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Tolerância de anfíbios e de bactérias simbiontes da pele a contaminação por salinidade e drenagem ácida

Tolerance of amphibians and its skin symbiotic bacteria to increased salinity and acid mine drainage contamination

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro

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“Até Hoje Fui Sempre Futuro.”
Almada Negreiros

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Palavras-chave Microbioma da pele, NaCl, drenagem ácida, girinos, ecotoxicidade.

Resumo

A salinização e drenagem ácida (AMD) constituem uma ameaça no meio ambiente repercutindo-se na perda de biodiversidade e equilíbrio nos ecossistemas.

O objetivo principal deste estudo consistiu em analisar os efeitos adversos de NaCl e AMD em anfíbios e na comunidade bacteriana da sua pele. Assim, a sensibilidade do microbioma, recolhido em diferentes populações de *Pelophylax perezi*, foi testada primeiramente a AMD, através de exposição a diferentes diluições recorrendo a duas metodologias: Difusão por poços e o teste por Microdiluição. O método de Microdiluição demonstrou ser mais adequado para avaliar a sensibilidade das bactérias uma vez que permitiu reunir mais informação da sensibilidade, resultados mais precisos e melhor discriminação das categorias de sensibilidade das bactérias a AMD. Posteriormente, e dadas as vantagens mencionadas, avaliou-se a sensibilidade das bactérias da pele a NaCl, apenas pelo método de Microdiluição. Observaram-se diferenças significativas na sensibilidade dos isolados a NaCl e AMD e diferenças entre os locais de amostragem. A maioria dos isolados revelou ser tolerante aos dois poluentes tendo-se calculado valores médios das diluições/concentrações que provocaram 20% de inibição no crescimento (EC_{20}) das bactérias de 62.4% e 13.5 g/L para AMD e NaCl, respetivamente. A sensibilidade de girinos foi testada por exposição a várias concentrações e diluições de NaCl e AMD, respetivamente, durante 168h. De uma forma geral a sensibilidade dos girinos de *P. perezi*, foi superior à sensibilidade da comunidade bacteriana intrínseca da pele. Comparando a sensibilidade dos isolados dois tipos de poluição com a sensibilidade de *X. laevis,* a sensibilidade do anfíbio foi maior que a dos isolados. Relativamente à sensibilidade das duas espécies de anfíbios não se verificaram diferenças significativas quando expostos aos dois tipos de contaminação.

Por fim, foi analisada a capacidade de uma bactéria do microbioma da pele de anfíbios, adquirir tolerância a NaCl, através da sua exposição continua a baixas concentrações deste composto. Para tal, o isolado *Erwinia toletana* foi exposto, durante seis semanas a LB ou à concentração de NaCl que induz 10% de inibição no seu crescimento (18 g/L). Após a exposição continua, foi realizado novamente um teste de sensibilidade a NaCl usando o método de Microdiluição. Seguidamente, realizou-se também um teste de metabolismo de compostos de carbono para analisar diferenças no processo metabólico. Os resultados confirmaram um aumento de tolerância da bactéria a NaCl, apresentando um aumento do EC_{20} de 20 g/L (18.5-21.9) (6ª geração exposta a meio LB) para 30.8 g/L (25.4-36.3) (6ª geração exposta ao EC_{10} de NaCl). As respostas a nível dos processos metabólicos apresentaram diferenças entre os isolados expostos durante seis semanas a LB ou a NaCl, no tipo de composto utilizado. Estes resultados sugerem que *E. toletana*, quando exposta a níveis baixos de NaCl (EC_{10}), utilize vias metabólicas que possam colmatar o stress induzido pela presença de NaCl no meio.

Assim, o estudo de sensibilidade de bactérias a contaminantes, para fins de bioaumentação em anfíbios expostos aos mesmos contaminantes no meio ambiente, poderá contribuir para uma maior defesa a nível do sistema imunitário.

Keywords Skin microbiome, NaCl, acid drainage, tadpoles, ecotoxicity.

Abstract

Salinity and Acid Mine Drainage (AMD) constitute environmental threats that may lead to biodiversity losses and ecosystems disturbances. The main goal of this study intended to assess the adverse effects that these two types of contamination may exert on amphibians and on its skin bacterial community. For this, the sensitivity of the skin microbiome, collected at different natural populations of *Pelophylax perezi*, was tested primarily to AMD, by exposing the skin bacteria isolates to serial AMD dilutions through two methodologies: Wells Diffusion and Microdilution methods. Microdilution method revealed to be more suitable to evaluate the sensitivity of the bacteria, since it allowed to better discriminating the AMD-sensitivity among bacteria. Therefore, only this method was used to proceed with the assessment of sensitivity of the skin bacterial community to NaCl. The majority of the isolates seemed to be tolerant to both pollutants (AMD and NaCl) revealing average values of the dilution/concentration causing a reduction of 20% in bacteria growth (EC_{20}) to AMD and NaCl of 62.4% and 13.5 g/L, respectively. Tadpole's sensitivity was assessed by exposing them, for 168 h, to different concentrations/dilutions of NaCl and AMD, respectively. In general, results showed that tadpole's sensitivity to these pollutants was higher than its intrinsic bacterial community, in what concerns to *P. perezi.* Additionally, *Xenopus laevis* tadpoles also showed a higher sensitivity to both stressors, comparatively to the skin bacterial isolates. Between amphibian species, no differences were observed in the sensitivity to NaCl and AMD sensitivity.

The capacity of amphibian skin bacteria to acquire tolerance to NaCl over a succession of exposures to low levels of this chemical was also assessed. For this, the bacteria isolate *Erwinia toletana* was exposed for six weeks to LB medium or to the EC_{10} for NaCl (18 g/L). After exposure for six weeks, the sensitivity to NaCl of the bacteria was reassessed by running again the Microdilution growth inhibition assay. Additionally, the metabolic degradation of carbon compounds were also tested to understand if there were differences on metabolic mechanisms. Results confirmed that tolerance to NaCl increased, by presenting a shift on EC_{20} from an initial value of 20 g/L (18.5-21.9) (for *E. toletana* exposed for six weeks to LB medium) to 30.8 g/L (25.4-36.3) (for *E. toletana* exposed for six weeks to the EC_{10} of NaCl). Also, the metabolic processes shown to be different between *E. toletana* continued exposed to LB or to NaCl, suggesting that *E. toletana,* when exposed to low levels of salinity (EC_{10}), use or activated different metabolic pathways to deal with osmotic stress caused by high salinity.

The study of bacterial isolates sensitivity to contaminants for bioaugmentation application in amphibians exposed to the same contaminants, present in the environment, could constitute an improvement of amphibian's immunity defense.

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CHAPTER 1

Main Introduction

1. Amphibians decline and major threats associated

First data observations concerning amphibian declines started in 1970, although alarming reports of amphibian population drops off, date back to 1989 (Stuart 2005; Hof *et al*. 2011). Since then, amphibians are considered the most threatened group of vertebrates entering in mass extinction (Lanyi 1974; Beebee and Griffiths 2005; Griffiths, Sewell, and McCrea 2010; Blaustein *et al*. 2011; Buckley, Beebee, and Schmidt 2014; Herkovits *et al*. 2015). The International Union of Conservation of Nature (IUCN), an environmental organization aiming at Nature's conservation and global assessment of species' extinction risk, performed a global assessment of amphibians. The obtained records indicated an imminent declining among amphibian's populations: in a total of 6000 amphibian species described worldwide, 32% are considered in extinction and 41% of the global amphibian taxonomic group whiteness's imminent declining populations (IUCN, 2008), foreseeing further extinctions in a near future.

Amphibians are capable to inhabit a large geographic area including different environments and ecosystems like wetlands or deserts, as well as diverse climatic regions (e.g., temperate, tropical, humid or Mediterranean climates) (Hamer and McDonnell 2008). Following this wide distribution, threats to amphibians could originate from several sources, leading to its exposure to vast scenarios of environmental perturbations. The intensity of the effects on amphibians, caused by these threats, depends on several factors, namely on organism life stages (e.g., embryo, larvae, juvenile, adult), since each stage holds different mechanisms of detoxification and will be exposed to the stressors through different pathways (Beebee and Griffiths 2005).

Amphibians decline cannot be attributed to just one single causative factor. In fact, some authors, as Beebee and Griffiths (2005) and Blaustein *et al*. (2011), highlighted an overall range of factors/threats that had been related with such declines. Furthermore, the IUCN (2008) published a report where influence of several threats on the declining of amphibians was described (Fig. 1).

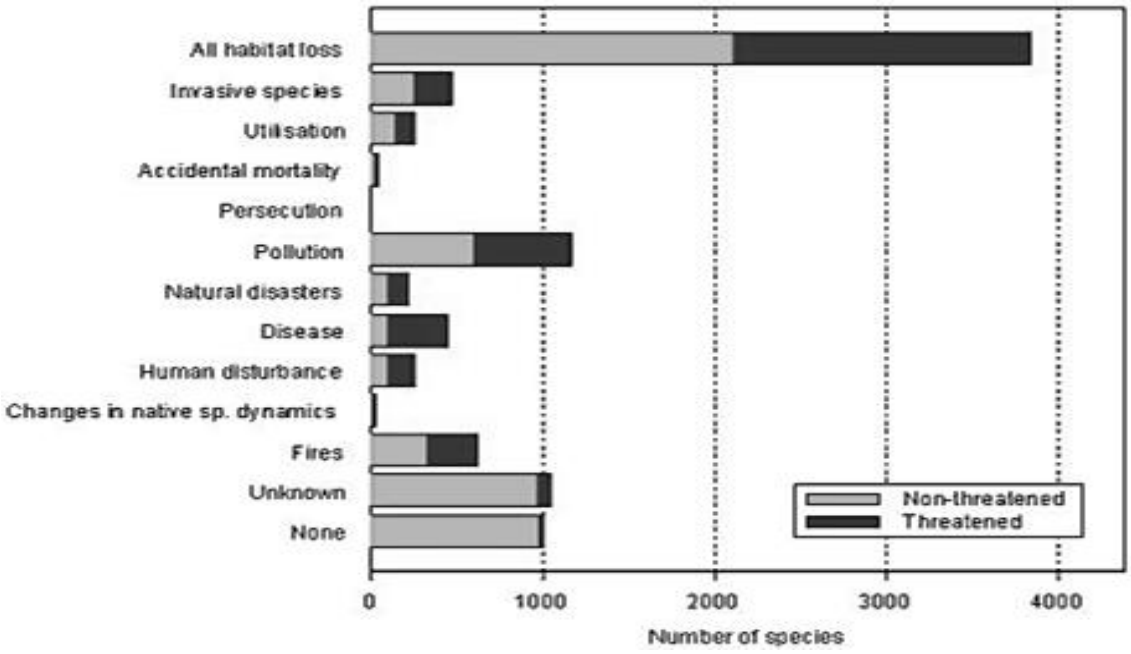

Figure 1: Representation of major threats concerning amphibians decline. Source: IUCN (2008).

These threats fall in two different groups: (i) indirect threats, including environmental causes and (ii) direct threats, including anthropogenic activities (D'Amen and Bombi 2009). The former group links with climate change, elevated levels of radiation (specifically UV-B), short-term fluctuations in rainfall and emerging infectious diseases caused by bacteria, fungus and virus (Rollins-Smith 2009; D'Amen and Bombi 2009; Griffiths, Sewell, and McCrea 2010; Blaustein *et al*. 2011). Nowadays the most studied and concerning indirect threat, due to its severe virulence and lethal effects, is *Batrachochytrium dendrobatidis* (Bd), a fungus which presence is associated with global warming (with subsequent temperature increase), chemical pollutants, and demographic alterations, (Daszak *et al*. 2003; Beebee and Griffiths 2005; Alford, Bradfield, and Richards 2007; D'Amen and Bombi 2009; Rollins-Smith 2009). In Figure 2 is illustrated the extension of declining amphibians projected for the year 2080 due to Chytridiomycosis caused by Bd infection and to climate change.

Despite of the wide range of factors identified to cause amphibians decline, it is consensual that *Bd* infection is the most dangerous and devastating threat known, to amphibians, being spread all over the world (Pimm *et al*. 2014; Longo *et al*. 2015).

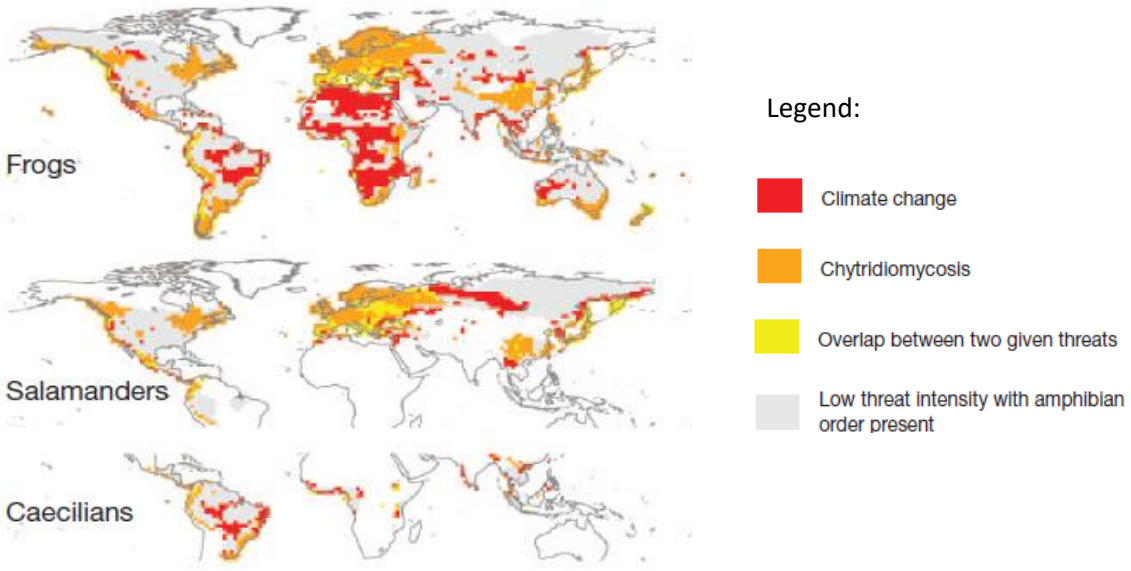

Figure 2: Worldwide distribution projection for the year 2080 of the two main threats of amphibian diversity: Climate change and Chytridiomycosis. Adapted from Hof *et al.* (2011).

In the second group, direct threats originate from anthropogenic activities that causes habitats destruction and alteration, pollutants releases (e.g. pesticides, pharmaceuticals and detergents), introduction of predators and alien species, and the massive capture of amphibians (Beebee and Griffiths 2005; Hamer and McDonnell 2008; Griffiths, Sewell, and McCrea 2010; Blaustein *et al*. 2011).

2. Environmental Contamination

2.1 Amphibians and Metal Contamination

Freshwater ecosystems all over the world have been one of the most affected environmental compartment concerning chemical contamination, both direct or indirectly (Sparling and Lowe 1996; Shuhaimi-Othman *et al*. 2012; Marques *et al*. 2013; Priyadarshani *et al*. 2015). Specifically, metals contamination has increased, over the years, in aquatic ecosystems mainly as a consequence of anthropogenic activities (Sparling and Lowe 1996; Zhou *et al*. 2008; Priyadarshani *et al*. 2015).

The anthropogenic activities that mostly contributes to the increased presence of metals in these aquatic systems are mine exploitation (resulting in acid mine drainage), industrial production and agriculture/domestic/industrial wastewaters (Sparling and Lowe 1996; Zhou *et al*. 2008; Priyadarshani *et al*. 2015). Almost all metallic elements are known

to be present in the environment, although some of them have been reported in water and/or aquatic systems worldwide in elevated concentrations (e.g. Fe, Al, Cd, Cu, Zn, Cr, As, Ag, Hg, etc.), that may constitute ecological risks (Sparling and Lowe 1996; Zhou *et al*. 2008; Shuhaimi-Othman *et al*. 2012).

The effects that metal contamination may posed to biota can vary from sublethal (e.g. physiological and behavioral abnormalities) to lethal (Sparling and Lowe 1996; Zhang *et al*. 2012; Marques *et al*. 2013; Priyadarshani *et al*. 2015). The intensity of such effects depends on several factors, namely: (i) intrinsic sensitivity of species, (ii) characteristics inherent to the organism (e.g. life stage at exposure, exposure pathway, mechanisms of detoxification/tolerance), (iii) mechanisms of defense and immunotoxicity process (iv) metals bioavailability in water, (v) other abiotic and biotic parameters (e.g. pH, Eh, nutrition and age) associated with the ecosystem that may alter the toxicity and speciation of metals (Sparling and Lowe 1996; Zhou *et al*. 2008; Marques *et al*. 2013; Flynn, Szymanowski, and Fein 2014).

Amphibians are sensitive to metals exposure and tend to bioaccumulate metals at high concentrations in their tissues, making them a reliable environmental sentinel for this type of contamination (Sparling and Lowe 1996; Lefcort *et al*. 1998; Parris and Baud 2004; Cooper and Fortin 2010; Xia *et al*. 2012; Priyadarshani *et al*. 2015).

Several studies dealing with the effects of metals on amphibians have been published in the scientific literature. As some examples, Yologlu and Ozmen (2015) exposed *Xenopus laevis*, at Gosner stages 46 to 48, to concentrations of Cd, Pb and Cu, both in single exposure and in binary and tertiary mixtures. Among the three metals, Cu presented the highest lethal toxicity to *X. laevis*, with a median lethal concentration (LC_{50}) of 0.85 mg AI/L (LC_{50} for Cd = 5.18 mg AI/L and for Pb = 123.05 mg AI/L). Furthermore, based on the observed responses of enzymatic activities, the authors reported that single exposure to metals induced lower toxicity than when in binary or tertiary mixtures. Interestingly, these authors observed an increase in effects at the biochemical level even between binary and tertiary mixtures, suggesting the occurrence of synergistic effects at sublethal effects. Santos *et al*. (2013) exposed embryos and tadpoles of *Pelophylax perezi* to NaCl and copper, both as single and mixture exposure. The authors observed that the two life stages of *P. perezi* exhibited different sensitivities to the chemicals and to their mixture. Tadpoles were more sensitive to lethal levels of copper than embryos: $LC_{50,96h}$ of 0.97 mg/L and 7.35 mg/L, respectively. On the contrary, embryos revealed to be more sensitive to lethal levels of NaCl than tadpoles: $LC_{50,96h}$ of 3.79 g/L and 7.40 mg/L, respectively. When *P. perezi* were expose to a mixture of Cu and NaCl the two life stages

exhibited different responses as well: for tadpoles no significant interactions were observed among NaCl and Cu in their lethal toxicity, but, for embryos an antagonistic effect was observed, i.e. lower concentrations of NaCl in the mixture acted as protective against the lethal effects of Cu. This study showed that different life stages of amphibians respond differently to chemical contamination, highlighting the need to deeply understand the biology of the whole life cycle of these organisms within the context of ecological risk assessment.

2.2 Amphibians and Increased Salinity

Within the past years an increase of salinity has been reported in freshwater systems (Sanzo and Hecnar 2006; Denoël *et al*. 2010; Bernabò *et al*. 2013; Hopkins, French, and Brodie 2013). Globally this increase has been associated mainly to anthropogenic activities (e.g., road de-icing application, mine exploration, discharges of saline wastewaters) and to the rise of seawater caused by climate changes (mainly for coastal lagoons) (Sanzo and Hecnar 2006; Collins and Russell 2009; Denoël *et al*. 2010; Shinn, Marco, and Serrano 2013). The effects of salinity fluctuations on aquatic systems, such as freshwaters ponds and wetlands, results in vegetation clearance, evapotranspiration, biodiversity losses, saline agriculture wastewaters and alterations of the river´s flow, among others (Kearney *et al*. 2014; Herbert *et al*. 2015). Additionally, variations on tidal flux, rainfall and evaporation as well as demographic alterations can implicate salinity fluctuations on aquatic systems (Kearney *et al*., 2015). One example of increased salinity caused by anthropogenic activity occurs in North America, where about 14 million tons of salt in water systems per year, due to the use to de-ice roads (being NaCl, $CaCl_2$ and $MgCl_2$ the most common forms of salt used), were reported (Sanzo and Hecnar 2006). Moreover, in Canada is possible to measure 4000 mg/L of NaCl on the roadside ponds, a very high concentration according to Environmental Canada (EC, 2001). Furthermore, Environmental Canada (2001) indicates that chronic exposure to Cl^- concentrations above 220 mg/L induces harmful effects to 10% of freshwater species (Collins and Russell 2009).

It is consensual that increased salinities provoke alterations on early life-stages of amphibians and affect not just their survival but also their behavior (Godwin, Hafner, and Buff 2003; Collins and Russell 2009; Karraker and Ruthig 2009; Denoël *et al*. 2010). Salts constitute a stressor to amphibians due to its conservative and soluble character (Sanzo and Hecnar 2006). The amphibian's high permeable skin and the salt tendency to

bioaccumulate in tissues, also contribute to enhance salts' toxicity to this group of organisms (Sanzo and Hecnar 2006; Bernabò *et al*. 2013; Hopkins, French, and Brodie 2013). Although being acknowledged that amphibians are not well adapted to salt water it's possible to find them in brackish waters, but they are absent in the oceans (Sanzo and Hecnar 2006).

Hopkins *et al.* (2013) exposed amphibian eggs of *Taricha granulosa* to two different types of salt: NaCl and $MgCl_2$. Results demonstrated that environmentally relevant concentrations of both salts (4.0 g/L Cl^- reported in ponds nearby the roads, in North of America; and 2.05 g/L Cl^- reported for habitats inhabited by *T. granulosa*) induced earlier hatching, delayed development and decreased body size, comparatively to the control. Additionally, Bernabó *et al.* (2013) observed that dilutions of seawater below 10% caused a decrease in growth and body mass and a delay in reaching metamorphosis in tadpoles of *Bufo bufo*. Karraker and Ruthing (2009) reported high mortality rates for *Ambystoma maculatum* embryos at 0.145 mg/L of chloride, while Denoël *et al.* (2010) observed behavior effects, as low speed and short distances in swimming, in tadpoles of *Rana temporaria* exposed to 1500 mg/L of NaCl.

According to the above mentioned and to reports predicting increased salinity in freshwater ecosystems, this environmental perturbation is alarming for amphibians once it can compromise amphibians resilience even at low levels.

3. Amphibian Defense Barriers

3.1 Amphibian Skin

Skin is one of the main organs responsible for organisms' immunity defense (Pessie 2002). In contact with external environment, skin constitute the primary barrier against external pressure, such as: water loss, invasion by pathogens, chemical exposure and substrate to household specific and natural symbiotic microbiome, responsible for pathogen defense, among others biological processes (Kueneman *et al*. 2014; Walke *et al*. 2014; Krynak, Burke, and Benard 2015). Anuran complex dermal tissue is responsible and the functional motor of several vital functions, as respiration, osmoregulation, thermoregulation, pigmentation, chemical communication, pathogen defense permeability and water absorption (Pessie 2002; Kueneman *et al*. 2014).

As dermal breathers, amphibians have high skin permeability and dependence on compounds as electrolytes, proteins like aquaporins (AQs) and differentiated cells as ionocytes. Aquaporins are involved in water exchanges and ionocytes are responsible for

ion exchanges and pH balance of the body fluid (Campbell *et al*. 2012; Haslam *et al*. 2014). These mechanisms are fundamental to conquer homeostasis and hydration of the skin and of the whole organism. The process of water intake is dependent of AQs activity and vasotocin hormone action (an neurohypophyseal hormone) (Campbell *et al*. 2012).

For amphibians being capable to perform gas and water exchanges it is necessary an hormonal function, dependent on vasotocin action in conjugation with skin glands secretions, to regulate cutaneous fluid and moisture, ion intake (e.g. Na^+, K^+ and Cl^-), and the ATPase and AQs pump activities (Campbell *et al*. 2012; Larsen and Ramløv 2013; Haslam *et al*. 2014). All these groups of transportation channels are responsible for gas exchange itself and are present in the membrane of dermal cells of amphibians (Haslam *et al*. 2014). So far, some specific AQs (e.g. AQPa1, AQPa2, AQP3) were identified as being involved in water absorption by amphibians, holding many similarities of functions with mammals AQs (Haslam *et al*. 2014).

Amphibians hold a skin that exhibits some similarities at the physiological, molecular and immunological level with Human skin. Similarly to mammals, amphibians' skin is composed by two layer: epidermis and dermis (Fig. 3 A, B). Epidermis comprehends tree layers (Fig. 3 B): stratum germinativum, stratum spinosum and corneum, which are responsible for mitotic activity ensuring epidermis layer, keratinization process and permeability to gas and fluids, respectively.

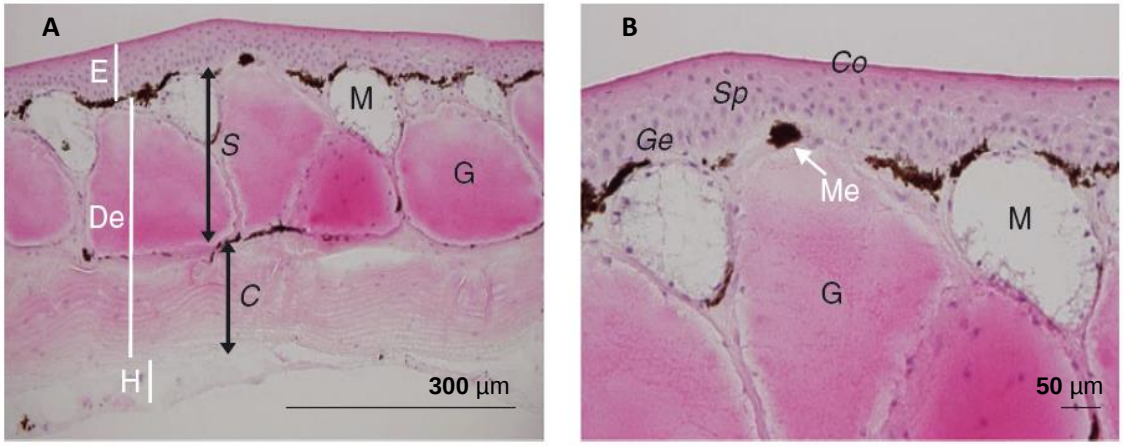

Figure 3 (A e B): Morphology of adult *Xenopus tropicalis* skin. In A white lines indicate epidermis (E), dermis (De) and hypodermis/subcutis (H). Black arrows indicate margins of the stratum spongiosum (S) and stratum compactum (C). M-small mucus gland; G-large granular/poison gland. B represents epidermis with indication of stratum germinativum (Ge), stratum spinosum (Sp), stratum corneum (Co) and small mucus gland (M). White arrow indicates a frog melanocyte (Me) at the dermal–epidermal junction. Adapted from Meier *et al*. (2013).

Epidermis possess different cell types, with specialized functions like for example: dendritic and Merkel cells responsible for immunity, Goblet cells consisting in mucus segregation, Flask and mitochondria-rich cells important in water, ions and organic molecules transportation and melanophores related to skin (Haslam *et al*. 2014). Anuran epidermis is covered by mucus, produced by glands that segregate this fluid into intracellular space leading to water accumulation (Haslam *et al*. 2014). This mucus is composed by glycoproteins, more specifically, antimicrobial peptides (AMPs), microbial symbionts, pathogens, lysozymes and mucosal antibodies (Kueneman *et al*. 2014; Colombo *et al*. 2015). Mucus has an important role against skin injury, infections protection, reducing dehydration and gas exchange (Colombo *et al*., 2015; Haslam *et al*., 2014).

In skin, dermis consists in collagen and fiber elastin, chromatophores, nerve fibers and blood vessels (Fig. 3). In depth it becomes denser and is constituted with collagen, fundamental to connect dermis to the muscle (Haslam *et al*., 2014). Along with mucus glands, granular glands are present in the dermis, being responsible for segregating substances into the epidermis (e.g. toxins, neurotoxins, cardiotoxins and hallucinogens or poison production). The characteristics of amphibian skin are important not only for organism defense to predators and external factors but also for basal metabolic mechanisms (Larsen and Ramløv, 2013; Colombo *et al*.; 2015; Haslam *et al*., 2014).

4. Immunity Defense

Anuran integument comprehends a large amount of secretions commanded by specialized glands and cells, active and crucial for homeostasis and organism maintenance. These secretions form the fundamental base of both Adaptive and Innate Immune System (Chinchar *et al*. 2004; Conlon 2011).

The adaptive immune system involves previous contact with potential pathogens or antigens, requiring a specific response (Carey *et al.*, 1999; Colombo *et al.*, 2015). This specific response is processed by T and B cells interaction through antigen-receptors. Once this cells contacts with antigens, they release antibodies and specific enzymes capable of neutralize them as it is assumed to occur in mammals (Colombo *et al*. 2015). On the other hand, innate immunity system does not need a previous contact with pathogens. Innate immunity comprise interactions within different cells and factors, defending or inhibiting infected cells through phagocytosis, macrophages mediation, neutrophils, dendritic cell or natural killer activity (Carey, Cohen, and Rollins-Smith 1999;

Colombo *et al*. 2015). The first line of innate immune defense is skin, acting as physical barrier to the external environment (Haslam *et al*. 2014). The immune defense acting through the skin structure involves antimicrobial peptides (AMPs), which are proteins with low molecular six capable of inhibiting a variety of microorganism such as bacteria, fungi and viruses (Chinchar *et al.*, 2004; Voyles *et al.*, 2011). Furthermore, immunity defenses at the skin level can be induced in amphibians through Biotherapy. Immunity defense inducement based on Biotherapy, involves the application or inoculum of living organisms, with beneficial properties to a subsequent host in order to protect this host from pathogen virulence or disease susceptibility (Woodhams *et al*. 2012). Some studies developed these induced immunity by using bacteria present on amphibian skin (e.g. *Janthinobacterium lividum* or *Pedobacter cryoconitis*) to reduce pathogen growth, allowing higher amphibian survival (Harris *et al*. 2009; Woodhams *et al*. 2012).

5. Amphibian' Skin Microbiome

5.1 Microbiome: Composition and Function

Each living organism, including amphibians, host a complex community of microbes, with an essential role in organism health and immune defense (McKenzie *et al*. 2012; Walke *et al*. 2014; Colombo *et al*. 2015). The amphibian microbiome community is represented by bacteria, archaea, fungi, viruses and protozoa groups living as symbionts or commensal microorganisms, co-habiting on skin and mucosal tissue with the host (Colombo *et al*. 2015). Microbiome is the base of the immune defense of the host, it provides homeostasis by helping in the control of metabolic and physiologic processes, disease susceptibility mediation and vitamin production, interaction with harmful pathogens by activating processes that compromise their survival releasing antimicrobial compounds or antifungal metabolites produced by some bacteria naturally present on amphibian skin or artificially inoculated, as *Janthinobacterium lividum* - (Harris *et al*. 2009; Jani and Briggs 2014; Kueneman *et al*. 2014; Colombo *et al*. 2015; Longo *et al*. 2015). The importance of an healthy and active cutaneous immunity in amphibian lays on the exposure, *via* epidermis, to several environmental perturbations like chemical contamination in water and soil, abiotic parameters associated with climate changes or pathogens that may infect them, among others (Hamer and McDonnell 2008). Skin microbiome of amphibians may change with environmental parameters, life cycle, metamorphosis, natural sloughing (which compromise both diversity and quantity of microbiota), alterations on diet, exposure to chemicals or pathogens capable to infect the

organism and that interact with the skin microbial community (Jani and Briggs 2014; Kueneman *et al*. 2014; Bataille *et al*. 2015; Colombo *et al*. 2015; Longo *et al*. 2015). McKenzie *et al*. (2012) carried out a study where they collected three species of amphibians (in pre-metamorphose stage) in three adjacent ponds and analyzed their skin microbiome community. Comparisons were made for microbiome community within species collected at the different ponds, among amphibian species within ponds and among species and ponds. Authors reported diversity of bacteria at the phyla level. Skin microbiome diversity did not vary across species inhabiting the same pond but differed when comparing species from different ponds, thus, suggesting that skin microbiome diversity is influenced by environmental conditions. Furthermore, these authors observed some consistency in skin microbiome composition within species from different ponds, suggesting some species specificity in the skin microbiome. They also reported that amphibians holding more skin anti-fungal bacteria were less susceptible to infections, highlighting the role of skin microbiome in the immunity defense mechanism of this group of organisms.

Several works on this research field have been carried out to investigate the composition of amphibian skin in order to comprehend its functions, microbial functions, immune defense role, antimicrobial peptides function and its applicability in Human health (Haslam *et al*. 2014; Becker *et al*. 2015; Colombo *et al*. 2015; Yasumiba, Bell, and Alford 2016).

5.2 Microbiome Responses to Metal Contamination

Some metals and metalloids constitute essential elements to organisms. To obtain enough amounts of these essential elements it is indispensable to acquire them through external sources (Nies 1999). Hence, some metals are essential and vital nutrients to maintain metabolic and molecular mechanisms such as redox process, gene expression control or osmoregulation (Nies and Silver 1995; Bruins, Kapil, and Oehme 2000).

Despite of their important role on vital functions, when present at high concentrations, they can induce direct toxic effects on the cells. Therefore, to avoid such adverse effects it is important to keep the balance of metals concentrations between the inner and outer membrane of the cell, which is kept by a chemiosmotic gradient through the membrane or ATP proteins activity (Nies and Silver 1995; Hobman and Crossman 2014).

Microorganisms, such as bacteria, can be adversely affected by exposure to high concentrations of metals (Gadd 1978; Hobman and Crossman 2014) since metals can

cause alterations on DNA structures, oxidative stress in the cell membrane and osmotic imbalance, among others (Bruins *et al.*, 2000). These processes involve biological configurations as bonding with thiol (SH) molecules inactivating them and forming reactive oxygen species (ROS), blocking molecules and inhibiting enzymatic or protein transporters activity (Nies and Silver 1995; Bruins, Kapil, and Oehme 2000; Hobman and Crossman 2014).

However, bacteria can activate and acquire tolerance mechanisms to metals by regulating intracellular and extracellular metal concentrations, or mechanism as methylation arrangements (Hobman and Crossman 2014). These mechanisms depends on codifying genes present on chromosomal, transposon and plasmid (Bruins *et al.*, 2000; Hobman and Crossman, 2014). Bruins *et al.* (2000) summarized mechanisms of tolerance to metals in bacteria, which consist in mechanisms going from metal exclusion by permeability barrier, active transport of the metal, intracellular sequestration of metals by protein binding, extracellular sequestration, enzymatic detoxication and reduction in metal sensitivity.

5.3 Microbiome Response to Salt Contamination

Salinity increment constitutes an environment concern since it affects several organisms in both freshwater and terrestrial systems (Zahran 1997). The normal range of salinity in freshwaters ecosystem without salt intrusions correspond approximately to 0.3 – 0.5 g/L of NaCl (Bernabò *et al*. 2013). Records on salinity contamination, reported elevated salt concentrations that overpass regular salinity of freshwater, ranging from 4 g/L to 30 g/L of NaCl (Bernabò *et al*. 2013; Cañedo-Arguelles *et al*. 2013). Despite these critical conditions, it is possible to find living microorganisms of *Archaea, Bacteria, and Eukarya* domains represented by *Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes*, and *Bacteroidetes* phyla in environments with elevated salt concentrations (Oren 2008; Margesin and Schinner 2001). Halophilic bacteria, as *Halobacterium salinarum*, can regulate osmotic activity or salt intake through two main mechanisms that require osmotic balance between membrane and cytoplasm and the surrounding medium (Zahran 1997; Margesin and Schinner 2001). The first mechanisms relies on the adjustment of intracellular enzymes that produce osmolytes (Zahran 1997; Margesin and Schinner 2001; Oren 2008; Ma *et al*. 2010). Salinity increments affect proteins and enzymes by compromising and altering their intrinsic structure. Thus, to keep intracellular normal salt concentration, enzymes must adapt to establish equilibrium between cell

cytoplasm and surrounding medium (Oren 2008; Margesin and Schinner 2001). The second mechanism consist in the production and accumulation of organic osmotic solutes that are responsible for energy conservation in the cell and subsequent exclusion of salt and/or ions from de intracellular structures (Zahran 1997; Oren 2008). Some consequences of an increase in salt or ion intake by bacteria, throughout the cell membrane, comprise: differences in metabolism and enzymes structure, physiological and structural modification of cell and cytoplasm volume imbalance (Zahran 1997; Margesin and Schinner 2001).

6. Objectives

The main objective of this study was to assess and compare the tolerance of amphibians and of its skin microbiome to two types of chemical stress: acid mine drainage and increased salinity. To achieve this major objective, the following specific goals were established:

- Determine a suitable method to test bacterial isolates sensitivity to chemical stress (acid mine drainage, AMD);
- Characterize the sensitivity of bacteria isolated from the skin of natural populations of the amphibian species *Pelophylax perezi* to AMD and NaCl;
- Assess the sensitivity of tadpoles of the amphibian species *Pelophylax perezi* and *Xenopus laevis* to both AMD and NaCl;
- Assess the capacity of amphibian skin bacteria to acquire an increase tolerance to NaCl after continuing exposure to low levels of this chemical.

This thesis is organized in five chapters. Chapter 1 aims at introducing the research thematic of this thesis and identifies the objectives of the present work. In Chapter 2, the first specific objective is addressed by assessing the sensitivity of bacteria from the skin of *P. perezi* to acid mine drainage by using two methodologies: Wells Diffusion method and microdilution protocols. In Chapter 3, the main objective was to analyze and compare the lethal sensitivity of tadpoles of *P. perezi* and of *X. laevis*, and of the skin symbiotic bacterial isolates to NaCl and AMD. Chapter 4 aimed at understanding if exposure of an intermediate sensitive skin bacterial isolate (*Erwinia toletana*) to low levels of NaCl, (corresponding to the concentration causing 10% of effects in the growth of *E. toletana*) through weeks, induced the acquisition of a higher tolerance to this chemical through the

evaluation of the occurrence of changes in its growth and in metabolic processes. Finally, Chapter 5 aimed to highlight the major findings and conclusions drawn from the present study and identify future perspectives on this research line.

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CHAPTER 2

Determination of the sensitivity of amphibian skin symbiotic bacteria to acid mine drainage by using two methodologies: Wells Diffusion and Microdilution protocols.

1. Introduction

Mining activities lead to the production of acid mine drainage (AMD) contamination that mainly results from the oxidation of sulphide minerals (e.g. pyrite, FeS_2;) (Akcil and Koldas 2006; Kuang *et al*. 2013; Méndez-García *et al*. 2014). This type of contamination is present worldwide and causes severe adverse effects in ecosystems due to its acidity and high metals contents. The adverse effects of AMD relies on corrosion damages, poor water quality, ecosystems destruction and high toxicity levels affecting biota communities (Kuang *et al*. 2013; Nieto *et al*. 2013; Simate and Ndlovu 2014). Regarding microbial communities and more specifically the bacteria group, exposure to AMD translates in decreased diversity, prevailing tolerant bacteria over sensitive ones (Zhang *et al*. 2007; Kuang *et al*. 2013). Consequently, environments contaminated with AMD tend to have lower bacterial diversity, being mainly constituted by acidophilic species, that are specifically adapted to these conditions (Zhang *et al*. 2007; Kuang *et al*. 2013; Méndez-García *et al*. 2014). The costs of such diversity losses, within microbial communities, can be of great concern since it may compromise ecological processes. Natural microbial populations have an important role in biological functions and mechanisms responsible for equilibrium imbalance such as photosynthesis (realized cyanobacteria), recycling nutrients, decomposition of dead organic matter, chemical processes including metal degradation (Zhang *et al*. 2007; Jackson *et al*. 2014). Furthermore, natural bacteria communities associated with other organisms may contribute to help the host dealing with chemical contamination; if the diversity of such community decreases then this role can be compromised. For example, symbiotic bacteria can decrease metals bioavailability for its host, by, for example, bioaccumulating the metals, changing their speciation, binding the metals in external matrices, among other processes (Jackson *et al*. 2014). Concerning the group of amphibians, their resilience depends greatly on the skin symbiotic microbiome, which function as a prime barrier defense from the surrounding environment (Colombo *et al*. 2015). The major functions of this microbiome consist in homeostasis regulation, control of metabolic and physiologic processes and interaction with harmful pathogens by the release of antimicrobial compounds or antifungal metabolites. Consequently, if exposure to chemical contamination depletes the diversity of this amphibian skin symbiotic microbiome, its functions, described above, may be impaired and may affect as

well the resilience/susceptible of amphibians to environmental perturbations (Kueneman *et al*. 2014; Colombo *et al*. 2015; Longo *et al*. 2015).

Therefore, the present work aimed at assessing the sensitivity of the skin microbiome of natural populations of the amphibian species *Pelophylax perezi* to contamination by AMD.

The assessment of the sensitivity of bacteria to chemicals may include two types of effects: (i) the chemical only affects the growth of the cells, maintaining them stationary (being a bacteriostatic agent), or (ii) the chemical leads to the death of the cells (being, a bactericidal agent) (Bernatová *et al*. 2013). Therefore, the use of rapid, cheap and sensitive methods that quantify and integrates the analysis of these two types of effects is important in order to obtain accurate results of bacterial sensitivity. The sensitivity of a bacteria to a chemical is frequently characterized by the minimum inhibitory concentration (MIC), which correspond to the minimal concentration of a chemical or antimicrobial agent that induce microbial growth inhibition (Mann and Markham 1998; Baquero *et al*. 2015; Fehlberg *et al*. 2016). There are innumerous different methods that can be applied to test the sensitivity of bacteria to chemicals, for example: E-Test, disk diffusion, microdilution methods (Matar *et al*. 2003), macrobroth or tube-dilution, antimicrobial gradient method and automated instrument systems (Biemer 1973). Although the majority of these methods are standardize for its use to determine susceptibility of bacteria to antibiotic agents and not for environmental samples such as mining effluents (Jorgensen and Ferraro 2009; Baquero *et al*. 2015). Consequently, in the present work, two standardized methods were used to access the sensitivity of the amphibian skin bacteria (Wells Diffusion and Microdilution methods) to AMD, in order to identify the method that allowed to better discriminating the sensitivity of bacteria to chemical contamination. After selecting the best method to assess the sensitivity of the bacteria to chemicals it was further used in chapter 3 to assess the sensitivity of bacteria to salinity (NaCl). Therefore, the discussion of bacteria sensitivity to AMD will be done in chapter 3 jointly with the discussion of their sensitivity to NaCl and in this chapter the discussion will be only focused in the selection of the best method to be used.

2. Material and Methods

2.1 Sampling sites

Bacterial collection of isolates were obtained through skin microbiome sampling of Perez' frog, *Pelophylax perezi*, from three different populations inhabiting aquatic ecosystems with distinct physico-chemical characteristics. The following three populations of *P. perezi* were sampled: Salreu (SL) (Brito, 2006, Cerqueira, 2008), Lagoa das Braças (LB) (Martins 2006; Castilho 2008) and Água Forte (TP) (Fig. 4; Costa *et al.*, 2016). Reference site (LB) was selected following the absent of chemical contamination (Martins 2006). Água Forte was chosen because it is impacted with metals (TP) due to mining activity (Luis 2007) and SL because it is impacted with salinity, since it is influenced by brackish water fluctuation of Ria de Aveiro (Brito and Pereira 2006). Coordinates and water site characterization are described in Table 1.

The acid mine drainage (AMD) effluent, used for the sensitivity assays, originates in the drainage of a mining settlement basin located near the TP site. This effluent meets the stream Ribeira da Água Forte (TP site).

Figure 4: Identification of the three sites where bacterial isolates, studied in this work, were sampled. Legend: Salreu (SL), Lagoa das Braças (LB) and Água Forte (TP). Adapted from Costa *et al*.,(2016)

Table 1: Values of physico-chemical parameters measured in the water column of the sample sites where adults of *Pelophylax perezi* were collected and swabbed. TSS: Total suspended solids; BOD5: Biochemical oxygen demand over five-days. Source: Costa *et al.* (2016).

Physico-chemical parameters	**Sample Sites**			
	LB	**SL**	**TP**	**AMD effluent**
Coordinates (Lat/ Long)	40°14'32 N/ 8°48'17 W	40°43'57 N/ 8°34'20 W	37°57'31 N/ 8°14'2 W	37°56'31 N/ 8°08'53 W
Dissolved oxygen (mg/l)	5.88 [a]	0.6	3.6	9.5
Conductivity (µS/cm)	293.0	5720	2210	4220– 4260
pH	8.8	7.7	4.9	2.1
Salinity	0	3	1	-
Turbidity (FTU)	31	37	73	-
TSS (mg/l)	9	13	113	-
BOD_5 (mg/l)	1.04	26.4	24	-
Nitrites (mg/l NO^-_2)	0.07	0.05	0.01	-
Nitrates (mg/l NO^-_3)	0.4	0.0	0.3	-
Nitrogen, Ammonia (mg/L NH_3-N)	0.27	1.06	5.7	-
Ca^{2+} (mg/l)	16	328	103	-
Mg^{2+} (mg/l)	5	96	31	-
Phosphorus, Reactive (mg/l PO_4^{3-})	0.04	0.43	0.41	-
Al (µg/l)	75	43	333	26000
As (µg/l)	16	5.4	87	1.3
Cd (µg/l)	0.31	0.05	0.84	310
Cu (µg/l)	5.2	2.1	148	2,800
Cr (µg/l)	2.8	1.0	1.6	-
Fe (µg/l)	5696	2976	48300	17000
Ni (µg/l)	1.80	4.00	16.0	61.0
Pb (µg/l)	0.59	0.95	19	3
Zn (µg/l)	22	9.2	575	15000

2.2 Model Organism: *Pelophylax perezi*

The Perez's frog *Pelophylax perezi* López-Seoane 1885 is an Iberian and Southern France frog present at high abundances in Portugal (Crochet *et al*. 1995; Egea-Serrano, Tejedo, and Torralva 2011). *Pelophylax perezi* inhabits a diverse type of aquatic and terrestrial environments (Ortiz-Santaliestra *et al*. 2007; Egea-Serrano, Tejedo, and Torralva 2011) as in larval and/or adult stage-life (Egea-Serrano, Tejedo, and Torralva 2011). Though amphibians are considered, in general, a very sensitive group, it has been observed that *P. perezi* may tolerate several types of contamination, namely agrochemicals and brackish water (Ortiz-Santaliestra *et al*. 2007; Egea-Serrano, Tejedo, and Torralva 2011). The use of *P. perezi* as a model species in this study relates with: (i) its abundance in Portugal geographic territory, being an autochthonous species, (ii) its ubiquity, being easily found in both reference and contaminated ecosystems, (iii) facility to capture and handle the adults to sample the skin microbiome, and (iv) lack of information on its skin microbiome.

2.3 Bacterial Isolates Collection and Storage

Bacterial isolates were collected from ten adult individuals of *Pelophylax perezi* skin in LB, TP, and SL (please see further details in Costa *et al*., 2016). In laboratory, the isolates were stored at -80°C in NB-medium with 15% glycerol (v/v). All bacterial isolates were identified through the 16S rRNA sequencing, Genbank accession numbers labeled on Table 6 of Annex I (please see also Costa *et al.*, 2016). Before running the assays, bacterial isolates were recovered in solid R2A medium (Oxoid, England) under a flow chamber. Every 96 to 120 hours, bacteria were re-cultured until normal growth and non-contamination were achieved. For later use, cultured bacteria in R2A Petri dishes were stored in a chamber at 4±1°C.

2.4. Sensitivity assay: Wells Diffusion Method

The Wells Diffusion method (Bauer *et al.*, 1966), with small changes, was used to expose 73 bacterial isolates to AMD. Diffusion disks were replaced by wells, according with the number of dilutions of the AMD being tested. To initiate the susceptibility test, each bacterium was suspended in previous autoclaved liquid NB medium (Nutrient Broth, Merck, Germany). The suspension intended to attain an optical density (O.D.) range of McFarland Standard No 0.5 corresponding to 1.5×10^8 CFU (McFarland J., 1907). This

O.D. was measured with a spectrophotometer (UVmini-1240, UV-vis Spectrophotometer, Shimadzu) at 600 nm of wavelength. After the measurement of McFarland O.D., 100 µL of each bacterial isolate suspension were uniformly cultured with sterile cotton swabs in sterilized solid R2A medium Petri dishes, in triplicate. The next step consisted in the AMD effluent addition at six increasing dilutions. Acid mine drainage was previously sterilized by filtration (Whatman™ Mixed Cellulose Ester Membrane Filter 0.2 µm) with a syringe. The AMD sterilized effluent was diluted with sterilized distilled water to obtain the following AMD test dilutions: 100%, 75%, 50%, 25%, 12.5%, and 6.25%. All bacterial isolates were also exposed to a negative control consisting of sterilized distilled water. All the exposures were performed by using a volume of test solutions of 50 µL in each test well of 0.38 cm^3.

For each bacterial isolate, three replicates were performed for the control and each dilution. Exposure took place at 23±1°C for 120 hours. At the end of this period, the minimal inhibition dilution (MID; the minimal tested dilution inducing microbial growth inhibition) was computed to categorize the sensitivity of bacteria to AMD.

Physical parameters as pH and conductivity of AMD dilutions were measured, at 23±1ºC, with a multiparameter equipment (WTW Multi 3410 SET C 2FD45C).

The sensitivity of the bacteria was categorized as follows: (i) **very sensitive**, for bacteria exhibiting a MID lower than the highest tested dilution (6.25%); (ii) **sensitive**, for bacteria exhibiting a MID equal or higher than the highest tested AMD dilution (6.25%) or equal to 50% of AMD; (iii) **tolerant**, for bacteria exhibiting a MID higher than 50% or equal or lower than 100% of AMD; and (iv) **very tolerant,** for bacteria exhibiting any growth inhibition when exposed to 100% of the AMD.

2.5 Sensitivity assay: Microdilution Method

Seventy three bacterial isolates previously cultured in R2A solid medium stored at 4°C were cultured in new Petri dishes with sterilized solid LB medium (Annex II, Table 11 and 13, respectively) under a flow chamber. The standard methodology (Clinical and Laboratory Standards Institute, 2012) was adapted by using 24-wells plate to culture bacterial exposed to AMD.

The bacterial isolates were suspended in sterilized liquid LB medium until reaching an O.D. of 1 (at 600nm) in order to allow starting the sensitive assay following the range of McFarland Standard No 0.5 corresponding to 1.5 x 10^8 CFU (McFarland J., 1907). After O.D. measurement, the sensitivity susceptibility assay was initiated. The following treatments were set in the 24-wells plates (Fig. 5 and Fig. 17 from Annex I): (i) the

negative control consisting of 2 mL of liquid LB medium plus 50 µL of bacteria isolate suspension; and (ii) each AMD dilution, consisting in 2 mL of each AMD dilution plus 50 µL of bacteria isolate suspension. All treatments were run in triplicate.

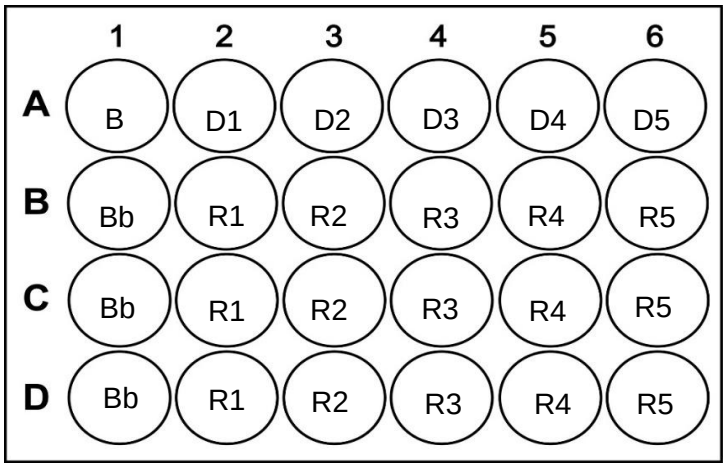

Figure 5: Representation of 24-wells plate filled with the tested solutions. Legend: B - LB medium, Bb - LB medium with Bacteria, D1 - Dilution 1 (6.25 % of effluent), R1 - replicates of D1 with Bacteria inoculum, D2 - Dilution 2 (12.5% of effluent), R2 - replicates of D2 with Bacteria inoculum, D3 - Dilution 3 (25 % of effluent), R3 - replicates of D3 with Bacteria inoculum, D4 - Dilution 4 (50 % of effluent), R4 - replicates of D4 with Bacteria inoculum, D5 - Dilution 5 (75 % of effluent), R5 - replicates of D5 with Bacteria plus D6 - Dilution 6 (100 % of effluent) which were placed in another 24-well plate with the exactly same method design.

Acid-mine drainage was sterilized by using the same methodology as that described in the section “Sensitivity assay: Disk Diffusion Method”. Also, bacteria isolates were exposed to the same AMD dilutions as for the Wells Diffusion Method. But here, the dilution media used was LB liquid medium instead of distilled water. The assay run for a period of 96 hours. Every 24h samples of 75 µL were taken from all wells and transferred to a 96-wells plate, under a sterilized flow chamber, to measure absorbance in a microplate reader (Jenway, 6505 UV/VIS spectrophotometer, Burlington, USA), at 600 nm. The sensitivity of bacteria was categorized accordingly the respective EC_{20} for growth (Annex I, Table 7). Four sensitive categories were established: (i) **very sensitive**, for bacteria exhibiting an effective dilution of 20% (considered the threshold for significant effects) for growth inhibition (ED_{20}) lower than the highest tested dilution (6.25%); (ii) **sensitive**, for bacteria exhibiting an ED_{20} equal or higher than the highest tested AMD dilution (6.25%) or equal or lower than the average ED_{20} of all tested isolates; (ii) **tolerant**, for bacteria exhibiting an ED_{20} higher than the average ED_{20} of all tested isolates or lower

than the lowest tested AMD dilution (100%); and (iv) **very tolerant,** for bacteria exhibiting any growth inhibition when exposed to 100% of AMD.

2.6 Statistical Analysis

The values of optical density were fitted to a logistic model to calculate the values of AMD dilution causing 10, 20 and 50% (EC_{10}, EC_{20} and EC_{50}) of growth inhibition of bacteria isolates, and the respective 95% confidence limits. Growth of bacteria was compared among treatments with a one-way analysis of variance (ANOVA) followed by the multicomparison Dunnett's test to assess significant differences between AMD dilutions and the respective control. Assumptions of ANOVA were tested with the Kolmogorov-Smirnov test for normality and Barttlet test for homoscedasticity of variances. All analyses were done with the software StatSoft, Inc. (2007) STATISTICA. Frequency of isolates per sensitive category was compared among sites using the chi-squared test with contingency tables.

3. Results

3.1 Physical-chemical Parameters

The pH and conductivity values of AMD decreased and increased with decreasing dilutions, respectively (Table 2). The pH value decreased about 0.200 units from 5.637 (6.25% AMD) to 5.44 (100% AMD), while conductivity increased one order of magnitude from 1.14 (6.25%) to 13.47 mS/cm (100% AMD). The pH and conductivity values of the control were 6.26 and 0.022 mS/cm, respectively.

Table 2: Values of pH and conductivity measured in the control and in the acid mine drainage dilutions that were tested, at 23 ±1ºC.

AMD Dilution (%)	0 (Ctr)	6.25	12.5	25	50	75	100
pH	6.26	5.64	5.59	5.55	5.49	5.47	5.44
Conductivity (mS/cm)	0.022	1.140	1.944	3.760	7.130	10.420	13.470

3.2 Sensitivity Wells Diffusion Method

The results obtained with the Wells Diffusion method are presented in Figure 6. Figure 7 illustrates an example of a test Petri dish used to run the assay. From the total of 73

tested bacterial isolates, none exhibited a MID lower than 6.25%, thus, any **very sensitive** bacterium was identified through the use of this method. However, 10.9% of the tested bacteria exhibited a MID of 50% of AMD, being considered as **sensitive;** 5.4% exhibited a MID within 50 and 100% of AMD, being considered as **tolerant**; and 83.5% presented no growth inhibition when exposed to 100% of AMD, being considered as **very tolerant**.

The pattern of categories of sensitivity per sampled populations of *P. perezi* were as follows: LB: VT-72%, T-8%, S-20%; SL: VT-91%, T-4.3%, S-4.3%; TP: VT-88%, T-4.0%, S-8.0%, The VT category being always at higher frequencies in all sites.

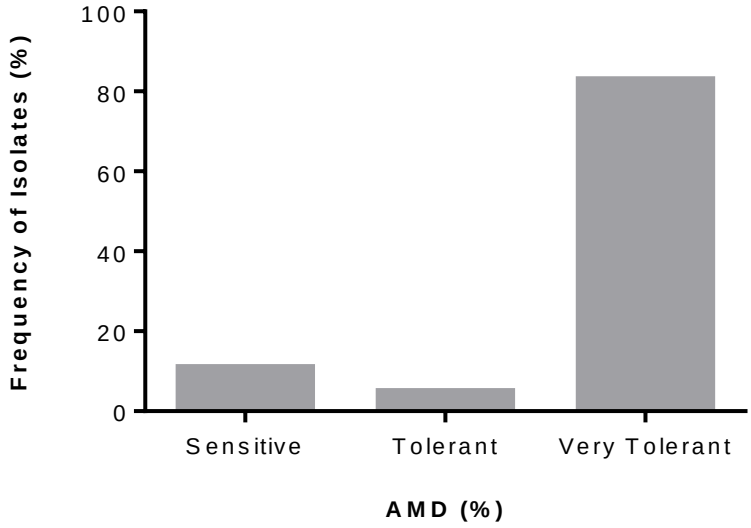

Figure 6: Frequency (%) of bacterial isolates sampled in the three populations of *Pelophylax perezi*, discriminated by categories of sensitivity to acid mine drainage, tested through the Wells Diffusion Method.

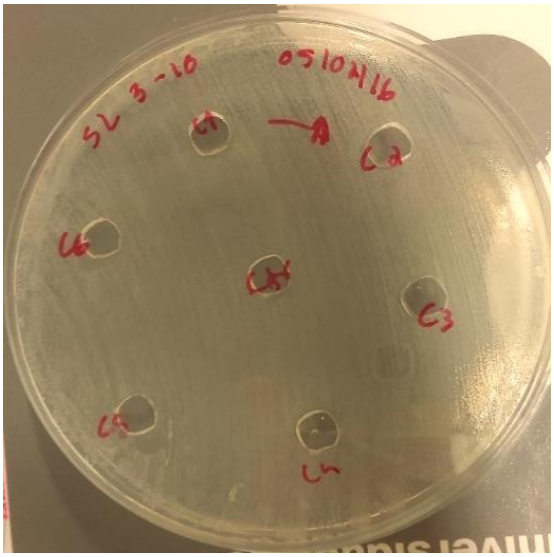

Figure 7: Photography of a test Petri dish corresponding to the exposure of the bacterium *Microvirga zambiensisa* to the negative control and to acid mine drainage dilutions by using the adapted Wells Diffusion method. Ctr- control; C1- 6.25%; C2-12.5%; C3- 25%; C4- 50%; C5- 75%; C6-100% of AMD.

3.3 Sensitivity assay: Microdilution method

The results obtained with the Microdilution method are shown in Figure 8. From all the tested bacteria isolates, 5.5% exhibited an effective dilution of 20% for growth inhibition (ED_{20}) lower than the highest tested AMD dilution (6.25%); 11% exhibited an ED_{20} lower than the average ED_{20} of all tested isolates (62.4%), but higher than 6.25% AMD; 23.3% showed an ED_{20} higher than the average ED_{20} of all tested isolates (62.4%), but lower than 100% AMD; and 60.3% exhibited any growth inhibition when exposed to 100% of AMD (Fig. 8). This pattern of categories of sensitivity was different among the three sampled populations of *P. perezi* ($p<0.001$; LB: VT-44%, T-36%, S-16%, VS-4%; SL: VT-57%, T-30%, S-13%, VS-0%; TP: VT-80%, T-4%, S-4%, VS-12%), although the category of very tolerant was the one present at the highest frequency in the three sampled sites. The values of EC_{10}, EC_{20} and EC_{50} for bacteria categorized as sensitive or tolerant are depicted in Table 7 of Annex I.

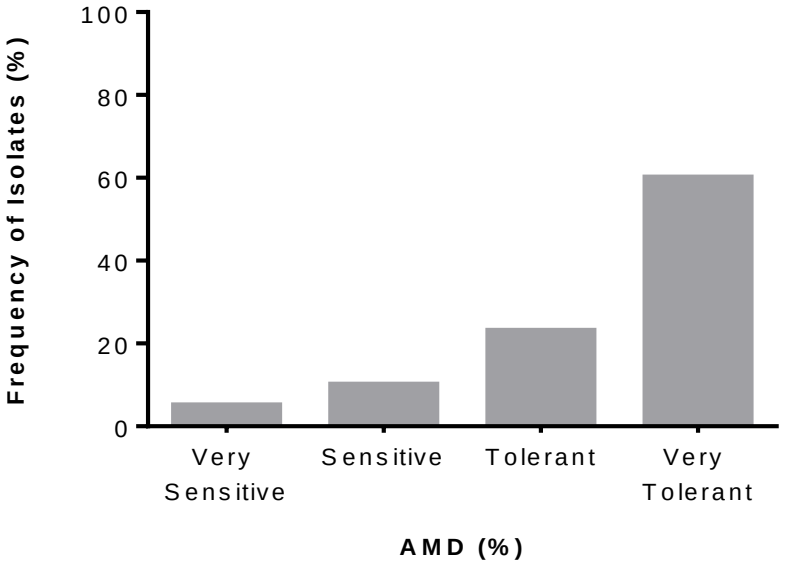

Figure 8: Frequency of bacterial isolates sampled in the three populations of *Pelophylax perezi*, discriminated by sensitivity to acid mine drainage, tested through the Microdilution Method**.**

In Figure 9 are shown the results of O.D. obtained for *Microvirga zambiensisa* with this method (Microdilution), aiming to allow a comparison with the results obtained for this bacterium with the Wells Diffusion method (Fig. 7).

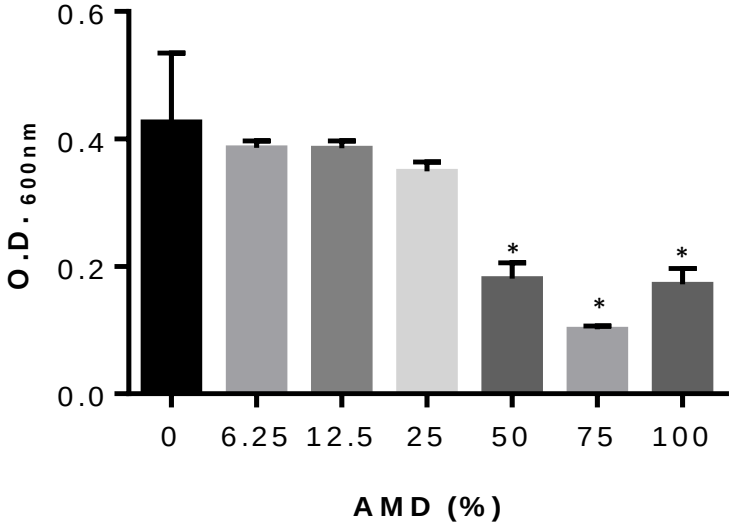

Figure 9: Results obtained for bacteria isolate *Microvirga zambiensisa* tested by the microdilution method. * - Represent significant differences relatively to the control (Dunnett's test: $p < 0.001$).

4. Discussion

The sensitivity of bacterial isolates, collected from the three natural populations of *P. perezi*, to contamination by AMD was assessed by using two methods: Wells Diffusion method and microdilution method. Globally, the most used test to identify antibiotic susceptibility of bacteria is the Wells Diffusion method which is already standardized (Jorgensen and Ferraro 2009; Baquero *et al*. 2015). Although, in the present work, this method only allowed to discriminate bacterial isolates that were sensitive, tolerant or very tolerant to AMD, not being capable of discriminating AMD-very sensitive bacteria. Furthermore, this method categorized more than 80% of the total bacteria isolates as VT, not being able to further discriminate the sensitivity of these isolates. This could be due to the fact that in this method the classification of effects is qualitative, in terms of the method itself, since the MID only classified the isolates as sensitive or not to the chemical being tested (Jorgensen and Ferraro, 2009). On the contrary, the microdilution method allowed to differentiate four categories of AMD sensitivity in the studied bacterial isolates: very sensitive, sensitive, tolerant and very tolerant. Thus, indicating that this method was more sensitive for the quantification of the effects caused by this type of contamination in bacteria, since it identified very sensitive bacteria to AMD. Furthermore, it was also able to better discriminate differences in sensitivity among the bacteria isolates, as a more even distribution of isolates through the categories attained (contrarily to the cluster of >80% VT formed by the Wells Diffusion method). These results suggest that the microdilution method offers a good option to test the sensitivity of bacteria to chemicals, being inline with the reports of other researchers (Matar *et al*. 2003; Jorgensen and Ferraro 2009; Fehlberg *et al*. 2016). Fehlberg *et al*. (2016) carried out a study where they compared several methodologies to assess bacteria sensitivity: the disk diffusion, E-test ®, agar dilution, and broth Microdilution methods. Those authors exposed 82 bacteria isolates to six antimicrobial agents, and concluded that the disk diffusion method worked poorly in comparison with the other dilution methods. This worst performance of the diffusion disk method was related with difficulties in reproducibility of the method and incongruent results obtained for minimum inhibitory concentrations (MICs) comparatively with MICs obtained with the broth dilution method.

Furthermore, in the present study, the Wells Diffusion method showed other disadvantages, namely, fungal and bacterial contaminations (due to the amount of material to proceed this method), that were more frequent comparatively to the microdilution method, and it was also more time and material-consuming. Actually, the later disadvantage has already been highlighted by other authors (Zgoda and Porter,

2001). Additional major advantages were identified for the microdilution method, namely its higher reproducibility, the facility in the observation of results, the quantification of daily bacterial growth, and the absence of some methodological difficulties, for example related with the spill of AMD or poor distribution of the inoculum in the exposure medium. Other advantages associated with this method have been reported in literature: it allows the measurement of more parameters as turbidity, growth and inhibition rate, conditions of growth such as time, concentration, agitation and temperature, the homogeneity of the inoculum is easier to guarantee because it is possible to control the concentration of the inoculum by pipetting it (Matar *et al*. 2003; Jorgensen and Ferraro 2009; Fehlberg *et al*. 2016). However, despite all the advantages highlighted for the Microdilution test, other methodologies should be studied to assess the sensitivity of bacteria to environmental chemical, aiming to avoid problems with pipetting of the inoculum in cases of bacteria that form sticky cultures making difficult to obtain homogeneous inoculum to start the assays.

5. Conclusions

After analyzing the two methods to assess the sensitivity to AMD of the microbiome collected from the skin of *P. perezi*, the microdilution method showed to be the most sensitive and the method that better discriminated the sensitivity of bacterial isolates to AMD. Although, this method also presented some disadvantages and, thus, it is suggested that other methods could be tested aiming at assessing if they can provide a more accurate quantification of the effects of environmental chemicals in bacteria (e.g. automated systems, biomarkers of oxidation).

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CHAPTER 3

Tolerance of amphibians and its own skin symbiotic bacteria to an acid mine drainage and increased salinity (NaCl).

1. Introduction

Chemical contamination in aquatic ecosystems has greatly increased over the past decades. Some of the most widely distributed types of chemical contamination involve salinization and metal contamination mainly associated with mining activities (Simate and Ndlovu 2014; Payen *et al*. 2016).

In freshwaters ecosystems salinity fluctuations can lead to serious environmental impacts affecting biodiversity, organisms life-cycle, growth, agriculture fertility and geochemical properties of rivers and soil (Cañedo-Arguelles *et al*. 2013; Kirwan and Megonigal 2013; Payen *et al*. 2016; Peng *et al*. 2016). Salinization is perceive as increased concentrations of salts (inorganic ions) dissolved in hydrological systems and soil (Cañedo-Arguelles *et al*. 2013; Payen *et al*. 2016). Increases of salinity and its fluctuations in the environment can be originated in natural causes like tidal flux/seawater intrusions, rainfalls, or anthropogenic (e.g. use of deicers to melt ice and snow) (Kearney *et al*. 2014; Dijk *et al*. 2015). Anthropogenic activities are identified as major causes of salinity increases in the environment, mainly those associated with the release of wastewaters from agriculture, mining and industry, use of de-icing agents (Cañedo-Arguelles *et al*. 2013). Other factors that are indirectly associated with anthropogenic activities may also cause the salinization of freshwater and terrestrial ecosystems, which is the case of sea level rise (associated with climate changes) that may lead to seawater intrusions in coastal regions (IPCC, 2013). Actually, several aquatic environments are already impacted with this type of contamination, some freshwater ecosystems exhibit salinity values corresponding to 50 g/L NaCl (caused by extensive mining activity) (Cañedo-Arguelles *et al.*, 2013); which is higher than seawater salinity corresponding to approximately of 32 g/L NaCl (Cowan and Cann 1988).

Regarding acid mine drainage (AMD) contamination, though it may occur due to natural processes, its main origin is associated with worldwide mine industry exploitation (Johnson and Hallberg 2005; Akcil and Koldas 2006). The AMD usually results from the oxidation, due to exposure to oxygen and water, of sulphidic minerals (e.g. FeS_2) (Akcil and Koldas 2006; Sánchez-Andrea *et al*. 2014), leading to the production of high hydrogen concentrations and dissolution of a wide range of metals. Therefore, AMD usually holds low pH, elevated conductivity and very high concentrations of a panoply of

metals (Lopes *et al*. 2000; Akcil and Koldas 2006; Sánchez-Andrea *et al*. 2014). Consequences of AMD in the environment are very alarming causing several alterations in the biota, soil degradation, water and soil pollution, biodiversity losses and ecosystem disturbance (Álvarez-Valero *et al*. 2008; Méndez-García *et al*. 2015).

These two types of environmental perturbations have been shown to severely impact amphibian populations. Amphibians are considered skin breathers, depending on its own skin functions not only to breath but as well for water regulation. Moreover, skin act as a prime barrier of these organisms immune system, being responsible for homeostasis maintenance, osmotic imbalance and control of ion efflux (Willumsen, Viborg, and Hillyard 2007; Colombo *et al*. 2015). Due to these functions, amphibians' skin is extremely irrigated and permeable, which facilitates the uptake of chemicals from the environment, making these organisms very sensitive to chemical contamination. As such, salinity increases constitute a stress to the amphibians group, leading to alteration on life-stages (e.g. early hatching and metamorphosis), survival and behaviour (e.g. swimming difficulties) (Karraker and Ruthig 2009; Denoël *et al*. 2010; Bernabò *et al*. 2013). For example, Collins and Russell (2009) studied the lethal toxicity of NaCl to tadpoles for five species of amphibians from Nova Scotia. These authors reported median lethal concentration of Cl^-, after 96h of exposure, ranging from 1178.2 mg/L Cl^- (corresponding to 1.94 g/L NaCl) for *Ambystoma maculatum* to 3925.8 mg/L (corresponding to 6.47g/L NaCl) for *Bufo americanus*. Concerning AMD, this type of contamination may induce effects in amphibians due to exposure to high hydrogen ions concentrations and/or to high metals concentrations. A recent study assessed the effects of an effluent originated from a coal mining (pH=7.6; conductivity=0.3 mS) on tadpoles of *Limnodynastes peronei* (Lanctôt *et al*. 2016). The authors reported a delay in tadpoles development and an increase in liver six when exposed to a dilution of 25% of the mining effluent. This type of contamination related to high levels of metals and metalloids can lead to amphibian physiological disruptions, metal bioaccumulation, toxicity, DNA damage, elevated mortality rates and others (Zocche *et al*. 2014; Lanctôt *et al*. 2016).

However, when amphibians are exposed to such contaminated environments their capacity to deal with it may be influenced by the microbial community inhabiting their skin. Amphibians skin comprehend an intrinsic microbiome that is considered a constituent of its immune system (e.g. salinity contamination, pathogens infection) (Colombo *et al*. 2015). For example, it is known that bacteria may produce external enzymes and other compounds that may hamper the uptake of chemicals from the environment (either by retaining them in an external matrix or by metabolizing them or changing their speciation)

(Bruins, Kapil, and Oehme 2000; Hobman and Crossman 2014). However, under contaminated environments, the skin microbiome of amphibians may as well be affected, if a loss of microbial diversity occurs, then the susceptibility of amphibians to the contamination may increase. Adverse effects of salinization or AMD have already been reported in, microbial communities, comprehending diversity losses, growth inhibition, alterations in enzymatic activities, plasmolysis of the cell, membrane damage, among others (Zhang *et al*. 2007; Méndez-García *et al*. 2015).

Therefore, the main goal of the present work was to compare the sensitivity of amphibians and its skin microbiome to chemical stress induced by increased salinity or by AMD. Two specific objectives were set: (1) assess the sensitivity of skin bacteria, collected from adults of *Pelophylax perezi*, to NaCl and AMD (for AMD already assessed in Chapter 2) and (2) determine the sensitivity of two amphibian species (*P. perezi* and *Xenopus laevis*) to the same environmental stressors. Three scenarios are expected: (i) skin bacteria and the two amphibian species exhibit similar sensitivity to chemical contamination; (ii) skin bacteria are less sensitive to chemical contamination relatively to amphibians, or (iii) skin bacteria is more sensitive to chemical contamination than amphibians, this would constitute the worst-case scenarios for the resilience of amphibians under contaminated environments.

2. Materials an Methods

2.1 Sampling site of amphibian skin microbiome

The skin microbiome of adults of *Pelophylax perezi*, inhabiting three natural freshwater ponds, was sampled. The description methodologies used for sampling, isolate and identify the microbial community are described on Chapter 2 (please see Material and Methods section).

2.2 Tested chemicals

The sensitivity of tadpoles and bacterial isolates was assessed for salinity and acid mine drainage (AMD). For salinity the salt NaCl was used as a surrogate of natural seawater. This salt was purchased as a powder to EMSURE® (Sodium chloride CAS 7647-14-5). Concentrations were made according to environmental relevance, being the higher concentration equivalent to seawater salt concentration (Cowan and Cann 1988; Sillero and Ribeiro 2010). The AMD effluent was sampled near Ribeira da Água Forte,

Portugal (37°56'31 N/ 8°08'53 W). This effluent originates in the drainage of a settlement basin of the Pirites Alentejanas mine (Aljustrel, Portugal) and exhibits high levels of several metals (please see Table 1 of Chapter 2 for further details).

2.3 Bacterial Isolates Culture

The methodology used to sample the amphibian skin microbiome is described in chapter 2 and in Costa *et al.* (2016). The bacterial isolates, previously stored at -80°C, were recovered in NB medium with 15 % Glycerol (v/v) in solid R2A medium (Oxoid, England) under a flow chamber and left at 23ºC for 120 h to normal growth. Bacteria were re-cultured three times, every 96 to 120 h, in solid LB medium in order to achieve normal growth and non-contamination of the cultures. For later use, cultured bacteria in LB medium Petri dishes were stored in a chamber at 4°C.

2.4 Bacteria Sensitivity assay: Microdilution Method

The microdilution method described in chapter 2 was here used to assess the sensitivity of skin bacteria isolates to salinity (for details on the sensitive assay to AMD, please see chapter 2). Each bacterium isolate was exposed to six concentrations of NaCl plus a control consisting in liquid LB medium. The following NaCl concentrations were tested: 5, 10, 15, 20, 25, and 35 g/L. The pH and conductivity of all concentrations were measured at room temperature of 23 ± 1 ºC with a multiparameter equipment (WTW Multi 3410 SET C 2FD45C). The assay ended after 120 hours of exposure, and the optical density of bacterium isolates was measured at 600 nm in a microplate reader (Jenway, 6505 UV/VIS spectrophotometer, Burlington, USA).

The sensitivity of the bacteria was categorized as follows: (i) **very sensitive**, for bacteria exhibiting an effective concentration of 20% (considered the threshold for significant effects) for growth inhibition (EC_{20}) lower than the lowest tested concentration (5 g/L); (ii) **sensitive**, for bacteria exhibiting an EC_{20} higher than the lowest tested concentration (5 g/L) and equal or lower than the average EC_{20} of all tested isolates; ; (ii) **tolerant**, for bacteria exhibiting an EC_{20} higher than the average EC_{20} of all tested isolates; and (iv) **very tolerant,** for bacteria exhibiting any growth inhibition when exposed to the highest tested concentration (35 g/L of NaCl).

2.5 Tadpoles Toxicity Assay

2.5.1 Model Organisms: *Pelophylax perezi* and *Xenopus laevis*

Two species of amphibians were selected to carry out this study: *Pelophylax perezi* and *Xenopus laevis.* The Green Frog, *P. perezi,* was selected because it is an autochthonous species in the Iberian Peninsula being widely and abundantly distributed in this region (Loureiro *et al*. 2010) and is one of the least concern amphibian species of the IUCN list (IUCN, 2008). This species inhabits a wide range of habitats varying from terrestrial to aquatic, although its breeding behavior depends entirely on aquatic systems (IUCN, 2008; Sillero and Ribeiro, 2010). Despite of being considered a resistant species in comparison to other amphibian species inhabiting the Iberian Peninsula area, *P. perezi* can face threats related with habitat destruction and introduction of invasive species such as *R. ridibunda* and *R. lessonae* (Loureiro *et al*. 2010). Studies with this species have been made related to pollution present in aquatic systems, pathogens like iridovirus (IUCN, 2008), skin microbiome composition and diversity (Costa *et al*. 2016) salt and metal contamination (B. R. D. F. Santos 2011), among others.

For the present work, egg masses of *P. perezi* were collected at a reference freshwater pond located in Aveiro (40°38'17.4"N 8°39'21.5"W). In the laboratory, the egg masses were transferred to FETAX medium and organisms were maintained at 23±1ºC and 16:8 h light:dark photoperiod until being used for toxicity assays (at Gosner stage 25; Fig.18 Annex I).

The African Clawed Frog *Xenopus laevis*, is an African native frog species (Chum *et al*. 2013). This frog is, as well, one of the least concern species of IUCN list due to its wide geographic distribution, capacity to inhabit diversified habitats and large population (IUCN, 2008). It is strictly a water-dependent species living in aquatic habitats and producing a large amount of eggs per brood (Tinsley, 2009). In Portugal is known as an exotic species (Rebelo *et al*. 2010). *Xenopus laevis* is one of the most used model system in development, immunology, toxicology, neurobiology, embryology regenerative biology, and ecotoxicology being a well-known biological system (Robert and Ohta 2009; Edholm and Robert 2013; Haynes-Gimore *et al*. 2015). Thus, this species constitute a (i) very well studied, (ii) easy to maintain in laboratory and (iii) known model for research (Edholm and Robert 2013), which constitute advantageous characteristic to be maintain in laboratories for research.

In this study, tadpoles of *X. laevis* were obtained from lab cultures. Female and males of *X. laevis* were injected with the hormone gonadotropin chorionic to induce amplexus

and reproduction. The layed eggs were transferred to an aquarium filled with Fetax medium and animals were maintained under the same conditions as those reported above for *P. perezi* tadpoles until being used for toxicity assays (at Gosner stage 25, Fig. 18 Annex I).

2.5.2 Exposure of Tadpoles to AMD and NaCl

Tadpoles at Gosner stage 25 (Gosner, 1960; Fig. 18 Annex I) were used to run the toxicity experiments. Tadpoles of *P. perezi* were only exposed to NaCl concentrations (due to constrains associated with the availability of egg masses) while tadpoles of *X. laevis* were exposed to the two types of contamination (salinity and AMD).

The assays were run with 3 replicates per treatment, each with five tadpoles at Gosner stage 25. Individuals were kept in plastic flask with 120 mL of test solution [a control (FETAX medium) and six dilutions/concentrations of AMD/NaCl, respectively], during 168 h at 23±1 ºC in 16:8 h light:dark photoperiod with food supply on oxygenation. Every other day, medium was changed completely and food supplement were provided. Mortality was registered every day and individuals were measure and observed for malformations in the first and last day of the assay, using a binocular microscope (Zeiss Stemi 2000-C Stereo Microscope).

For the assays with AMD, the following dilutions (with Fetax medium) were tested: 10%, 15%, 18.8%, 23.4%, 29.3% and 36.6% (dilution factor of 1.25x). For NaCl two sets of concentrations were tested (using a dilution factor of 1.1x): (i) 4.18 g/L, 4.64 g/L, 5.15 g/L, 5.71 g/L, 6.28 g/L and 8.36 g/L of NaCl for *P. perezi* (ii) and 4.68 g/L, 5.14 g/L, 5.66 g/L, 6.22 g/L, 6.88 g/L, 7.53 g/L for *X. laevis* tadpoles. Tested concentrations were obtained by dissolving NaCl in Fetax medium.

2.6 Statistical Analysis

The concentrations causing 10, 20 and 50 % (LC_{10}, LC_{20} e LC_{50}) of mortality in tadpoles, and the respective confidence limits, were compute by using a probit model with the Priprobit Software. For the bacteria, the effective concentrations causing 10, 20 and 50% (EC_{10}, EC_{20} e EC_{50}) of effect in growth were computed by fitting the optical density values into a logistic model by using the StatSoft, Inc. (2007) STATISTICA software.

Frequencies of bacterial isolates, per sensitivity category to AMD and NaCl, were compared among sites through the Chi-squared test by using contingency tables.

3. Results

3.1 Sensitivity of Bacterial Isolates to AMD

The pH and conductivity values measured in the tested AMD dilutions are described in Chapter 2 (Table 2).

The EC_{20} average for growth inhibition of all bacterial isolates was 62% of AMD (EC_{10} and EC_{50} for each isolate are shown in Table 7, Annex I). From the 73 bacterial isolates tested 5.5% exhibited an effective dilution of 20% for growth inhibition (ED_{20}) lower than the highest tested AMD dilution (6.25%) being classified as very sensitive; 11% exhibited an EC_{20} lower than the average EC_{20} (62%) but higher than 6.25%, being considered sensitive; 23% exhibited an EC_{20} higher than the average EC_{20} and lower than the lowest tested dilution of AMD (100%), being considered tolerant; and 60% were considered very tolerant, as they exhibited any growth inhibition when exposed to 100% of the AMD. All the bacterial isolates tested are listed in Table 3 with the respective category of sensitivity for AMD and NaCl.

Table 3: List of all bacterial isolates categorized according to their sensitivity to AMD per sampling site (LB, SL and TP). Legend VS -very sensitive; S - Sensitive; T - tolerant; VT- very tolerant. * - not tested. *(continue on the next page)*

Isolate	Classification		Isolate	Classification		Isolate	Classification	
	NaCl	AMD		NaCl	AMD		NaCl	AMD
LB13-1	S	S	**SL3-8**	S	T	**TP10-5**	T	T
LB13-11	T	VT	**SL3-10**	T	S	**TP10-6**	VS	VT
LB1-5	T	T	**SL12-1**	T	VT	**TP1-4**	T	VS
LB7-3	T	S	**SL12-11**	T	VT	**TP1-5**	VT	VS
LB7-1	T	VS	**SL12-2**	T	T	**TP1-6**	T	VS
LB1-1	VT	VT	**SL12-3**	S	T	**TP3-5**	S	S
LB1-10	T	VT	**SL12-5**	T	VT	**TP10-10**	*	VT
LB1-3	S	VT	**SL12-6**	VT	VT	**TP10-11**	S	VT
LB13-10	T	T	**SL12-7**	T	VT	**TP10-13**	S	VT
LB13-12	T	VT	**SL12-9**	T	VT	**TP10-7**	VS	VT
LB13-2	T	VT	**SL2-2**	T	VT	**TP10-8**	T	VT
LB13-5	S	VT	**SL2-4**	T	T	**TP1-1**	T	VT
LB13-6	VT	T	**SL2-5**	T	T	**TP11-2**	S	VT
LB13-7	T	T	**SL2-6**	T	VT	**TP11-3**	T	VT
LB1-6	T	T	**SL2-7**	T	VT	**TP11-4**	VT	VT
LB1-7	T	VT	**SL3-1**	T	VT	**TP1-2**	T	VT
LB1-8	VT	VT	**SL3-4**	T	T	**TP1-3**	T	VT
LB1-9	T	T	**SL3-5**	S	VT	**TP2-1**	T	VT
LB7-2	T	VT	**SL3-6**	S	T	**TP2-2**	T	VT
LB7-6	VS	T	**SL3-9**	S	VT	**TP2-4**	T	VT
LB7-8	T	VT	**SL2-8**	T	VT	**TP2-5**	S	VT

Isolate	Classification		Isolate	Classification		Isolate	Classification	
	NaCl	AMD		NaCl	AMD		NaCl	AMD
LB1-11	T	S	**SL12-8**	T	S	**TP3-1**	T	VT
LB1-4	VS	S	**SL2-1**	S	S	**TP3-2**	S	VT
LB7-4	T	T				**TP3-3**	T	VT
LB7-9	S	T				**TP11-6**	S	VT
LB7-10	T	*						

In Table 8 and 10 (Annex I) are indicated the p-values resulting from the one-way analysis of variance followed by the Dunnett's identify significant differences between the AMD dilutions or the NaCl concentration and the respective control.

In Figure 10 are represented the frequencies of each category of sensitivity to AMD per sampling site. Very tolerant bacterial isolates were present at larger frequencies in the three sampling sites 44%, 57% and 80% for LB, SL and TP, respectively. Samples collected from SL and LB had similar proportions of sensitive and tolerant isolates, respectively: 13% and 30% for SL and 16% and 36% for LB. No very sensitive bacterial isolates were observed in SL. In TP, the categories of sensitive and tolerant bacteria exhibited similar frequencies (4%) (Fig. 10).

Significant differences were observed in the frequencies of the four categories of sensitivity to AMD when comparing the three sites (Fig. 10; $p<0.001$).

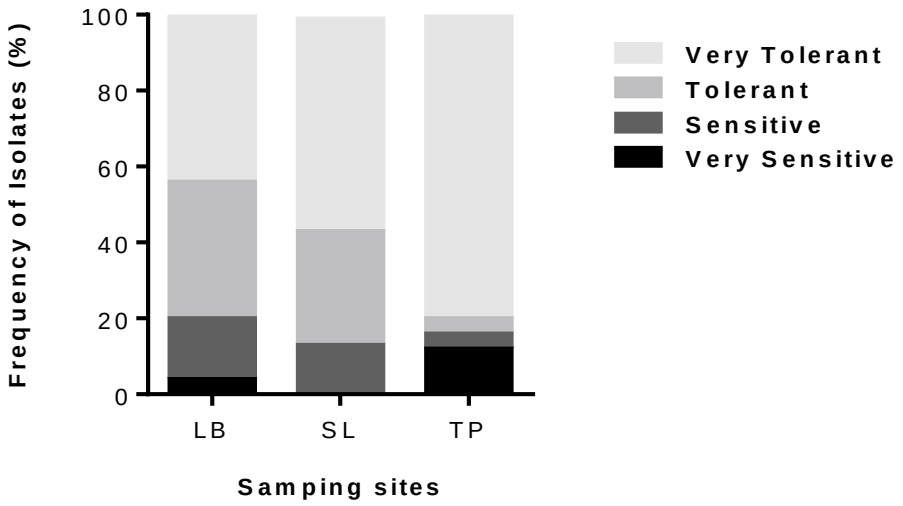

Figure 10: Frequency (%) of the four sensitivity categories, when the bacterial isolates were exposed to AMD, per sampled site ($p<0.001$).

3.2 Sensitivity of Bacterial Isolates to NaCl

The pH and conductivity values of the tested NaCl concentrations decreased and increased with increasing concentrations, respectively (Table 4). The pH decreased about 0.24 units from 7.0 (5 g/L of NaCl) to 6.86 (35 g/L of NaCl), while conductivity increased from 22.5 mS/cm (5 g/L of NaCl) to 61.4 mS/cm (35 g/L of NaCl). The pH and conductivity values of the control were 7.10 and 11.72 mS/cm, respectively.

Table 4: Values of pH and conductivity measured in the control and in the NaCl concentrations at 23 ±1ºC.

NaCl (g/L)	LB medium (Ctr)	5	10	15	20	25	35
pH	7.10	7.00	7.01	7.00	6.94	6.91	6.86
Conductivity (mS/cm)	11.72	22.5	28.7	35.9	42.2	51.3	61.4

The EC_{20} average for growth inhibition of all bacterial isolates was 13.5 g/L of NaCl (EC_{10} and EC_{50} for each isolate are shown in Table 9, Annex I). From the total of 73 bacterial isolates tested, 5.5% exhibited an effective concentration of 20% for growth inhibition (EC_{20}) lower than the lowest tested concentration (5 g/L), being considered as very sensitive to NaCl; 23% exhibited and EC_{20} lower than the average value of EC_{20} (13.5 g/L) but higher than the lowest tested concentration (5 g/L), being considered as sensitive; 63% showed an EC_{20} higher than the average EC_{20} and lower than the highest tested concentration (35 g/L), being considered tolerant; and 8.2% were able to grow even at the highest tested concentration of NaCl (35 g/L), being considered very tolerant. The categories of sensitivity to NaCl of all the bacterial isolates are listed in Table 3.

In Figure 11 are represented the frequencies for each category of sensitivity to NaCl per sampling site. The four categories of sensitivity to NaCl were present only in LB and TP sampling sites. The category of tolerant to NaCl was the one present at a highest frequency in the three sampling sites: 65%, 70% and 54% for LB, SL and TP, respectively. For LB site the category of very sensitive to NaCl was the one presenting the lowest frequencies (7.7%), being absent in SL site. For TP site equal frequencies were observed for very sensitive and very tolerant category (8.3%).

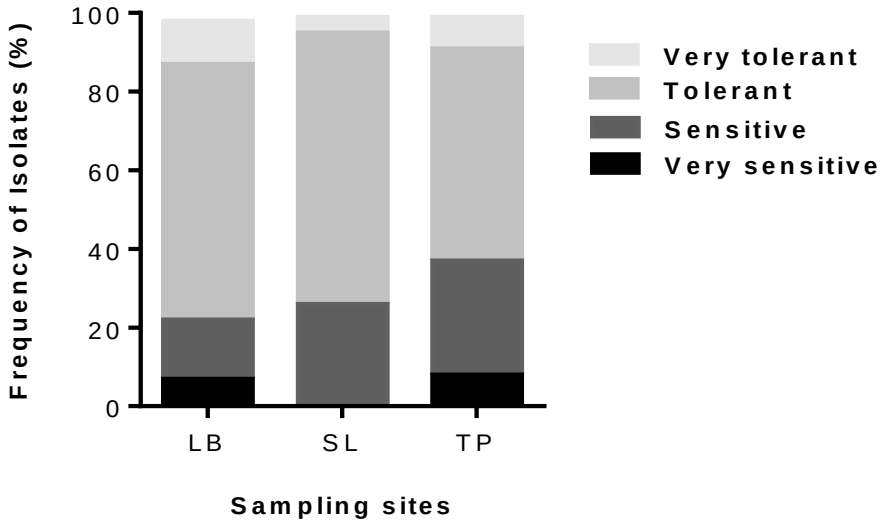

Figure 11: Frequency (%) of the four sensitivity categories, when the bacterial isolates were exposed to NaCl, per sampled sites (p=0.008).

3.4 Comparison of Bacterial Sensitivity to the two Stressors

A significant difference was observed between the frequencies of sensitivity categories, of bacterial isolates exposed to AMD and NaCl ($p<0.001$). In the former a higher number of very tolerant bacterial isolates was present comparatively to the latter (60% versus 8%, respectively) (Fig. 12). The percentage of isolates categorized as very sensitive was similar for both contaminants (5.5% for both) and were present at the lowest frequency for both contaminants. On the contrary, the percentage of sensitive isolates was higher for NaCl comparatively to AMD (23% versus 11%, respectively). Additionally, tolerant category was present at higher frequency to isolates exposed to NaCl (63%).

Furthermore, it was observed that 23% of the tested bacterial isolates showed an inversion in sensitivity to AMD and NaCl, i.e. the bacteria being sensitive to one stressor were tolerant or very tolerant to the other stressor or the bacteria being very tolerant to one stressor were very sensitive or sensitive to the other one (Table 3, Fig. 12).

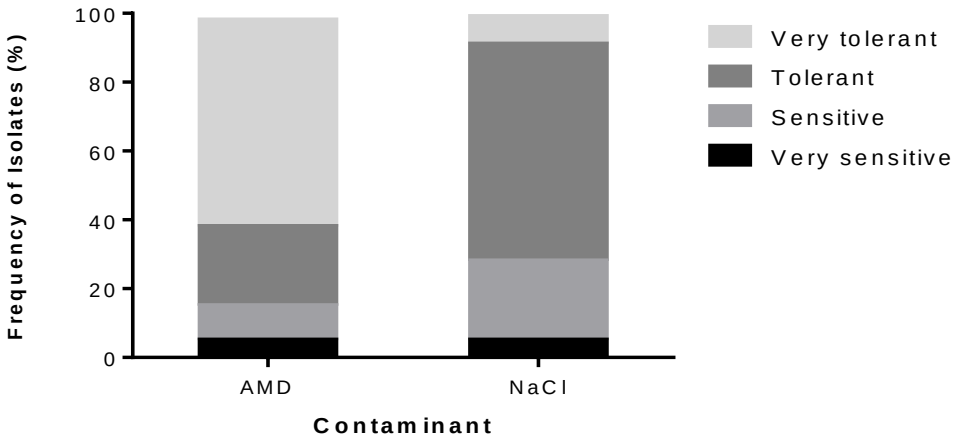

Figure 12: Frequency (%) of the four sensitivity categories when the bacterial isolates were exposed to AMD and NaCl ($p < 0.001$).

3.6 Tadpoles Sensitivity to AMD and NaCl

The average cumulative mortality of tadpoles of *Xenopus laevis* after being exposed for 168 h to AMD dilutions are represented in Figure 13.

After 168h of exposure to AMD, 20% mortality of tadpoles of *X. laevis* was observed at AMD dilutions of 10 and 15%, while 100% of mortality was observed for AMD dilutions equal or above 18.8%. The AMD dilution causing 20% of mortality was 10.8% (Fig. 13).

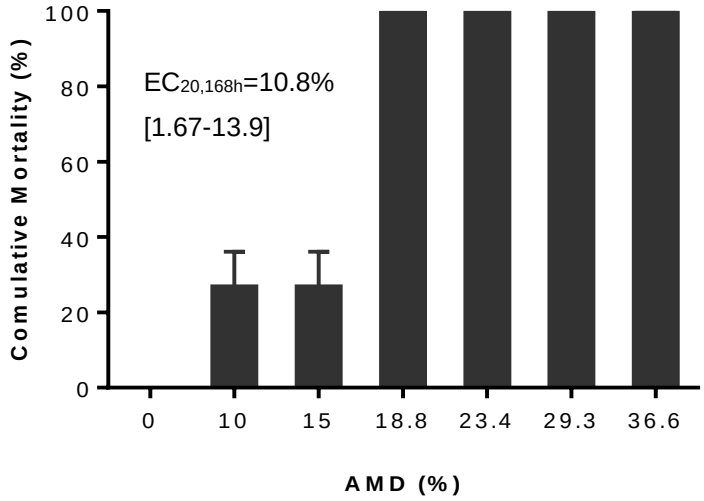

Figure 13: Average of cumulative mortality of *X. laevis* after 168h of exposure to AMD dilutions. Error bars represent standard deviation. The concentration causing 20% of mortality ($EC_{20,168h}$) and the respective 95% confidence limit is also depicted.

Mortality rates of tadpoles of *P. perezi* and *X. laevis* exposed to NaCl are shown in Figures 14 and 15, respectively. The two species exhibited a similar lethal sensitivity to NaCl. For *P. perezi* tadpoles, the concentration of NaCl causing 20% of mortality after an exposure period of 168h was 4.61 g/L, while for *X. laevis* the $EC_{20,168h}$ was 4.98 g/L of NaCl.

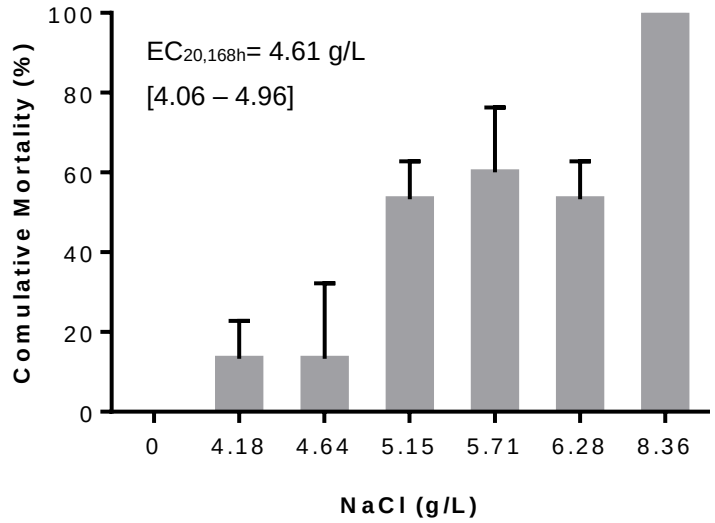

Figure 14: Average of cumulative mortality of *P. perezi* after being exposed for 168h to NaCl concentrations. Error bars represent standard deviation. The concentration causing 20% of mortality ($EC_{20,168h}$) and the respective 95% confidence limit is also depicted.

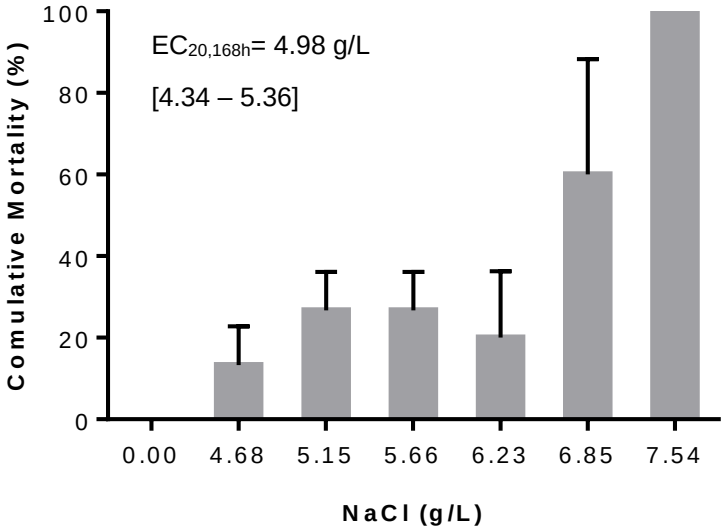

Figure 15: Average of cumulative mortality of *X. laevis* tadpoles after being exposed for 168h to NaCl. Error bars represent standard deviation. The concentration causing 20% of mortality ($EC_{20,168h}$) and the respective 95% confidence limit is also depicted.

4. Discussion

Bacterial isolates, collected from the skin of adult *P. perezi* and exposed in the laboratory to AMD and NaCl, presented different sensitivity responses to these stressors. However, the highest percentage of bacterial isolates, independently of their site of origin, showed to be very tolerant and tolerant to AMD and NaCl, respectively. The higher frequency of VT and T bacteria, in the populations of *P. perezi* collected at SL and TP, was expected since these populations have been historically exposed to this type of contaminants (SL to increased salinity and TP to AMD). In fact, some works reported that long-term exposure to chemical contamination might lead to an increased tolerance of the bacterial community to that type of contamination (e.g. Hobman and Crossman, 2014). Díaz-Raviña and Bååth (1996) observed that the development of an increased tolerance to metal contamination by a soil bacteria community was due to the death of the most sensitive species to this type of contamination. It is hypothesized that the elimination of the most sensitive species could have occurred in SL and TP, since both communities may be exposed to extreme pulses of seawater or AMD effluent that could lead to the death of very sensitive bacteria species. In fact, population SL did not exhibited any bacterial isolate categorized as very sensitive (either to AMD or NaCl), suggesting that those bacteria could have disappeared from an initial bacterial community. However, since the exposure of these bacterial communities to AMD and increased salinity is occurring for several decades, it is suggested that other tolerance acquisition mechanism may also be here involved. For example, the acquisition of tolerant genes from the external environment or from horizontal gene transfer, mechanisms that have also been associated with bacteria tolerance acquisition to chemical stress (e.g. Bruins *et al*. 2000; Barlow, 2009; Zhang *et al*., 2009). The higher frequencies of tolerant and very tolerant bacteria in LB site was not expected since this is considered a reference site relatively to metal and salinity contamination. However, as this pond is located near small human settlements it is hypothesized that the pond could be exposed to pulses of input of some type of anthropogenic contamination (e.g. antibiotics) that could explain the higher frequencies of tolerant and very tolerant bacteria, since multiple-co/tolerance between antibiotics and metals or salinity have already been reported for bacteria (e.g. Hood *et al.* 2009). Most of the studied bacteria isolates showed a similar category of sensitive to AMD and to NaCl, which was expected considering the existence of multiple/co-tolerance to several chemicals. For example, Hood *et al.* (2009) reported that tolerance in the bacteria *Acinetobacter baumannii* was correlated with the tolerance of this bacterium to a range of antibiotics (e.g. aminoglycosides, carbapenems, quinolones, and colistin).

When comparing the overall sensitivity of the skin bacterial community to AMD and NaCl with that of tadpoles of *P. perezi* and *X. laevis* it was observed that, in general, the, bacterial isolates exhibited a more than 2-fold higher tolerance to the two stressors than the amphibian species. For AMD, *X. laevis* showed an EC_{20} of 10.8% while the average EC_{20} of the bacteria isolates was 62%. For NaCl, tadpoles of *X. laevis* and of *P. perezi* exhibited EC_{20} of 4.98 and 4.41 g/L, respectively, while the average EC_{20} for bacteria was 13.5 g/L. Furthermore, more than 70% of the studied isolates exhibited and EC_{20} equal or higher than the average of the EC_{20} computed for all bacteria. This shows that for the here studied amphibian species, the skin bacterial isolates exhibits a much higher tolerance to AMD and NaCl, thus under such scenarios of contamination the higher tolerance of bacteria could help the amphibians to increase their tolerance to this type of contamination. Since amphibians depends greatly on their skin functions to maintain their fitness, intrinsic bacteria exhibiting an increased tolerance to environment contamination, could be approached to enhance amphibian's tolerance (McKenzie *et al*. 2012). Very tolerant bacteria species could be selected from the pool of the amphibian skin microbiome to proceed with bioaugmentation methodologies aiming to achieve a higher resilience of the amphibian population under scenarios of chemical contamination. The usage of microbial benefits has been practiced to increase organisms survival, an example is the usage of selected tolerant non-pathogenic bacteria to salt and metals to improve plant growth exposed to metal and salinity contamination in the environment (Mayak, Tirosh, and Glick 2004; Rajkumar and Freitas 2008). As well, for amphibians microorganism have been used to improve amphibian's survival to fungal pathogens (Bletz *et al*. 2013; Harris *et al*. 2009; Holden *et al*. 2015).

5. Conclusions

In this present work both AMD and increased salinity induced higher toxicity to the tadpoles of the two studied amphibian species comparatively to the overall skin bacterial community, suggesting that under this type of contamination bacteria can help amphibians to deal with the chemicals and, as well, be used to promote bioaugmentation treatments in these organisms.

Both amphibian species exhibited similar sensitivities to AMD and NaCl, however, other amphibian species must be studied since inter-species sensitivity to environmental perturbations may differ greatly.

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CHAPTER 4

Effects of continuing exposure to increased salinity in the amphibian skin bacterium *Erwinia toletana.*

1. Introduction

Skin is one of the most important organs concerning amphibian's immune system constituting a premier barrier to the surrounding environment (Colombo *et al*. 2015). It holds diverse components essential for amphibians' survival and health, namely: mucus glands, toxins and venom substances, antimicrobial peptides and a microbial community that help the organisms to deal with pathogens and chemical contaminants (Kueneman *et al*. 2014; Colombo *et al*. 2015; Costa *et al*. 2016). This skin microbiome community has been studied in order to better understand it's potential role in the amphibian tolerance to fungal infection (e.g. *Batrachochytrium dendrobatidis*) and it's use for probiotic bioaugmentation treatments (Harris *et al*. 2009; Holden *et al*. 2015; Woodhams *et al*. 2016). Bioaugmentation correspond to a probiotic biotherapy using native and beneficial microorganism (e.g. capable of producing protective metabolites, properties as tolerance to a chemicals) from the individual, in order to improve its immunity to external threats (Harris *et al*. 2009; Bletz *et al*. 2013; Woodhams *et al*. 2016). In the case of amphibians, if their skin bacteria are tolerant to some environmental pollutants (e.g. agrochemicals, chemical pollutants, salt and metal contamination, among others), then they could be used to apply biotherapy and improve the tolerance of amphibians to those pollutants (Beebee and Griffiths 2005; Woodhams *et al*. 2016).

Bacteria have been developing metal contamination tolerance evolving different mechanisms of resistance (Bruins, Kapil, and Oehme 2000; Hobman and Crossman 2014). Reviewed by Bruins *et al.,* (2000) bacteria can control the passage of metal ion through different mechanisms. Given an example of plasmid or chromosome-encoded resistance adaptation to chromate toxicity, bacteria can exclude it from the cell through electron-transport system and enzymatic reduce mechanism. Since bacteria can adapt and evolve mechanisms to expel and exclude metallic ion from the cell, it could be possible to select from amphibian skin microbiome, a tolerant isolate for bioaugmentation application, in order to improve amphibian organism defense to metal contamination.

Amphibians are very sensitive to salinization, which is considered for extended time a global destabilizer of ecosystems equilibrium (Hart *et al*. 1990; Bernabò *et al*. 2013; Cañedo-Arguelles *et al*. 2013). Salinity increment, or elevated concentrations of dissolved salts in ecosystems have been caused by different sources: natural causes (e.g. rainfall season), climatic changes (e.g. raise temperature causing evapotranspiration) and

anthropogenic activities (e.g. mining activity) (Cañedo-Arguelles *et al*. 2013; Payen *et al*. 2016).

Despite the fact that microorganisms may be affected by salinity fluctuations, it is possible to distinguish well-adapted microbial communities to hyper salinity conditions, classified as halophiles (Margesin and Schinner 2001). Halophiles, are frequently used as a bioremediation tool and in bioaugmentation therapy (Geider *et al*. 2006; Miliute *et al*. 2015; Waditee-Sirisattha, Kageyama, and Takabe 2016). Bioremediation can be applied to treat oil-wastewaters present on the sea, using classified halophiles microorganisms that can metabolize this type of pollution (Oren 2008). Although, according to He *et al*. (2016) it is also possible to acclimate microbes, such as bacteria, to salinity stress conditions, in order to apply bioremediation to wastewater treatments in brackish conditions.

The present work aimed at assess if a skin bacterial isolate already tolerant to NaCl could increase its tolerance to this salt after continuing exposures to low levels of NaCl The NaCl-tolerant bacterial isolate *Erwinia toletana* was selected as the study species.

Rojas *et al* (2004) and Silva *et al* (2014) described *Erwinia toletana* as “non-pathogen, Gram-negative, oxidase-negative and catalase-positive bacteria from *Enterobacteriaceae* family”. *Erwinia toletana* is usually reported as being a co-existent bacteria with olive tree knot *Pseudomonas savastanoi* pv. *Savastanoi* pathogen, although little is known about *E. toletana* pathogenicity (Rojas *et al*. 2004). Furthermore, scarce information is gathered about *E. toletana* NaCl toxicity, however, some studies with different *Erwinia* species have been made to investigate and apply bioaugmentation in plants (Geider *et al*. 2006; Mills, (Bud) Platt, and Hurta 2006; Miliute *et al*. 2015).

2. Material and Methods

2.1 Bacterium Isolate Selection

The bacterium *Erwinia toletana* Gene Bank Accession Number KT720377, was selected to carry out this study. This choice was based on: (i) its origin, being collected at a reference site it is not expected to have been exposed to salinity stress previously; (ii) it exhibits a good growth rate and cell viability in liquid medium.

2.2 Continuing exposure to salinity

To determine if continuing exposure of *E. toletana* to low levels of salinity could cause an increase on its tolerance to this chemical stress, the bacterium was exposed for six

weeks to its EC_{10} of NaCl (18 g/L; computed from data obtained in chapter 3). For this, *E. toletana*, stored at -80ºC in NB-medium with 15% glycerol, were recovered in sterilized solid LB medium and cultures were renewed three times to ensure no contamination and the viability of the cells. To determine if the time to achieve an O.D. of 1, in liquid medium, was similar in the absence and presence of NaCl, a pre-inoculum (with O.D. of 1) was set by exposing *E. toletana* to 15 mL sterilized LB + NaCl medium on Falcon tubes at 23 ±1ºC and 70 rpm agitation, in triplicate. Once confirmed this correspondence, the bacterium was cultured, in triplicate, for approximately 6 weeks in: (i) LB medium, consisting in 375 µL of bacterial suspension, with O.D. of 1, in 15 mL sterilized liquid LB medium (Et-LBM); and (ii) salinized medium, consisting in 375 µL of bacterial suspension, with O.D. of 1, in 15 mL sterilized liquid LB medium with a concentration of 18 g/L of NaCl (EC_{10} for *E. toletana* computed from data obtained in chapter 3) (Et-NaCl). To control for possible contaminations of the media, a treatment with LB medium with no bacterial inoculation was also performed. Every 120 to 144 hours, medium was replaced once achieved an O.D. of 1 and no contamination observed.

To assess the capacity of *E. toletana* to recover from continuing exposure to increased salinity, the isolate exposed for six weeks to 18 g/L of NaCl, was afterwards transferred to 15 mL of liquid LB medium free of NaCl for four weeks (Et-R).

2.3 Bacterial Growth Assay

The sensitivity of *E. toletana* after exposure for six weeks to LB medium or to NaCl was compared in order to determine its capacity to acquire tolerance to this chemical. For this, the isolates of this bacterium maintained for six weeks in LB medium and in LB medium containing NaCl were exposed to six concentrations of NaCl (5 g/L; 10 g/L; 15 g/L; 20 g/L; 25 g/L; 35 g/L) plus a control (LB medium), by using the Microdilution method (please see description of the method in chapter 2). For each treatment 5 replicates were carried out. Exposure occurred for 120 hours in a photoperiod of 16:8 hours light at 23±1ºC and 70 rpm.

At the end of exposure the O.D. was measured at 600 nm in a UV-vis Spectrophotometer (UVmini-1240, UV-vis Spectrophotometer, Shimadzu).

2.4 Bacterial Isolate Metabolic Analysis: Biolog “Fingerprint” Analysis

The effect of successive exposure to increased salinity was also assessed by comparing the metabolic degradation of carbon compounds (through Biolog Plates). Biolog (Biolog

Inc., Hayward, CA, USA) is commonly used to differentiate and identify microbial communities, based on 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity assays that results in different patterns responses for different microorganisms (Stefanowicz, 2006; Guckert *et al*., 1996). Though this method is mostly used for identification of bacteria, here it was used to compare the capacity of a single bacterial isolate to metabolize different carbon sources when previously exposed to different conditions. The application of Biolog method analysis followed the instructions of GEN III MicroPlate^{TM} protocol test. All procedures were executed by adapting the step 1 of the protocol, i.e., instead of using solid medium to isolate cultures; the LB liquid medium was used (favorable to the bacterium growth). Also step 4 of the same protocol was altered, here incubation time was extended for 48 h at 23±1ºC since all isolates were cultured at this temperature, and stress caused for altering the incubation period could affect the results. This procedure was performed for the *E. toletana* isolates exposed for six weeks to LB medium; to LB medium containing NaCl and to LB medium containing NaCl + 4 weeks in LB medium.

2.5 Statistical Analysis

The results obtained in the growth assay for optical density were adjusted to a logistic model to calculate the values of EC_{10}, EC_{20} and EC_{50} and respective 95% confidence limits, for the *E. toletana* isolates. These calculations were made via StatSoft, Inc. (2007) STATISTICA. To assess significant effects NaCl exposure on growth relatively to the control a one-way variance analysis (ANOVA) was performed followed by the comparison Dunnett's test. Assumptions were tested with Kolmogorov-Smirnov test for normality and with the Barttlet tests for variances homogeneity.

3. Results

3.1 Bacterial Growth Assay

No contaminations were observed during the growth assays with *E. toletana*.

Significant differences were observed in the sensitivity to NaCl between *E. toletana* exposed for six weeks to LB medium (Et-LBM) or to NaCl (Et-NaCl). In the former case, a significant decrease in growth, relatively to the control, was observed at concentrations 15 and 35 g/L, while for the latter case a significant reduction of growth was only observed at 35 g/L NaCl (Fig. 16; $p<0.001$). Furthermore, the $EC_{20,120h}$ values computed for *E. toletana*

successively exposed to LB medium was lower than that computed for *E. toletana* successively exposed to 18 g/l of NaCl: 20 (18.5-21.9) g/L and 30.8 (25.4-36.3) g/L, respectively.

Within the tested NaCl concentration, significant differences were observed between *E. toletana* exposed for six weeks to LB medium and to NaCl, the later exhibiting higher O.D. values at 15, 25 and 35 g/L of NaCl (Fig.16; p<0.001).

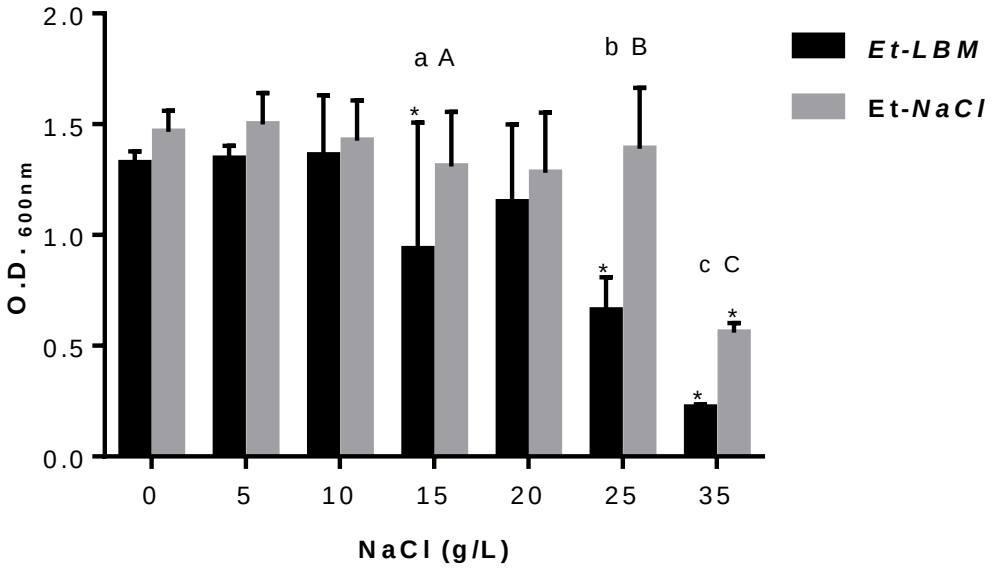

Figure 16: Average O.D (and standard deviation) of *Erwinia toletana* after exposure for six weeks to LB medium (Et-LBM) or 18 g/L of NaCl (Et-NaCl). aA, bB and cC – significant differences between isolates within each concentration and * - represent significant differences comparatively to the respective control (P<0.001).

3.2 Biolog Carbon Compounds Metabolic Analysis

Contaminations in cultures of the three treatments (Et-LBM, Et-NaCl and Et-R), were not observed. Cultures in Petri dish only presented one type of colonies of white-yellow color, corresponding to the same previous *E. toletana* stored at 4ºC ±1 stored in solid medium.

In Table 5 are shown the changes in the rates of carbon compounds, metabolized by *E. toletana* (submitted to the three exposure scenarios: Et-LBM, Et-NaCl and Et-R). From the 71 carbon sources tested, differences between Et-LBM and Et-NaCl were only found for ten of them. The Et-NaCl exhibited higher growth rate, comparatively to Et-LBM, when having the following carbon substrates: D-Cellobiose, D-Salicin, D-Serine, D-Aspartic Acid, L-Arginine, Pectin, β-Hydroxy- Butyric Acid, α-Keto-Butyric; and exhibited lower

growth rate when having as carbon substrate L-Galactonic Acid Lactone and Quinic Acid (Table 5).

Comparing Et-LBM with Et-R, the later showed a higher growth rate in the presence of the following carbon subtracts: D-Cellobiose, N-Acetyl-D-Mannosamine, L-Arginine and 2 and a lower growth rate in the presence of inosine and quinic acid (Table 5).

Table 5: Percentage of changes, relatively to the control (Et-LBM), observed in the rates of carbon substrate metabolism of the bacterial skin frog isolate *Erwinia toletana* (Et-NaCl and Et-R). Et-LBM- *E. toletana* exposed for six weeks to LB medium, Et-NaCl *E. toletana* exposed for six weeks to 18g/L NaCl, and Et-R – *E. toletana* exposed for six weeks to NaCl followed by a four generation exposure to LB medium. Dark grey cells- growth inhibited relatively to Et-LBM; Light grey cells- growth stimulated relatively to LBM; White cells – similar growth relatively to Et-LBM, considering 20% as the threshold to consider changes relatively to Et-LB as significant.

Class of substrates	Carbon Substrates	Et-LBM vs Et-NaCl (%)	Et-LBM vs Et-R (%)
Sugars	D-Cellobiose	-35,1	-23,7
	D-Salicin	-32,3	3,90
	N-Acetyl-b-DMannosamine	-15,6	-22,0
	Inosine	1,41	21,0
Other sugars	D-Serine	-23,9	-1,96
	D-Aspartic Acid	-210	2,13
Aminoacids	L-Arginine	-26,6	-57.0
Hexose acids	L-Galactonic Acid Lactone	26,5	2,99
	Quinic Acid	74,8	77,0
	Pectin	-21,7	2,62
Carboxylic Acids, testers and fatty acids	β-Hydroxy- Butyric Acid	-61,9	-10,8
	α-Keto-Butyric	-141	1,05

Comparing results obtained for Et-NaCl and Et-R, recovery seems to have occurred for metabolic pathways involving the following substrats: D-Salicin, D-serine, D- Aspartic Acid, L- Galactonic Acid Lactone, Pectin, β-Hydroxy- Butyric Acid and α-Keto-Butyric, since changes relatively to Et-LBM were below 20%.

Relatively to the results obtained with the chemical sensibility test, only Et-NaCl showed differences relatively to Et-LBM, the former revealed higher sensitivity to aztreonam (19.7%) (a monobactam antibiotic used to treat infections with Gram-negative bacteria) and potassium tellurite (21.4%) (also used as an anti-microbial agent).

4. Discussion

Successive exposure of *Erwinia toletana* to low levels of NaCl allowed the bacterium to acquire tolerance to this chemical stressor. After being exposed, for six weeks, to the EC_{10} for NaCl (18 g/L), the $EC_{20,120h}$ for NaCl increased from 20 (18.5-21.9) g/L to 30.8 (25.4-36.3) g/L. These results were expected since, in the scientific literature, several works have already reported the capacity of bacteria to acquire increased tolerance to salinity (e.g., Dahr *et al.*, 2011; Zhou *et al.*, 2013). Such increased tolerance to salt stress has been associated with a set of physiological responses. The biosynthesis of osmolytes and intracellular accumulation of mixtures of organic compounds (aiming to maintain the cytoplasm in an isosmotic state), has been reported to be one of the major pathways to cope with salt stress (Oren, 2008; Galinski *et al.*, 2010), mainly in Gram-negative bacteria (Baumann and Marschner 2013). Osmolytes are compatible solutes with osmoprotectants properties produced by bacteria without disturbing cell functions (Saum and Müller 2007; Qurashi and Sabri 2013). A 8-fold accumulation of glutamate (8-fold) and of alanine (1.8-fold) was observed in the bacterium *Desulfovibrio vulgaris* after being exposed for 100-h to 250 mM NaCl (He *et al.*, 2010). Within the genus of *Erwinia*, Goude *et al.* (2004) detected that *E. chrysanthemi* accumulated mainly glutamine when exposed to high salinity levels, while alpha-glucosylglycerate and glutamate were the predominant osmolytes when exposure occurred at low salt concentrations. It is then hypothesized that in the present work, one of the major mechanisms involved in the acquisition of salinity tolerance by *E. toletana* involved the production and intracellular accumulation of a mixture of osmolytes. Another mechanism identified to be involved in tolerance acquisition to salinity includes the changes in the lipid composition of the cell membrane. The increase in unsaturated branched fatty acids, in bacteria exposed to salt stress, was associated with higher membrane fluidity (e.g. Zhou *et al*., 2013). In the present work, no direct evidence for the occurrence of this mechanism in NaCl-exposed *E. toletana* was observed; nevertheless, the possibility of its occurrence is not excluded here.

Successive exposure of *E. toletana* to low levels of NaCl also induced changes in the rate of consumption of carbon sources. In general, *E. toletana* exposed for six weeks to NaCl showed a higher consumption of carbon sources than that exposed to LB medium. This could be related with the activation of metabolic pathways involving enzymes associated with the use of those carbon sources to produce energy. This extra need for energy could be associated with the activation of detoxification mechanisms, like the production of osmolytes. However, other energetic demanding mechanisms could also have been activated to deal with osmotic stress. For example, the basal increase of cation exchange proteins or other mechanisms of active transport across the cell membrane.

Zhou *et al*. (2013) reported an increase in basal expression of the Na^+/H^+ antiporter and of a cation efflux protein encoding genes in response to NaCl stress in bacteria. The results obtained for *E. toletana,* after being able to recover for four weeks from NaCl exposure, support this hypothesis since, the differences in the used of carbon sources between Et-R and Et-LBM were fewer than between Et-NaCl and Et-LBM, suggesting that the bacteria, in the absence of salt stress, was restoring its basal energetic metabolism. Possibly, if the period of continued exposure of *E. toletana* to LB medium, following six weeks exposure to NaCl was longer than four weeks, a complete recovery of the basal energetic metabolism would have occurred.

Furthermore, it was observed that Et-NaCl exhibited a higher sensitivity to the antimicrobial chemicals aztreonam and potassium tellurite comparatively to Et-LBM and Et-R. Aztreonam as a monobactam with great antibiotic activity against Gram-negative bacteria of *Enterobacteriaceae* family (Ramsey and MacGowan 2016; Singh et al. 2015; Stutman et al. 1984) inhibited Et-NaCl growth. Although Et-LBM and Et-R didn't seemed to be affected by the antibiotic. These results could indicate that Et-NaCl triggered mechanisms involved to process NaCl increment probably were activating only to manage NaCl stress leading Et-NaCl susceptible to the antibiotic. Also, the energy used by the cell to tolerate this antibiotic, or the tolerant encoding genes of aztreonam resistance while NaCl is increased in the medium could be affected, leading to diminishing activity. Relating to potassium tellurite, Et-NaCl once more shown to be more sensitive revealed by growth inhibition. Potassium tellurite has been investigating and reveled an antimicrobial with toxic effects related to oxyanion in the cells (Alavi, Amoozegar, and Khajeh 2014; Taylor 1999). Pérez *et al.* (2007) reported toxic damaging effects on *E.coli* when in the presence of potassium tellurite. The authors reported that the toxicity were due to oxyanion what could lead to inactivation of metabolic processes and dehydratases, inactivation of enzyme and protein carboxylation and also inactivation of membrane peroxidation.

Following the same occurrence with aztreonam in Et-NaCl, it's possible that increased salinity in the cells could lead to damages in cell membranes, inactivation of mechanisms involving both antimicrobial agents resistance, or the energy from the cell to manage the osmotic stress could be used to process the increment of NaCl failing Et-NaCl tolerance to these antimicrobial agents.

5. Conclusion

Erwinia toletana revealed to be able to acclimatize and acquire an increased tolerance to low levels of salinity. These processes were associated with physiological alterations that were reversible after transferring the bacteria to LB medium. This ability of no-pathogens to acquire tolerance and multiple tolerance to environmental stress could constitute a promising tool in bioaugmentation in amphibian's skin, aiming the improvement of the tolerance of these organisms to chemical contamination.

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CHAPTER 5

Conclusions and major findings

The results obtained in chapter 2 revealed the Microdilution methodology to be more suitable than the Wells Diffusion Disk (a standardized methodology commonly used to susceptibility of bacteria to antibiotics), to assess the sensitivity of amphibian skin microbiome to chemical contamination. The former method showed to be more sensitive and discriminative, since was the only that allowed identifying bacterial isolates within the category of very sensitive (both to AMD and to NaCl). Considering other major advantages of the microdilution method (e.g. higher reproducibility) it is suggested as a suitable test to characterize the sensitivity of skin microbiome to chemical contamination. Nevertheless, the optimization of the methodology is required to improve its accuracy, namely the procedure related with the pipetting of the inoculum in cases of bacteria that form sticky cultures making difficult to obtain homogeneous inoculum to start the assays. Furthermore, the suitability and advantages provided by the microdilution method could be compared with other dilution methods in order to attest if this is indeed the most suitable test to assess the sensitivity of skin bacteria to chemicals.

The microdilution test allowed to distribute the skin bacteria isolates into four categories of sensitivity to AMD and to NaCl: VS, S and T and VT. The four categories were present in the three sampling sites with the exception of VS that did not appeared in SL (with increased salinity), suggesting that long-term exposure to increased salinity could have wiped out some species of bacteria (the most sensitive) from the skin of *P. perezi* inhabiting this site. The distribution of these sensitive categories showed that the VT and T were present at higher frequencies in LB, SL and LB. The high frequency of VT or T bacteria in the skin of *P. perezi* suggests that the role of these bacteria as part of the premier barried to chemicals (and other environmental stressors like pathogens) is ensured even in populations of amphibians inhabiting chemically impacted sites. Further supporting this finding, the overall sensitivity of the skin microbiome was lower than that of tadpoles of *X. laevis* and of *P. perezi*, indicating that bacteria will prevail in the amphibian's skin even at levels of contamination that may severely affect amphibians. Therefore, it is expected that under such scenarios the bacteria will contribute to an increased tolerance of the amphibian to the chemical, through, for example, processes associated with changes in the speciation of metals (altering its bioavailability and toxicity not only for the bacteria but as well for the amphibian).

Finally, this work also demonstrated the possibility to improve the tolerance of *Erwinia toletana* to NaCl, through successive exposure to low levels of this chemical (corresponding to the EC_{10} to the bacteria) stressor. This increased tolerance seemed to

be associated with physiological adjustments to salinity since effects observed at the metabolic level were reversible after removing NaCl from the medium for four weeks. This type of bacterial responses may contribute as well for a higher tolerance in amphibians. Therefore, bacteria like *E. toletana* (that not only are tolerant to a chemical but also capable of acquiring an even higher increased tolerance to that chemical) constitute promising tools to be used in bioaugmentation aiming at an increased resilience of natural populations of amphibians inhabiting contaminated sites.

Further Perspectives

Bioaugmentation

Following the work of this thesis and taking into consideration published literature (Harris *et al*. 2009; Bletz *et al*. 2013; Loudon *et al*. 2014; Yasumiba, Bell, and Alford 2016) further goals could direct this investigation to amphibian's biotherapy application through bioaugmentation. Biaugmentation in amphibian' skin has been applied, using probiotic bacteria with anti-pathogen properties, allow amphibians to resist pathogen infections, like the *Batrachochytrium dendrobatidis* (Bletz *et al*. 2013; Loudon *et al*. 2014; Woodhams *et al*. 2016). Becker *et al*., (2009) through biotherapy tried to inoculate in amphibians skin the bacteria *J. lividum* producer of anti-Bd metabolite violacein to unable *Bd* infection. The study revealed mortality and morbidity decrease with this bioaugmentation method. Despite of this promising method, there are some difficulties to overpass as: total number spores present in ponds, microorganism dilution in pond after being inoculated and how it could compromise non-target living pond organisms (Bletz *et al*. 2013).

Considering the results obtained here, a follow-up work should involve bioaugmentation of the tadpoles skin with AMD or NaCl-VT bacteria in order to understand if the bacteria could contribute to an increased tolerance of the tadpoles to AMD, NaCl or both (considering the existence of multiple or co-tolerance) contamination.

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ANNEX I

Table 6: Bacterial Isolates studied Identification by 16S rRNA sequency and correspondent Gene bank accession numbers. Legend: * Identification were not possible, (*continue on the next page*).

Bacterial Code	GenBank Accession Number	Closest EzTaxon (nº)
LB1-1	KT720415	*Exiguobacterium undae* DSM 14481(T) (DQ019165)
LB1-10	KT720377	*Erwinia toletana* A37(T) (AF130910)*et al* *Erwinia toletana* A64 (AF130963)
LB1-11	KT720378	*Porphyrobacter tepidarius* DSM 10594(T) (AB033328)
LB1-3	KT720379	*Acinetobacter beijerinckii* 58a(T) (AJ626712)
LB13-1	KT720416	*Frigoribacterium faen*i 801(T) (Y18807)
LB13-10	KT720380	*Rhizobium rosettiformans* W3(T) (EU781656)
LB13-11	KT720381	*Azohydromonas* lata IAM 12599(T) (D88007) *Pelomonas aquatica* CCUG52575(T) (AM501435)
LB13-2	KT720382	*Aquabacterium parvum* B6(T) (AF035052)
LB13-5	KT720376	*Azorhizobium doebereinerae* BR 5401(T) (AF391130)
LB13-6	KT720383	*Piscinibacter aquaticus* IMCC1728(T) (DQ664244)
LB13-7	KT720418	*Staphylococcus warneri* ATCC 27836(T) (L37603)
LB1-4	KT720384	*Bosea lathyri* LMG26379(T) (FR774993)
LB1-5	KT720419	*Microbacterium testaceum* DSM 20166(T) (X77445)
LB1-6	KT720385	*Sphingomonas faeni* MA-olki(T) (AJ429239)
LB1-7	KT720386	*Piscinibacter aquaticus* IMCC1728(T) (DQ664244)
LB1-8	KT720420	*Nocardioides furvisabuli* SBS-26(T) (DQ411542)
LB1-9	KT720387	*Roseomonas stagni* HS-69(T) (AB369258)
LB7-1	KT720421	*Geodermatophilus obscurus* DSM 43160(T) (CP001867)
LB7-2	KT720422	*Nocardioides alpinus* Cr7-14(T) (GU784866)
LB7-3	KT720423	*Microbacterium lacus* A5E-52(T) (AB286030)
LB7-4	KT720424	*Microbacterium lacus* A5E-52(T) (AB286030)
LB7-6	KT720425	*Lapillicoccus jejuensis* R-Ac013(T) (AM398397)
LB7-8	KT720390	*Brevundimonas nasdae* GTC 1043(T) (AB071954)
LB7-9	KT720426	*Microbacterium lacus* A5E-52(T) (AB286030)
SL12-1	KT720427	*Bacillus vietnamensis* 15-1(T) (AB099708)
SL12-2	KT720428	*Paenibacillus pabuli* JCM 9074(T) (AB073191)
SL12-3	KT720392	*Sphingomonas faeni* MA-olki(T) (AJ429239)
SL12-5	KT720429	*Paenibacillus arybhattai* B8W22(T) (EF114313)
SL12-6	KT720393	*Bacillus vietnamensis* 15-1(T) (AB099708)
SL12-7	KT720430	*Bacillus aerophilus* 28K(T) (AJ831844)
SL12-8	KT720394	*Aquabacterium parvum* B6(T) (AF035052)
SL12-9	KT720395	*Moraxella osloensis* AerLab-37 (EU499677)
SL2-1	KT720396	*Methylobacterium marchantiae* JT1(T) (FJ157976)
SL2-2	KT720431	*Bacillus mycoides* ATCC 6462(T)(AF155956)
SL2-4	KT720432	*Micrococcus antarcticus* T2(T) (AJ005932)
SL2-5	KT720397	*Paracoccus marinus* KKL-A5(T) (AB185957)
SL2-6	KT720433	*Deinococcus grandis* DSM 3963(T) (Y11329)
SL2-7	KT720398	*Rhizobium rosettiformans* W3(T) (EU781656)

Bacterial Code	GenBank Accession Number	Closest EzTaxon (nº)
SL2-8	KT720399	*Flavobacterium tegetincola* ACAM 602(T) (U85887)
SL3-1	KT720434	*Arthrobacter oxydans* DSM 20119(T) (X83408)
SL3-10	KT720401	*Microvirga zambiensis* WSM3693(T) (HM362433)
SL3-5	KT720403	*Massilia aerilata* 5516S-11(T) (EF688526)
SL3-8	KT720404	*Sphingomonas glacialis* C16y(T) (GQ253122)
SL3-9	KT720405	*Brevundimonas bullata* IAM 13153 (D12785)
TP10-10	KT720435	*Cellulomonas fimi* ATCC 484(T) (CP002666)
TP10-5	KT720437	*Microbacterium testaceum* DSM 20166(T) (X77445)
TP10-6	KT720438	*Cellulomonas composti* TR7-06(T)(AB166887)
TP10-8	KT720406	*Ensifer adhaerens* LMG 20216(T) (AM181733)
TP1-1	KT720440	*Microbacterium testaceum* DSM 20166(T) (X77445)
TP11-2	KT720407	*Sphingomonas cynarae* SPC-1(T) (HQ439186)
TP11-4	KT720442	*Staphylococcus saprophyticus* subsp. bovis GTC 843(T) (AB233327)
TP11-6	KT720443	*Leifsonia shinshuensis* JCM 10591(T) (DQ232614)
TP1-2	KT720444	*Mycobacterium frederiksbergense* DSM 44346(T) (AJ276274)
TP1-3	KT720408	*Pseudomonas koreensis* Ps 9-14(T) (AF468452)
TP1-4	KT720445	*Bacillus aerophilus* 28K(T) (AJ831844)
TP1-5	KT720446	*Bacillus safensis* FO-036b(T) (AF234854)
TP1-6	KT720447	*Bacillus simplex* NBRC 15720(T) (AB363738)
TP2-1	KT720409	*Brevundimonas nasdae* GTC 1043(T) (AB071954)
TP2-2	KT720410	*Brevundimonas nasdae* GTC 1043(T) (AB071954)
TP2-4	KT720412	*Pseudomonas syringae* ATCC 19310(T) (AJ308316)
TP2-5	KT720413	*Moraxella osloensis* NCTC 10465(T) (X74897) *Moraxella osloensis* AerLab-37 (EU499677)
TP3-2	KT720414	*Hydrotalea flava* CCUG 51397(T) (FN665659)
TP3-3	KT720448	*Micrococcus luteus* NCTC 2665(T)(CP001628)
TP3-5	KT720449	*Janibacter terrae* CS12(T) (AF176948)
TP10-7	*	
TP10-11	*	
TP10-13	*	
TP11-3	*	
TP3-1	*	
SL12-11	*	
SL3-4	*	
SL3-6	*	

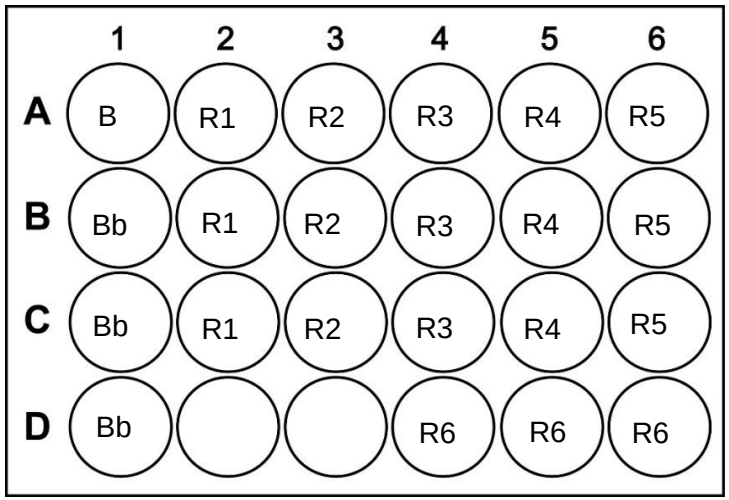
Table 7: Representation of EC_{10}, EC_{20} and EC_{50} of all sensitive bacterial isolates to acid-mine drainage contamination. Legend: - 95% confidence interval could not be computed.

	AMD									
Isolate	**EC10**	**Low config limit**	**Up conf limit**	**EC20**	**Low config limit**	**Up conf limit**	**EC50**	**Low config limit**	**Up conf limit**	**R**
LB1-11	54.67	34.92	74.42	61.42	45.52	77.31	74.75	65.03	84.47	0.88
LB13-1	4.31	-	11.91	8.96	-	19.69	50.04	33.89	66.19	0.87
LB1-4	23.45	8.57	38.32	31.80	16.94	46.66	53.20	40.35	66.05	0.92
LB1-5	68.21	-	-	83.96	68.86	99.07	85.04	-	-	0.78
LB7-3	-	-	-	-	-	-	36.23	16.84	55.60	0.89
LB7-3	6.84	-	20.50	10.37	-	27.05	20.87	0.71	41.04	0.99
LB7-4	63.81	59.07	68.56	69.24	65.72	72.77	79.48	76.83	82.13	0.98
LB7-9	68.84	-	-	70.25	-	-	72.49	-	-	0.75
SL12-8	14.16	-	29.03	21.57	4.68	38.45	43.88	25.61	62.14	0.86
SL2-1	35.61	11.90	59.33	43.14	22.02	64.26	59.65	43.17	76.13	0.79
SL2-8	72.60	58.39	86.80	76.43	64.05	88.80	83.36	67.46	99.26	0.79
SL3-10	11.78	1.12	22.44	20.17	6.87	33.47	50.04	33.89	66.19	0.91
TP10-5	56.94	18.13	95.76	88.59	60.64	116.54	-	33.18	-	0.76
TP11-6	97.49	-	-	99.28	-	-	-	99.01	-	0.40
TP1-4	32.92	-	209.83	108.39	-	410.16	-	-	-	0.27
TP3-3	-	-	-	93.07	65.29	120.85	-	-	-	-
TP3-5	88.59	56.35	120.83	-	83.72	116.86	-	-	-	0.69

Table 8: Representation of p-values from one-way analysis of variance (ANOVA) followed by the multicomparison Dunnett's test to assess significant differences between the lowest dilution of AMD dilutions and the respective control of very sensitive and very tolerant bacterial isolates to acid-mine drainage.

Isolate	Classification	p-value	Isolate	Classification	p-value	Isolate	Classification	p-value
LB7-1	VS	0.9984	**SL12-11**	VS	0.7724	**TP 10-7**	VS	0.0371
LB 13-2	VS	0.643	**SL12-3**	VS	<0.0001	**TP10-11**	VS	<0.0001
LB 7-10	VS	0.0946	**SL2-1**	VS	<0.0001	**TP10-6**	VS	0.0068
LB1-11	VS	0.9996	**SL2-2**	VS	0.7997	**TP11-2**	VS	0.0481
LB13-11	VS	0.2399	**SL2-6**	VS	0.6235	**TP1-3**	VS	0.3393
LB13-12	VS	0.9622	**SL3-1**	VS	> 0.9999	**TP2-1**	VS	0.9581
LB13-5	VS	0.0006	**SL3-10**	VS	0.8427	**TP2-5**	VS	<0.0001
LB1-4	VS	0.0001	**SL3-5**	VS	0.8202	**TP3-3**	VS	0.798
LB1-5	VS	0.9979	**SL12-1**	VT	0.6621	**TP 3-5**	VS	0.0013
LB1-7	VS	0.9982	**SL12-2**	VT	0.1675	**TP 1-4**	VT	> 0.9999
LB7-3	VS	0.9997	**SL12-7**	VT	0.4253	**TP11-3**	VT	0.7514
LB7-6	VS	0.3587	**SL12-8**	VT	0.9996	**TP11-4**	VT	0.0035
LB1-1	VT	0.0381	**SL2-4**	VT	0.7756	**TP1-2**	VT	0.9979
LB13-10	VT	0.9598	**SL2-5**	VT	0.181	**TP1-5**	VT	0.0258
LB13-6	VT	0.0196	**SL2-7**	VT	0.9792	**TP1-6**	VT	0.9698
LB1-8	VT	0.0475	**SL12-11**	VS	0.7724	**TP2-4**	VT	0.9268
LB7-2	VT	0.073	**SL12-3**	VS	<0.0001	**TP 10-7**	VS	0.0371
LB7-1	VS	0.9984				**TP10-11**	VS	<0.0001
LB 13-2	VS	0.643						

Figure17: Illustration of a second Microdilution test using a different disposal of the bacteria culture exposed to concentrations. Legend: B - LB medium. Bb - LB medium with Bacteria. D1 - Dilution 1 (6.25 % of effluent). R1 - replicates of D1 with Bacteria inoculum. D2 - Dilution 2 (12.5% of effluent). R2 - replicates of D2 with Bacteria inoculum. D3 - Dilution 3 (25 % of effluent). R3 - replicates of D3 with Bacteria inoculum. D4 - Dilution 4 (50 % of effluent). R4 - replicates of D4 with Bacteria inoculum. D5 - Dilution 5 (75 % of effluent). R5 - replicates of D5 with Bacteria plus D6 - Dilution 6 (100 % of effluent). R6- replicates of D6 with Bacteria inoculum.



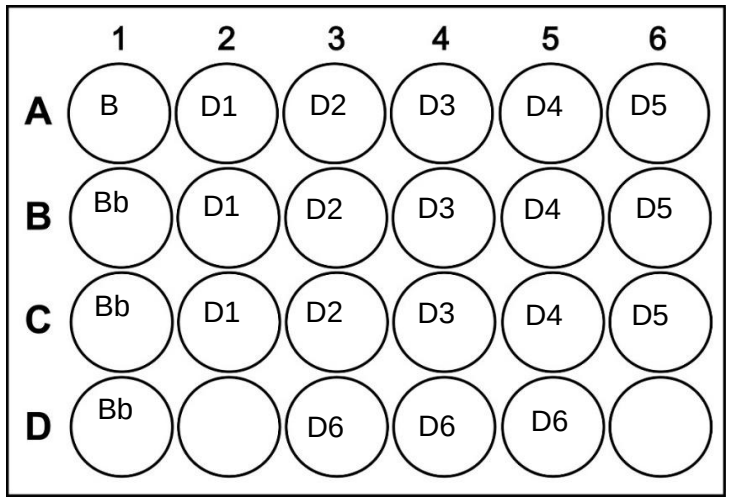


Table 9: Representation of EC_{10}. EC_{20} and EC_{50} of all sensitive bacterial isolates to NaCl contamination. Legend: - 95% confidence interval could not be computed. (*continue on the next page*).

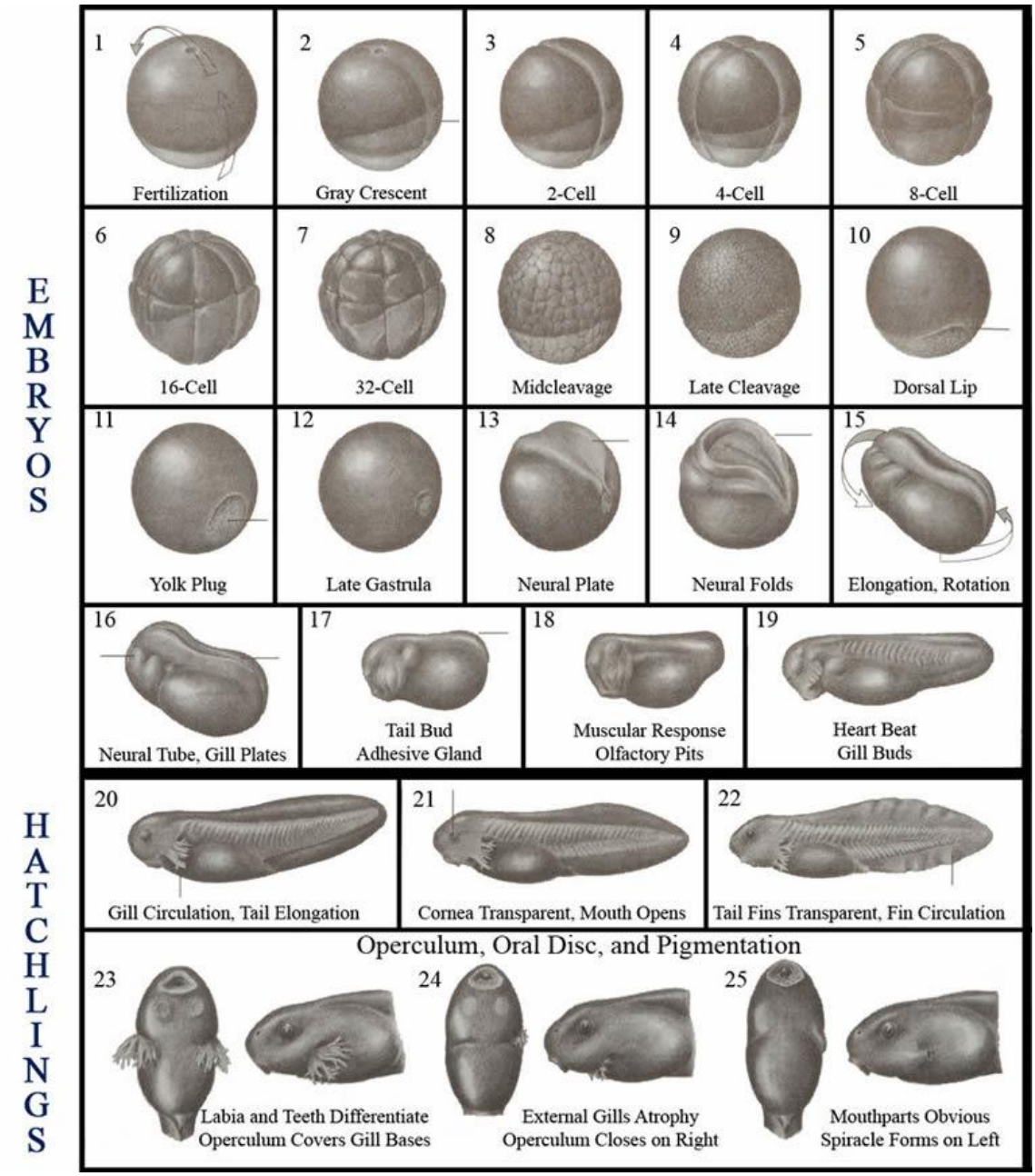
	NaCl									
Isolate	**EC10**	**Low config limit**	**Up conf limit**	**EC20**	**Low config limit**	**Up conf limit**	**EC50**	**Low config limit**	**Up conf limit**	**R**
LB 1-10	15.28	8.16	22.40	18.27	12.20	24.34	24.70	19.95	29.45	0.83
LB7-8	16.91	8.32	25.50	19.78	12.59	26.96	25.78	20.06	31.49	0.75
TP2-2	16.39	13.00	19.78	18.66	15.92	21.41	23.23	21.28	25.18	0.95
TP10-5	13.48	-	41.35	21.08	-	45.61	211.98	-	-	0.52
TP3-1	14.34	-3.09	31.77	19.10	3.58	34.62	31.02	16.65	45.38	0.53
SL3-9	3.59	-	9.36	6.43	-	13.87	17.24	7.67	26.82	0.78
LB7-9	-	-	-	5.97	-	21.33	28.86	-	64.74	0.53
LB13-1	-	-	-	12.26	4.36	20.16	-	-	-	0.81
LB1-6	10.52	1.13	19.92	14.52	5.46	23.57	25.04	17.17	32.90	0.75
LB1-3	8.17	-	17.69	11.41	1.82	21.01	20.04	11.60	28.47	0.72
TP11-6	5.30	2.15	8.45	7.61	4.22	10.99	13.99	10.68	17.30	0.94
LB13-1	-	-	-	3.44	-	8.73	42.86	7.12	78.61	0.85
LB13-7	-	-	-	16.35	2.55	30.14	20.40	10.95	29.86	0.45
SL2-8	13.37	-	-	13.88	-	-	14.25	-	-	0.49
LB7-4	9.51	4.07	14.95	14.19	8.69	19.69	27.89	22.54	33.24	0.89
SL3-4	21.34	18.19	24.49	23.25	20.88	25.62	26.87	24.68	29.05	0.94
SL3-6	-	-	-	5.76	3.03	8.50	7.08	4.47	9.69	0.91
SL12-5	11.39	4.91	17.87	22.89	16.11	29.68	74.48	-	-	0.91
TP1-1	14.94	7.59	22.28	22.89	16.11	29.68	25.05	20.03	30.08	0.81
SL3-8	-	-	-	1.11	-	4.53	-	-	-	-
TP10-8	18.59	-	-	18.81	-	-	19.31	18.09	20.53	0.93
TP10-13	6.09	2.32	9.87	7.90	4.20	11.60	12.25	9.05	15.45	0.92
SL12-6	3.72	-	7.98	6.26	1.00	11.51	14.96	8.64	21.28	0.85
TP3-2	5.46	-	20.99	11.10	-	31.18	36.79	-	74.06	0.54
LB7-10	22,87	8,35	37,39	25,86	13,83	37,89	31,82	23,69	39,94	0,80
LB13-5	1,39	-	3,35	2,13	-	4,56	4,41	1,41	7,41	0,99
SL12-3	2,71	1,87	3,56	3,37	2,64	4,09	4,84	4,42	5,26	0,98
SL2-1				0,62	-	2,41	1,76	-	4,67	0,97
SL3-5	1,10	-	2,72	2,29	-	4,75				0,94
TP10-11	8,65	7,48	9,82	9,40	8,63	10,16	10,81	9,99	11,63	0,98

NaCl										
Isolate	**EC10**	**Low config limit**	**Up conf limit**	**EC20**	**Low config limit**	**Up conf limit**	**EC50**	**Low config limit**	**Up conf limit**	**R**
TP1-3	23,41	13,93	32,89	25,96	17,99	33,93	30,93	25,35	36,50	0,84
TP3-5	23,41	13,93	32,89	25,96	17,99	33,93	30,93	25,35	36,50	0,84
TP2-5				0,60	-	4,60				0,98
TP11-2	3,74	-	8,72	6,68	0,65	12,71	17,79	14,34	21,24	0,99

Table 10: Representation of p-values from one-way analysis of variance (ANOVA) followed by the multicomparison Dunnett's test to assess significant differences between the highest NaCl concentration and the respective control of very sensitive and very tolerant bacterial isolates to NaCl. *(continue on the next page)*

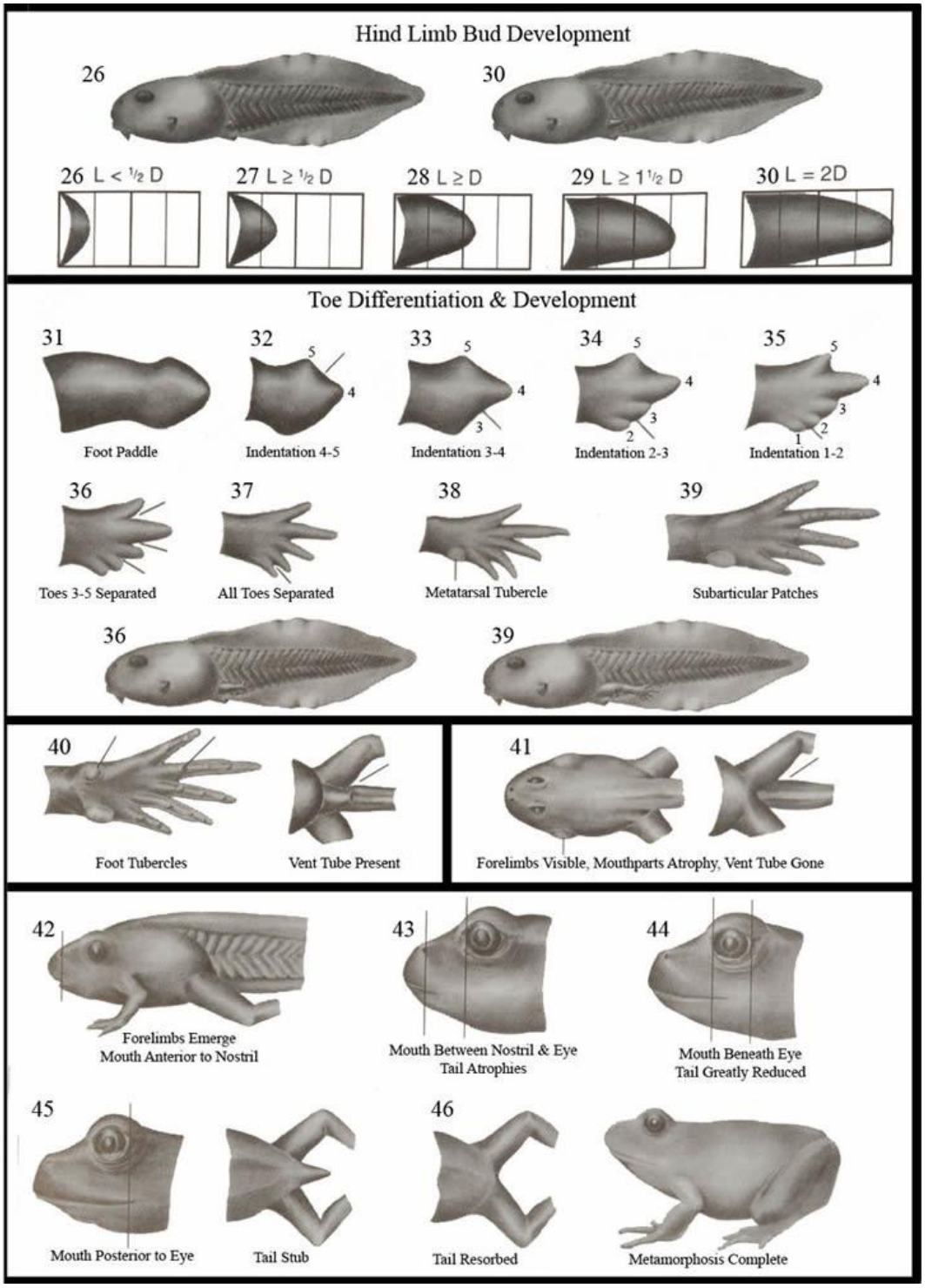
Isolate	Classification	p-value	Isolate	Classification	p-value	Isolate	Classification	p-value
LB7-1	T	0.9984	**SL12-11**	T	0.7724	**TP 10-7**	VS	0.0371
LB 13-2	T	0.643	**SL12-3**	S	< 0.0001	**TP10-11**	S	< 0.0001
LB 7-10	T	0.0946	**SL2-1**	S	< 0.0001	**TP10-6**	VS	0.0068
LB1-11	T	0.9996	**SL2-2**	T	0.7997	**TP11-2**	S	0.0481
LB13-11	T	0.2399	**SL2-6**	T	0.6235	**TP1-3**	T	0.3393
LB13-12	T	0.9622	**SL3-1**	T	> 0.9999	**TP2-1**	T	0.9581
LB13-5	S	0.0006	**SL3-10**	T	0.8427	**TP2-5**	S	< 0.0001
LB1-4	VS	0.0001	**SL3-5**	S	0.8202	**TP3-3**	T	0.798
LB1-5	T	0.9979	**SL12-1**	T	0.6621	**TP 3-5**	S	0.0013
LB1-7	T	0.9982	**SL12-2**	T	0.6894	**TP 1-4**	T	> 0.9999
LB7-3	T	0.9997	**SL12-7**	T	0.4253	**TP11-3**	T	0.7514
LB7-6	VS	0.3587	**SL12-8**	T	0.9996	**TP11-4**	VT	0.0035
LB1-1	VT	0.0381	**SL2-4**	T	0.7756	**TP1-2**	T	0.9979
LB13-10	T	0.9598	**SL2-5**	T	0.181	**TP1-5**	VT	0.0258
LB13-6	VT	0.0196	**SL2-7**	T	0.9996	**TP1-6**	T	0.9698
LB1-8	VT	0.0475	**SL3-8**	S	0.0031	**TP2-4**	T	0.9268
LB7-2	T	0.5103	**SL2-8**	T	0.9629	**TP11-6**	S	< 0.0001
LB13-1	S	< 0.0001	**SL12-5**	T	< 0.0001	**TP10-13**	S	< 0.0001
LB7-4	T	0.0003	**SL12-6**	VT	0.8962	**TP10-8**	T	< 0.0001
LB7-9	S	0.0678	**SL3-4**	T	< 0.0001	**TP1-1**	T	0.0129
LB1-10	T	< 0.0001	**SL3-6**	S	< 0.0001	**TP2-2**	T	< 0.0001
LB1-3	S	0.0673	**SL3-9**	S	0.0024	**TP3-1**	T	0.4906
LB13-7	T	0.8816	**SL12-9**	T	0.3596	**TP3-2**	S	< 0.0001
LB1-6	T	0.0352				**TP10-5**	T	0.072
LB7-8	T	0.0035						
LB1-9	T	0,9937						

Figure 18: Illustration of Gosner stages development of amphibians. from egg to adult stage. (*continue on the next page*). Source Gosner et (1960).



LARVAE

METAMORPHS



ANNEX II

Medium composition approached in this work:

Table 11: Typical composition of R2A medium (Oxoid. England).

R2A Agar	Formula (g/L)
Yeast extract	0.5
Proteose peptone	0.5
Caseín hydrolysate	0.5
Glucose	0.5
Starch	0.5
Di-potassium phosphate	0.3
Magnesium sulphate	0.024
Sodium pyruvate	0.3
Agar[a]	15

Table 12: Composition of liquid nutrient broth NB.

NB Medium	Formula (g/L)
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride (NaCl)	5
Demineralized Water	1

Table 13: Typical composition of Luria Broth medium (LB).

LB medium	Formula (g/L)
Tryptone	10
Yeast Extract	5
NaCl	5
Agar[a]	15

[a] Agar was added only when necessary for to bacterial culture in Petri Dishes.

Table 14: Composition of Fetax Solution

Substance	(mg per 20 L)
NaCl	1250
$NaHCO_3$	1920
KCl	600
$CaCl_2$	468
$CaSO_4H_2O$	1200
$MgSO_4$	2901