuerst, J.A., 1995. The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology 141, 1493-506.

Fuerst, J.A., 2011. Microbial diversity beyond *E. coli*: new microbial worlds, new concepts in biology. Microbiology 32, 73-76.

Fuerst, J.A., Gwilliam, H.G., Lindsay, M., Lichanska, A., Belcher, C., Vickers, J.E. and Hugenholtz, P., 1997. Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. Appl. Environ. Microbiol. 63, 254–262.

Fukunaga, Y., Kurahashi, M., Sakiyama, Y., Ohuchi, M., Yokota, A. and Harayama, S., 2009. *Phycisphaera mikurensis* gen. nov., sp. nov., isolated from a marine alga, and proposal of Phycisphaeraceae fam. nov., Phycisphaerales ord. nov. and Phycisphaerae classis nov. in the phylum Planctomycetes. J. Gen. Appl. Microbiol. 55, 267-275.

Gade, D., Stuhrmann, T., Reinhardt, R. and Rabus, R., 2005. Growth phase dependent regulation of protein composition in *Rhodopirellula baltica*. Environ. Microbiol. 7, 1074-1084.

Gall, M.L., Poore, A.G. and Johnston, E.L., 2012. A biomonitor as a measure of an ecologically-significant fraction of metals in an industrialized harbour. J. Environ. Monit. 14, 830-838.

García-García, G., Nandini, S., Sarma, S.S.S., Martínez-Jerónimo, F. and Jiménez-Contreras, J., 2012. Impact of chromium and aluminium pollution on the diversity of zooplankton: A case study in the Chimaliapan wetland (Ramsar site) (Lerma basin, Mexico). J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 47, 534-547.

Gaze, W.H., Zhang, L., Abdouslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J. and Wellington, E.M., 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J. 5, 1253-1261.

Geremias, R., Bortolotto, T., Wilhelm-Filho, D., Pedrosa, R.C. and de Fávere, V.T., 2012. Efficacy assessment of acid mine drainage treatment with coal mining waste using *Allium cepa* L. as a bioindicator. Ecotoxicol. Environ. Saf. 79, 116-121.

Geslin, C., Llanos, J., Prieur, D. and Jeanthon, C., 2001. The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. Res. Microbiol. 152, 901-905.

Gibson, A.M., Morgan, R.M., MacDonald, N. and Nikitin, A.G., 2012. Possible effects of the presence of common household chemicals in the environment: the growth of an aquatic bacterial species on high concentrations of caffeine. J. Biotech. Res. 4, 72-79.

Gilbert, N., 2012. Water under pressure. Nature, 483, 256-57.

Giovannoni, S.J., Godchaux, W., Schabtach, E. and Castenholz, R.W., 1987. Cell wall and lipid composition of *Isosphaera pallida*, a budding eubacterium from hot springs. J. Bacteriol. 169, 2702–2707.

Glockner, F.O., Kube, M., Bauer, M., Teeling, H., Lombardot, T., Ludwig, W., Gade, D., Beck, A., Borzym, K., Heitmann, K., Rabus, R., Schlesner, H., Amann, R. and Reinhardt, R., 2003. Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc. Natl Acad. Sci. USA. 100, 8298–8303.

Glockner, J., Kube, M., Shrestha, P.M., Weber, M., Glöckner, F.O., Reinhardt, R. and Liesack, W., 2010. Phylogenetic diversity and metagenomics of candidate division OP3. Environ. Microbiol. 12, 1218–1229.

Gordon, D.M. and Cowling, A., 2003. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology 149, 3575-3586.

Graves, G.A., Wan, Y. and Fike, D.L., 2004. Water quality characteristics of stormwater from major land uses in South Florida. J. Am. Water. Resour. Assoc. 40, 1405–1419.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C. and Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27, 1972-1978.

Gupta, K., Gaumat, S. and Mishra, K., 2011. Chromium accumulation in submerged aquatic plants treated with tannery effluent at Kanpur, India. J. Environ. Biol. 32, 591-597.

Gupta, R.S., Bhandari, V. and Naushad, H.S., 2012. Molecular signatures for the PVC clade (Planctomycetes, Verrucomicrobia, Chlamydiae, and Lentisphaerae) of bacteria provide insights into their evolutionary relationships. Front. Microbiol. 3, 327.

Harrenga, A. and Michel, H., 1999. The cytochrome c oxidase from *Paracoccus denitrificans* does not change the metal center ligation upon reduction. J. Biol. Chem. 274, 33296-33299.

Hayashi, H., Seiki, H., Tsuneda, S., Hirata, A. and Sasaki, H., 2003. Influence of growth phase on bacterial cell electrokinetic characteristics examined by soft particle electrophoresis theory. J. Colloid Interface Sci. 264, 565–568.

Hering, D., Borja, A., Carstensen, J., Carvalho, L., Elliott, M., Feld, C. K., Heiskanenf, A-S., Johnsong, R.K., Moeh, J., Ponti, D., Solheimh, A.L. and de Bund, W.V., 2010. The European Water Framework Directive at the age of 10: a critical review of the achievements with recommendations for the future. Sci. Total Environ. 408, 4007-4019.

Hiemenz, P.C. and Rajagopalan, R., 1997. Principles of colloid and surface chemistry, 3rd edn. Marcel Dekker, New York.

Hieu, C.X., Voigt, B., Albrecht, D., Becher, D., Lombardot, T., Glöckner, F.O., Amann, R., Hecker, M. and Professor, T.S., 2008. Detailed proteome analysis of growing cells of the planctomycete *Rhodopirellula baltica* SH1T. Proteomics 8, 1608–1623.

Hogan, C.M., 2010. Water pollution. *Encyclopedia of Earth*. Topic ed. Mark McGinley, ed. in chief C. Cleveland, National Council on Science and the Environment, Washington DC. Retrieved March 29, 2011.

Hu, N. and Zhao, B., 2007. Key genes involved in heavy-metal resistance in *Pseudomonas putida* CD2. FEMS Microbiol. Lett. 267, 17-22.

Hughes, M.N. and Poole, R.K., 1989. Metal toxicity. In: Hughes, M.N.; Poole, R.K. (Eds), Metals and microorganisms. Chapman and Hall, London, pp. 252-301.

Hussein, H., Ibrahim, S.F., Kandeel, K. and Moawad, H., 2004. Biosorption of heavy metals from waste water using *Pseudomonas* sp. Electron. J. Biotechnol. 7, 30-37.

Ivanova, A.O. and Dedysh, S.N., 2012. Abundance, diversity, and depth distribution of planctomycetes in acidic northern wetlands. Front. Microbiol. 3, 5.

Jastrzębska, A. and Buszewski, B., 1999. Application of biomonitorig in ecoanalytics. Chem. Inż. Ekol. 6, 1097.

Jenkins, J.S., Flickinger, M.C. and Velev, O.D., 2012. Deposition of composite coatings from particle–particle and particle–yeast blends by convective-sedimentation assembly. J. Colloid Interface Sci. 380, 192-200.

Jetten, M.S., 2008. The microbial nitrogen cycle. Environ. Microbiol. 10, 2903–2909.

Jiang, W., Mashayekhi, H. and Xing, B., 2009. Bacterial toxicity comparison between nano- and micro-scaled oxide particles. Environ. Pollut. 157, 1619-1625.

Jogler, C., Waldmann, J., Huang, X., Jogler, M., Glöckner, F.O., Mascher, T. and Kolter, R., 2012. Identification of proteins likely to be involved in morphogenesis, cell division, and signal transduction in planctomycetes by comparative genomics. J. Bacteriol. 194, 6419-6430.

Jones, D.S., McGovern, J.G., Woolfson, A.D. and Gorman, S.P., 1997. Role of physiological conditions in the oropharynx on the adherence of respiratory bacterial isolates to endotracheal tube poly(vinyl chloride). Biomaterials 18, 503–510.

Jones, J.F., Feick, J.D., Imoudu, D., Chukwumah, N., Vigeant, M. and Velegol, D., 2003. Oriented adhesion of *Escherichia coli* to polystyrene particles. Appl. Environ. Microbiol. 69, 6515–6519.

Jordaan, K. and Bezuidenhout, C.C., 2013. The impact of physico-chemical water quality parameters on bacterial diversity in the Vaal River, South Africa. Water SA, 39, 385-396.

Jordan, M.A., Welsh, D.T., Teasdale, P.R., Catterall, K. and John, R., 2010. A ferricyanide-mediated activated sludge bioassay for fast determination of the biochemical oxygen demand of wastewaters. Water Res. 44, 5981-5988.

Jubeaux, G., Simon, R., Salvador, A., Quéau, H., Chaumot, A. and Geffard, O., 2012. Vitellogenin-like proteins in the freshwater amphipod *Gammarus fossarum* (Koch, 1835): Functional characterization throughout reproductive process, potential for use

as an indicator of oocyte quality and endocrine disruption biomarker in males. Aquat. Toxicol. 112, 72-82.

Judy, J.D., Unrine, J.M., Rao, W., Wirick, S. and Bertsch, P.M., 2012. Bioavailability of gold nanomaterials to plants: importance of particle size and surface coating. Environ. Sci. Technol. 46, 8467-8474.

Jun, S.R., Sims, G.E., Wu, G.A. and Kim, S.H., 2010. Whole proteome phylogeny of prokaryotes by feature frequency profiles: An alignment-free method with optimal feature resolution. Proc. Natl Acad. Sci. USA 107, 133–138.

Kartal, B., Kuenen, J.G. and van Loosdrecht, M.C., 2010. Engineering. Sewage treatment with anammox. Science 328, 702–703.

Kennedy, A.J., Johnson, D.R., Seiter, J.M., Lindsay, J.H., Boyd, R.E., Bednar, A.J. and Allison, P.G., 2012. Tungsten Toxicity, Bioaccumulation, and Compartmentalization into Organisms Representing Two Trophic Levels. Environ. Sci. Technol. 46, 9646-9652.

Kerger, B.D., Mancuso, C.A., Nichols, P.D., White, D.C., Langworthy, T., Sittig, M., Schlesner, H. and Hirsch, P., 1988. The budding bacteria, Pirellula and Planctomyces, with atypical 16S rRNA and absence of peptidoglycan, show eubacterial phospholipids and uniquely high proportions of long chain beta-hydroxy fatty acids in the lipopolysaccharide lipid A. Arch. Microbiol. 149, 255– 260.

Kim, C.R. and Marshall, D.L., 1999. Microbiological, colour and sensory changes of refrigerated chicken legs treated with selected phosphates. Food Res. Int. 32, 209-215.

Kirby, B.J. and Hasselbrink, E.F., 2004. Zeta potential of microfluidic substrates: 1. Theory, experimental techniques, and effects on separations. Electrophoresis 25, 187-202.

Kirby, B.J., 2010. Micro- and Nanoscale Fluid Mechanics: Transport in Microfluidic Devices. Cambridge University Press, Cambridge, UK. ISBN 978-0-521-11903-0.

Kleinman, P.J., Sharpley, A.N., McDowell, R.W., Flaten, D.N., Buda, A.R., Tao, L., Bergstrom, L. and Zhu, Q., 2011. Managing agricultural phosphorus for water quality protection: principles for progress. Plant Soil 349, 169-182.

Knight, A.W., Keenan, P.O., Goddard, N.J., Fielden, P.R. and Walmsley, R.M., 2004. A yeast-based cytotoxicity and genotoxicity assay for environmental monitoring using novel portable instrumentation. J. Environ. Monit. 6, 71-79.

Kohler, T., Stingl, U., Meuser, K. and Brune, A., 2008. Novel lineages of Planctomycetes densely colonize the alkaline gut of soil-feeding termites (*Cubitermes* spp.). Environ. Microbiol. 10, 1260–1270.

Komlos, J., Rowe, A., Clark, S., Traver, R. and Stinson, M., 2010. Assessing BMP Performance Using Microtox Toxicity Analysis. In: World Environmental and Water Resources Congress 2010@ sChallenges of Change, ASCE, pp. 3209-3217.

König, E., Schlesner, H. and Hirsch, P., 1984. Cell wall studies on budding bacteria of the Planctomyces/Pasteuria group and on a *Prosthecomicrobium* sp. Arch. Microbiol. 138, 200–205.

Kuenen, J.G., 2008. Anammox bacteria: from discovery to application. Nature Rev. Microbiol. 6, 320–326.

Kungolos, A., Emmanouil, C., Tsiridis, V. and Tsiropoulos, N., 2009. Evaluation of toxic and interactive toxic effects of three agrochemicals and copper using a battery of microbiotests. Sci. Total Environ. 407, 4610-4615.

Lage, O.M. and Bondoso, J., 2011. Planctomycetes diversity associated with macroalgae. FEMS Microbiol. Ecol. 78, 366-375.

Lage, O.M., Bondoso, J. and Catita, J.A., 2012. Determination of zeta potential in Planctomycetes and its application in heavy metals toxicity assessment. Arch. Microbiol. 194, 847-855.

Lage, O.M., Bondoso, J. and Lobo-da-Cunha, A., 2013. Insights into the ultrastructural morphology of novel Planctomycetes. Antonie van Leeuwenhoek 1-10.

Langsrud, S., Sidhu, M.S., Heir, E., Holck, A.L., 2003. Bacterial disinfectant resistance—a challenge for the food industry. Int. Biodeterior. Biodegradation 51, 283-290.

Lau, F.W., Nauli, S., Zhou, Y. and Bowie, J.U., 1999. Changing single side-chains can greatly enhance the resistance of a membrane protein to irreversible inactivation. J. Mol. Biol. 290, 559-564.

Laughinghouse IV, H.D., Prá, D., Silva-Stenico, M.E., Rieger, A., Frescura, V.D.S., Fiore, M.F. and Tedesco, S.B., 2012. Biomonitoring genotoxicity and cytotoxicity of *Microcystis aeruginosa* (Chroococcales, Cyanobacteria) using the *Allium cepa* test. Sci. Total Environ. 432, 180-188.

Lawrence, J.R., Zhu, B., Swerhone, G.D., Roy, J., Tumber, V., Waiser, M.J. and Topp, E., Korber, D.R., 2012. Molecular and microscopic assessment of the effects of caffeine, acetaminophen, diclofenac, and their mixtures on river biofilm communities. Environ. Toxicol. Chem. 31, 508- 517.

Lee, K.C., Webb, R.I., Janssen, P.H., Sangwan, P., Romeo, T., Staley, J.T. and Fuerst, J.A., 2009. Phylum Verrucomicrobia representatives share a compartmentalized cell planwith members of bacterial phylum Planctomycetes. BMC Microbiol. 9, 5.

Lehman, J.T., 1976. Ecological and nutritional studies on *Dinobryon Ehrenb*.: Seasonal periodicity and the phosphate toxicity problem. Limnol. Oceanogr. 21, 646-658.

Lei, Y., Chen, W. and Mulchandani, A., 2006. Microbial biosensors. Anal. Chim. Acta 568, 200-210.

Letson, D., 1992. Point/nonpoint source pollution reduction trading: An interpretive survey. Nat. Resources J. 32, 219.

Lewis, P.J., Nwoguh, C.E., Barer, M.R., Harwood, C.R. and Errington, J., 1994. Use of digitized video microscopy with a fluorogenic enzyme substrate to demonstrate cell- and compartment-specific gene expression in *Salmonella enteritidis* and *Bacillus subtilis*. Mol. Microbiol. 13, 655–662.

Li, H., Chen, S., Mu, B.Z. and Gu, J.D., 2010. Molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in high-temperature petroleum reservoirs. Microb. Ecol. 60, 771–783.

Li, W. and Palmer, G., 1993. Spectroscopic characterization of the interaction of azide and thiocyanate with the binuclear center of cytochrome oxidase: evidence for multiple ligand sites. Biochemistry 32, 1833-1843.

Liesack, W., König, H., Schlesner, H. and Hirsch, P., 1986. Chemical composition of the peptidoglycan-free cell envelopes of budding bacteria of the Pirella/ Planctomyces group. Arch. Microbiol. 145, 361–366.

Lin, Y.S.E., Vidic, R.D., Stout, J.E. and Yu, V.L., 1996. Individual and combined effects of copper and silver ions on inactivation of *Legionella pneumophila*. Water Res. 30, 1905-1913.

Lindsay, M.R., Webb, R.I. and Fuerst, J.A., 1997. Pirellulosomes: a new type of membrane-bounded cell compartment in planctomycete bacteria of the genus *Pirellula*. Microbiology 143, 739–748.

Lindsay, M.R., Webb, R.I., Strous, M., Jetten, M.S., Butler, M.K., Forde, R.J. and Fuerst, J.A., 2001. Cell compartmentalisation in planctomycetes: novel types of structural organization for the bacterial cell. Arch. Microbiol. 175, 413–429.

Lindström, E.S., Kamst-Van Agterveld, M.P. and Zwart, G., 2005. Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. Appl. Environ. Microbiol. 71, 8201–8206.

Little, R.H., Cheesman, M.R., Thomson, A.J., Greenwood, C. and Watmough, N.J., 1996. Cytochrome bo from *Escherichia coli*: binding of azide to CuB. Biochemistry 35, 13780-13787.

Liu, F.H., Lin, G.H., Gao, G., Qin, B.Q., Zhang, J.S., Zhao, G.P., Zhou, Z.H. and Shen J.H., 2009. Bacterial and archaeal assemblages in sediments of a large shallow freshwater lake, Lake Taihu, as revealed by denaturing gradient gel electrophoresis. J. Appl. Microbiol. 106, 1022–1032.

Liu, Z., Zeng, Z., Zeng, G., Li, J., Zhong, H., Yuan, X., Zhang, J., Chen, M., Liu, Y. and Xie, G., 2012. Influence of rhamnolipids and Triton X-100 on adsorption of phenol by *Penicillium simplicissimum*. Bioresour. Technol. 110, 468-473.

Lu, G., Yang, X., Li, Z., Zhao, H. and Wang, C., 2013. Contamination by metals and pharmaceuticals in northern Taihu Lake (China) and its relation to integrated biomarker response in fish. Ecotoxicology 22, 50-59.

Lyklema, J., 1995. Fundamentals of Interface and Colloid Science, vol.2, page.3.208.

Lytle, D.A., Rice, E.W., Johnson, C.H. and Fox, K.R., 1999. Electrophoretic mobilities of *Escherichia coli* 0157:H7 and wild-type *Escherichia coli* strains. Appl. Environ. Microbiol. 65, 3222–3225.

Mahaut, M.L., Basuyaux, O., Baudinière, E., Chataignier, C., Pain, J. and Caplat, C., 2013. The porifera *Hymeniacidon perlevis* (Montagu, 1818) as a bioindicator for water quality monitoring. Environ. Sci. Pollut. Res. Int. 20, 2984-2992.

Malhotra, A. and Coupland, J.N., 2004. The effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates. Food Hydrocoll. 18, 101-108.

Malvern Instruments Ltd., 2004. Chapter 16: Zeta Potential Theory. In: ZetaSizer Nano Series User Manual, MAN0317. Malvern, Worcestershire WR14 1XZ, UK, pp. 1-12.

Mamber, S.W., Bryson, V. and Katz, S.E., 1983. The *Escherichia coli* WP2/wp100 rec assay for detection of potential chemical carcinogens. Mutat. Res. 119, 135–144.

Mangia, A.H.R., Teixeira, L.M. and Filho, F.C.E.S., 1995. The electrokinetic surface of five enteropathogenic *Escherichia coli* serogroups. Cell Biophys. 26, 45–55.

Marsalek, R., 2012. Zeta Potential-Applications. 2nd International Conference on Environment and Industrial Innovation IPCBEE vol.35 (2012) © (2012) IACSIT Press, Singapore.

Martinez, R.E., Pokrovsky, O.S., Schott, J. and Oelkers, E.H., 2008. Surface charge and zeta-potential of metabolically active and dead cyanobacteria. J. Colloid Interface Sci. 323, 317-325.

Matias, V.R. and Beveridge, T.J., 2005. Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. Mol. Microbiol. 56, 240-251.

Matthews, E.K. and O'Connor, M.D., 1978. Correlation spectroscopy and Laser Doppler anemometry; a new method for determining secretory particle electrophoretic properties [proceedings]. J. Physiol. (Lond.) 278, 1P-2P.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N., 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. U.S.A. 72, 5135- 5139.

McDonnell, G. and Russell, A.D., 2001. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. 12, 147-179.

Michel, H., Behr, J., Harrenga, A. and Kannt, A., 1998. Cytochrome c oxidase: structure and spectroscopy. Annu. Rev. Biophys. Biomol. Struct. 27, 329-356.

Moriel, D.G., Rosini, R., Seib, K.L., Serino, L., Pizza, M. and Rappuoli, R., 2012. *Escherichia coli*: great diversity around a common core. mBio 3, e00118-12.

Morris, C.M., George, A., Wilson, W.W. and Champlin, F.R., 1995. Effect of polymyxin B nonapeptide on daptomycin permeability and cell surface properties in *Pseudomonas aeruginosa*, *Escherichia coli*, and *Pasteurella multocida*. J. Antibiotics 48, 67–72.

Morris, R.M., Longnecker, K. and Giovannoni, S.J., 2006. *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. Environ. Microbiol. 8, 1361–1370.

Mosleh, Y.Y., Ismail, S.M.M., Ahmed, M.T. and Ahmed, Y.M., 2003. Comparative toxicity and biochemical responses of certain pesticides to the mature earthworm Aporrectodea caliginosa under laboratory conditions. Environ. Toxicol. 18, 338–346.

Moyer, L.S., 1936. Changes in the electrokinetic potential of bacteria at various phases of the culture cycle. J. Bacteriol. 32, 433–464.

Musat, N., Werner, U., Knittel, K., Kolb, S., Dodenhof, T., van Beusekom, J.E., de Beer, D., Dubilier, N. and Amann, R., 2006. Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Romo Basin, Wadden Sea. Syst. Appl. Microbiol. 29, 333–348.

Nakajima, H., Kobayashi, K., Kobayashi, M., Asako, H. and Aono, R., 1995. Overexpression of the robA gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. 61, 2302-2307.

Nałęcz-jawecki, G., 2005. Spirotox test—*Spirostomum ambiguum* acute toxicity test. In: Blaise, C., Férard, J.F. (Eds.), Small-scale freshwater toxicity investigations. Springer Netherlands, pp. 299-322.

Namieśnik, J. and Wardencki, W., 2000. Application of vegetation in environmental biomonitoring. Chem. Inż. Ekol. 7, 189.

National Pollutant Inventory (NPi), Australian Government, Department of Sustainability, Environment, Water, Population and Communities. Table 1 - the 93 substances listed on the NPI from the tenth reporting year (2007- 08). From: http://www.npi.gov.au/substances/list-ofsubst.html

Noumi, T., Maeda, M. and Futai, M., 1987. Mode of inhibition of sodium azide on H^+-ATPase of *Escherichia coli*. FEBS lett. 213, 381-384.

Nwuche, C.O. and Ugoji, E.O., 2008. Effects of heavy metal pollution on the soil microbial activity. Int. J. Environ. Sci. Tech. 5, 409-414.

Oh, S.E., Hassan, S.H. and Van Ginkel, S.W., 2011. A novel biosensor for detecting toxicity in water using sulfur-oxidizing bacteria. Sens. Actuators B Chem. 154, 17-21.

Oliver, D.B., Cabelli, R.J., Dolan, K.M. and Jarosik, G.P., 1990. Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery. Proc. Natl. Acad. Sci. U.S.A. 87, 8227-8231.

Ore, S., Mertens, J., Brandt, K.K. and Smolders, E., 2010. Copper toxicity to bioluminescente *Nitrosomonas europaea* in soil is explained by the free metal ion activity in pore water. Environ. Sci. Technol. 44, 9201-9206.

Oukarroum, A., Polchtchikov, S., Perreault, F. and Popovic, R., 2012. Temperature influence on silver nanoparticles inhibitory effect on photosystem II photochemistry in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. Environ. Sci. Pollut. Res. Int. 19, 1755-1762.

Oztetik, E., Cicek, A. and Arslan, N., 2013. Early antioxidative defence responses in the aquatic worms (*Limnodrilus* sp.) in Porsuk Creek in Eskisehir (Turkey). Toxicol. Ind. Health 29, 541-554.

Palais, F., Dedourge-Geffard, O., Beaudon, A., Pain-Devin, S., Trapp, J., Geffard, O., Noury, P., Gourlay-Francé, C., Uher, E., Mouneyrac, C., Biagianti-Risbourg, S. and Geffard, A., 2012. One-year monitoring of core biomarker and digestive enzyme responses in transplanted zebra mussels (*Dreissena polymorpha*). Ecotoxicology 21, 888-905.

Park, J.H., Lee, B.J., Lee, S.K., Kim, K., Lee, K.H., Che, J.H., Kang, K.S. and Lee, Y.S., 2000. Genotoxicity of drinking water from three Korean cities. Mutat. Res. 466, 173–178.

Paulsson, M., Nyström, B. and Blanck, H. 2000. Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosm study. Aquat. Toxicol. 47, 243-257.

Pearson, A., Budin, M. and Brocks, J.J., 2003. Phylogenetic and biochemical evidence for sterol synthesis in the bacterium *Gemmata obscuriglobus*. Proc. Natl Acad. Sci. 100, 15 352–15 357.

Petzold, G.L., Swenberg, J.A., 1978. Detection of DNA damage induced in vivo following exposure of rats to carcinogens. Cancer Res. 38, 1589-1598.

Pimenta, F.M., 2010. Biofluorescência no meio marinho: estrutura e propriedades de uma Pioverdina. Master's thesis. University of Lisbon, Faculty of Sciences, Department of Chemistry and Biochemistry, Portugal.

Prajongtat, P., Phromyothin, D.S.T. and Hannongbua, S., 2013. Key role of hydrazine to the interaction between oxaloacetic against phosphoenolpyruvic carboxykinase (PEPCK): ONIOM calculations. J. Mol. Model. 1-10.

Pulvertaft, R.J.V. and Lumb, G.D., 1948. Bacterial lysis and antiseptics. J. Hyg. 46, 62–64.

Purchase, I.F.H., Longstaff, E., Ashby, J., Styles, A., Anderson, D., Lefevre, P.A. and Westwood, F. R., 1978. An evaluation of 6 short-term tests for detecting organic chemical carcinogens. Br. J. Cancer. 37, 873-959.

Puustinen, A., Finel, M., Haltia, T., Gennis, R.B. and Wikstrom, M., 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry 30, 3936-3942.

Rachel, R., 2009. Another way to divide: the case of anammox bacteria. Mol. Microbiol. 73, 978–981.

Radecki, J. and Radecka, H., 1995. Biosensors in environmental protection. Polish J. Environ. Studies 4, 13.

Rajkumar, M., Ae, N., Prasad, M.N.V. and Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol. 28, 142-149.

Ramanavičienė, A., Mostovojus, V., Bachmatova, I. and Ramanavičius, A., 2003. Anti-bacterial effect of caffeine on *Escherichia coli* and *Pseudomonas fluorescens*. Acta Medica Lituanica. 10, 185-188.

Ravera, O., 2001. Monitoring of the aquatic environment by species accumulator of pollutants: a review. J. Limnol. 60, 63-78.

Richardson, D.J., 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology 146, 551-571.

Rodrigues, A.C., Wuertz, S., Brito, A.G. and Melo, L.F., 2005. Fluorene and phenanthrene uptake by *Pseudomonas putida* ATCC 17514: kinetics and physiological aspects. Biotechnol. Bioeng. 90, 281-289.

Rodriguez-Mozaz, S., Lopez de Alda, M.J. and Barceló, D., 2007. Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography–mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water. J. Chromatogr. A 1152, 97-115.

Rosato, V., Pucello, N. and Giuliano, G., 2002. Evidence for cysteine clustering in thermophilic proteomes. Trends Genet. 18, 278-281.

Rosenholm, J.M., Peuhu, E., Bate-Eya, L.T., Eriksson, J.E., Sahlgren, C. and Lindén, M., 2010. Cancer-Cell-Specific Induction of Apoptosis Using Mesoporous Silica Nanoparticles as Drug-Delivery Vectors. Small 6, 1234-1241.

Sadowski, Z., 2001. Effect of biosorption of Pb (II), Cu (II) and Cd (II) on the zeta potential and flocculation of *Nocardia* sp. Miner. Eng. 14, 547-552.

Salton, M.R.J., 1968. Lytic agents, cell permeability and monolayer penetrability. J. Gen. Physiol. 52, 227-252.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J. and Solomon, K.R., 2004. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul. Toxicol. Pharmacol. 39, 158–183.

Santarella-Mellwig, R., Pruggnaller, S., Roos, N., Mattaj, I.W. and Devos, D.P., 2013. Threedimensional reconstruction of bacteria with a complex endomembrane system. PLoS Biol. 11, e1001565.

Santo, C.E., Lam, E.W., Elowsky, C.G., Quaranta, D., Domaille, D.W., Chang, C.J. and Grass, G., 2011. Bacterial killing by dry metallic copper surfaces. Appl. Environ. Microbiol. 77, 794-802.

Sarathchandra, S.U., Ghani, A., Yeates, G.W., Burch, G. and Cox, N.R., 2001. Effect of nitrogen and phosphate fertilisers on microbial and nematode diversity in pasture soils. Soil Biol. Biochem. 33, 953-964.

Schlesner, H., 1994. The Development of Media Suitable for the Microorganisms Morphologically Resembling *Planctomyce*s spp., *Pirellula* spp., and other *Planctomycetales* from Various Aquatic Habitats Using Dilute Media. Syst. Appl. Microbiol. 17, 135-145.

Schneider, D., Liu, Y., Gerstein, M. and Engelman, D.M., 2002. Thermostability of membrane protein helix–helix interaction elucidated by statistical analysis. FEBS lett. 532, 231-236.

Schott, H. and Young, C.Y., 1973, Electrokinetic studies of bacteria. 3. Effect of polyvalent metal ions on electrophoretic mobility and growth of streptococcus faecalis. J. Pharm. Sci. 62, 1797–1801.

Schwegmann, H., Feitz, A.J. and Frimmel, F.H., 2010. Influence of the zeta potential on the sorption and toxicity of iron oxide nanoparticles on *S. cerevisiae* and *E. coli*. J. Colloid Interface Sci. 347, 43-48.

Sellin Jeffries, M.K., Mehinto, A.C., Carter, B.J., Denslow, N.D. and Kolok, A.S., 2012. Taking microarrays to the field: differential hepatic gene expression of caged fathead minnows from Nebraska watersheds. Environ. Sci. Technol. 46, 1877-1885.

Serafim, A., Lopes, B., Cravo, A., Gomes, T., Sousa, V. and Bebianno, M.J., 2011. A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. Ecotoxicology 20, 1959-1974.

Sheng, L., Aijia, Z., Rongyu, W., Ting, C., Mingming, Z., Zhaonian, Y. and Wenxiong, L., 2012. Effects of Sugarcane Ratooning Cultivation on the Alteration of Bacterial Communities in the Rhizosphere Soil. Sugar Tech. 14, 275-283.

Sherbet, G.V., Lakshmi, M.S. and Rao, K.V., 1972. Characterisation of ionogenic groups and estimation of the net negative electric charge on the surface of cells using natural pH gradients. Exp. Cell Res. 70, 113–123.

Smith, K.F., Schmidt, V., Rosen, G.E. and Amaral-Zettler, L., 2012. Microbial Diversity and Potential Pathogens in Ornamental Fish Aquarium Water. PloS one 7, e39971.

Soni, K., Balasubramanian, A., Beskok, A. and Pillai, S., 2008. Zeta potential of selected bacteria in drinking water when dead, starved, or exposed to minimal and rich culture media. Curr. Microbiol. 56, 93–97.

Soon, R.L., Nation, R.L., Cockram, S., Moffatt, J.H., Harper, M., Adler, B., Boyce, J.D., Larson, I. and Li, J., 2011. Different surface charge of colistin-susceptible and-resistant *Acinetobacter baumannii* cells measured with zeta potential as a function of growth phase and colistin treatment. J. Antimicrob. Chemother. 66, 126-133.

Speth, D.R., van Teeseling, M.C., Jetten, M.S., 2012. Genomic analysis indicates the presence of an asymmetric bilayer outer membrane in Planctomycetes and Verrucomicrobia. Front. Microbiol. 3, 304.

Srivastava, R.B. and Thompson, R.E.M., 1965. Influence of bacterial cell age on phenol action. Nature (Lond.) 206, 216.

Srivastava, R.B. and Thompson, R.E.M., 1966. Studies in the mechanism of action of phenol on *Escherichia coli* cells. Br. J. Exp. Pathol. 67, 315–323.

Stendahl, O., Edebo, L., Magnusson, K.-E., Tagesson, C. and Hjerten, S., 1977. Surface-charge characteristics of smooth and rough Salmonella typhimurium bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta Path. Microbiol. Scand. Sect. B 85, 334–340.

Stoltz, J.F., Janot, C., Saur, F., Weber, M., Malher, E. and Duvivier, C., 1984. Use of a laser-Doppler electrophoresis method in bacteriology (preliminary results). Biorheology Suppl. 1, 303-307.

Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., Dutilh, B.E., Op den Camp, H.J., van der Drift, C., Cirpus, I., van de Pas-Schoonen, K.T., Harhangi, H.R., van Niftrik, L., Schmid, M., Keltjens, J., van de Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M.A., Mewes, H.W., Weissenbach, J., Jetten, M.S., Wagner, M. and Le Paslier, D., 2006. Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440, 790–794.

Su, C.C., Long, F., Zimmermann, M.T., Rajashankar, K.R., Jernigan, R.L. and Edward, W.Y., 2011. Crystal structure of the CusBA heavy-metal efflux complex of *Escherichia coli*. Nature 470, 558- 562.

Sugimura, T., Sato, S., Nagao. M., Yahagy, T., Matsushima, T., Seino, Y., Takeuchi. M. and Kawachi, T., 1976. Overlapping of carcinogens and mutagens. In: Magee, P.N., Takayama, S., Sugimura T., Matsushima, T., (Eds.), Fundamentals in Cancer Prevention. University of Tokyo Press, Tokyo, pp. 191-215.

Sukul, P. and Spiteller, M., 2000. Metalaxyl: persistence, degradation, metabolism, and analytical methods. Rev. Environ. Contam. Toxicol. 164, 1-26.

Summers, R.M., Louie, T.M., Yu, C.L., Gakhar, L., Louie, K.C. and Subramanian, M., 2012. Novel, highly specific N-demethylases enable bacteria to live on caffeine and related purine alkaloids. J. Bacteriol. 194, 2041-2049.

Suresh, A.K., Pelletier, D.A., Wang, W., Moon, J.W., Gu, B., Mortensen, N.P., Allison, D.P., Joy, D.C., Phelps, T.J. and Doktycz, M.J., 2010. Silver nanocrystallites: biofabrication using *Shewanella oneidensis,* and an evaluation of their comparative toxicity on gram-negative and gram-positive bacteria. Environ. Sci. Technol. 44, 5210-5215.

Swanson, J., Dorward, D., Lubke, L. and Kao, D., 1997. Porin polypeptide contributes to surface charge of gonococci. J. Bacteriol. 179, 3541–3548

Sze, A., Erickson, D., Ren, L. and Li, D., 2003. Zeta-potential measurement using the Smoluchowski equation and the slope of the current–time relationship in electroosmotic flow. J. Colloid Interface Sci. 261, 402-410.

Tang, C., Madigan, M.T. and Lanoil, B., 2013. Bacterial and Archaeal Diversity in Sediments of West Lake Bonney, McMurdo Dry Valleys, Antarctica. Appl. Environ. Microbiol. 79, 1034-1038.

Tariq, M., Bruijs, C., Kok, J. and Krom, B.P., 2012. Link between Culture Zeta Potential Homogeneity and Ebp in *Enterococcus faecalis*. Appl. Environ. Microbiol. 78, 2282-2288.

Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuku, M., Takaya, N., Nakajima-Kambe, T., Uchiyama, H. and Nomura, N., 2010. Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 76, 3732-3739.

Tenaillon, O., Skurnik, D., Picard, B. and Denamur, E., 2010. The population genetics of commensal *Escherichia coli*. Nat. Rev. Microbiol. 8, 207-217.

Thomson, N., Sebaihia, M., Cerdeño-Tárraga, A., Bentley, S., Crossman, L. and Parkhill, J., 2003. The value of comparison. Nat. Rev. Microbiol. 1, 11-12.

Titley-O'Neal, C.P., Munkittrick, K.R. and MacDonald, B.A., 2011. The effects of organotin on female gastropods. J. Environ. Monit. 13, 2360-2388.

Torres-Cortés, G., Millán, V., Fernández-González, A.J., Aguirre-Garrido, J.F., Ramírez-Saad, H.C., Fernández-López, M., Toro, N. and Martínez-Abarca, F., 2012. Bacterial community in the rhizosphere of the cactus species *Mammillaria carnea* during dry and rainy seasons assessed by deep sequencing. Plant Soil 357, 275-288.

Tsuneda, S., Jung, J., Hayashi, H., Aikawa, H., Hirata, A. and Sasaki, H., 2003. Influence of extracellular polymers on electrokinetic properties of heterotrophic bacterial cells examined by soft particle electrophoresis theory. Colloids Surf. B Biointerfaces 29, 181–188.

U.S. Environmental Protection Agency (EPA) Washington, D.C. The National Water Quality Inventory: Report to Congress for the 2002 Reporting Cycle–A Profile. Document No. EPA-841-R-07-001.October 2007.

Urbieta, M.S., Toril, E.G., Aguilera, A., Giaveno, M.A. and Donati, E., 2012. First prokaryotic biodiversity assessment using molecular techniques of an acidic river in Neuquén, Argentina. Microb. Ecol. 64, 91-104.

Van der Schalie, W.H., Shedd, T.R., Knechtges, P.L. and Widder, M.W., 2001. Using higher organisms in biological early warning systems for real-time toxicity detection. Biosens. Bioelectron. 16, 457-465.

van Elsas, J.D., Semenov, A.V., Costa, R. and Trevors, J.T., 2010. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J. 5, 173-183.

van Niftrik, L., Geerts, W.J., van Donselaar, E.G., Humbel, B.M., Webb, R.I., Harhangi, H.R., Camp, H.J., Fuerst, J.A., Verkleij, A.J., Jetten, M.S. and Strous M., 2009. Cell division ring, a new cell division protein and vertical inheritance of a bacterial organelle in anammox planctomycetes. Mol. Microbiol. 73, 1009–1019.

van Niftrik, L.A., Fuerst, J.A., Sinninghe Damsté, J.S., Kuenen, J.G., Jetten, M.S. and Strous, M., 2004. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. FEMS Microbiol. Lett. 233, 7–13.

Wagner, M. and Horn, M., 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr. Opin. Biotechnol. 17, 241–249.

Wanner, B.L., 1993. Gene regulation by phosphate in enteric bacteria. J. Cell. Biochem. 51, 47-54.

Ware, B.R. and Flygare, W.H., 1971. The simultaneous measurement of the electrophoretic mobility and diffusion coefficient in bovine serum albumin solutions by light scattering. Chem. Phys. Lett. 12, 81–85.

Warnes, S.L., Caves, V. and Keevil, C.W., 2012. Mechanism of copper surface toxicity in *Escherichia coli* O157: H7 and Salmonella involves immediate membrane depolarization followed by slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. Environ. Microbiol. 14, 1730-1743.

Watt, J.M., Swiatlo, E., Wade, M.M. and Champlin, F.R., 2003. Regulation of capsule biosynthesis in serotype A strains of *Pasteurella multocida*. FEMS Microbiol. lett. 225, 9-14.

Webster, N.S. and Bourne, D., 2007. Bacterial community structure associated with the Antarctic soft coral, *Alcyonium antarcticum*. FEMS Microbiol. Ecol. 59, 81–94.

Weijs, L., Covaci, A., Yang, R.S., Das, K. and Blust, R., 2011. A non-invasive approach to study lifetime exposure and bioaccumulation of PCBs in protected marine mammals: PBPK modeling in harbor porpoises. Toxicol. Appl. Pharmacol. 256, 136-145.

Westerhoff, P., Yoon, Y., Snyder, S. and Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649-6663.

White, J.R., Patel, J., Ottesen, A., Arce, G., Blackwelder, P. and Lopez, J.V., 2012. Pyrosequencing of bacterial symbionts within *Axinella corrugata* sponges: diversity and seasonal variability. PloS one 7, e38204.

Winkelmann, N., Jaekel, U., Meyer, C., Serrano, W., Rachel, R., Rosselló-Mora, R. and Harder, J., 2010. Determination of the diversity of *Rhodopirellula* isolates from European seas by multilocus sequence analysis. Appl. Environ. Microbiol. 76, 776-785.

Wnek, W.J. and Davies, R., 1977. An analysis of the dependence of the zeta potential and surface charge on surfactant concentration, ionic strength, and pH. J. Colloid Interface Sci. 60, 361-375.

Woebken, D., Teeling, H., Wecker, P., Dumitriu, A., Kostadinov, I., Delong, E.F., Amann, R. and Glöckner, F.O., 2007. Fosmids of novel marine Planctomycetes from the Namibian and Oregon coast upwelling systems and their cross-comparison with planctomycete genomes. ISME J. 1, 419–435.

Woese, C.R., 1987. Bacterial evolution. Microbiol. Rev. 51, 221–271.

Wood, J.M., 1980. The interaction of micro-organisms with ions exchange resins. In: Berkeley, R.C.W., Lynch, J.M., Melling, J.,Rutter, P.R., Vincent, B. (Eds.), Microbial Adhesion To Surfaces. Ellis Harwood, Chichester, pp. 163–185.

World Health Organization, 1987. Environmental Health Criteria 68: Hydrazine. International Programme on Chemical Safety, Geneva, Switzerland, section 9.2.1, 1-89.

World Health Organization, 2003. Guidelines for Safe Recreational Water Environments, Volume 1: Coastal and Fresh Waters. World Health Organization.

World Health Organization, 2012. UN-Water Global Analysis and Assessment of Sanitation and Drinking-Water (GLAAS) 2012. Report: The Challenge of Extending and Sustaining Services.

Woznica, A., Nowak, A., Ziemski, P., Kwasniewski, M. and Bernas, T., 2013. Stimulatory Effect of Xenobiotics on Oxidative Electron Transport of Chemolithotrophic Nitrifying Bacteria Used as Biosensing Element. PloS one 8, e53484.

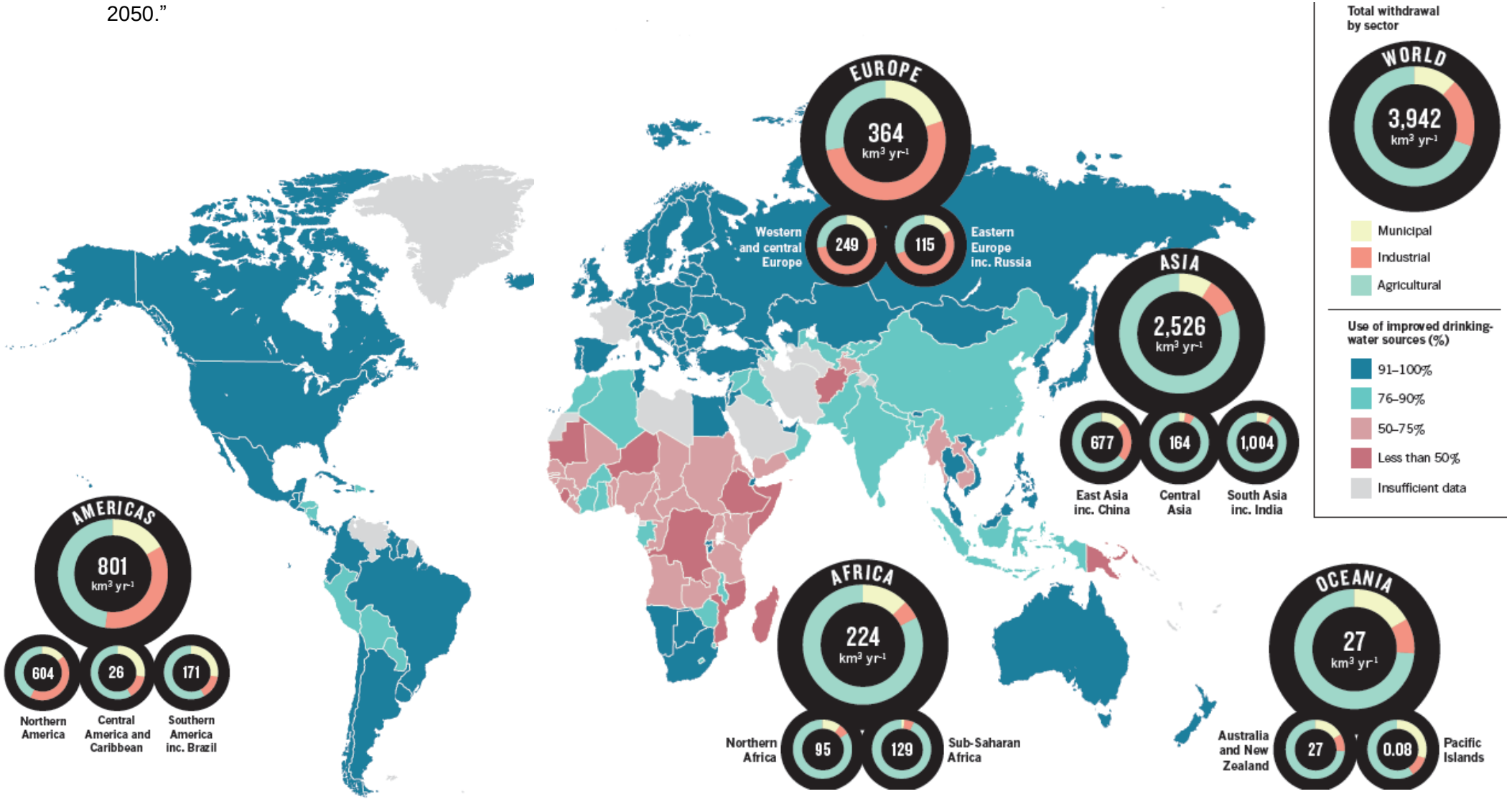
Wu, T., Xie, A.G., Tan, S.Z. and Cai, X., 2011. Antimicrobial effects of quaternary phosphonium salt intercalated clay minerals on *Escherichia coli* and *Staphylococcus aureus*. Colloids Surf. B Biointerfaces, 86, 232-236.

Yan, Z., Lu, G., Liu, J. and Jin, S., 2012. An integrated assessment of estrogenic contamination and feminization risk in fish in Taihu Lake, China. Ecotoxicol. Environ. Saf. 84, 334-40.

Yu, H., Nie, E., Xu, J., Yan, S., Cooper, W.J. and Song, W., 2013. Degradation of Diclofenac by Advanced Oxidation and Reduction Processes: Kinetic Studies, Degradation Pathways and Toxicity Assessments. Water Res. 47, 1909-1918.

8. Annexes

Annex 1. Representation of water management around the world. The graphic below was published in a *Nature* report (Gilbert 2012), as a synthesis of the fourth *World Water Development Report* by the UN Educational, Scientific and Cultural Organization (UNESCO), launched at the World Water Forum in Marseilles, France, March. The analysis reported that "more than 80% of the world's waste water is not collected or treated, causing millions of deaths from waterborne diarrhoeal diseases every year in the developing world. Urban settlements are the main source of pollution, and the challenge will grow as the world's urban population almost doubles to 6.3 billion by 2050."



Annex 2. Examples of organisms from different phyla reported as biomonitors or source of different biosensors in 2011 and 2012.

Source sample	Pollutants analyzed	Bioindicators / Biomarkers	Organism affiliation	References
Salinelle (volcanoes); surface outflow of a hydrothermal system located below Mount Etna	metals (Al, As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, V, and Zn) and hydrocarbons	*Bryum argenteum*	Moss	Bonanno et al. 2012
Coast of the Persian Gulf and areas close to sources of anthropogenic pollution (Ganaveh and Nuclear power facilities)	nickel, cadmium, copper and lead	Seaweed: green - *Ulva interstinalis*, brown - *Padina pavonica*, *Cystoseira myrica*, *Sargassum angustifolium*, and red - *Acanthophora spicifera, Gracilaria corticata, Hypnea cervicornis*	Macroalgae	Alahverdi et al. 2012
Treated tannery effluent from Jajmau, Kanpur (India)	chromium	*Vallisneria spiralis* and *Hydrilla verticillata*	Aquatic plants	Gupta et al. 2012
Methanolic extracts from two strains of *Microcystis aeruginosa*	microcystin, aeruginosin,	*Allium cepa*	Plant	Laughinghouse et al. 2012
Carboniferous underground mining region of Criciúma (Santa Catarina state, South Brazil); acid mine drainage (AMD)	aluminum, iron, manganese, zinc, copper, lead and sulfate	*Allium cepa* / Biomarkers of lipid peroxidation (LPO) and antioxidant defenses: TBARS, protein carbonyls, catalase activity (CAT) and glutathione levels (GSH)		Geremias et al. 2012
Chimaliapan wetland (Lerma basin, Mexico)	aluminium (Al) and chromium (Cr)	92 species of rotifers, cladocerans and copepods	Zooplankton	García-García et al. 2012
Drinking water and soil	metallic tungsten	*Brassica oleracae* and *Otala lactea*	Plant and mollusk (bivalve)	Kennedy et al. 2012
Intertidal zones on the Lower Normandy coast (France)	copper, zinc and the hydrocarbon: fluoranthene	*Hymeniacidon perlevis*	Porifera	Mahaut et al. 2012
Estuarine; industrial and agricultural sources; Bages-Sigean lagoon (Western Europe)	heavy metals (Cu and Cd)	*Ammonia* spp. , *Quinqueloculina bicostata* and *Quinqueloculina seminula*	Foraminifera	Foster et al. 2012
Bizerte bay, northeast coast of Tunisia	Petroleum pollution	*Odontophora villoti*	Nematode	Boufahja et al. 2012
Porsuk Creek in Eskisehir (Turkey)	metals (Fe, Al, Zn, Mn, Pb, Cu, Ni, B, Cd, Cr and Hg)	*Limnodrilus hoffmeisteri* and *Limnodrilus udekemianus* / antioxidant defenses: GSH and glutathione S-transferase (GST) activity	Oligochaeta	Oztetik et al. 2012
Lake Trasimeno, littoral zone (Italy)	heavy metals and other toxicants	*Chironomus plumosus*	Insect	Di Veroli et al. 2012
Seawater and sediments of Bizerta Lagoon (northeast of Tunisia)	metals (Al, Co, Cu, Fe, Cd, Pb, Mn, Zn, Ni and Cr) and hydrocarbons	*Carcinus maenas* (gills and digestive gland) / antioxidant defenses (CAT, GST, TBARS, AChE), LPO and metallothioneins (MTs)	Crustacean	Ben-Khedher et al. 2012

Annex 2 (continued)

Not applicable	the insecticide methoxyfenozide, the fungicide propiconazole and pharmaceutical products: benzophenone, carbamazepine, cyproterone, and R-propranolol	Male *Gammarus fossarum* / vitellogenin-like proteins	Crustacean	Jubeaux et al. 2012
South coast of Portugal	metals (Cd, Cu, Ni, Pb and Zn) and a wide range of polycyclic aromatic hydrocarbons (PAHs)	*Mytilus galloprovincialis* / metallothioneins, oxidase system (MFO) and the enzyme d-aminolevulinic acid dehydratase (ALAD), biomarker for oxidative stress: GST, acetylcholinesterase (AChE), superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx) and LPO	Mollusk bivalve	Serafim et al. 2011
Vesle river basin (Champagne-Ardenne, France)	PAHs, heavy metals (Cu, Pb, Zn and Ni), pesticides, chlorides, phosphates, nitrates, ammonium and sulfates	*Dreissena polymorpha* / biomarkers: AChE, GST, MTs, amylase, endocellulase		Palais et al. 2012
Not applicable	tributyltin, other tin (Sn) compounds, steroids and other contaminants such as Aroclor 1260	Female gastropods	Mollusk gastropod	Titley-O'Neal et al. 2011
Taihu Lake (shallow freshwater lake in eastern China)	copper (Cu), cadmium (Cd), lead (Pb), arsenic (As), chromium (Cr), zinc (Zn) and pharmaceuticals: sulfamethoxazole, caffeine, norfloxacin and ofloxacin	*Carassius auratus* / antioxidant enzymes (Brain AChE and liver EROD, GST, GPx and SOD)	Fish	Lu et al. 2012
Taihu Lake	hormones; estrone, 17β-estradiol (E2), estriol, 17α-ethinylestradiol (EE2), bisphenol-A and diethylstilbestrol	Male *Carassius auratus* / enzymatic biomarkers		Yan et al. 2012
South America industrial and sewage effluents (Itata River)	hydrocarbons, metals (Al, Zn and Fe), nitrogen, silica, sulphur and ammonia	*Trichomycterus areolatus* and *Percilia gillissi* / enzymes (EROD and brain AChE)		Chiang et al. 2012
Luvuvhu River	DDT and related metabolites: p,p'-and o,p'-forms of DDT, DDE and DDD	*Clarias gariepinus*		Brink et al. 2012
Nebraska, USA watersheds: the Niobrara and Dismal Rivers (low-impact agricultural sites) and the Platte and Elkhorn Rivers (high-impact agricultural sites)	ammonia, phosphorus, chloride, nitrate and pesticides: acetochlor, alachlor, atrazine, simazine, metolachlor,	hepatic gene expression of *Pimephales promelas*		Sellin Jeffries et al. 2012
Aqueous solutions	stannum dioxide (SnO_2), cerium dioxide (CeO_2) and iron oxide (Fe_3O_4) nanoparticles	*Paracentrotus lividus* / cholinesterase, HSC70 and GRP78	Echinoderm	Falugi et al. 2012
Southwest Atlantic	mercury (Hg)	Feathers of *Spheniscus magellanicus*	Mammal	Frias et al. 2012
Black Sea, North sea	PCB compounds	*Phocoena phocoena*		Weijs et al. 2011

Annex 3. Summary of bacterial growth levels observed after pollutant exposure.

Pollutant	time (min)	[ ]*	*R. baltica*	LF2	Sm4	UC9	UC17	MsF5	*E. coli*	*P. putida*	*V. anguillarum*
Arsenic	30	59 µM	4	4	4	4	4	4	4	4	4
		293 µM	4	4	4	4	4	4	4	4	4
		587 µM	4	4	4	4	4	4	4	4	4
		1466 µM	4	3	4	4	4	4	4	4	4
		2933 µM	4	2	3	1	4	4	4	3	0
		4399 µM	4	1	2	1	3	3	4	1	0
		5866 µM	2	0.5	0.5	0	0.5	2	4	0	0
Cadmium	30	59 µM	4	4	3	4	3	4	4	4	0.5
		293 µM	4	3	0.5	3	0	3	4	4	0
		587 µM	3	2	0	1	0	1	4	4	0
		1466 µM	3	1	0	0.5	0	0	4	4	0
		2933 µM	2	0.5	0	0	0	0	4	4	0
		4399 µM	1	0	0	0	0	0	4	4	0
		5866 µM	0	0	0	0	0	0	4	2	0
Chromium	30	59 µM	4	4	4	4	4	4	4	3	3
		293 µM	4	3	4	3	4	4	4	2	0
		587 µM	4	2	3	2	3	4	4	2	0
		1466 µM	2	1	0	1	0	1	4	1	0
		2933 µM	1	0.5	0	0.5	0	0	4	0.5	0
		4399 µM	1	0	0	0	0	0	4	0	0
		5866 µM	0.5	0	0	0	0	0	4	0	0
Copper	30	59 µM	0.5	3	0	3	1	0.5	4	2	1
		293 µM	0	0	0	1	0	0	4	0	0
		587 µM	0	0	0	1	0	0	4	0	0
		1466 µM	0	0	0	0.5	0	0	4	0	0
		2933 µM	0	0	0	0.5	0	0	4	0	0
		4399 µM	0	0	0	0	0	0	4	0	0
		5866 µM	0	0	0	0	0	0	4	0	0
Nickel	30	59 µM	4	4	4	4	4	4	4	4	4
		293 µM	4	4	4	4	4	4	4	4	3
		587 µM	4	4	4	4	4	4	4	4	3
		1466 µM	4	3	4	4	4	4	4	4	0
		2933 µM	4	2	4	4	4	4	4	4	0
		4399 µM	4	2	4	4	4	4	4	4	0
		5866 µM	4	0.5	2	3	3	3	4	4	0
Zinc	30	59 µM	4	4	4	4	4	4	4	4	0
		293 µM	4	3	4	4	2	4	4	4	0
		587 µM	4	3	1	4	1	4	4	4	0
		1466 µM	3	3	0	2	0.5	4	4	4	0
		2933 µM	3	2	0	1	0.5	3	4	3	0
		4399 µM	2	2	0	1	0	2	4	2	0
		5866 µM	0.5	1	0	0.5	0	0.5	4	1	0
Nitrates	30	0.35 M	4	4	4	4	4	4	4	3	4
		1.77 M	4	4	4	4	4	4	4	2	3
		3.53 M	4	4	4	4	4	4	4	0.5	2
	60	0.35 M	4	4	4	4	4	4	4	4	4
		1.77 M	4	4	4	4	4	4	4	0	4
		3.53 M	4	3	4	4	4	4	4	0	3
Nitrites	30	0.14 M	4	4	4	4	4	4	4	4	4
		0.73 M	4	4	4	4	4	4	4	3	3
		1.45 M	4	4	4	4	4	4	4	1	2
	60	0.14 M	4	4	4	4	4	4	4	4	4
		0.73 M	4	4	4	4	4	4	4	1	3
		1.45 M	4	4	4	4	4	4	4	0.5	2
Ammonium	30	0.02 M	4	4	4	4	4	4	4	4	4
		0.19 M	4	4	4	4	4	4	4	1	2
		1.87 M	4	2	4	4	2	4	4	1	0
	60	0.02 M	4	4	4	4	4	4	4	4	4
		0.19 M	4	4	4	4	3	4	4	3	0.5
		1.87 M	3	3	3	4	1	4	4	1	0
Phosphates	30	0.08 M	2	4	2	4	4	2	4	2	0
		0.42 M	0	1	1	0.5	1	1	4	0.5	0
		0.83 M	0	0.5	1	0.5	0.5	0.5	4	0	0
	60	0.08 M	0	2	2	1	3	2	4	3	0
		0.42 M	0	0	0.5	0.5	0.5	1	4	0.5	0
		0.83 M	0	0	0.5	0	0	0.5	4	0	0
Hand cleanser	30	1 %	4	3	4	4	4	4	4	4	2
		5 %	3	3	3	4	2	4	4	4	0
		10 %	2	2	1	4	2	4	4	4	0

Annex 3 (continued)

Hand cleanser	60	1 %	4	3	3	4	3	4	4	4	0
		5 %	3	2	0,5	4	2	0,5	4	3	0
		10 %	2	1	0	4	1	0	4	2	0
Dish detergent	30	1 %	4	3	4	4	4	4	4	4	1
		5 %	3	3	4	4	4	4	4	3	1
		10 %	2	2	4	4	4	4	4	3	0.5
	60	1 %	4	4	4	4	4	4	4	3	0.5
		5 %	2	3	4	4	4	4	4	2	0.5
		10 %	1	2	3	4	4	3	4	1	0
IGEPAL CA-630	30	1 %	4	3	4	4	4	2	4	4	0.5
		5 %	3	2	4	4	2	0,5	4	4	0.5
		10 %	3	2	3	1	0,5	0	4	4	0
	60	1 %	4	3	4	4	4	1	4	4	0
		5 %	3	2	4	2	2	0	4	4	0
		10 %	2	0.5	3	0,5	0,5	0	4	3	0
Ridomil®	30	0.1 %	4	4	4	4	4	4	4	4	4
		0.5 %	1	2	2	2	1	1	4	4	0
		1 %	0	0	0	0	0	0	4	1	0
	60	0.1 %	4	3	4	4	4	4	4	4	4
		0.5 %	2	0.5	0	2	2	3	4	4	0
		1 %	0	0	0	0	0	0	4	1	0
Previcur N®	30	1 %	4	4	4	4	4	4	4	4	4
		5 %	4	3	3	4	4	3	4	4	4
		10 %	0	0.5	1	4	3	1	4	4	4
	60	1 %	4	4	4	4	4	4	4	4	4
		5 %	3	0.5	2	4	4	2	4	4	4
		10 %	0	0	1	3	0.5	0	4	4	4
Phenol	30	0.01 M	4	4	4	4	4	4	4	4	0.5
		0.11 M	0	0	0	0	0	0	0	0	0
		1.06 M	0	0	0	0	0	0	0	0	0
	60	0.01 M	4	4	4	4	4	4	4	4	0
		0.11 M	0	0	0	0	0	0	0	0	0
		1.06 M	0	0	0	0	0	0	0	0	0
Hydrazine	30	0.15 mM	4	2	2	4	4	4	4	1	0
		1.54 mM	0	0	0.5	0	0	0	4	0	0
		15.4 mM	0	0	0	0	0	0	4	0	0
	60	0.15 mM	0.5	1	0.5	0.5	0	0	4	0	0
		1.54 mM	0	0	0	0	0	0	4	0	0
		15.4 mM	0	0	0	0	0	0	4	0	0
Sodium Azide	30	0.15 M	4	4	4	4	4	4	4	4	3
		0.77 M	4	4	4	4	4	4	3	0	2
		1.54 M	4	4	4	4	4	4	2	0	1
	60	0.15 M	4	4	4	4	4	4	4	4	3
		0.77 M	4	4	4	4	4	4	2	0	2
		1.54 M	4	4	4	4	4	4	2	0	0
Diclofenac	30	3.1 mM	4	4	4	4	4	2	4	3	0
		31.4 mM	1	0	0	0	0	0	4	1	0
Acetaminophen	30	6.6 mM	4	4	4	4	4	4	4	4	4
		66.2 mM	4	4	4	4	4	3	4	4	2
Caffeine	30	5.2 mM	4	4	4	4	4	4	4	4	4
		51.5 mM	4	4	4	4	4	4	4	4	0

* Concentrations tested.
0 – Absence of growth; 0.5 to 4 – Different levels of growth.

Annex 4. List of cytochrome c-like proteins with a role or potential role in cell respiration of *P. putida* and *V. anguillarum*.

FIGfam assignment	Protein	Length (aa)	Bacteria	Genomes
FIG00000640	Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	295 ± 8.7	*P. putida* (17 genomes)	17
FIG00001108	Cytochrome c oxidase subunit CcoO (EC 1.9.3.1)	202 ± 36.5		17
FIG00001139	Cytochrome c oxidase subunit CcoP (EC 1.9.3.1)	326 ± 71.3		17
FIG00002166	Cytochrome c oxidase subunit CcoQ (EC 1.9.3.1)	65 ± 2.6		17
FIG00041887	Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	363 ± 66.6		17
FIG00078450	Cytochrome c2	132		1
FIG00103126	Cytochrome c oxidase subunit CcoN (EC 1.9.3.1)	480 ± 139.9		17
FIG00132629	Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	529 ± 100.7		17
FIG00134827	Cytochrome c4	200 ± 1.7		17
FIG00136407	FIG135464: Cytochrome c4	216 ± 1.7		17
FIG00149502	FIG002261: Cytochrome c family protein	304 ± 77.6		17
FIG00953232	cytochrome c-type protein	96		2
FIG01278337	Cytochrome c family protein	266 ± 169.9		17
FIG01308127	Cytochrome c5	140 ± 40.4		17
FIG01345229	cytochrome c, putative	500 ± 130.3		16
FIG00001108	Cytochrome c oxidase subunit CcoO (EC 1.9.3.1)	204	V. anguillarum (3 genomes)	3
FIG00001139	Cytochrome c oxidase subunit CcoP (EC 1.9.3.1)	325		
FIG00002166	Cytochrome c oxidase subunit CcoQ (EC 1.9.3.1)	58		
FIG00103126	Cytochrome c oxidase subunit CcoN (EC 1.9.3.1)	475		
FIG00134827	Cytochrome c4	205 ± 8.7		
FIG01308127	Cytochrome c5	146		

Annex 5. List of cytochrome c and cytochrome bd proteins (or putative) with a role or potential role in cell respiration of Planctomycetes.

FIGfam assignment	Protein	Length (aa)	Genomes (out of 23)
FIG00000390	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.)	339 ± 67.5	14
FIG00080642	putative Cytochrome bd2, subunit I	449 ± 2.8	2
FIG00096593	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	429 ± 20.9	14
FIG01634385	putative cytochrome d ubiquinol oxidase subunit II	345	1
FIG00032582	putative Cytochrome bd2, subunit II	351	1
FIG00147534	probable cytochrome oxidase (cbb3-type)	1556 ± 303.9	14
FIG01898829	Probable cytochrome oxidase (Cbb3-type) (EC 1.9.3.1)	896 ± 10.6	2
FIG00000640	Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	297 ± 97.2	19
FIG00001108	Cytochrome c oxidase subunit CcoO (EC 1.9.3.1)	272 ± 28.9	4
FIG00001139	Cytochrome c oxidase subunit CcoP (EC 1.9.3.1)	205 ± 11.4	10
FIG00029484	Cytochrome c oxidase polypeptide I (EC 1.9.3.1) / Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	833 ± 7.5	10
FIG00041887	Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	333 ± 46.8	19
FIG00073282	Cytochrome c oxidase polypeptide IV (EC 1.9.3.1)	123 ± 33.4	3
FIG00103126	Cytochrome c oxidase subunit CcoN (EC 1.9.3.1)	484 ± 22.1	5
FIG00132629	Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	566 ± 39.9	18
FIG00132938	Cytochrome c oxidase subunit CcoN (EC 1.9.3.1) / Cytochrome c oxidase subunit CcoO (EC 1.9.3.1)	832 ± 164.4	12
FIG00149218	FixO3 cytochrome-c oxidase subunit	474 ± 34.0	9
FIG00340871	Ubiquinol-cytochrome C reductase complex core protein I, mitochondrial precursor (EC 1.10.2.2)	419 ± 8.0	9
FIG00538303	cytochrome c, class I	247 ± 78.6	7
FIG00929168	probable cytochrome c oxidase, subunit III	187 ± 8.9	12
FIG01278337	Cytochrome c family protein	458 ± 66.4	12
FIG01331832	cytochrome c family protein	657	1
FIG01585259	Cytochrome c precursor	429	1
FIG01934443	Probable cytochrome c	1141 ± 337.8	16
FIG01949722	probable c-type cytochrome precursor	698 ± 146.6	8