Universidade de Aveiro Departamento de Biologia
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Beatriz
Agostinho da Cruz

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Avaliação dos efeitos e interações de nitratos e salinidade em *Echinogammarus meridionalis*, isolados e em misturas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizado sob a orientação científica do Doutor António José Arsénia Nogueira, Professor Associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro e Co-orientação de Doutora Ana Isabel Lillebø Batista, Investigadora principal no Departamento de Biologia da Universidade de Aveiro.

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DEDICATÓRIA

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O júri

Presidente	**Professor Doutor Fernando José Mendes Gonçalves** **Professor Associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro**
Arguente	**Doutora Maria de Fátima Tavares de Jesus** **Investigadora em Pós-Doutoramento, Departamento de Biologia da Universidade de Aveiro**
Orientador	**Professor Doutor António José Arsénia Nogueira** **Professor Associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro.**

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Palavras –chave

Alterações climáticas, intrusão salina, ecossistemas aquáticos, anfípodes, *Echinogammarus meridionalis*, salinidade, nitratos, toxicidade aguda, toxicidade de misturas, efeitos biológicos, necrose.

Resumo

Os ecossistemas de água doce são dos ecossistemas mais vulneráveis do planeta às alterações climáticas. Estas mudanças climáticas têm vindo a provocar vários efeitos nos ecossistemas, entre eles, o aumento do nível médio do mar que poderá levar à intrusão salina em cursos de água doce. Por outro lado, as práticas agrícolas intensivas e a produção animal fez com que fossem atingidas concentrações elevadas de nitratos nas águas subterrâneas e nos solos, em várias zonas do planeta. Assim, devido a estas perturbações e desequilíbrios que se têm verificado nos ecossistemas, em particular nos ecossistemas aquáticos, estudou-se as variáves salinidade e concentratação de nitratos, isoladas e em mistura (cenário mais realista dos ecossistemas). O objetivo do trabalho focou-se em avaliar a tolerância de *Echinogammarus meridionalis* à variação de cada uma das variáveis e à sua variação conjunta. Durante 96 horas, os organismos foram expostos a diferentes concentrações. Os organismos não foram alimentados durante o período de exposição. Todos os dias, a mortalidade e os parâmetros físico-químicos, tais como a condutividade, pH, concentração de oxigénio e temperatura foram registados. No final de cada ensaio, os organismos foram separados em machos e fêmeas e foi determinado o comprimento do corpo. O LC_{50} dos testes de toxicidade aguda para a salinidade foi de 25.38 ± 0.33g L^{-1} e para os ensaios de nitratos o LC_{50} resultante da primeira amostragem de organismos foi de 121.59 ± 11.71 mg NO_3-N L^{-1} e para a segunda amostragem, o LC_{50} foi de 116.99 ± 3.67 mg NO_3-N L^{-1}. Os ensaios de toxicidade da mistruras binárias de salinidade e nitratos evidenciou um padrão antagonista em baixas concentrações no modelo S/A (sinergismo/antagonismo) e no modelo DL (desvio do nível da dose) verificou-se antagonismo em concentrações baixas e sinergismo em concentrações altas. Os resultados obtidos neste trabalho mostram uma grande tolerância à salinidade, assim espera-se que estes anfípodes portugueses se consigam adaptar às mudanças climáticas. Relativamente à tolerância aos nitratos, houve também uma resistência alta dos organismos a concentrações ambientais realistas e concentrações mais elevadas. As combinações binárias das misturas um efeito antagonista em baixa concentração, sugerindo assim uma boa adaptabilidade desse organismo a esses distúrbios ambientais. Ao realizar estudos de biomonitorização ambiental em Portugal, é útil o uso de espécies nativas em vez de espécies modelo, pois desempenham um papel ecológico relevante nos ecossistemas inerentes, e as informações obtidas acabam por ser mais confiáveis e realistas.

Keywords

Climate change, saline intrusion, aquatic ecosystems, amphipods, *Echinogammarus meridionalis*, salinity, nitrates, acute toxicity, toxicity of mixtures, biological effects, necrosis.

Abstract

Freshwater ecosystems are among the world's most vulnerable ecosystems to climate change. These climatic changes have affected negatively the ecosystems, with the mean sea level rise leading to saline intrusion into freshwater ecosystems. Additionally, intensive agricultural practices and livestock production have been the cause of high concentrations of nitrates in groundwater and soil in various parts of the world. Due to these disturbances, namely in aquatic ecosystems, and imbalances, the variables salinity and nitrates were studied separately and combined (a more realistic scenario for ecosystems). The objective of this work was to evaluate the tolerance of *Echinogammarus meridionalis* to each of the selected variables seperately and in combination (mixture). The organisms were exposed to different concentrations for 96 hours. No food was given during the exposure period. Every day, mortality and physico-chemical parameters, such as conductivity, pH, oxygen saturation and temperature were recorded. At the end of each test, the organisms were separated into males and females and their body lengh measured. The LC_{50} of the acute toxicity tests for salinity was 25.38 ± 0.33 g L^{-1} and for the nitrate assays the LC_{50} resulting from the first collection of organisms was 121.59 ± 11.71 NO_3-N L^{-1} and for the second collection the LC_{50} was of 116.99 ± 3.67 NO_3-NL^{-1}. The toxicity tests of the binary mixtures salinity and nitrate showed an antagonistic pattern at low concentrations in the S/A model (synergism /antagonism) and in the DL model (Dose Level deviation) there was antagonism at low concentrations and synergism at high concentrations. The results obtained in this work suggest a great tolerance to the salinity, hence it is expected that these Portuguese amphipods will be able to adapt to the climatic changes. Regarding nitrate tolerance, there was also a high resistance of the organisms in realistic concentrations and higher concentrations. The binary combinations of the mixture have an antagonistic effect at low concentration, thus suggesting a good adaptability of that organism to these environmental disturbances. When carrying out environmental biomonitoring studies in Portugal, it is useful to use native species rather than model species, since they play a crucial ecological role in the inherent ecosystem and the information obtained becomes more reliable and realistic.

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1. General introduction

Water is essential for the maintenance of life on Earth. Our existence and our economic activities depend thoroughly on this resource. However, water is a limited resource. Seventy percent (70%) of the planet Earth is composed of water and the other 30% is composed of land, the continents. Of these 70%, 97% refers to salt water and only 3% to fresh water (Rachwal and Souza, 2003). The presence of water in good quality and quantity is an essential factor for socioeconomic development. As the result of the human population increase, there is a need to manage the water resources responsably, in order to reduce the resource degradation (Karr, 1991).

Various authors have tried for several years to define their own ideas about Ecotoxicology and Environmental Toxicology (Table 1.I). Newman (2009), combined the many definitions and defined ecotoxicology or environmental toxicology as the science of contaminants in the biosphere and their effects on the constituents of the biosphere, including humans.

Table 1.I- Definitions of Environmental Toxicology and Ecotoxicology (Newman, 2009)

Definition	**Reference**
Environmental Toxicology	
1. The study of the effects of toxic substances occurring in both natural and manmade environments.	Duffus (1980)
2. The study of the impacts of pollutants upon the structure and function of ecological systems (from molecular to ecosystem).	Landis and Yu (1995)
Ecotoxicology	
1. The branch of ecotoxicology concerned with study of toxic effects, caused by natural and synthetic pollutants, to the constituents of ecosystems, animals (including human), vegetable and microbial in an integrated context.	Truhaut (1977)
2. The natural extension from toxicology, the science of poisons on individual organisms, to the ecological effects of pollutants.	Moriarly (1983)
3. The science that seeks to predict the impacts of chemicals upon ecosystems.	Levin *et al.*, (1989)
4. The study of the fate and effect of toxic agents in ecosystems.	Cains and Mount (1990)
5. The science of toxic substances in the environment and their impact on living organisms.	Jorgensen (1990)
6. The study of toxic effects on non-human organisms populations and communities.	Suter (1993)
7. The study of the fate and effects of a toxic compound on an ecosystem.	Shane (1994)
8. The field of study, which integrates the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment.	Forbes and Forbes (1994)
9. The science of predicting effects of potentially toxic agents on natural ecosystems and nontarget species.	Hofman (1995)
10. The study of the pathways of exposure, uptake and effects of chemical agents on organisms, populations, communities, and ecosystems.	Connell (1990)
11. The study of harmful effects of chemicals on ecosystems; the harmful effects of chemicals (toxicology) with in the context of ecology.	Walker *et al.*, (2001)
12. The study of harmful effects of chemicals upon ecosystems and includes effects on individuals and consequent effects at the levels of population and above.	Walker *et al.*, (2002)

Ecotoxicological assessment is necessary to safeguard the ecosystems. It is relevant to reflect on existing tools for assessing the ecological effects of contaminants to improve the capacity to predict the impacts of these substances on ecosystems (Newman, 2009).

Aquatic ecotoxicology is a science that aims to solve problems when there is contamination of water by toxic compounds. This science helps understand the toxicity of chemical compounds, signalling the ecotoxicological effects and mechanisms of action in living organisms. Concentration-effect and concentration-response curves can be obtained through an ecotoxicological evaluation whose goal is to identify the toxicity in the impacted environment (Magalhães and Filho, 2008).

In aquatic ecotoxicology are inserted two ecosystems, freshwater ecosystems and marine water. Freshwater ecosystems have a relevant role in maintaining biodiversity, 9.5% of all species on the planet occupy these environments. Of the 126,000 species of animals described for freshwater, 60.4% are insects, 14.5% are vertebrates, 10% are crustaceans (Balian et al., 2008). These ecosystems are characterized as having salinities below 0.5 gL^{-1} (Hammer, 1986).

1.1. Saline intrusion in freshwater ecosystems

Natural and anthropogenic processes are affected by the saline intrusion. Freshwater ecosystems and the resources inherent to these systems are included among the most vulnerable to climate change (Kundzewicz et al., 2008; Werner and Simmons, 2009). This has induced a variety of effects, such as raising the temperatures in river waters, increasing the evaporation, increasing the precipitation during the winter and decreasing it during the summer, among others. However, one of the most visible and most concerning effects at international level is the mean sea level rise associated with the climate change process since it is causing increased saline intrusion in freshwater ecosystems (Robins et al., 2016; Werner and Simmons, 2009), resulting in more saline environments.

Due to the increase in salinity, a decrease in diversity may occur as well as a number of species. These can be expected, within the aquatic system, to move from freshwater to transitional water or seawater ecosystem. Salinity changes, during a short period of time, can lead to problems in osmotic regulation for organisms coexisting in freshwater environments and those living in marine waters. Thus, the organisms try to avoid an increase of the saline concentration inside their bodies or tend to develop mechanisms of adaptation so that they can inhabit in these environments with variations of salinity (Flöder and Burns, 2004). Changes in salinization can cause harm to the biota of freshwater ecosystems, directly or indirectly. Increased salinity can cause toxic effects to organisms (physiological or genetic changes), leading to a decrease in species richness. Indirect changes can occur when salinity increases and these changes have the ability to modify the structure and function of the community (e.g. removing taxonomic groups that serve as food, altering/modifying predation pressure) (Campbell, 1994; Nielsen et al., 2003; Savage, 1981).

Therefore, saline intrusion can lead to serious disturbances in freshwater ecosystems, since organisms coexisting in this habitat usually do not have the capacity to tolerate high salinity values, salinity levels are practically zero. These environments are characterized by having salinities below 0.5 gL^{-1} (Hammer, 1986). However, the communities that occupy these environments are characterized by numerous species and these have different salinity tolerances. Thus, the sensitivity of these species may be different as some species are more halotolerant than others (Boronat et al., 2001; Kefford et al., 2007).

Freshwater ecosystems are affected by various environmental factors, such as, temperature, dissolved oxygen concentration, the percentage of oxygen saturation, pH, among others. However, in these environments salinity variation turns out to be a crucial parameter for the ecological balance of these ecosystems. In the long term, the consequences of changes in salinity and saline intrusion in freshwater environments are poorly understood, hence future studies are needed to develop sustainable management strategies to safeguard the ecological quality of these ecosystems (Little et al., 2016).

For an efficient study of salinity tolerance one must understand and try to predict the effect of salinity on the variety of organisms coexisting in a given habitat, identify some generic responses within taxonomic groups, test organisms at various stages of life cycles and, if possible, identify the salinity tolerance of the organisms tested (Kefford et al., 2007).

1.2. Nitrates in the aquatic environment

Nitrogen compounds are essential for life, as N is a ‘building block’ for aminoacids and proteins. However, in high concentrations in aquatic systems, namely in the dissolved inorganic forms, it may pose a risk to the environmental health and to human health and wellbeing. Within the different species of inorganic nitrogen (ammonium, nitrites and nitrates), in surface running or well aerated waters, nitrate (NO_3^-) is the most abundant form (Alaburda and Nishihara, 1998; Rabalais, 2002). Human health intake of water contaminated with nitrates can lead to serious dysfunction after it is reduced to nitrite ions (NO_2^-), especially in infants during digestion (in the gastrointestinal tract). In the blood, these ions prevent haemoglobin from carrying oxygen and transforming in methemoglobin. Small children are more susceptible to the development of methemoglobin due to conditions of the gastrointestinal system, since it is more alkaline than in adults (Alaburda and Nishihara, 1998; Bouchard et al., 1992).

Nitrogen occurs naturally in the environment, however, inorganic nitrogen species can be found in aquatic ecosystems resulting from other source of pollution (anthropogenic sources). When mentioning anthropogenic sources, it refers mainly to man-made disturbances (e.g. urban agricultural run-offs, sewage effluents, industrial wastes, inadequate wastewater treatment plants, etc.) (Bouchard et al., 1992; Camargo et al., 2005; Rabalais, 2002).

Since the 19th century, intensive agriculture practices have led to the occurrence of excessive concentrations of nitrates due to excessive use of synthetic fertilizers in soils and groundwater in various regions of the planet. Nitrate is considered the most ubiquitous contaminant in groundwater, on a planetary scale, due to its increasing use in agricultural activities (Robertson et al., 2008). Agriculture accounts for more than 50% of the total discharge of nitrogen into surface waters. Thus, at a European level, the Nitrates Directive (1991) (91/676 / EEC) was created, with the following main objectives:

1. Protect water quality throughout Europe by preventing pollution of groundwater and surface water from the use of nitrates from agricultural sources.
2. Promote the use of good agricultural practices.

Directive 91/676 / EEC, has established criteria for identifying waters polluted or at risk of being polluted by nitrates from agricultural sources. Groundwater and surface water (which may be used for human consumption) must not exceed the concentration of 50 mg L^{-1} of nitrates. This directive aims to identify surface waters, estuaries, coastal and marine waters that are eutrophic or may become eutrophic in the short term. At EU level there are also two other relevant Directives, the Water Framework Directive (WFD) and the Drinking Water Directive (DWD). The WFD (2000/60/EC), as amended by Directives 2008/105/EC, 2013/39/EU and 2014/101/EU, established a new integrated approach to the protection of the water environment, whilst the DWD, (Council Directive 98/83/EC) concerns the quality of water intended for human consumption and forms part of the regulation of water supply and sanitation in the European Union. The nitrate concentration (NO_3-N) limit for drinking water in the United States of America is 10 mgL^{-1} (Spalding and Exner, 1993).

The trophic relationships in aquatic ecosystems are delimited by two nutrients, phosphorus (P) and nitrogen (N). Excessive use of fertilizers can lead to the increase of these nutrients and consequently cause eutrophication, which will favour the overgrowth of algae and cyanobacteria, which include harmful forms and may be toxin producers. This increased algae growth will decrease the penetration of light into the water, therefore a reduction in oxygen availability will occur. Thus, due to the shortage of oxygen and deterioration of water quality, some mortality of aerobic organisms will ensue and therefore causing an imbalance of these ecosystems (Rabalais, 2002; Ryther and Dunstan, 1971).

Harmful effects on the environment and risks to human health resulting from high concentrations of nitrates in soil and water are considered relevant environmental issues. Bouwman et al. (2013) performed projections for the coming decades and it has been found that globally the nutrients will continue to increase (N and P). Furthermore, the authors recommend an improvement in agricultural management. If there is adequate use of synthetic fertilizers there will be a greater regulation of the nutrients flux (N and P) into the environment. Regarding livestock production, the study proposes a change in the human diet, encouraging the consumption of poultry or pork instead of cattle production to reduce the presence of nutrients in ecosystems.

1.3. Importance of benthic organisms in freshwater ecosystems

Benthic species help to understand how organic matter is processed in freshwater ecosystems. However, in these communities, even though some species are related, food resources can be obtained from different sources. Thus, according to their function in the ecosystem these organisms have different roles (e.g. primary producers, herbivores, detritivores or predators.). In these freshwater ecosystems, some shredded and suspended fragments are transported downstream, so in the feeding process, the species that filter have the tendency to inhabit downstream of the shredders. If shredders species decrease, the food available for the filter species will also decrease and may even lead to an imbalance in the ecosystem (Covich et al., 1999).

Benthic macroinvertebrates (e.g. bivalves or crawfish) contribute to nutrients dynamics in ecosystems. These organisms enhance the activity of microorganisms that have the ability to convert organic detritus (sediments organic matter) into dissolved macronutrients (nitrogen and phosphorus), and organic carbon, and micronutrients (trace elements). These nutrients can be returned to the water column and enable algae growth (phytoplankton) and rooted plants (macrophytes) and consequently increasing primary productivity. Regarding the feeding behaviour, one can find some omnivorous benthic species, which feed on macrophytes, algae, and zooplankton, and many of those omnivorous benthic species are predated by fish. Some macroinvertebrates (shrimps, amphipods, and gastropods) can increase microbial growth through sediment mixing and may influence the release of greenhouse gases (CO_2 and CH_4), toxic gases (H_2S and NH_4) and nitrogen (N_2) (Figure 1.1) (Covich et al., 1999).

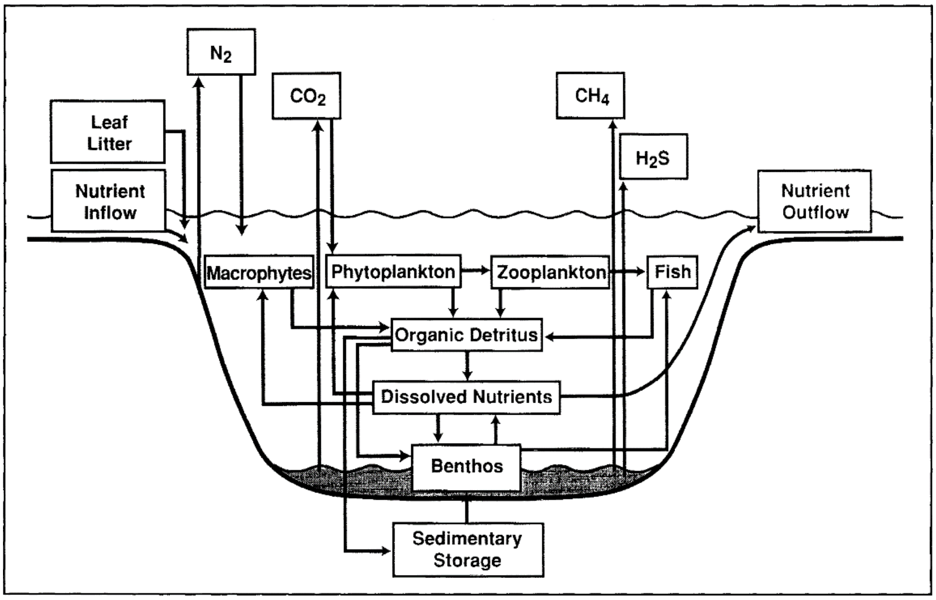

Figure 1.1- The role of benthic organisms in nutrient cycling in freshwater ecosystems (Covich et al., 1999).

Detritivorous organisms have a very important role in the processing of detritus and food webs in freshwater ecosystems. These organisms are shredders, which break down the plant material into smaller particles making them more accessible to other organisms (e.g. insect larvae) and they are still available for predation (e.g. shrimp, crabs, fish, amphibians and birds). Nutrient input to ecosystems can be increased, so nutrients for primary producers would become more available. However, while improving productivity and nutrient flow, this variation might occur because of unusual events often related to natural disturbances (fallen trees, landslides, and hurricanes) (Crowl et al., 2001). Therefore, the structure of the food chain can be altered and biomonitoring of these ecosystems is recommended to have a good environmental management since its main objectives are to identify the impact of contaminants on ecosystems and to evaluate the effectiveness of measures taken to control pollution (Maltby et al., 2002).

1.4. Freshwater amphipods

Gammarids are freshwater members of the class Malacostraca, superorder Peracarida and order Amphipoda (Figure 1.2). About 85% of the Amphipoda order corresponds to the Gammaridea suborder. Gammaridea are mostly marine species but also live in freshwater environments. These organisms are diversified and they are classified as epibenthic, nektobenthic (more common in marine water) and terrestrial (talitrids) (MacNeil et al., 1997).

The females of the amphipods transport the brood through the pereipods (thoracic legs). The juveniles reach maturity after accomplishing several moults (growth occurs through moult). These organisms have a curved and flattened shape, a segmented body (13 segments) and seven pairs of legs (Väinölä et al., 2008; Wade et al., 2004). The gnathopds (third thoracic appendages) are very versatile since they are used for feeding, grooming, burrowing and precopulatory pairing (MacNeil et al., 1997). The three tail appendages are named uropods (located at the end of the abdomen), these appendages help in swimming. Amphipods have an open circulatory system and gills are located in the thoracic segments (Wade et al., 2004). The length of these organisms in freshwater can range from 2-40mm but the most frequent range is 5-15mm (Väinölä et al., 2008).

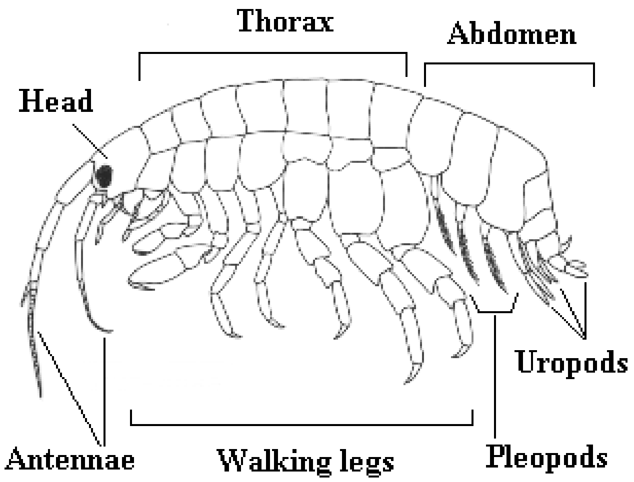

Figure 1.2- Representation of a gammarid, these are divided into three part head, thorax, and abdomen. On the head, there are two pairs of antennas, the eyes and the mouthparts. The amphipod is composed of 13 segments (7 thoracics and 6 abdominals). In the abdominal part, there are 3 pairs of pleopods and 3 pairs of uropods that differentiates them from other organisms. Available at - http://media.museum.vic.gov.au/discoverycentre/websites-mini/crustaceans-of-southern-australia/amphipods-of-southern-australia/amphipod-biology/

The amphipods minimize the effects of dispersion, since they do not have pelagic larval stage and they are benthic recruiters. These benthic organisms are the main constituents of aquatic ecosystems, both in terms of biomass and in species diversity. In addition to their ecological importance and numerical abundance, amphipods are also sensitive to toxic substances and pollutants and play a key role as an environmental bioindicator (Thomas, 1993). These organisms are considered a good bioindicator for the sediment because they are abundant organisms, they have a very relevant role in the benthic communities, they have a large spatial distribution, they are easy to handle and they are tolerant to a great range of environmental variables (Casado-Martinez et al., 2007). Moreover, they are also useful bioindicators of water quality as they can undergo changes in their structure and composition according to environmental changes that may result from both natural and anthropogenic disturbances (Conradi et al., 1997; Guerra-Garcia and Garcia-Gomez, 2001).

In general, the use of amphipods as bioindicators was mainly to evaluate the toxicity of the sediments, they were considered the best choice to perform this type of tests, once the results obtained were positively correlated with the changes of the benthic communities (Costa et al., 1998; Long et al., 2001; Swartz et al., 1994). Yet, other types of tests should be carried out, for instance, the development of models that can be used for risk assessment related to fluctuations in concentrations of chemicals. Floating concentrations and repeated pulses of pollutants are more easily found in organisms that live longer (e.g. *Gammarus pulex*) than in those living in the same environment but have a shorter life cycle (e.g. *Daphnia magna*) (Ashauer et al., 2011, 2010). In addition to these types of tests, bioassays have also been carried out to evaluate the effects of contaminants based on physiological responses (respiration, growth, behaviour, feeding) (Gerhardt et al., 2004; Maltby et al., 1990; Pestana et al., 2007).

Due to increased pressures on freshwater ecosystems, such as saline intrusion and nitrate pollution, it is necessary to assess the tolerance of freshwater amphipods to salinity and the presence of nitrates, as these organisms have a crucial role in these ecosystems.

Freshwater amphipods, due to their natural habitat are exposed to low osmotic pressure, tolerate low salinities but still there should be more studies on the lack of tolerance of high salinity levels by these organisms, as these ecosystems are threatened not only by sea level rise but also by anthropogenic causes (e.g. industrialization, urbanization) (Grzesiuk and Mikulski, 2006). Some examples of species of amphipods that coexist in these environments (0-0.5 and 0.6-1) are shown in table 1.II. Within these species there is one that has great European importance which is *Gammarus pulex*. This specie has been widely used as a test organism, with sensitivity varying according to the type and concentration of chemicals. This organism exists in freshwater, occurring naturally in lotic water bodies and it has been used in toxicity tests (Ashauer et al., 2011, 2010; Galic et al., 2010). *G. pulex* are common in Great Britain, Western Europe, as well as, in Northern Ireland (Dick, 2008).

Table 1.II- Representation of some species of amphipods living in waters with salinities of 0-0.5 and 0.6-1 Adapted from Grabowski et al (2007)

Salinities	
0-0.5	**0.6-1**
• *Gammarus fossarum* • *Gammarus varsoviensis* • *Gammarus leopoliensis* • *Gammarus roeseli*	• *Gammarus lacustris* • *Gammarus pulex* • *Gammarus balcanicus* • *Dikerogammarus haemobaphes* • *Echinogammarus ischnus*

For freshwater amphipods, one of the most important abiotic factors is salinity. However, despite scarce information about freshwater ecosystems, these organisms have different tolerances to salinity because some are more halotolerant than others (Table 1.III). Table 1.III demonstrates that the amphipod more tolerant to salinity is *G. pulex*. Despite having salinity tolerance, this disturbance causes stress to organisms and can cause physiological and behavioural changes (e.g. when salinity increases these organisms have a tendency to decrease respiration and ammonia excretion rates) (Grzesiuk and Mikulski, 2006).

Table 1.III- Tolerance to salinity of some European amphipods inhabiting freshwater ecosystems.

Species	**LC_{50} (gL^{-1})**	**Reference**
Chelicorophium curvispinum	10.8±1.5	(Piscart et al., 2011)
Gammarus fossarum	9.9±1.4	(Piscart et al., 2011)
Gammarus pulex	12.8±1.7	(Piscart et al., 2011)
Gammarus roeseli	12.6	(Piscart et al., 2007)
Gammarus roeseli	8.1±1	(Piscart et al., 2011)

Excessive use of fertilizers with nitrogen composition, the combustion of fossil fuels and urbanization have led to the increase of nitrogen in various forms (e.g. nitrate) available in aquatic ecosystems. Acute and chronic toxicity caused by excess nitrates in the environment can affect several aquatic organisms (e.g. amphibians, fish, insect larvae, crustaceans, etc.). Unfortunately, in aquatic organisms there is still very little information about nitrate toxicity (especially sublethal effects on organisms in the presence of high nitrate concentrations) (Stelzer and Joachim, 2010).

Camargo et al. (2005) evaluated the toxicity of nitrate in three species of amphipods (*Eulinomnogammarus toletanus*; *Echinogammarus echinosetosus* and *Hydroyche exocellata*). The LC_{50} for the three species was 73.1; 56.2 and 230.3 mg NO_3-N L^{-1}, respectively. The maximum concentration of nitrates in drinking water in the United States of America is 10 mg NO_3- N L^{-1} but in Camargo et al. (2005) study this concentration may negatively affect the amphipods, especially when the exposure to nitrates is chronic. For the species *E. echinosetosus* LC_{10} was 8.5 mg NO_3-N L^{-1}, which means this concentration causes 10% of mortality to the organisms. The author concludes that, in order to protect the most sensitive freshwater species, the concentration nitrates should not exceed 2 mg NO_3-N L^{-1}.

To perform this kind of studies in Portugal native species should be used, which is the case of *Echinogammarus meridionalis*. In ecotoxicological tests, the use of native organisms is a better option than standard species. Native species play an ecological role in their aquatic ecosystems which are under study and more reliable and realistic information is acquired during this research (Macedo-Sousa et al., 2007).

1.4.1. *Echinogammarus meridionalis*

Maintaining the structure of freshwater ecosystems can be facilitated by the presence of detritivores in food webs, which recycle nutrients by making them available to producers and can serve as prey for predators. Thus, these organisms allow the connection between different groups in the food network (e.g. producers or consumers) (Quintaneiro et al., 2014).

The amphipod *E. meridionalis* (Figure 1.3) is a benthic freshwater crustacean that occupies an important position in the food chain, since they serve as food for several species of fish. This organism is detritivor that feeds on coarse organic matter particles (COMP, >1 mm). Therefore, it has major importance on processing detritus and on recycling nutrients in the Portuguese rivers. *E. meridionalis* is a fragmentor that comes, generally, from habitats with slowly running waters or sometimes it can be found in waters with some domestic effluent pollution (Pestana et al., 2007; Quintaneiro et al., 2016, 2014).

Figure 1.3- *Echinogammarus meridionalis:* juvenile photographed under a magnification of 6.3 x.

There isn't much information about *E. meridionalis*, as only a few studies have used this organism as a model organism. Further ecotoxicological studies with this species are essential. Even though the studies are scarce, in the literature, some scientific studies can be found which used this model organism to evaluate the rate of ingestion. The ingestion tests are rapid, inexpensive, an efficient tool and they should be used in biomonitoring studies in Portuguese freshwaters (Pestana et al., 2007).

Feeding inhibition can be used as a biological response to the stress caused by the exposure of organisms to contaminants (McWilliam and Baird, 2002). Behavioural, feeding or mobility studies allow to understand the different organisms' responses to anthropogenic stress (Macedo-Sousa et al., 2007).

In a study performed by Pestana et al. (2007), lethal and sub-lethal effects of cadmium and zinc on two crustaceans *Atyaephyra desmarestii* and *E. meridionalis* were evaluated. The acute tests occurred during 96 hours to verify the sensitivity of the two crustaceans to cadmium and zinc metals and to identify the sub-lethal concentration ranges for later use in feeding experiments. For the two metals, concentrations below the LC_{10} were chosen to perform feeding inhibition tests. The results obtained for the two species indicated that as the concentration of the metals increased there were significant reductions in the feeding rate.

Macedo-Sousa et al. (2007) published a work on behavioural and feeding responses in *E. meridionalis* when exposed to the acid drainage of the São Domingos mine. The acid mine drainage (AMD) was collected at a pH of 2.4. The AMD was diluted with the aim of obtaining a realistic chemical approach (populations were subject to a mixture of contaminants). The results obtained evidenced a positive relation between AMD and the toxic effects caused to *E. meridionalis*. Thus, if there had been an increase in AMD concentration, pH would have decreased. There was also an increase in mortality, reduction in locomotion and inhibition of feeding rate.

In Quintaneiro et al. (2014) study, the main objective was the evaluation of the feeding preference of *A. desmarestii* and *E. meridionalis* between contaminated and uncontaminated leaves and different leaf sizes. Two types of tests (no choice and multiple choice) were performed to evaluate the preferred leaf size of the organisms. In the non-choice tests, the organisms were given a single size of leaf, there was no opportunity of choice. On the other hand, in the multiple choice tests, different leaf sizes were used which represented a more realistic river scenery where there is a wide variety of leaf types and sizes. Organisms were offered four different sizes of leaf and one with an irregular form. For each metal (zinc and copper), two tests of dual choice were done to verify the feeding preference of the organisms to determine if they preferred discs of contaminated or uncontaminated leaves. The results of this work did not reveal any significant preference for a specific leaf size for the organisms under study. However, the increase in ingestion rate happened when the leaf disc area was larger. The authors recommended, for future studies, the use of leaves with an area of 1.767 cm^2. Considering that this amphipod is a shredder, the size of the leaf is rather relevant since smaller leaves provide a smaller area to fragment. Regarding the preference for contaminated leaves, the amphipod preferred the ones contaminated with copper (with a concentration of 2.19 $\mu g.L^{-1}$), which may be due to the metabolic needs of this organism. This result highlights the need to include this metal in the diet of these organisms.

More recently, Quintaneiro et al. (2016) evaluated the physiological effects of copper and zinc on *A. desmarestii* and *E. meridionalis*. Hence, acute tests were performed for each species in order to determine the LC_{50} for each metal and sublethal feeding tests. These were performed in four phases. The first phase was acclimatization (the organisms were placed in clean artificial pond water with uncontaminetd food); In the second phase (exposure), the organisms were exposed to several treatments with different concentrations and the food distributed was contaminated with the same concentration as the medium used in each test; In the third phase, depuration occured and in the fourth phase (recovery), the organisms were inserted in clean water and they were given uncontaminated food, once again. The results showed that the most toxic metal for the two species was zinc. This metal showed inconclusive data regarding the feeding rate of the amphipods. However, when exposed to copper, their feeding rate was lower.

1.5. Importance of the study of multiple stressors in freshwater ecosystems

Catastrophes and imbalances in ecosystems have become more evident on the planet and these phenomena may result from anthropogenic activities. Therefore, environmental problems do not occur in isolation and sometimes a combination of problems occur. For example, urbanization affects the amount of runoff, water quality, habitat availability, among other effects. In addition, climate change is complicating the problems highlighted in ecosystems (Ormered et al., 2010).

The increased use of pesticides and fertilizers in agriculture affected the quality of aquatic ecosystems. However, these environmental contaminants are not the only ones that have been causing environmental problems. Currently, climate change is expected to be the main cause of biodiversity loss and consequently causing degradation in these aquatic environments (Davis et al. 2010; Strayer and Dudgeon 2010). In ecosystems, there are physical, chemical and biological stressors, which exist simultaneously in the environment and have been increasing rapidly. Thus, for a more realistic study it is convenient to approach multiple stressors and individual stressors to avoid redundant conclusions (Folt et al., 1999). Example of some drivers and stressors that occur in natural landscapes in urban and agricultural regions are represented in table 1.V. Changes in hydrological processes cause disruption in urban systems and agricultural systems, such as salinization, eutrophication and acidification (Davis et al. 2010).

Table 1.IV- Some drivers and stressors that occur in natural landscapes in urban and agricultural regions (Davis et al. 2010)

Drivers				Stressors		
Land use	**Modification of natural landscape**	**Change in water source**	**Water regime**	**Nutrients (N&P)**	**Salinity**	**pH**
Urban	Deep-rooted vegetation replaced by impermeable surfaces	Change from groundwater (GW) to surface water (SW) dominated	Change from seasonal to permanent	Increase	No change	No change
Urban	Groundwater extraction for domestic water supply	GW inputs decrease	Change from seasonal to waterlogged to dry	Decrease	Slight increase	Reduction where acid sulphate soils present
Urban	Stormwater runoff	SW inputs increase	Change from seasonal to permanent	Increase	No change	No change
Agricultural	Deep-rooted vegetation replaced by shallow-rooted crops	Change from SW to GW dominance	Change from seasonal to permanent, dry to flooded.	No change	Increase	Decrease where drains intercept acidic GW
Agricultural	Fertiliser use + reduced interception of surface water	SW inputs increase	Increased depth	Increase	Slight decrease	No change

Combinations of multiple stressors might have more serious consequences to ecosystems than the presence of a single stressor as they might interact with each other. The interactions between multiple stressors can be classified as antagonistic additive, antagonistic or synergistic. Synergistic interactions are more harmful. Identifying and distinguishing the interactions (synergism/antogonism) in the environment is very relevant because it classifies the effects according to their severity to the ecosystems

(Vinebrooke et al., 2004; Folt et al., 1999).

Due to the importance of freshwater, its management is crucial for the future of living organisms. Management approaches to this natural resource should understand the nature of the effects of multiple stressors on populations, communities and ecosystems. The negative impacts in the ecosystems should be identified and diagnosed as soon as possible to mitigate the effects of the manifested multiple stressors. Furthermore, when management measures are defined, they must also be adaptable to all regions that may suffer from the changes (Davis et al., 2010; Ormered et al., 2010; Strayer and Dudgeon, 2010).

1.6. Rationale and aims

The rationale behind this work was:

- The *Echinogammarus meridionalis* is not well studied, even though it has a very important role in the Portuguese rivers;
- Climate change might disturb the ecosystems such as increasing saline intrusion and nitrate pollution in freshwater. It would be pertinent to realize if this specie, endemic in Portugal, has the capacity to tolerate these changes;

Hence, according to the above, the dissertation work will focus on the evaluation of the tolerance of *E. meridionalis* to salinity, nitrate concentration and the mixture of both, to answer the following research question:

"Does the *Echinogammarus meridionalis* amphipod adapt to salinity and nitrate pollution?"

To address this question the following hipothesys were tested:

1. *E. meridionalis* amphipod is very sensitive to salinity.
2. *E. meridionalis* amphipod is sensitive to nitrate concentration.
3. The presence of salinity decreases the toxicity of nitrates.

1.7. Dissertation organization

The present dissertation was organized in 3 chapters:

- **Chapter 1:** refers to the general introduction, addresses the issue of saline intrusion into freshwater ecosystems, the presence of nitrates in the same ecosystems and the choice of the test organism used.
- **Chapter 2:** “Assessment of the joint effects of salinity and nitrate pollution on the freshwater amphipod *Echinogammarus meridionalis*”. It presents the results obtained in acute toxicity tests performed with various concentrations of salinity and nitrates and the results of the binary mixture toxicity test between these two stressors in *Echinogammarus meridionalis*.
- **Chapter 3:** Presents a general discussion and conclusion of the results obtained in the work. It describes the main conclusions of chapters 1 and 2, comparing the results obtained in this work with some of the data of other authors and reports about future work perspectives.

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CHAPTER 2

Assessment of the joint effects of salinity and nitrate pollution on the freshwater amphipod *Echinogammarus meridionalis*

2. Assessment of the joint effects of salinity and nitrate pollution on the freshwater amphipod *Echinogammarus meridionalis*

(Submitted to Science of the Total Environment)

Abstract

Climate change has been increasing and has caused some nefarious effects to freshwater ecosystems as a result of sea level rise. Furthermore, intensive agricultural practices and animal production led to high concentrations of nitrates in groundwater and soil in various parts of the world. Thus, due to these disturbances and imbalances that have occurred in these ecosystems, it was intended to evaluate the tolerance of *Echinogammarus meridionalis* to salinity, nitrate concentration and the mixture of both. Acute tests were performed during ninety-six hours to determine the median lethal concentration (LC_{50}) values for this specie of amphipod. The LC_{50} of the acute toxicity tests for salinity was 25.38± 0.33 g L^{-1} and in the nitrate assays the LC_{50} resulting from the first collection of organisms was 121.59 ± 11.71 mg NO_3-N L^{-1} and for the second collection, the LC_{50} was 116.99 ± 3.67 NO_3-N L^{-1}. The toxicity tests of the binary mixtures salinity and nitrate showed an antagonistic pattern at low concentrations in the S/A model (synergism / antagonism) and in the DL model (dose level deviation) there was antagonism at low concentrations and synergism at high concentrations. The results obtained in this work show a great tolerance to salinity and nitrate, thus suggesting a good adaptability of this organism to these environmental disturbances.

Keywords: Climate change, saline intrusion, aquatic ecosystems, amphipods, *Echinogammarus meridionalis*, salinity, nitrates, acute toxicity, toxicity of mixtures, biological effects, necrosis.

2.1. Introduction

Freshwater ecosystems play a key role in maintaining biodiversity (Balian et al., 2008). These ecosystems are characterized by having salinities below 0.5 g.L^{-1} (Hammer, 1986). Nowadays, they are exposed to several pressures, both natural and anthropogenic.

Climate change has been disrupting aquatic ecosystems, but in freshwater ecosystems one of the most notorious and worrying effects inherent to these changes is saline intrusion (Robins et al., 2016). Due to saline intrusion, the freshwater environments have become more saline. These salinity changes may decrease species diversity, since the species that coexist in this environment tend to tolerate very little salinity (Flöder and Burns, 2004). Direct and indirect effects might appear in the communities of organisms because of the salinity increase this type of environment. The direct effects are evident when there are physiological or genetic alterations and consequent reduction of species richness. The indirect effects could occur when the structure and function of communities are altered (Campbell, 1994; Nielsen et al., 2003; Savage, 1981).

Furthermore, nitrogen occurs naturally (inorganic forms: NH_4^+, NO_3^-, NO_2^-) and may appear in the environment through anthropogenic sources (e.g. urban agricultural runoff, sewage effluents, industrial waste, (Bouchard et al., 1992; Camargo et al., 2005; Rabalais, 2002). There are two main sources of nitrate pollution: one of them is the intensive agricultural practices that have been observed since the 19th century, with excessive concentrations of nitrates in soils and water, which is inherent of fertilizers, herbicides and pesticides and the other harmful source of nitrate pollution is animal production on an industrial scale. There is great concern about the pollution of ecosystems by nitrates as this is considered to be the most ubiquitous contaminant in water (Bouwman et al., 2013; Robertson et al., 2008). For the next decades, globally, is expected to increase phosphorus and nitrogen nutrients (N and P), so an improvement in agricultural management is needed. If there is adequate use of synthetic fertilizers, there will be greater control of the nutrients flow (N and P) into the environment. In animal production in countries with intensive ruminant production, a change in the human diet is recommended, encouraging the consumption of poultry or pork to reduce the presence of nutrients in ecosystems (Bouwman et al., 2013). Nitrate regulation policy at European level is acquired through Directive 91/676 / EEC (1991),which has created a number of criteria for identifying polluted waters or at risk of being polluted by nitrates of agricultural origin (nitrates higher than 50 mg L^{-1}).

Benthic macroinvertebrates recycle nutrients and control their output in ecosystems. These organisms have the ability to transform organic waste into dissolved macronutrients and micronutrients. Within the benthic macroinvertebrates, shredders are very important to the freshwater ecosystems because they break down plants into smaller particles making them available to other organisms. They also serve as prey and thus connecting different trophic levels (producer or consumer), and facilitating the maintenance of these ecosystems (Covich et al., 1999; Crowl et al., 2001; Quintaneiro et al., 2014).

The benthic macroinvertebrate selected for this study was *Echinogammarus merionalis.* This organism is a freshwater amphipod, endemic to Portugal that comes, generally, from habitats with slowly running waters or may appear sometimes in waters with some pollution from domestic effluents. It feeds on organic matter particles and so it is a fragmentor that has a great importance in recycling the nutrients in the Portuguese rivers (Pestana et al., 2007; Quintaneiro et al., 2016, 2014).

The increase of disturbances in freshwater ecosystems has become increasingly evident, in particular saline intrusion and nitrate pollution, hence it is very pertinent to evaluate the tolerance of *E. meridionalis* to salinity and the presence of nitrate, since this organism has a crucial role in these ecosystems. Thus, the main objective of this study was to evaluate *E. meridionalis* tolerance to salinity, nitrate concentration and the mixture of both and understand the ability of this organism to respond to changes in the parameters in the environment. Therefore, the following hipothesys were tested:

1. E. meridionalis amphipod is very sensitive to salinity.
2. E. meridionalis amphipod is sensitive to nitrate concentration.
3. The presence of salinity decreases the toxicity of nitrates.

2.2. Materials and methods

2.2.1. Sampling and acclimation of organisms

Juveniles and adults of *E. meridionalis* were collected in January, March an May with a hand net, at a reference site of the river Alcaide, Porto de Mós, district of Leiria, Portugal (39°35'29.5"N, 8°48'31.5"W). In the process of acclimation partial water exchanges were made, with the gradual transition of water from the reference point to the artificial pond water (APW) (ASTM, 1980). The acclimation had a duration of one week, the organisms were maintained with constant aeration, a temperature of 20 °C and a photoperiod of 16 / 8h (light / dark). The physical-chemical parameters were controlled (conductivity, pH, temperature and oxygen saturation) (Table 2.I). Individuals were fed *ad libitum* with dried alder leaves (*Alnus aglutinosa*).

Table 2.I- Mean values of the physical chemical parameters controlled and their respective standard deviation of the various acclimatization performed during various assay.

Physical-chemical parameters	**Values**
pH	8.09 ± 0.09
Conductivity (µS/Cm)	736.46 ± 79.64
Oxygen saturation (%)	92.62 ± 1.24
Temperature (ºC)	19.26 ± 0.76

2.2.2. Acute tests

To evaluate the sensitivity and tolerance of *E. meridionalis* amphipods to salinity, nitrate concentration and the mixture, lethality tests were performed. The organisms were exposed to different concentrations for 96h. The amphipods were maintained with aeration and without food, along the assay. Each replicate contained 5 organisms and 100 mL of the solution in test. Every day the physical-chemical parameters (conductivity, salinity, pH, oxygen saturation and temperature) were registered, as well as mortality. After 96 hours, all organisms were photographed, measured, the gender was identified using Stemi 508 Stereo Microscope from Zeiss and the body weight of each organism was registered (KERN ABT 100-5NM).

After the tests, all organisms were photographed and classified according with a necrosis index. Six categories were considered:

0- No dark spots (no necrosis)

1- Few localised spots (head, antennae, pleopods, pereopods, uropods, gnathopods and telson)

2- Several localised dark spots

3- Large dark spots without loss of sensory and prehensile appendages

4- Severe necrosis (large dark spots) with loss of sensory and prehensile appendages

5- Dark body or almost completely dark with or without loss of sensory and prehensile appendages

2.2.2.1. Salinity

Salinity toxicity was evaluated, therefore different salinity concentrations were tested. Control treatment was performed from synthetic medium and 9 treatments, the salinity ranged from 5.1 to 31.5 g L^{-1}. For each treatment and control, 3-4 replicates were set up. The different concentrations of salinity were obtained from a stock solution. This stock solution was made as of a sea salt (Ocean fish), with a grade of purity of almost 100% .Successive dissolutions were prepared to obtain the desired concentration for each treatment and were supplemented with artificial medium (APW).

2.2.2.2. Nitrate

The bioassays were prepared with control and 21 different nominal concentrations of nitrates (mg NO_3 L^{-1}), each treatment existed 3-4 replicates. The concentrations of nitrate varied from 0 to 760 mg of $NaNO_3$ L^{-1}. These solutions were prepared from a stock solution of sodium nitrate ($NaNO_3$) (MERCK). A sample of each treatment was saved for further analysis. The values of each medium tested were read in the FIASstar 5000 analyzer to know the actual NO_3-N L^{-1} values present in each treatment. Each analyzed medium was collected at the beginning of each experiment.

2.2.2.3. Mixtures

Mixtures tests were performed by concentration addition method, so the various concentrations of salinity and nitrates were added together with the purpose of predicting their toxicity (Norwood et al., 2003). The objective was to evaluate the response (antagonism or synergism) which occurred in the addition of the concentration. 16 binary combinations of salinity and nitrate were prepared for the mixtures tests. The values of salinity varied from 11.33 to 22.47 g.L^{-1} and the nitrate concentrations varied from 28.1 to 194.8 NO_3-N L^{-1}.

The toxicity of the mixtures may differ, a high quantity but slightly toxic chemical may have a less harmful effect than another chemical in small amounts but toxic. The dimensionless toxic unit (TU xi) quantifies the relative contribution of the toxicity of the individual chemical (i) in the mixture of (n) chemical products, is calculated by the following formula (Jonker et al. 2005):

$$z_i = \frac{\mathrm{TU}x_i}{\sum_{j=1}^{n} \mathrm{TU}x_j}, \quad \text{where} \quad \mathrm{TU}x_i = \frac{c_i}{\mathrm{EC}x_i}$$

2.2.3. Statistical analysis

The concentration-response relationship for salinity, nitrates and for the binary mixture was studied. The response (mortality) was recorded at various exposure concentrations in order to determine LC_{50}. The data of the binary mixtures were used to identify possible deviations of each model (IA and CA). In excel, it was added the solver to run the ToxCalc spreadsheet. In figure 2.1, outlines the decision process for the best model, which is given automatically by the ToxCalc spreadsheet (Nogueira, in prep.).

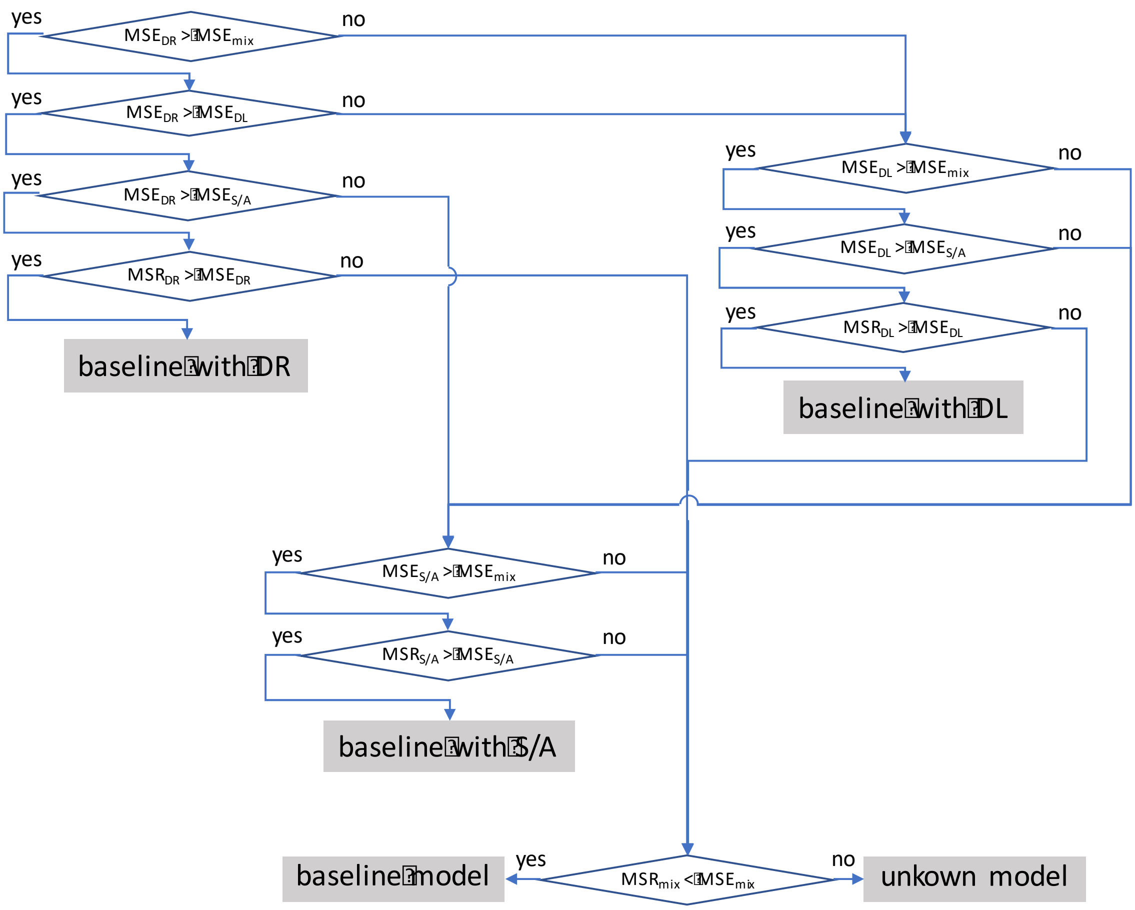

Figure 2.1- Flowchart for the decision process of the best model to be used. CA = Concentration addition; S / A = synergism / antagonism; DR = Dose ratio deviation; DL = Dose Level deviation (Nogueira, in prep).

The Minitab 16 statistical package was used to perform a General Linear Model ANOVA, in order to verify if there were significant differences between the body length and body weight of the organisms exposed to the different concentrations of salinity, nitrate and binary mixtures. Logarithmic transformation was used to mormalize the data and homogenize its variance prior to ANOVA.

2.3. Results

2.3.1. Acute tests

2.3.1.1. Salinity

The physical-chemical parameters of the 9 treatments and controls were registered daily, in the experiences performed. All the amphipods exposed had identical body length and body weight ($p>0.05$; more information in supplementary data Table S I and Table S II). Mortality in the controls in the various assays was zero. There was a higher mortality in the higher salinity of 30.9 g L^{-1} (Table 2.II). **Erro! A origem da referência não foi encontrada.** shows the sensitivity of the organism changes as the salinity increases, and a very abrupt response can be verified when the salinity reaches 22.74 g L^{-1} (LC_5). The lethal concentration (LC_{50}) at the end of the 96 hour test was 25.382 ± 0.333, with 95% confidence intervals. Although the LC_{50} value is high, since the amphipod under study is characteristic of freshwater environment there was an increase in the appearance of necrosis which was observed as the salinity rose. At the salinity of 31.5 g L^{-1}, the organism was completely filled with necrosis (figure 2.3).Using the index of necrosis defined and with the photographs taken in the salinity experiences, one graph was obtained (figure 2.4) and it is evident that as the salinity increases the number of organisms classified at the lower levels of the index decreases while the number of organisms at the higher levels increases. In figure 2.3, for the last concentration (salinity 30.10 g L^{-1}), all the organisms had a dark body which means they were full of necrosis.

Table 2.II- Survival rate of the amphipods exposed to the 9 treatments and in the controls, in the experiences performed. Mean values and respective standard deviation of each treatment (g L^{-1}) tested are represented.

Salinity (g L^{-1}) nominal	**Salinity (g L^{-1}) measured**	% Survival
Control	0.39±0.07	100
5.5	4.78±0.04	100
8.7	8.12±0.15	100
13.7	12.71±0.15	93
21.6	20.41±0.20	73
24.2	23.68±0.08	85
25	24.75±0.03	55
25.8	25.40±0.06	45
27	26.95±0.04	5
34	30.10±0.00	0

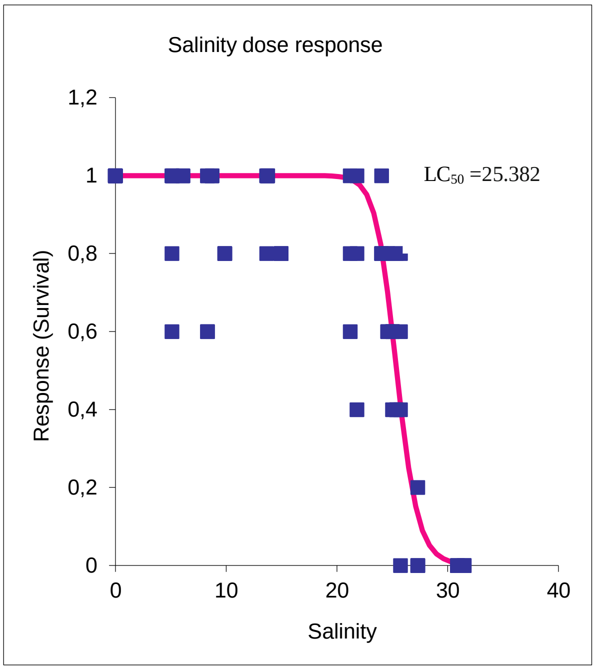

Figure 2.2- Dose-response curve according to the different values of salinities and respective LC_{50} in $g.L^{-1}$

Figure 2.3- Images of *Echinogammarus meridionalis* that were subjected to different salinities, control, 5.17, 8.73, 13.67, 21.8 and 31.5 g L^{-1}, respectively (photographs were taken under a magnification of a 6.3x). As the salinity rose, the increase in the appearance of necrosis was also evident.

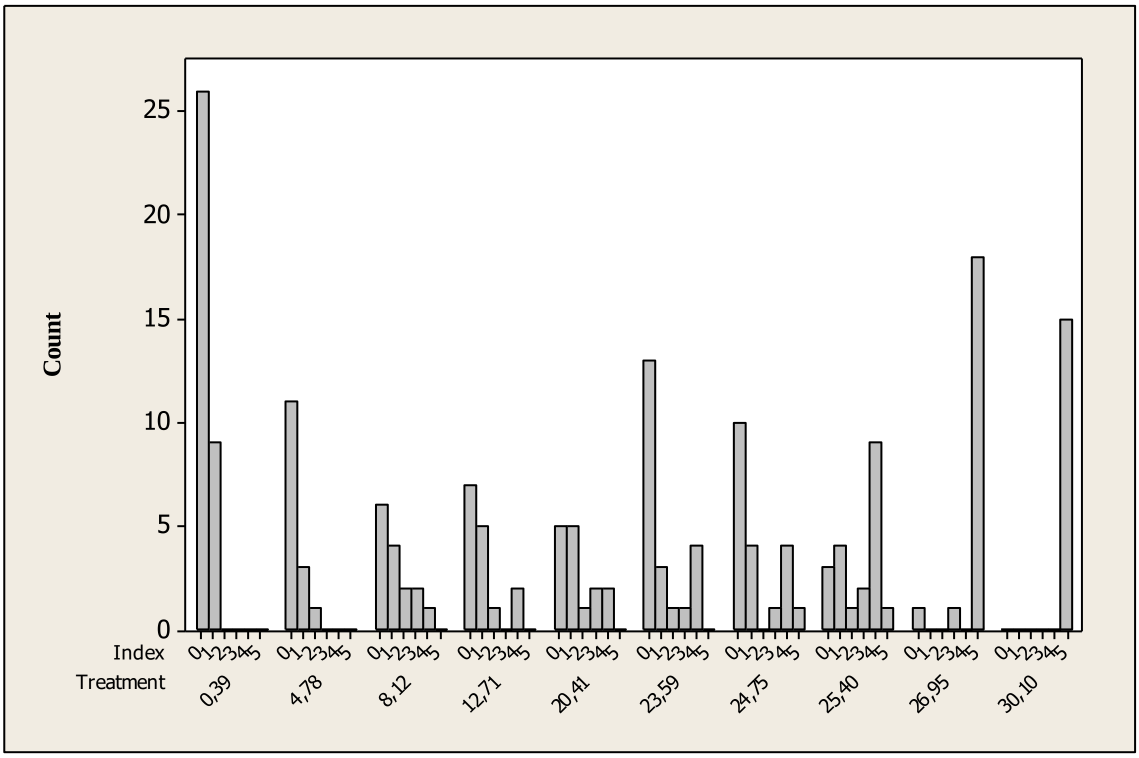

Figure 2.4- Classification of the organisms *Echinogammarus meridionalis* tested, in each treatment (0.39 to 30. 10 g.L^{-1}) according with the necrosis index in the experiences of salinity.

2.3.1.2. Nitrate

In the 3 experiences performed all the amphipods exposed had identical body length and body weight ($p>0.05$), however in the preliminary test, the organisms had significant differences to body length ($p<0.05$). This was due to the presence of bigger amphipods in the treatment 56.74 mg NO_3-N L^{-1} (275 mg $NaNO_3$ L^{-1}) than in order treatments, more information in supplementary data (Table S III and Table S IV). The nitrate results had to be divided in two since the parameters of the reference site were different as it could modify the sensitivity of the amphipods. The nitrate value registered in the first field collection was 0.941 mg NO_3-N L^{-1} and in the second collection was 2.035 mg NO_3-N L^{-1}. No mortality was verified in the control samples. The mortality of the organisms exposed to the 21 treatments performed are shown in table 2.III. The LC_{50} for the assays resulting from the first collection was 121.59 ± 11.71 and for the second collection of organisms, the LC_{50} was 116.99 ± 3.67, with 95% confidence intervals. The organisms that were collected at the first sampling, when subjected to acute tests had a slight increase in LC_{50} than those collected at the second sampling (Figure 2.5). It is possible to observe in figure 2.5 that the organisms collected in the first sampling are more sensitive to low concentrations than those collected in the second sampling (they are more tolerant to low concentrations). Analyzing the photographs taken in the three nitrates experiences, it was possible to draw 2 graphs (figures 2.6 and 2.7). In the three figures, it is evident that the level of necrosis present in the organisms is low, compared to the levels obtained in the salinity tests. The great majority of the amphipods that were present in the three nitrate experiences were classified as 0 or 1 (0- no dark spots and 1- few localized spots). Of the three assays performed, no organism was classified with the last level of the necrosis index (5- dark body or almost completely dark)

Table 2.III- Survival rate of *Echinogammarus meridionalis* in the 21 treatments tested. 14 treatments were performed in the first collection and 7 were performed in the second collection. Values are represented for each treatment in mg $NO_3 – N\ L^{-1}$.

Field collection	**Nominal concentration (mg $NaNO_3\ L^{-1}$)**	**Measured concentration (mg NO_3-N L^{-1})**	**% Survival**
1	Control	0.37	100
	2.75	1.12	100
	2.75	1.30	100
	7	2.16	100
	17	2.99	100
	45	8.89	100
	110	17.66	100
	175	45.08	100
	210	51.85	100
	275	56.74	73
	470	98.23	53
	470	116.90	80
	700	170.27	0
	759	191.55	0
2	Control	0	100
	100	17.51	100
	150	36.24	90
	225	52.94	80
	309	77.15	90
	509	115.50	50
	760	167.30	0

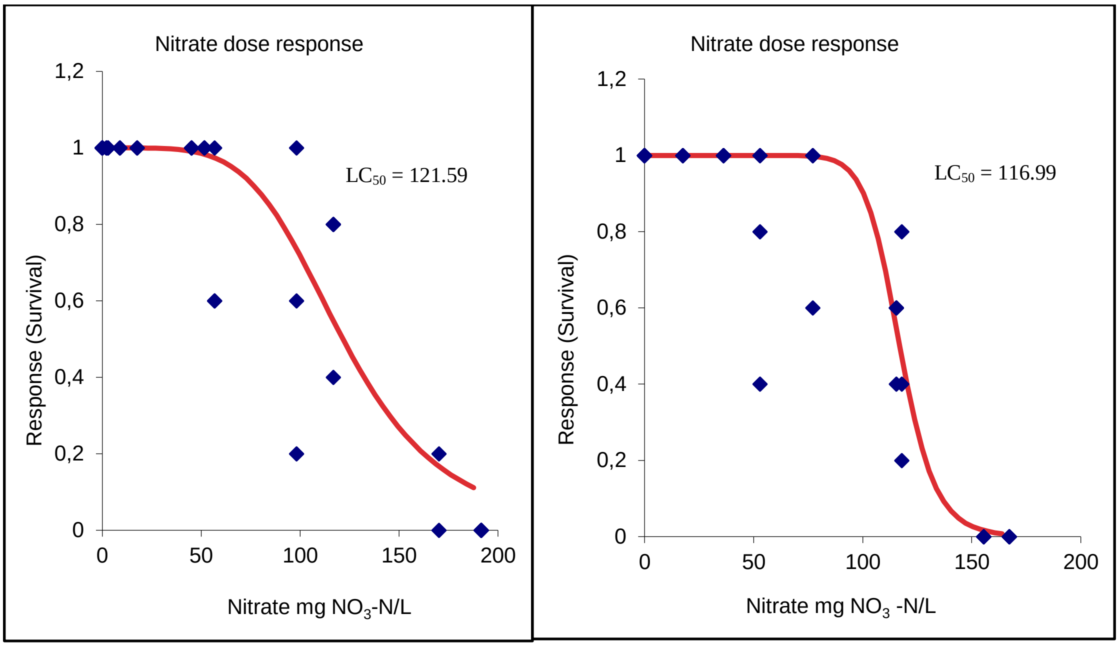

Figure 2.5- Dose response curves inherent to the exposure of different concentrations of nitrates in *Echinogammarus meridionalis*, the organisms were collected in the first and second sampling, respectively. The LC$_{50}$ for the organisms resulting from the first sampling was 121.59 mg NO$_3$-N L^{-1} and for the second LC$_{50}$ was 116.99 mg NO$_3$-N L^{-1}.

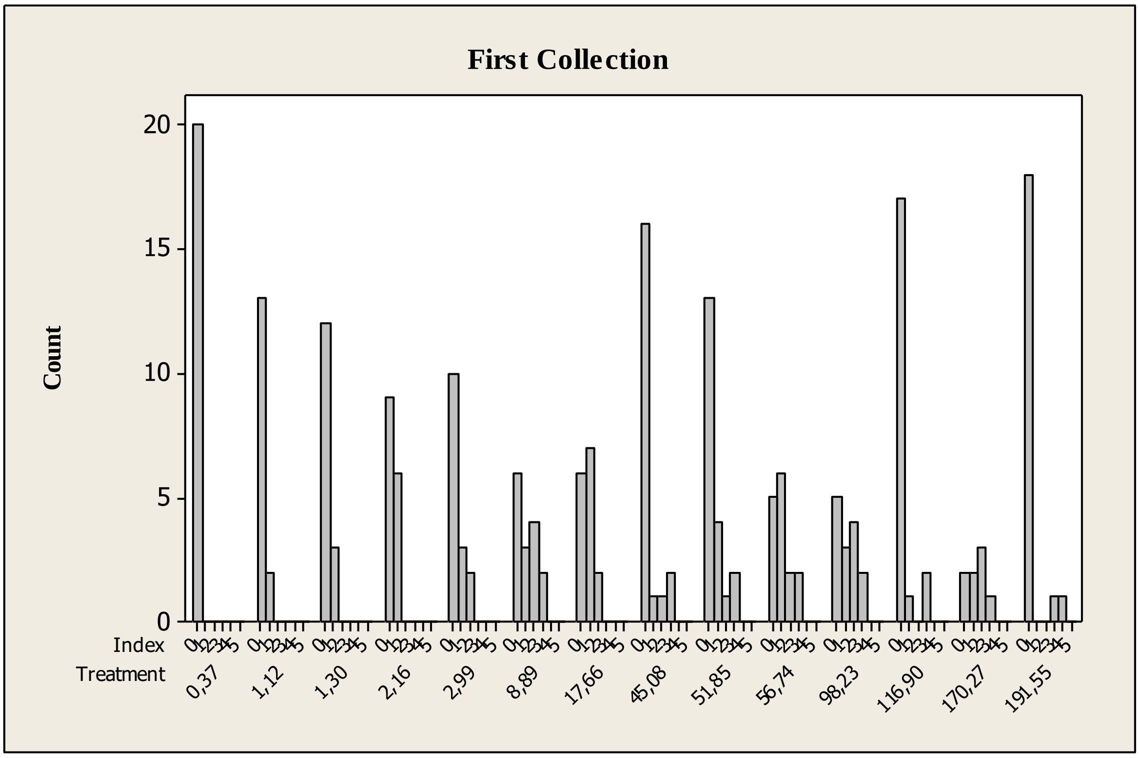

Figure 2.6- Classification of the organisms *Echinogammarus meridionalis* tested, in each treatment (0.37 to 191.55 mg NO$_3$-N L^{-1}) according to with the necrosis index in the experiences of nitrates. The organisms were collected in the first sampling.

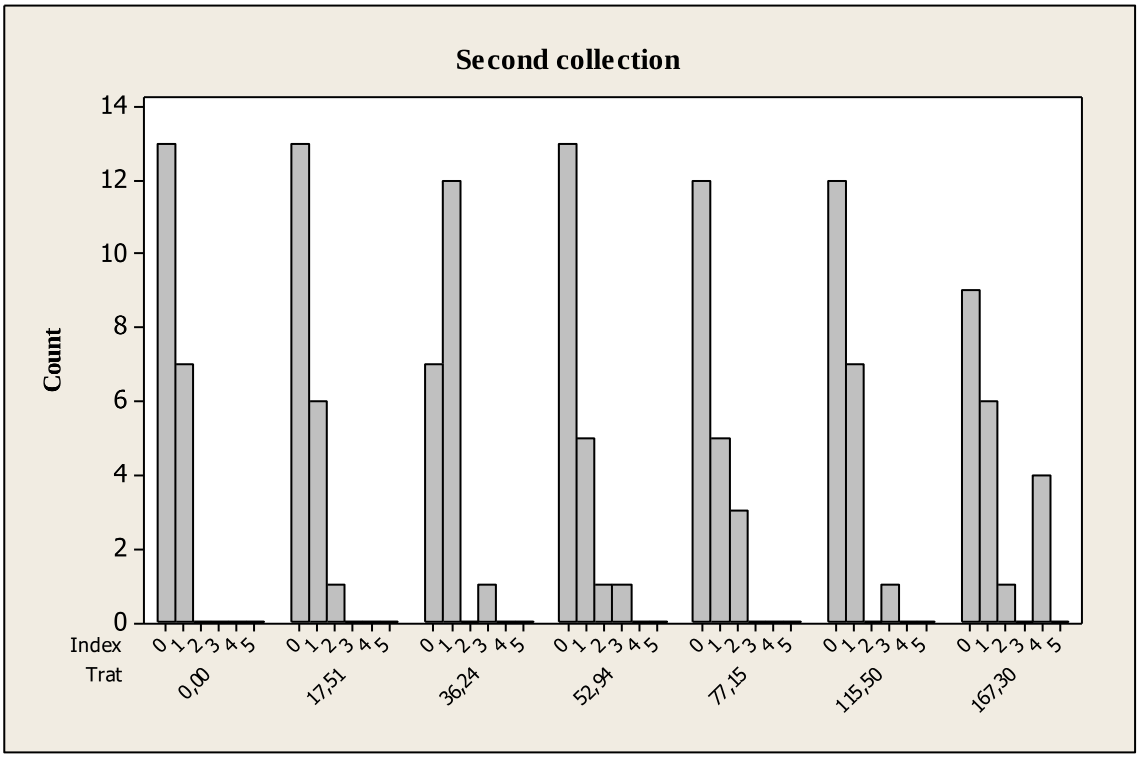

Figure 2.7- Classification of the organisms *Echinogammarus meridionalis* tested, in each treatment (0 to 167.30 mg NO_3-N L^{-1}) according with the necrosis index in the experience 3 of nitrates. The organisms were collected in the second sampling.

2.3.1.3. Mixtures

In the 2 experiences performed, the amphipods exposed in the first experience had the similar body length and body weight ($p > 0.05$), however, in the second experience, the organisms presented significant differences in body length and body weight ($p < 0.05$) (Table S V available in supplementary data). The body length in the 900 mg NO_3 L^{-1} treatment was different from the nominal salinity treatments 8.5, 15 and 25 g L^{-1} and for the two binary mixtures (5 g L^{-1} and 650 mg NO_3 L^{-1} and 15 g L^{-1} and 650 mg NO_3 L^{-1}). In the five treatments mentioned above, the organisms have identical body length. The body weight in the 900 mg NO_3 L^{-1} treatment was different from the nominal salinity treatments 8.5 and 25 g L^{-1} and also in one binary mixture (5 g L^{-1} and 650 mg NO_3 L^{-1}). In the three treatments aforementioned the amphipods have identical body weight (more information available in supplementary data in table S VI, figure S 1 and figure S 2).The survival rates of *E. meridionalis* at the end of the experiments are represented in table 2.IV. Table 2.IV shows that in mixtures the mortality was higher when the salinity levels were higher combined with high nitrate concentrations, for example, in the treatment of salinity 22.47 g L^{-1} and with nitrate concentration of 175.1 mg NO_3-N L^{-1} the survival rate was 40%. Mixtures toxicity test evidenced an antagonistic pattern at low concentrations in the model S/A (synergism / antagonism) and in the model DL (Dose Level deviation) there was antagonism at low concentrations and synergism at high concentrations (Figure 2.8). This can be observed in table 2.V, once *a* was greater than zero (*a* S/A =32; *a* DR=21.86 and *a* DL=1.99) and the bDL was lower than zero (bDL=-8.33). In Figures 2.9 and 2.10, it is evident that the level of necrosis in organisms decreased with the addition of nitrates to the mixture. Nitrates reduced the appearance of necrosis as in treatments where only concentrations of salinities were tested, a higher incidence of necrosis was observed when compared to the tested mixtures. No individual was classified as having level 5 in the necrosis index in all mixtures treatments tested and only one organism was classified with that level in a salinity treatment (22.43 g L^{-1}) as shown in figure 2.10.

Table 2.IV- The survival of *Echinogammarus meridionalis* resulting from the 25 treatments tested, in the 2 experiences. Mean values and respective standard deviation for each treatment (g L^{-1}) and (mg NO_3-N L^{-1}), body length (mm) and body weight (mg) are represented. All organisms were collected in the second sampling.

Nominal Concentration	**Treatment (g L^{-1})**	**Nominal concentration (mg NO_3 L^{-1})**	**Treatment (mg NO_3-N L^{-1})**	**% Survival**
Control	0.36 ±0.06	Control	0.22	100
S=11.5	11.33±0.12	235	52.49	100
S=11.5	11.63±0.14	250	28.1	80
S=11.5	11.29±0.06	340	50.55	100
S=14	13.90±0.05	235	31.48	87
S=14	13.73±0.03	250	123.1	74
S=17	16.56±0.07	235	60.3	74
S=17	16.53±0.21	340	90.5	87
S=20	19.36±0.16	450	104.3	60
S=0	0.99±0.18	450	118	47
S=25	20.43±0.21	0	0	80
Control	0.36±0.06	Control	0.082	100
S=0	1.1±0	900	155.6	0
S=5	5.1±0.11	400	86.95	95

Nominal Concentration	**Treatment (g L^{-1})**	**Nominal concentration (mg NO_3 L^{-1})**	**Treatment (mg NO_3-N L^{-1})**	**% Survival**
S=5	5.01±0.06	450	106.95	85
S=5	5.11±0.05	650	149.6	40
S=8.5	8.09±0.01	400	82.90	100
S=8.5	8.10±0.05	450	102.25	85
S=15	13.77±0.08	400	106.95	95
S=15	14.05±0.25	650	194.8	45
S=25	22.47±0.6	900	175.1	40
S=5	4.89±0.07	0	0	85
S=8.5	8.11±0.15	0	0	85
S=15	13.57±0.28	0	0	80
S=25	22.98±0.47	0	0	70

Table 2.V- Summary of the analysis on the effects of salinities and nitrates binary mixtures in *Echinogammarus meridionalis*. CA = Concentration addition; S/A= synergism/antagonism; DR= Dose ratio deviation; DL= Dose Level deviation; a, b DR1, b DR2 and b DL are parameters in the deviation function, which are then used to the biological understanding of the mixtures; r^2= coefficient of determination; AIC= Akaike information criterion

Parameter	**CA model fit**	**Deviation from CA model**		
	SS	S/A	DR	DL
A		32	21.86	1.99
b DR1			8.06	
b DR2			13.79	
b DL				-8.33
AIC		-161.44	-156.44	-194.55
r^2	0.791	0.255	0.256	0.606
p	1.2 $*10^{-41}$	0.009	0.037	1,60$*10^{-08}$

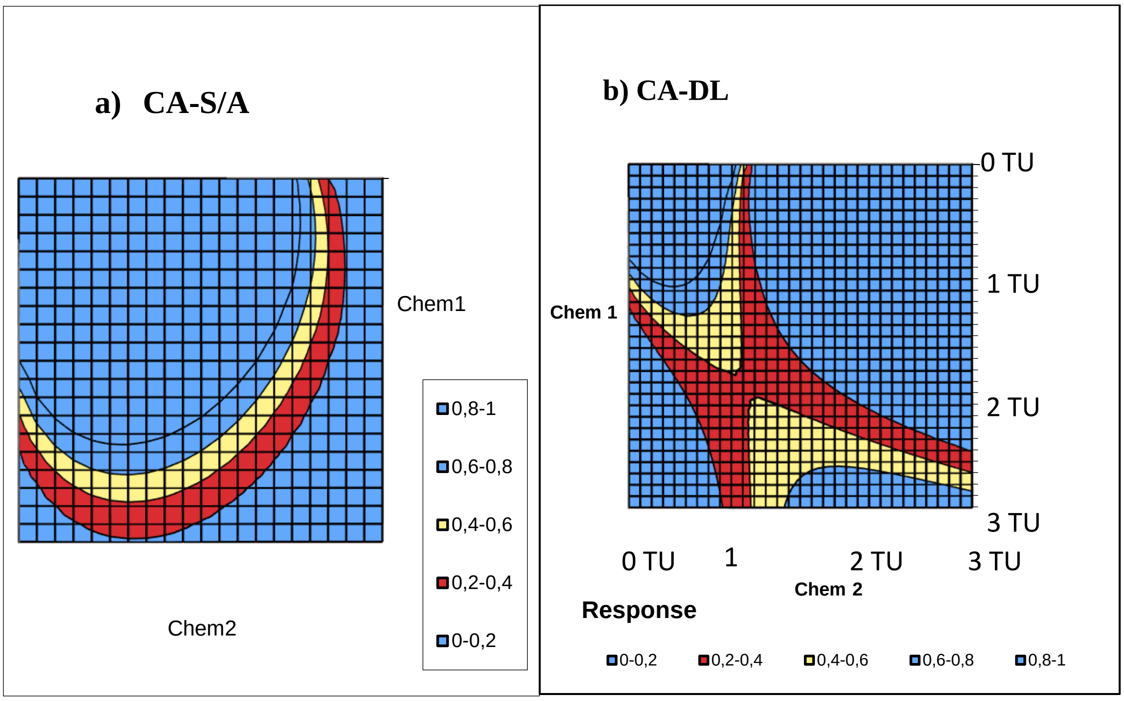

Figure 2.8- a) An antagonistic pattern results from the tests of toxicity in mixtures, in lower concentrations. CA = Concentration addition; S/A= synergism/antagonism; Chem 1= Nitrates (mg NO_3-N L^{-1}) and Chem 2= Salinity (g L^{-1}); b) A pattern of synergisms for high concentrations and antagonism for low concentrations were obtained in mixtures essays. CA = Concentration addition; DL= Dose Level deviation; Chem 1= Nitrates (mg NO_3-N L^{-1}) and Chem 2= Salinity (g L^{-1})

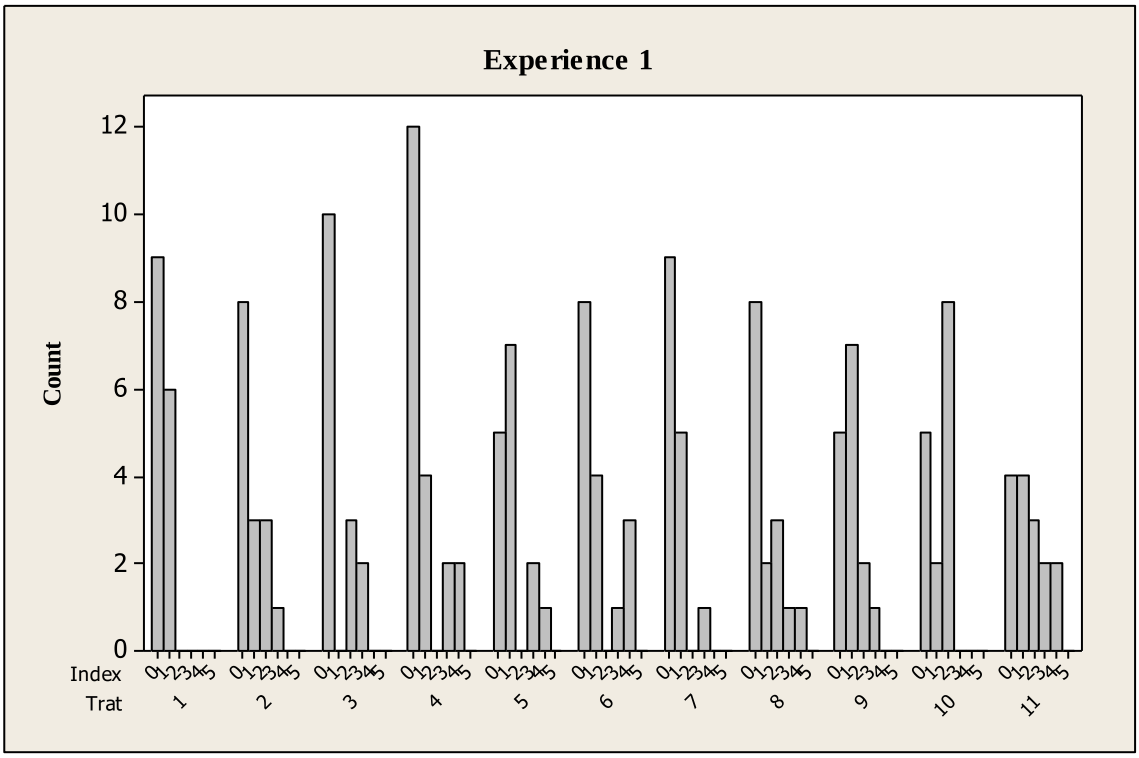

Figure 2.9- Classification of the *Echinogammarus meridionalis* organisms tested in each mixing treatment (1 to 11) according to the necrosis index in the experience 1. The organisms were collected in the second sampling. The treatments correspond to the mixture of various concentrations of salinity (g L^{-1}) and nitrates (mg NO_3-N L^{-1}).**1** S = 0.36 and 0.22; **2** S=11.33 and 52.49; **3** S=11.63 and 28.1; **4** S=11.29 and 50.55; **5** S=13.90 and 31.48; **6** S=13.73 and 123.10 ; **7** S=16.56 and 50.30; **8** S=16.53 and 90.5; **9** S=19.36 and 104.3; **10** S=0.99 and 118; **11** S=20.43

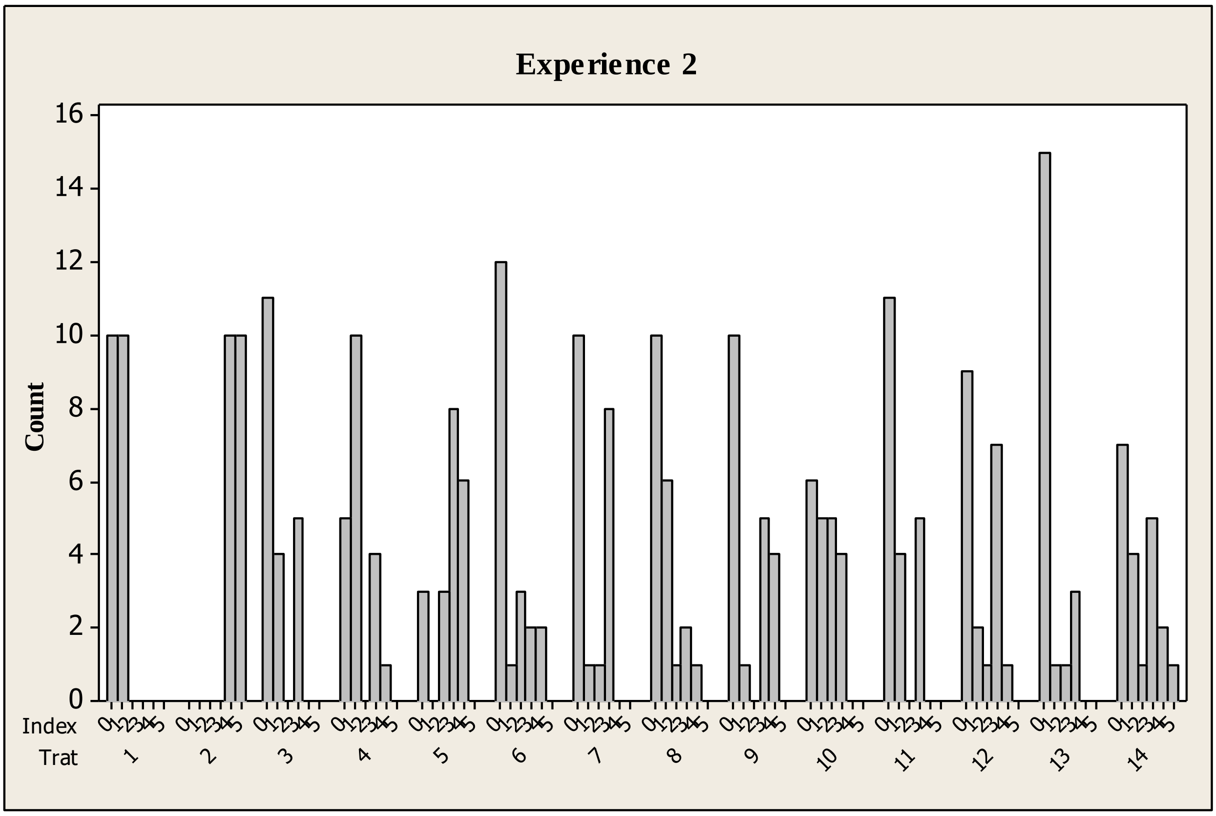

Figure 2.10- Classification of the *Echinogammarus meridionalis* organisms tested in each mixing treatment (1 to 14) according to the necrosis index in the experience 2. The organisms were collected in the second sampling. The treatments correspond to the mixture of various concentrations of salinity (g L^{-1}) and nitrates (mg NO_3-N L^{-1}).**1** S = 0.36 and 0.082; **2** S=1.1 and 155.6; **3** S=5.10 and 86.95; **4** S=5.01 and 106.95; **5** S=5.11 and 149.6; **6** S=8.09 and 82.9 ; **7** S=8.10 and 102.25; **8** S=13.71 and 106.95; **9** S=14.05 and 194.80; **10** S=22.47 and 175.1; **11** S=4.89; **12** S=8.11;**13** S=13.57;**14** S=22.98.

2.4. Discussion

2.4.1. Salinity

There are different projections about what will happen until 2100 as a result of climate change. The sea surface temperature is expected to increase by 1.5-4 ° C, but this depends on carbon emissions, hence international agreements on emissions are extremely important (Lowe et al., 2009; Robins et al., 2016). The rising temperature in the atmosphere can affect the hydrological cycle. This cycle might be intensified, increasing rainfall and river flow in winter (more than 25%) and decrease in summer (40-80%) (Hannaford, 2015; Robins et al., 2016). Several authors claim that the sea level will increase from 0.44-0.74m (Lewis et al., 2011; Lowe et al., 2009; Robins et al., 2016; Woth et al., 2006), however some argue that the sea level rise will be even greater reaching over 1.9m as a consequence of melting ice caps (Jevrejeva et al., 2014; Robins et al., 2016). The sea level rise associated with the climate change process is among the most concerning effects, as it is causing increased saltwater intrusion into freshwater ecosystems (Robins et al., 2016; Werner and Simmons, 2009). Therefore, it is rather pertinent to evaluate the tolerance to salinity in species described for freshwater environments.

Identifying the tolerance of freshwater species is very important, as salinity levels have been increasing. The ability of the osmotic concentration regulation indicates the tolerance of the species to the salinity. Normally, freshwater species are poorly tolerant to increased salinity, since the body's cells may exhibit excess ions and lack of water. Freshwater invertebrates are hyperosmotic regulators. These excrete dilute urine, while marine water organisms usually have more body fluids and are hypo-osmotic (urine is concentrated and in small amounts) (Hart et al., 1991)

In this study, the tolerance and sensitivity of the *E. meridionalis* amphipod was determined. At the end of the 96 hour toxicity tests LC_{50} was 25.38 ± 0.33 g L^{-1}, with 95% confidence intervals (table 2.VI.). This value was quite high for a species that was described for freshwater environments. Although the information is scarce relative to the salinity tolerance for freshwater amphipods. Piscart et al. (2011) tested the tolerance to salinity for several groups of animals among them amphipods. The LC_{50} (in g L^{-1}) results for freshwater amphipod species were: *Chelicorophium curvispinum* 10.8 ± 1.5; *Gammarus fossarum* 9.9 ± 1.4; *Gammarus pulex* 12.8 ± 1.7 and *Gammarus roeseli* 8.1 ± 1. Despite the results presented in the literature, the results obtained for the LC_{50} of the *E. meriodinalis* were much higher, this suggests that this endemic amphipod of Portugal is more resistant than expected.

A possible justification for the tolerance of this amphipods to salinity may be the evolutionary history of this species. Many invertebrates that coexist in freshwater ecosystems have a marine ancestry. Thus, a part of their evolutionary history has been passed in the marine environment, which means that this species has colonized freshwater ecosystems more recently are less permeable to salts and water and require less energy to maintain ionic balance than the species established in these environments the longest (Hart et al., 1991).

Table 2.VI- The 96-h lethal concentration (LC_5; LC_{10};LC_{20} ;LC_{50};LC_{80} and LC_{90}) values with 95% confidence intervals for *Echinogammarus meridionalis* exposed to NaCl (gL^{-1}).

	NaCl ($g L^{-1}$)
LC_5	22.75±7.30
LC_{10}	23.35±3.15
LC_{20}	24.08±1.10
LC_{50}	**25.38±0.34**
LC_{80}	26.75±1.41
LC_{90}	27.59±4.71

The organism under study showed a great tolerance to salinity which is very important since it indicates good adaptive capacities to saline intrusion resulting from sea-level rise. Though, despite the survival of the organisms, it was possible to verify loss of appendices (e.g. antennas, telson, gnatopods, pleopods), necrosis in several areas of pleon, pereon and cephalon. Due to the appearance of necrosis, there was a need to classify the organisms according to a necrosis index. Using the necrosis index, it was possible to prove that the increase in salinity caused a raise in the number of organisms classified at the highest levels (in figure 2.3, for the last salinity concentration 30.10 g L^{-1}, all the organisms were classified with the highest level of the index).

The two processes of cell death, cell necrosis and cellular apoptosis (programmed cell death) have already been observed for several multicellular organisms, such as invertebrates (Bergmann et al., 1999; Hengartner, 1996; Wagner et al., 1998). Necrosis is a form of cell injury that results in premature cell death. This arises due to a bioenergetic disorder resulting from a reduction of ATP, usually to a level where there is no possibility of cell survival. External factors to the cell cause necrosis (pathogenic activity; acute changes in environmental conditions and physical damage). Morphologically, necrosis is characterized by the vacuolation of the cytoplasm, degradation of the plasma membrane and inflammation of the surrounding tissues. This inflammation in the surrounding tissues, when left untreated, accumulates damage and increases the accumulation of decomposing dead tissue (Dunn et al., 2002; Edinger and Thompson, 2004; Proskuryakov et al., 2003).

According with the necrosis index (figure 2.3), one organism was classified as level 4 and two organisms with level 3 (which measured 6.45, 8.96 and 6.95 mm and weighed 5.06, 6.85, 5.58 mg, respectively) were subjected to the salinity of 8.12 g L^{-1} (that is below LC_5) and looking at table 2.II it is possible to verify that all the organisms survived, however some showed really noticeable necrosis. The loss of sensory appendages (antennas) evident in these 3 organisms is very relevant since they have several important functions. The antennas have antennal glands that are involved in the absorption and regulation of ions (Na^+ and K^+) and also they are excretory organs (Lignot et al., 2000). Several spots appeared in the region of the thorax, which is where the gills of the amphipods are located (Wade et al., 2004). The gills in crustaceans are of great importance as they act as the main organ of osmotic and ionic regulation and also aid in respiration and excretion (Lignot et al., 2000). In the thorax, some minor leg injuries were also observed. The third pairs of legs, the gnatopods, are the most versatile and they are related to feeding, grooming, burrowing and precopulatory pairing (MacNeil et al., 1997). In the abdomen, more specifically in the uropods and pleopods, necrosis were also observed, these appendages have their importance to emphasize (these organs are help swimming) (Wade et al., 2004).

Regardless of the tolerance of *E. meridionalis* to salinity, this organism must be studied more thoroughly. Above all, in the context of feeding trials with sublethal salinities, since necrosis were present in these salinities and it would be interesting to see if in the long run the feed rate would be modified. This type of tests will allow to determine in the amphipods the feeding rate and verify if the mouthparts will be affected in case necrosis appear. Ingestion trials are a fast, inexpensive and effective tool and should be used in studies of Portuguese freshwater biomonitoring (Pestana et al., 2007).

2.4.2. Nitrate

Water pollution resulting from overuse of fertilizers and pesticides in agriculture is a serious problem at international level. These substances have a negative impact on water quality, increasing the concentration of nutrients in the water, such as nitrates and phosphorus, diminishing the ability to sustain plant and animal life and the affected areas becomer less atractive (Cooper, 1993; Geiger et al., 2010; Wilson and Tisdell, 2001). Thus, due to the importance of amphipods in food networks in freshwater ecosystems, it is important to determine the tolerance of *E. meriodinalis* to nitrates.

The two sampling events were months apart but the organisms were taken from the same site and that led to different nitrate concentrations. In the first collection, the reference value was 0.94 mg NO_3-N L^{-1} and in the second collection it was 2.04 mg NO_3-N L^{-1}. Hence, the organisms had to be separated according to the different sampling events and treated separately since they could have different sensitivities. In the first sampling, the LC_{50} was 121.59 mg NO_3-N L^{-1} ± 11.71 and for the second collection the value was 116.99 mg NO_3-N L^{-1} ± 3.67, with 95% confidence intervals. The sensitivity of the organisms is slightly different, although the LC_{50}'s are similar. The organisms collected on the first sampling showed a lower nitrate tolerance for low concentrations than the those collected in the second sampling (Figure 2.5). This suggests that organisms from the second sampling were acclimated to tolerate some nitrate concentration, therefore, they are less sensitive to lower concentrations as mentioned above.

Despite the toxicity tests performed in the laboratory, it is always recommended to monitor physical-chemical variables because the assessment of environmental impacts on aquatic ecosystems could be more precise (e.g. immediate identification of changes in the physical and chemical properties of water) (U.S.EPA, 1998). Thus, through the analysis of water collected from the reference site it was possible to quantify the background levels of nitrates present. The difference of 1.09 mg NO_3-N L^{-1} detected between consecutive sampling periods of field organisms suggests an episodic release of nitrates into the river, supported by declarations of a local inhabitant. This difference in nitrates level, and the concomitant exposure to nitrates in the field, has altered the sensitivity of amphipods at low concentrations. As such it is critical to quantify the background level of nitrates, to which organisms were previously exposed, before conducting experiments. Camargo et al. (2005) suggested that in order to protect the most sensitive freshwater species, the value of 2 mg of NO_3-N L^{-1} for freshwater environments must not be exceeded, however, the value recorded in the second sampling was already slightly above that recommended by these authors.

Carmago et al. (2005) determined the LC_{50} for 3 species of amphipods, *Eulinomnogammarus toletanus*, *Echinogammarus echinosetosus* and *Hydroyche exocellata* which values of LC_{50} were 73.10; 56.20 and 230.30 mg NO_3-N L^{-1}, respectively. Within these 3 species of amphipods, the amphipod *E. echinosetosus* is the closest phylogenetically to *E. meriodionalis*. *E. echinosetosus* is the endemic amphipod of Spain (Martinez et al., 1996). Comparing the LC_{50} obtained for *E. meridionalis*, these values more than doubled the LC_{50} of *E. echinosetosus* (56.20 mg NO_3-N L^{-1}), thus showing that very similar species can have different tolerance to nitrates (the Portuguese species shows a greater tolerance to nitrates).

Regarding the appearance of necrosis resulting from exposure to nitrates, it was observed that necrosis appeared in the organisms at the lower levels of the index when compared to the levels obtained from organisms exposed to salinity. Revealing, therefore, that the exposure of amphipods to nitrates causes fewer necrosis than exposure to salinity. A study by Stelzer and Joachim (2010) also observed the non-effect of high concentrations of nitrates on mortality, molt, ingestion rate or C:N ratio in *Gammarus pseudolimnaeus*. A possible explanation for these events is that the amphipods as increasing the concentration of nitrates in the water they did not increase the nitrate absorption.

The information on nitrate tolerance in amphipods is very scarce, with only few studies in this area. Even so, the results obtained in this work are identical with the ones Stelzer and Joachim (2010). Camargo et al. (2005) determined that amphipods were very sensitive to nitrates (LC_{50} = 56.2 mg NO_3-N L^{-1}). Concentrations of 56.74, 51.85 and 52.94 mg NO_3-N L^{-1}, near to the LC_{50} obtained by Camargo et al. (2005) resulted in survival rate of 73.33, 80 and 100%, respectively (Table 2.II). Our results are in agreement with studies that did not find such notorious effects for high concentrations of nitrates in aquatic invertebrates (Alonso and Camargo, 2003; Corrao et al., 2006; Stelzer and Joachim, 2010).

There is a need for future experiments on nitrate toxicity in aquatic invertebrates, since more knowledge on this subjetc must be acquired in order to implement sustainable measures and decisions. Measures to control water pollution will depend heavily on strategies defined at the political level. Therefore, each country must have a good environmental management to control pollution, promote good environmental practices and minimize the impact of polluting substances in already affected areas removing those pollutants.

2.4.3. **Mixtures**

Most of the nitrate concentration used in the toxicity tests are high and can not be treated as environmentally relevant concentrations. The toxicity of nitrates is generally more noticeable in freshwater invertebrates (e.g. shrimp larvae are sensitive to the presence of nitrates) (Muir et al.1991). Several authors have estimated that increased salinity would decreased nitrates toxicity (Camargo et al. 2005; Tsai and Chen 2002). A study by Isnansetyo et al. 2014 documented that the nitrification rate increased for salinities between 13 and 20 g L^{-1} reaching the optimum at 19 g L^{-1}, and the nitrification rate decreases abruptly in higher salinities. Hence the interest in combining these two conditions (nitrates and salinity) in the form of binary mixtures.

In aquatic ecosystems, the risk assessment of toxic substances is usually carried out for single chemicals. However, in a more realistic scenario, the contaminants are mixed and therefore the organisms that coexist in these environments are exposed to several mixtures, the effect of the isolated contaminants is quite pertinent because without this information it would not be possible to determine the toxic effects caused by mixtures of contaminants. Without this information, it was not possible to verify if the effects of the mixtures were additive, synergistic or antagonistic (Barata et al. 2006).

It is important to understand how nitrate toxicity increases in freshwater organisms when compared to marine organisms. Excessive levels of nitrate can significantly affect the abundance and physiological condition of crustaceans. But for this, we have to focus on the nitrogen cycle. The dissolved inorganic nitrogen ions, in aquatic ecosystems, are usually present as ammonium, nitrate and nitrite. Aerobic nitrification is a two-step process, converting ammonium into nitrate (Isnansetyo et al. 2014; Romano and Zeng 2013):

- In the first step: most of the ammonium is not absorbed by the plants, being oxidized to nitrite by *Nitrosomonas* bacteria.

  ($NH_4^+ + CO_2 + 1.5O_2$ + Nitrosomonas $\longrightarrow$ $NO_2^- + H_20 + H^+$)

- In the second step: the nitrite formed by the nitrous bacteria is released and oxidized by *Nitrobacter* bacteria.

  ($NO_2^- + 0.5\ O_2$ + *Nitrobacter* $\longrightarrow$ NO_3^-)

In the nitrogen cycle, nitrification connects N mineralization to denitrification (the phenomenon of coupled nitrification-denitrifications with transformation of nitrate into nitrogen gas by the action of denitrifying bacteria). The two biochemical processes coupled nitrification-denitrification, are quite relevant since they can remove between 10-80% of the nitrogen resulting from anthropogenic pollution (Romano and Zeng 2013).

Of the three inorganic forms of nitrogen, the most toxic to decapods is usually ammonium-N and the least toxic is nitrate-N (Fanjul et al. 2008; Romano and Zeng 2013). Ammonium-N, nitrate-N and nitrite-N may affect the survival and growth of aquatic organisms, however, elimination and capping of nutrients may be influenced by biotic factors (e.g., osmoregulation capacity) and abiotic factors (e.g. temperature, pH and salinity) (Romano and Zeng 2013). These chemical elements are passed on to the organisms through the ecological relations maintained through the food chain along the different trophic levels (producer, primary consumer, secondary consumer, tertiary ...), so it is important to check how these organisms behave to these parameters' environmental impacts.

The mechanism of nitrate detoxification is less complex compared to ammonia/ammonium and nitrites mechanisms, the permeability of gills to N-nitrate is low, thus reducing passive diffusion (Jensen 1996). Formation of nitrate is the last step of nitrification and since it has a much lower toxicity compared to ammonium and nitrite, there are fewer published studies. Therefore the cause of a higher nitrate toxicity in freshwater organisms, when compared to saltwater organisms, is not yet fully understood. Nevertheless, the species under study was very tolerant to nitrates and it could still be verified that the presence of nitrates decreased the number of more serious occurrences, in the levels of the necrosis identified in the mixtures of salinity and nitrate.

Nowadays, it is possible to predict the effects of contaminants in mixtures. Two type effect models are commonly used based on the different modes of action of the contaminants: the Concentration Addition (CA) (Loewe and Muischnek 1926) and Independent Action (IA) (Bliss, 1939). The CA model was defined as a sum of the relative toxicities of the individual toxicants in the mixture (Loewe and Muischnekand, 1926) and the IA model was based on the idea of a different action of the mixtures components (Bliss 1939), generating independence toxicities probabilities of mixture components (Jonker et al. 2005).

Through the predicted CA effect, a dataset was analyzed and synthesized in table 2.V. These values allow to characterize the toxicity of the mixture. Several effects may be observed to be of greater biological importance:

1. Synergism / antagonism (S/A): the observed effect was antagonism (a = 32).

2. Dose-Level dependent deviation (DL): the deviation of the reference models was different at low and high dose levels. An antagonistic behavior (a = 1.99) and a synergism effect at higher dose levels (b = -8.33) were found at lower dose levels; The antagonistic effect is less severe, reduces the effect of the other toxic while synergism causes more severe effects increases or potentiates the consequences of the other (Jonker et al. 2005).

3. Dose-ratio dependent deviation (DR): The deviation depends on the composition of the mixture. Both toxins caused antagonism (toxic 1 being nitrate obtained

a bDR1= 8.06 and toxic 2 being salinity obtained a bDR2=13.79).

The model selected as more suitable to explain data variability is CA. Within the three deviation functions tested (S/A, DL and DR), the best model suggested by ToxCalcMix spreadsheet is the DL (Dose-Level Dependence). Our results revealed an antagonistic effect for lower concentrations binary mixtures. Isnansetyo et al. (2014) verify for lower salinities (13-19 g L^{-1}) an increase in nitrification was verified. This increase in nitrification will lead to the conversion of ammonia (which is highly toxic to aquatic organisms) into nitrates (which is less toxic than ammonia), it can be said that salinity decreases the toxicity of nitrates, this is the antagonistic effect. At salinities higher than 25 g L^{-1} the nitrification rate begins to decrease abruptly, this effect is synergism (environmentally responsible for effects more harmful to organisms than antagonism as synergism increases or potentiates the consequences of toxicants).

In the DL an antagonistic behavior was verified for low concentrations (1 toxic unit), this is the most important information to retain on the behavior in binary mixtures. There was a synergistic effect when concentrations were very high but in environmental terms, these concentrations are unrealistic. DR identified which of the tested variables was most toxic to organisms which was salinity, both at the level of toxicity and at the level of necrosis. Thus, the results obtained give us a good indication of the adaptability of these amphipods to these two environmental variables.

2.5. Conclusion

The acute tests of salinity, nitrate and binary mixtures were performed in *E. meridionalis*. This work shows that Portuguese amphipods have great tolerance to salinity, therefore it is expected that these organisms can adapt to climate change, namely to sea level rise. However, due to necrosis at very low salinities (below LC 5), additional studies on the rate of ingestion of the organism are recommended. Salinity tolerance has been assessed, but it is necessary to understand whether long-term organisms have the ability to feed properly. As for nitrate tolerance, there was also a high resistance of amphipods at realistic environmental concentrations and higher concentrations. The maximum permissible nitrate value for inland waters (rivers) in the European Union is 50 mg NO_3-N L^{-1} and the survival rate of *E. meridionalis* in similar values was on average 85%. The mixture of both stressors showed an antagonistic effect at low concentration (the most realistic scenario at the environmental level), thus suggesting a good adaptability of this organism to these environmental disturbances.

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Supplementary data

Salinity

Log Body length (mm)

Table S I 1- Mean Log body length (mm) of organisms with respective satandard error.

Treatment_1 Nominal concentration (gL^{-1})	**Mean**	**SE Mean**
Control	1.012	0.021
5.5	1.016	0.012
8.7	0.992	0.013
13.7	0.987	0.013
21.6	1.025	0.023
34	1.008	0.021
Treatment_2 Nominal concentration (gL^{-1})	**Mean**	**SE Mean**
Control	0.993	0.028
5.5	0.959	0.022
8.7	0.883	0.022
13.7	0.969	0.023
21.6	0.924	0.397
34	0.967	0.039
Treatment_3 Nominal concentration (gL^{-1})	**Mean**	**SE Mean**
Control	0.994	0.015
24.2	0.969	0.015
25	0.985	0.017
25.8	0.966	0.015
27	1.077	0.015

Log Body weight (mg)

Table S II 1- Mean Log body weight (mg) of organisms with respective standard error.

Treatment_2 Nominal concentration (gL^{-1})	**Mean**	**SE Mean**
Control	0.843	0.089
5.5	0.876	0.073
8.7	0.726	0.073
13.7	0.992	0.073
21.6	0.798	0.090
34	0.7618	0.089
Treatment_3 Nominal concentration (gL^{-1})	**Mean**	**SE Mean**
Control	0.976	0.053
24.2	0.930	0.054
25	0.940	0.062
25.8	0.842	0.053
27	1.020	0.053

Nitrates

Log Body length (mm)

Table S III 1 - Mean Log body length (mm) of organisms with respective standard error.

Treatment_1 Nominal concentration mg $NaNO_3$/L	Mean	SE Mean
2.75	0.915	0.016
7	0.985	0.022
17	0.882	0.022
45	0.920	0,022
110	0,880	0,022
275	1.019	0,022
470	0.848	0.024
700	0.822	0.028
Treatment_2 Nominal concentration mg NaNO3/L	**Mean**	**SE Mean**
Control	0.979	0.016
175	0.973	0.016
210	0.979	0.016
470	1.004	0.018
759	0.985	0.156
Treatment_3 Nominal concentration mg $NaNO_3$/L	**Mean**	**SE Mean**
Control	0.858	0.024
100	0.900	0.023
150	0.896	0.024
225	0.897	0.025
309	0.845	0.024
509	0.811	0.026
760	0.853	0.024

Log Body weight (mg)

Table S IV 1- Mean Log body weight (mg) of organisms with respective standard error.

Treatment_1 Nominal concentration mg $NaNO_3$/L	Mean	SE Mean
2.75	0.8039	0.043
7	0.949	0.060
17	0.671	0.060
45	0.786	0.060
110	0.762	0.060
275	0.999	0.060
470	0.611	0.062
700	0.574	0.077
Treatment_2 Nominal concentration mg $NaNO_3$/L	**Mean**	**SE Mean**
Control	0.919	0.050
175	0.984	0,050
210	1.007	0,050
470	0.942	0,050
759	1.039	0,050
Treatment_3 Nominal concentration mg $NaNO_3$/L	**Mean**	**SE Mean**
Control	0.714	0.064
100	0.792	0.064
150	0.825	0.064
225	0.848	0.064
309	0.715	0.065
509	0.648	0.064
760	0.695	0.064

Mixtures

Table S V 1 - Mean Log body length (mm) and Log Body weight (mg) of organisms with respective standard error**.** In the first column, the values in the left correspond to the salinity nominal concentration (g L^{-1}) and the the values in the right correspond to the nitrate nominal concentration (mg NO_3 L^{-1}).

Treatment_1	Log Body length (mm)		Log Body weight (mg)	
	Mean	SE Mean	Mean	SE Mean
Control-Control	0.9316	0.022	0.841	0.0653
0-450	0.8918	0.023	0.686	0.0653
11,5-235	0.8850	0.023	0.747	0.0653
11,5-250	0.8952	0.023	0.719	0.0653
11,5-340	0.8563	0.023	0.650	0.0653
14-235	0,8219	0.023	0.648	0.0653
14-250	0.8496	0.023	0.742	0.0653
17-235	0.8849	0.023	0.669	0.0653
17-340	0.8577	0.023	0.671	0.0653
20-415	0.8584	0.023	0.652	0.0653
25-0	0.8703	0.023	0.652	0.0653

Table S VI 1- Mean Log body length (mm) and Log Body weight (mg) of organisms with respective standard error**.** In the first column, the values in the left correspond to the salinity nominal concentration (g L^{-1}) and the the values in the right correspond to the nitrate nominal concentration (mg NO_3 L^{-1}).

Treatment_2	Log Body length (mm)		Log Body weight (mg)	
	Mean	SE Mean	Mean	SE Mean
Control-Control	0.889	0.018	0.859	0.051
0-900	0.974	0.018	0.945	0.051
15-0	0.877	0.018	0.756	0.051
15-400	0.899	0.018	0.856	0.051
15-650	0.834	0.203	0.720	0.051
25-0	0.868	0.020	0.6925	0.051
25-900	0.888	0.020	0.715	0.051
5-0	0.913	0.19	0.816	0.051
5-400	0.912	0.019	0.872	0.051
5-450	0.901	0.020	0.871	0.051
5-650	0.860	0.020	0.685	0.051
8.5-0	0.869	0.019	0.733	0.051
8.5-400	0.920	0.019	0.900	0.051
8.5-450	0.906	0.019	0.859	0.051

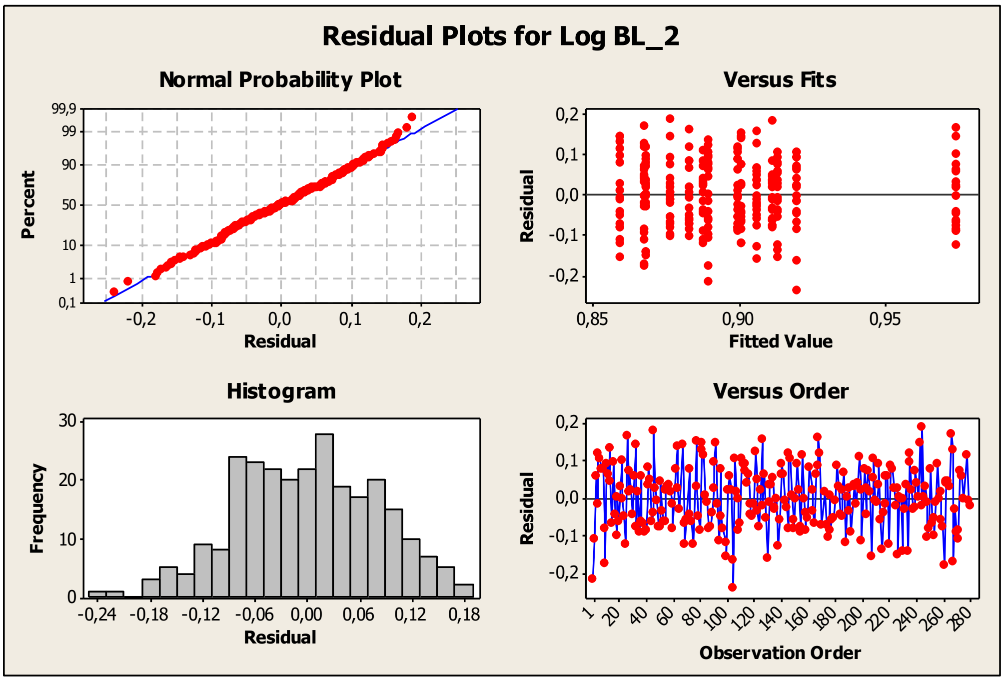

Figure S 1 1- Residual plots for Log Body Length (mm) in the experience 2 of binary mixture.

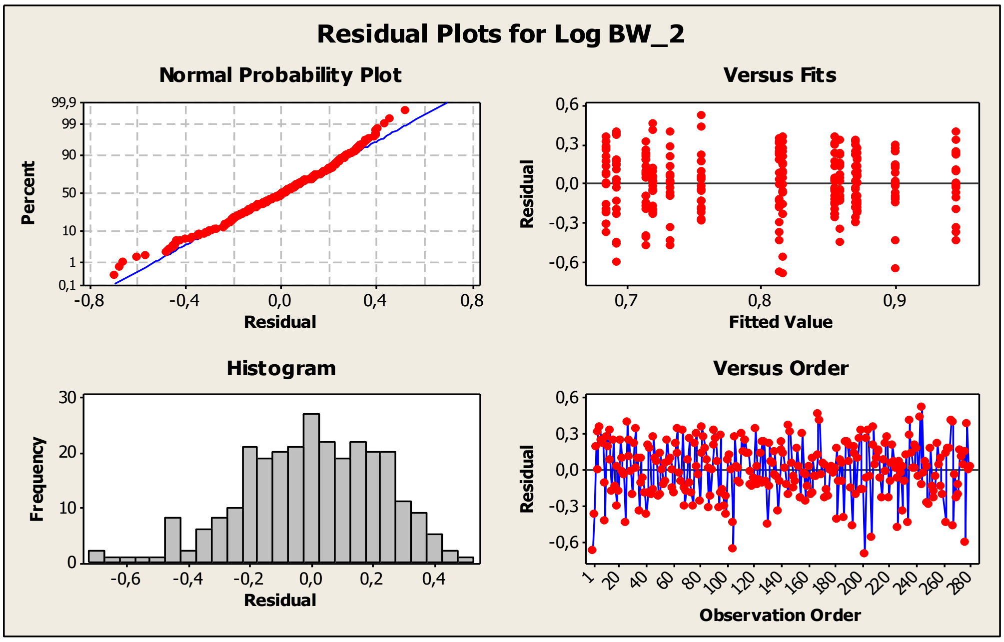

Figure S 2 1 - Residual plots for Log Body weight (mg) in the experience 2 of binary mixture.

3. Conclusions and Final remarks

Climate change is becoming increasingly evident and several harmful effects emerge in ecosystems. Among them, a very worrying one is the rising sea-level, this is causing saltwater intrusion into freshwater ecosystems (Robins et al., 2016; Werner and Simmons, 2009). In another perspective, freshwater ecosystems are threatened by the overuse of fertilizers due to excess nutrients (N and P) in water. This increase in nutrients can lead to the eutrophication of environments and deterioration of the quality of freshwater, thus arising imbalance the ecosystems (Rabalais, 2002; Ryther and Dunstan, 1971).

The organism under study was an amphipod endemic to Portugal, *E. meridionalis*. There is little information in the literature on the tolerance of amphipods to salinity and nitrates, although these organisms play a very important role in ecosystems. These organisms recycle the nutrients and make them available to other organisms, serve as prey and allow the connection between different trophic levels (producer or consumer), therefore facilitating the maintenance of these ecosystems (Covich et al., 1999; Crowl et al., 2001,Quintaneiro et al, 2014). Moreover, amphipods can be considered useful bioindicators of water quality because they can undergo changes according to the environmental modifications that can result from natural or anthropogenic disturbances (Conradi et al., 1997; Guerra-Garcia and Garcia-Gomez, 2001). The choice for an individual response (only a species of amphipod) was due to the fact that individual responses are more sensitive and can be obtained more quickly than those acquired at the level of populations, communities and ecosystems. Therefore, individual responses are very useful as early indicators and may further help identifying the mechanism that causes environmental disruption (Magalhães and Filho, 2008).

The results obtained in this work show a great tolerance to salinity contrary to the one described by Piscart et al (2011) which the amphipods evidenced a greater sensitivity to the salinity, so it is hoped that Portuguese amphipod will be able to adapt to the climatic changes, namely to rising sea level. However, the appearance of necrosis at very low salinities below LC_5 indicates the need for further studies about the ingestion rate of the organism. Salinity resistance is not that important if the organisms can not endure chronic exposure to high salt concentratrions which can not feed themselves properly. Regarding nitrate tolerance, there was also a high resistance of amphipods in realistic environmental concentrations and higher concentrations. The maximum permitted nitrate value for inland water (rivers) in the European Union is 50 mg NO_3-N L^{-1} above this level, the rivers are considered at risk of being polluted or polluted (91/676/EEC). The survival rate of *E. meridionalis* to values close to 50 mg NO_3-N L^{-1} was on average 85%. The mixture of both stressors showed an antagonistic effect at low concentration (a more realistic scenario at the environmental level), thus suggesting a good adaptability of this organism to these environmental disturbances.

Future works with this organism should take into account ingestion tests previously mentioned. It would also be interesting to check their resistance to the tolerance of polycyclic aromatic hydrocarbons since this year, in Portugal, there were numerous occurrences of forest fires, one of the most affected districts was Leiria. Porto de Mós was the reference site for the collection of the organisms under study and severely affected by these disturbances. It is expected that climate change will increase the global temperature and cause longer drought periods (this phenomena will increase up to 140% by the end of the 21st century) which will increase the number forest fires (Wotton et al., 2010).

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