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Assessment of *Daphnia magna* as a toxicity bioindicator for wastewaters

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Abstract

Toxicity tests on complex wastewater effluents have been considered as an important complement to emission limit values (ELV) based on physico-chemical and microbiological parameters in recent years. However, relatively few studies have been conducted so far evaluating the toxicity of effluents with aquatic organisms, and it remains unclear which test species should be used in such evaluations. The first aim of this dissertation was therefore to assess the potential of the crustacean *Daphnia magna* as a bioindicator for the toxicity of a domestic effluent disinfected with peracetic acid (PAA), a disinfect that has received increasing attention in recent years as an alternative disinfectant for chloride. To this end, bioassays were performed with *D. magna* on the secondary effluent from the WWTP of Beirolas, with and without disinfection by 5 $mg.L^{-1}$, 10 $mg.L^{-1}$ and 15 $mg.L^{-1}$ PAA. These PAA concentrations were selected since they were shown in a parallel MSc study to have high removal efficacy of coliform and faecal bacteria.

Exposure to the secondary effluent without disinfection caused no mortality or immobility on the organisms. Although the disinfected effluent adhered to all the standards set in current Legislation, even the lowest PAA concentration resulted in 100% daphnid mortality within 48h. Subsequently, efforts should be made to evaluate whether lower PAA concentrations or a longer residual time after PAA treatment may ensure disinfection efficacy without exerting toxicity to aquatic organisms like *D. magna*.

The second aim of this dissertation was to compare the sensitivity of *D. magna* to wastewater with that of other species commonly used in bioassays. This was done to evaluate which test species are the most appropriate for use in wastewater toxicity testing. To this end, a literature search was conducted by collecting data from papers where the toxicity of effluents was tested to *D. magna* and at least one other species. This thus allowed to evaluate the relative tolerance (Trel) of these species as compared to *D. magna*.

The taxonomic groups that appeared to be more sensitive to effluents than *D. magna* were bacteria and rotifers. On the other hand, macrophytes, insects and fish were found to be generally less sensitive than *D. magna*. Since no single species was aways the most sensitive species to the wide range of effluents (e.g. different sources, compositions and sampling periods) included in the dataset, a test battery including species from different taxonomic groups is recommended for effluent testing.

Keywords: *Daphnia magna,* toxicity tests, wastewater, disinfection, relative tolerance (Trel).

Resumo

Os testes de toxicidade em efluentes constituem um importante complemento aos valores limite de emissão (VLE), baseados em análises físico-químicas e microbiológicas, por forma a garantir a conservação do meio recetor. No entanto, poucos estudos foram realizados com o fim de avaliar a toxicidade de efluentes em organismos aquáticos, não tendo ainda sido definidas quais as espécies mais adequadas à realização destes testes. Desta forma, o primeiro objetivo deste trabalho foi avaliar o potencial do crustáceo *Daphnia magna* como bioindicador de toxicidade para águas residuais domésticas desinfetadas com ácido peracético (PAA). Para tal, foram realizados testes de toxicidade com a *D. magna* no efluente secundário proveniente da ETAR de Beirolas, e após desinfeção com diferentes concentrações de PAA - 5 mg. L^{-1}, 10 mg. L^{-1} e 15 mg. L^{-1}. Estas concentrações foram escolhidas com base num estudo efetuado paralelamente numa dissertação de Mestrado, onde demonstraram elevada eficácia de desinfeção.

A exposição dos organismos ao efluente secundário sem desinfeção não resultou em mortalidade ou imobilidade nos organismos testados, mostrando, aparentemente, ausência de toxicidade do efluente para a *D. magna*. Por outro lado, relativamente ao efluente desinfetado, obteve-se, para todas as concentrações testadas, uma mortalidade de 100% dos organismos, após um tempo de exposição de 48h. Desta forma, estudos futuros deverão ser desenvolvidos que avaliem se concentrações mais baixas de PAA possam assegurar a eficácia da desinfeção sem provocar efeitos tóxicos em organismos aquáticos como, por exemplo, a *D. magna*.

O segundo objetivo do presente estudo foi comparar a sensibilidade a águas residuais da *D. magna* com outras espécies comummente utilizadas em testes de toxicidade. Desta forma, foi realizado um estudo de revisão bibliográfica onde foram recolhidos dados de toxicidade de artigos científicos que avaliassem a toxicidade de efluentes na *D. magna* e de, pelo menos, mais uma espécie. Posteriormente, perante os dados de toxicidade, foi calculada a tolerância relativa (Trel) aos diferentes tipos de efluente para cada espécie comparativamente com a *D. magna*.

Os grupos taxonómicos que demonstraram ter uma maior sensibilidade aos efluentes, quando comparados com a *D. magna,* foram as bactérias (mais especificamente a espécie *Vibrio fischeri*) e os rotíferos. Por outro lado, os grupos que demonstram ter uma menor sensibilidade comparativamente à *D. magna* foram os peixes, os insetos e as macrófitas. No entanto, nenhuma espécie demonstrou ter a mesma sensibilidade a efluentes com caraterísticas diferentes (natureza, concentrações ou amostragem do mesmo efluente em períodos distintos, entre outros) sendo recomendada a realização de uma bateria de testes utilizando espécies de diferentes grupos taxonómicos.

Palavras-chave: *Daphnia magna*, testes de toxicidade, desinfeção, tolerância relativa (Trel).

List of Abbreviations and Acronyms

BOD	Biochemical oxygen demand
ChV	Chronic value
CI	Confidence Interval
COD	Chemical oxygen demand
EC	European Commission
EC_{50}	Half maximal effective concentration
ELV	Emission limit values
EU	European Union
DO	Dissolved oxygen
IQR	Interquartile range
ISO	International Organization for Standardization
LC50	Median lethal concentration
LD50	Median lethal dose
LOEC	Lowest-observed-effect concentration
MAV	Maximum allowable value
NOEC	No-observed-effect concentration
OECD	Organisation for Economic Co-operation and Development
PAA	Peracetic acid
RMV	Recommended maximum value
Trel	Relative tolerance
TSS	Total suspended solids
US EPA	United States Environmental Protection Agency
WWTP	Wastewater treatment plant

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1. Introduction

1.1. Research scope

Domestic and industrial wastewaters are a byproduct of anthropogenic activities, being produced everyday around the world. If left untreated, they may negatively affect both human and environmental health. Globally, approximately 80% of the produced wastewater is released in the environment without adequate treatment (Jackson et al., 2008; UNESCO, 2017).

Untreated wastewater discharges have been identified as one of the major sources of aquatic pollution in industrialized countries, mainly due to the presence of organic matter and nutrients. Carbon, being the primary constituent of organic matter, can negatively impact the aquatic ecosystems, as excessive amounts of oxidizable organic matter can significantly decrease oxygen concentrations. Additionally, excessive nitrogen and phosphorus have been identified as the main causes for water quality degradation in Europe (more specifically, eutrophication) (Lema et al., 2017; Prasse et al., 2015; Sepp et al., 2018).

Poor sanitation management is linked to transmission of diseases such as cholera, diarrhoea, dysentery, hepatitis A, typhoid and polio. Annually, inadequate sanitation is estimated to cause 280 000 diarrhoeal deaths and is a major factor in several neglected tropical diseases (WHO, 2019b).

Wastewater treatment plants (WWTP) aim to receive and treat wastewater effluents and subsequently transfer it to an adequate destination (Águas de Portugal, 2018). The main constituents of concern in wastewater treatment effluents are suspended solids, biodegradable organics, dissolved inorganics, pathogens, nutrients and heavy metals (Metcalf & Eddy, 2003). Additionally, the presence of emerging pollutants in effluents has been increasing in recent decades, with traditional wastewater treatment systems showing to be insufficient in the removal of these pollutants (Deblonde et al., 2011) .

Wastewater effluents quality assessment is currently primarily based on physico-chemical parameters such as pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total nitrogen and total phosphorus, as well as microbiological parameters, such as faecal and total coliforms (Mara, 2003; Metcalf & Eddy, 2003). In order to counteract the continuous release of organic and inorganic pollutants, the Water Framework Directive requires a good status in terms of quantity and quality (chemical and ecological), by implementing the most efficient technique available to control their emissions in the receptor bodies (Bundschuh et al., 2011).

Since wastewater treatment effluents include a complex mixture of several organic and inorganic compounds, as well as macro and micronutrients, the determination of only some parameters is insufficient for a complete assessment of the effluent quality. Therefore, ecotoxicological bioassays

have been suggested as an important supplement to chemical analysis as the means of evaluation of biological effects caused by wastewater treatment effluents to the aquatic ecosystems. WWTP are not able to (fully) eliminate all possible contaminants, so it is important to assess if the cleaning processes in WWTP are sufficient to provide a satisfactory decrease of environmental impact on the species living in the receiving ecosystem (Gargosova & Urminska, 2017).

In Portugal, there is no legislation available on monitoring effluents through bioassays. However, when the conditions of effluent discharge for the aquatic ecosystems are considered, and it is aimed to protect the integrity of the aquatic environment, a methodology based only on ELV for pollutants specifically identified in those effluents, may present some gaps (Picado et al., 2008).

Toxicity tests use several species to assess and characterize acute and chronic effects of toxic agents in receptor bodies. The most common organisms used in ecotoxicology are invertebrates, fish, aquatic plants (i.e. macrophytes), algae and bacteria. The criteria to be considered when choosing the test organism must include the availability, abundance, ecological representativeness, economic viability and knowledge of the species regarding its biology (Magalhães & Ferrão-Filho, 2008; Tothill & Turner, 1996). Ecotoxicological bioassays are not legally applied in the European Union to evaluate effluent toxicity, although many countries have started to implement some toxicity tests in order to assess the impact of wastewater discharges and to establish ELV to protect aquatic ecosystems (Power & Boumphrey, 2004).

Daphnia magna is one of the most common invertebrate species used in toxicity tests. It is a planktonic crustacean and belongs to the Daphniidae family, being commonly called “water flea”. *D. magna* inhabits permanent water bodies, such as ponds and lakes and is usually the dominant zooplankter in temperate freshwaters. The frequent use of daphnids in toxicity tests is due to its high sensitivity to environmental changes, short doubling time and simple handling (Hoffman et al, 2003; Movahedian & Bina, 2005).

1.2. Document structure

This dissertation is structured in 9 chapters.

Chapter 1 presents an introduction to the ecotoxicological tests used in wastewater treatment effluents.

Chapter 2 consists of a literature review. This section presents relevant scientific publications that sustain the elaboration of this dissertation.

Chapter 3 describes the aims of this research work.

Chapter 4 presents the methods adopted to conduct the thesis work. In this section, all the steps and methods conducted during the elaboration of the laboratory and sensitivity analysis studies are detailed.

Chapter 5 comprises the results obtained throughout the development of the dissertation and discusses these results in view of other scientific studies.

Chapter 6 presents the conclusions and limitations of this study.

Chapter 7 discusses possible future studies for further investigation that could be deducted from the thesis work.

Chapter 8 presents the bibliographic references which supported this study.

Chapter 9 contains the annexes, which exhibit additional information of the laboratory experimental work and the bibliographic research study carried out for this dissertation.

2. Literature review

2.1. Wastewater treatment

Wastewaters are characterized in terms of their physical, chemical and biological composition, which depend on the domestic and industrial contribution, as well as the precipitation that flows into the sewage systems. Wastewater main constituents are solid matter, nutrients, pathogenic organisms and a large variety of chemical substances. The physico-chemical parameters traditionally used to characterise wastewater effluents are: pH, temperature, colour, turbidity, total and suspended solids (TS and TSS), volatile suspended solids (VSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), alkalinity and nutrients, such as total nitrogen and total phosphorus (Mara, 2003, Metcalf & Eddy, 2003).

The main constituents of concern in wastewater treatment are listed in Table 2.1.

Table 2.1 - Principal constituents of concern in wastewater treatment (Source: Metcalf & Eddy, 2003)

Constituent	Reasons for importance
Suspended solids	Suspended solids can enhance the development of sludge deposits and anaerobic conditions when untreated wastewater is discharged in aquatic ecosystems.
Biodegradable organics	Biodegradable organics are composed primarily of proteins, carbohydrates and fats and are most commonly measured in terms of BOD and COD; if discharged untreated in the aquatic systems can lead to the depletion of natural oxygen resources and to the development of septic conditions.
Pathogens	Diseases can be transmitted by pathogenic organism that may be present in wastewater effluents.
Nutrients	When discharged to que aquatic environment, nutrients such as nitrogen and phosphorus can lead to the growth of undesirable algae and contribute to the contamination of groundwater, when discharged in excessive amounts on land.
Priority pollutants	Many organic and inorganic compounds suspected of carcinogenicity, mutagenicity, teratogenicity or high acute toxicity are frequently found in wastewater.
Refractory organics	These organics (e.g. surfactants phenols and agricultural pesticides) tend to resist conventional wastewater treatment methods.
Heavy metals	Heavy metals are usually added to wastewater from commercial and industrial activities and it may be recommended to be removed if the wastewater is to be reused.
Dissolved inorganics	Inorganic constituents are added to the original domestic water supply as a result of water use, and may have to be removed if the wastewater is to be reused. Calcium, sodium and sulphate are examples of these constituents.

The protection of surface water and groundwater resources from wastewater pollution is imperative for the effective management of risks posed to both human health and the environment. The discharge of insufficiently treated domestic and/or industrial effluents to aquatic ecosystems may for example lead to excess nutrient enrichment, algal blooms and eutrophication. It can also cause waterborne diseases through outbreaks of WWTP effluents into drinking water sources (Naughton & Hynds, 2014).

Wastewater treatment methods consist of operation units and processes. Operation units include treatment methods in which the application of physical forces predominate, while processes include methods in which the removal of contaminants is brought about by chemical and/or biological

reactions. The type of treatment depends on the influent characteristics and the effluent quality required (Metcalf & Eddy, 2003; Qasim & Zhu, 2018). Table 2.2 lists the principal levels of wastewater treatment with the respective description.

Table 2.2 - Levels of wastewater treatment (Source: Metcalf & Eddy, 2003)

Treatment level	Description
Preliminary	Removal of solid constituents such as rags, sticks, floatables, grit and grease that may cause problems at a maintenance and/or operational level of the treatment operations and processes.
Primary	Removal of a portion of the suspended solids and organic matter from the wastewater.
Advanced primary	Enhanced removal of suspended solids and organic matter from the wastewater, usually through chemical addition or filtration.
Secondary	Removal of biodegradable organic matter (in solution or suspension) and suspended solids. It might also include removal of nutrients (nitrogen and phosphorus).
Tertiary	Removal of residual suspended solids, usually by granular medium filtration or microscreens and/or disinfection.
Advanced	Removal of dissolved and suspended materials remaining after normal biological treatment, typically when the effluent is required for carious reuse applications.

2.2. Disinfection

Disinfection is the primary mechanism for inactivation and destruction of disease-causing organisms present in the wastewater, representing a fundamental treatment to guarantee the discharge quality and minimise potential risks to humans and the environment. This treatment level is especially relevant when the discharge is near beaches or is used directly or indirectly for agricultural, domestic or recreational purposes (Qasim & Zhu, 2018; Ragazzo et al., 2017).

Nowadays, around 2 billion people do not have access to basic sanitation facilities and 432 000 people living in middle-income countries die annually as a result of bad sanitation through diseases such as cholera, diarrhoea, hepatitis A and typhoid fever (WHO, 2019a). The infectious agents responsible for these diseases include bacteria, viruses, protozoa and a variety of helminths (Shannon et al., 2008; Victoria, 2002).

Table 2.3 summarizes the common pathogens that may be present in raw domestic sewerage, as well as their infectivity and associated diseases.

Table 2.3 - Common pathogens that may be present in raw domestic wastewater (Source: Qasim & Zhu, 2018)

Organism	Species	Relative Infectivity	Associated waterborne disease
Bacteria	*Escherichia coli, Salmonella* spp.*, Salmonella typhi, Shigella* spp.*, Vibrio cholerae*	Low to moderate	Gastroenteritis, salmonellosis, Typhoid fever, Shigellosis, Cholera
Viruses	Hepatitis A virus, Parvoviruses, Calciviruses, Enteric viruses, among others	High	Infectious hepatitis, gastroenteritis, paralysis, fever.
Protozoa	*Cryptosporidium parvum, Entamoeba histolytica, Giardia lamblia*	High	Cryptospordiosis, Amebiasis, Guardiasis
Helminths	*Ascaris lumbricoides, Schistosoma mansoni, Trichuris trichiura*	High	Acariasis, Schistosomiasis, Trichuriasis

Coliform bacteria, members of Enterobacteriaceae, are widely used to evaluate microbiological quality of drinking water, specifically as an indicator of water treatment efficiency and distribution system integrity and as a screen for faecal contamination. These bacteria are present in the normal intestinal flora of humans and other warm-blooded animals, and are usually found in large numbers in faecal waste and wastewater. With the exception of a few strains, coliforms are not considered pathogenic and most species are free-living in the environment, thus their presence in water

masses does not necessarily represent a risk. Also, waterborne pathogens are not necessarily present when coliforms are detected. Coliform bacteria are easily detected and quantified and comprise total coliforms and faecal coliforms (Craun et al., 1997; Mara, 2003).

2.2.1. Conventional disinfection treatments

There are several techniques for wastewater disinfection that include chemical, physical and biological processes that aim the neuralization of pathogenic organisms. These techniques can be used individually or in combination, depending on the discharge quality requirements and the influent quality (Eckert, 2013; Luukkonen et al., 2014). Table 2.4 presents some of the most commonly used disinfection methods.

Table 2.4 - Common disinfectants and respective characteristics (Source: Metcalf and Eddy, 2003)

Characteristics	Free and combined chlorine species	Chlorine dioxide	Ozone	UV light
Hazardous chemicals that threat surrounding population	Yes (in case of chlorine gas)	No	No	No
Energy intensive	No	No	Yes	Yes
High contact time	Yes	Yes	No	No
Effective in the destruction of resistant organic constituents	No	No	No (but can reduce concentration when applied with a higher dosage)	Yes (at very high dosages)
Residual disinfectant	Yes	Yes	No	No

Chemical disinfectants are commonly used in wastewater treatments, not only because of their high effectiveness in the reduction of microorganisms, but also because of their oxidation potential, enabling the destruction of emerging contaminants (e.g. micropollutants, pharmaceuticals, such as endocrine disrupting chemicals (ECDs)) by converting them into biodegradable and/or less toxic compounds (Acero et al., 2013; Ahmed et al., 2017; Bolong et al., 2009).

Chlorination is the most widely used method for disinfecting effluents from WWTP and from drinking water treatment plants (DWTP), mainly for being one of the most economically advantageous options and for allowing the maintenance of a disinfectant residual in the distribution system. However, in the presence of organic matter, chlorine reacts with the organic compounds and forms

trihalomethanes, which are mutagenic/carcinogenic and other toxic by-products that are potentially harmful to human and aquatic organisms (EPA, 1999; Fiessinger et al., 1981). Even at low concentrations, chlorine is toxic to water life and residual chlorine can persist in the effluent for many hours. Subsequently, the use of chlorine to disinfect wastewater effluents requires dichlorination prior to the effluent release in the receptor body, even though the long-term effects of discharging dechlorinated compounds into the environment are unknown (Collivignarelli et al., 2017; De Souza et al., 2015).

Ultraviolet irradiation is one of the most commonly used alternatives to chlorination with a comparable and often more effective disinfection for viruses and bacteria. UV light penetrates the cell wall of the microorganism and is absorbed by the nucleic acids, which can either prevent replication or cause cell death. The effectiveness of UV radiation as a disinfectant depends on the characteristics of the UV disinfection system, the overall system hydraulics, the presence of particles, the characteristics of the microorganism and the chemical characteristics of the wastewater (Metcalf & Eddy, 2003, Lazarova et al., 1999). Even though UV light has some advantages as compared to chemical disinfectants, such as the absence of disinfection byproducts and improved safety regarding toxicity to humans and aquatic species, it also presents some disadvantages, being relatively expensive and not allowing any residual effect (Koivunen & Heinonen-Tanski, 2005).

The ideal disinfection system should guarantee the maximum efficiency in pathogenic microorganism removal without generating toxic by-products. Moreover, it should be inexpensive and technologically compatible with the other treatment processes. Some of the main characteristics to be considered when choosing a disinfection system are the availability, safety, stability, toxicity to microorganisms and other forms of life, and interaction with extraneous material (Metcalf & Eddy, 2003; Veschetti et al., 2003).

In applying a disinfection system, some factors must be considered that influence its action, namely contact time, concentration of the disinfectant, intensity and nature of the disinfection agent, temperature, types of organism and the nature of the suspending liquid (Metcalf & Eddy, 2003).

2.2.2. Peracetic Acid

Peracetic Acid (PAA) is a strong disinfectant with a wide spectrum of antimicrobial properties. In recent years, it has been used in several industries (including food processes, beverage, medical and pharmaceutical, and as a decolouring agent in textile and pulp and paper industries) demonstrating high bactericidal, virucidal, fungicidal and sporicidal effectiveness. Therefore, the use of PAA as a disinfectant for wastewater effluents has been studied and explored (Gehr et al., 2003; Kitis, 2004).

PAA is commercially available at 5-40% w/w in a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, peracetic acid and water, as described in the equation below (Gehr et al., 2003; Henao et al., 2018):

$$CH_3CO_2H + H_2O_2 \leftrightarrow CH_3CO_3H + H_20 \qquad (1)$$

Even though the information concerning the mode of action of PAA as an antimicrobial agent is still limited, it is speculated that it functions as other peroxides and oxidizing agents, being that its disinfectant activity is based on the release of active oxygen. It is also suggested that PAA disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of the cell wall (Kitis, 2004).

Doses of 1.5 to 2 mg/L with a contact time of 15 to 20 minutes have been proven to be enough for bacteria removal of tertiary effluent wastewater, while a 60 minutes contact time provides coliphage virus removal. For secondary effluents, a dose of 2 to 7 mg/L and a contact time of 30 minutes are required for a 3-log reduction in total coliform number. However, the desired dose and contact time depend on the wastewater effluent quality (Luukkonen et al., 2015).

PAA disinfection may be conducted in the presence of organic matter, since it has been shown that it produces no to little toxic or mutagenic byproducts after reaction with organic material present in wastewater effluents or surface waters used for drinking water. The decomposition products of PAA are acetic acid, hydrogen peroxide, oxygen and water. Although any formation of halogen-containing disinfection by-products (DBP's) has not demonstrated, this possibility cannot be completely discarded (Kitis, 2004).

Emerging pollutants are new products or chemicals with no regulatory status and whose effects on ecosystems and human health are still not totally known. In recent years, there has been an increasing awareness regarding some of these compounds, such as micropollutants and endocrine disruptors. Therefore, the European Commission has created a list of certain substances that must be monitored. PAA has shown potential in the removal of certain pharmaceuticals (four anti-inflammatory drugs and two lipid-regulating agents) (European Comission, 2018; Deblonde et al., 2011; Hey et al., 2012; Luukkonen et al., 2015) and appeared to be highly efficient in the removal of the sex hormone 17α-ethinylestradiol (EE2) in a study conducted by Maurício et al. (2019).

The presence of acetic acid (which is present both in the PAA commercial solution and as a product of its decomposition) has been linked to a potential microbial regrowth. However this can be avoided by using higher doses in the range of 2-15 mg/L (Antonelli et al., 2013). The disinfection with PAA is also linked to an increase in organic matter in treated wastewater effluents, resulting in an increase in TOC and BOD. Additionally, the use of PAA can also cause a decrease in pH values, as peracetic acid is prepared from acetic acid and hydrogen peroxide (Cavallini et al., 2013; Lazarova et al., 1999; Lefevre et al., 1992). Factors such as temperature (microbial reductions increase with increasing water temperature during the disinfection process), pH (PAA activity is greater at neutral or mildly acidic conditions), TSS (disinfection efficiency of PAA increases with decreasing TSS) can affect PAA disinfection efficiency (McFadden et al., 2017; Kitis, 2004).

Pilot plant experiments have shown a higher efficiency in disinfection when PAA is combined with UV radiation, due to the formation of free radicals as a consequence of photolysis of the PAA when in presence of the UV light rays (Caretti & Lubello, 2003).

The cost of a disinfection system depends on its availability, the composition of the wastewater to be treated, the final effluent quality required, the plant size and configuration and the market situation (Liberti & Notarnicola, 1999). The investment cost inherent to disinfection systems with PAA is generally lower than UV light and ozone and higher than chlorine and chlorine dioxide. The investment costs regarding equipment and construction of a new system in order to implement disinfection with PAA are very low when the plant already functions with chlorine, as contact times and handling equipment are similar (Collivignarelli et al., 2000; Nurizzo et al., 2001).

2.3. Water Framework Directive

The European Water Framework Directive (Directive 2000/60/EC of 23rd October of 2000; EC, 2000; WFD) is a legally binding document that requires European Commission member states to implement water management measures and policies in order to achieve good overall quality of water bodies within a 15 years' timeframe. It aims to complement a number of legislative instruments that existed before its implementation including Bathing (77/160/EEC), Drinking (98/83/EC), Fish (78/659/EEC) and Shellfish (79/923/EEC) Water Directives, as well as those based on chemical substances or sources of pollution (e.g. Dangerous Substances (76/464/EC), Groundwater (80/68/EEC), and Pesticide (91/414/EEC) Directives) .The WFD resulted from a joint decision and policy-making process involving the European Parliament and environmental NGO's, and is built upon two main approaches: more integrated ecological definitions of water and the introduction of the notion of public participation for policy implementation (Allan et al., 2006; Steyaert & Ollivier, 2007).

The WFD is the main community water policy instrument in the European Union, promoting a transparent, effective and coherent legislative framework, changing the water quality control for all the EU member states (De Stefano, 2010). The Directive aims the maintenance and improvement of the aquatic environment of the Community, being its purpose primarily concerned with the quality of the water within the EU territory, and encourages Member States to achieve the objective of at least good water status, by defining and implementing the necessary measures through integrated programmes. Areas where good water status already exists, it should be maintained. Regarding groundwater, in addition to the requirements of good status, any significant upward trend in the concentration of any pollutant should be identified and reversed (EC, 2000).

The ultimate goal of the WFD is to achieve the elimination of priority hazardous substances and contribute to achieving concentrations in the marine environment near background values for naturally occurring substances based on the precautionary, polluter pays, correction at the source and prevention principles (EC, 2000).

Annex V of the WFD states the quality elements for the classification of ecological status for surface water and groundwater. The classification considers biological, hydromorphological and physico-chemical elements. The biological elements considered in the assessment of water quality are the composition and abundance of phytoplankton, aquatic flora and benthic invertebrate fauna as well as the abundance and age structure of fish fauna. The hydromorphological elements considered are the hydrological regime, continuity and morphological conditions. On the other hand, the general physico-chemical elements accessed are thermal and oxygenation conditions, salinity, acidification status and nutrient conditions. Additionally, some specific pollutants are also considered, such as priority substances identified as being discharged into the body of water. The priority substances are approached in Annex X. Before the implementation of the WFD, water quality monitoring in most EU Member States was mainly based on chemical and physical

parameters, with only about half of the states using biological parameters as part of their water quality assessment system (Hering et al., 2003).

Three modes of monitoring regimes are specified in the Water Frame Directive (Allan et al., 2006):

a) surveillance monitoring, which aims long-term assessment of water quality changes and provides baseline data on river basins, allowing the design and implementation of other types of monitoring;
b) operational monitoring, aimed at providing data on water bodies at risk or failing objectives of the WFD;
c) investigative monitoring aimed at assessing causes of such failure.

The WFD has been transposed into Portuguese law by Law nº 58/2005 from 29th of December, establishing the institutional frame for the sustainable management of water bodies, being rectified by the Rectification Declaration nº 11-A/2006, from 23rd of February and modified by the Decrees-Law nº 245/2009 from 22 September, nº 60/2012 from 14th of March, nº130/2012 from 22 of June and changed by the Laws nº 17/2014 from 10th of April, nº 42/2016 from 28th of December and nº 44/2017, from 19th of June (Law nº58/2005 de 29th of December, 2005; Rectification Declaration nº 11-A/2006, 2006; D.L. nº 245/2009 de 22nd of September, 2009; D.L. nº 60/2012 de 14th of March, 2012; D.L. nº 130/2012 de 22nd of June, 2012; Law nº17/2014 de 10th of April, 2013; Law nº 42/2016 de 28th of December, 2016; Law nº 44/2017 of 19th de June, 2013). Subsequently, the WFD has been under constant evaluation and revision over the past two decades.

Until now, water quality monitoring has heavily relied on spot sampling followed by instrumental measurements to determine pollutant concentrations. These procedures can be advantageous; however, it presents limitations in terms of temporal and spatial variation that may be achieved at reasonable costs and the information on bioavailability that might be obtained. Therefore, a successful implementation of the WFD across the EU member states relies on the establishment and use of emerging and low-cost tools as part of monitoring programmes as a complement , providing additional information with the aim to obtain a more representative perspective of water bodies quality (Allan et al., 2006)

When implementing the WFD, the deadlines for each of the requirements were set. The key milestones are listed on Table 2.5.

Table 2.5 - Water Framework Directive milestones (EC, 2019)

Year	Issue	Reference
2000	Directive entered into force	Art. 25
2003	Transposition in national legislation Identification of River Basin Districts and Authorities	Art. 23 Art. 3
2004	Characterisation of river basin: pressure, impacts and economic analysis	Art. 5
2006	Establishment of monitoring network Beginning of public consultation	Art. 8 Art. 14
2008	Presentation of draft river basin management plan	Art. 13
2009	Finalisation of river basin management plan, including programme of measures	Art. 13 & 11
2010	Introduction of pricing policies	Art. 9
2012	Development of operational programme of measures	Art 11.
2015	Meeting of environmental objectives End of first management cycle Second river basin management plan and first flood risk management plan	Art. 4
2021	Ending of second management cycle	Art. 4 & 13
2027	Ending of third management cycle, final deadline for meeting objectives	Art. 4 & 13

Despite the effort invested in the coordination of the WFD implementation across the EU Member States and the strict timetable, the implementation process has shown to be challenging towards achieving the WFD objectives and improving ecological status of waters in Europe has been slow across all the Member States. In 2015, nearly half of the EU surface waters did not reach good ecological status and the chemical status of 40% of EU water bodies was unknown. Additionally, 73 infringement cases on non-implementation of water legislation against Member States were open (Giakoumis & Voulvoulis, 2018).

2.4. Legislation and water quality control in Portugal

Wastewater effluent discharges into the receiving environment, under regular operating conditions, are regulated using ELV as set in the reference legislation, the Decree-Law nº 152/97 of 19th of June (changed by D.L nº 384/98 of the 9th of November, D.L nº 149/2004 of 22nd of June and D.L. nº 198/2008 of 8th of October) and the Decree-Law nº 238/98 of 1st of August. All discharge licenses, also called water use licenses, are issued by the Portuguese Environment Agency (APA) (Perdigão, 2018).

D.L. nº 152/97 from 19th of June defines the physical and chemical requirements, namely BOD_5, COD, TSS, total nitrogen and total phosphorus, for domestic wastewater treatment plants effluents after secondary treatment. ELV for parameters such as pH, temperature and colour can be found in D.L. nº 236/98 from 1st of August. Table 2.6 presents some of the ELV for physical and chemical parameters of secondary wastewater effluents, according to the reference legislation Table 2.6.

Table 2.6 - Emission limit values for physical and chemical parameters of wastewaters (Source: D.L n.º 152/97 de 19 de Junho, 1997)

Parameters	**ELV**	**Minimum percentage reduction(1)**
pH	6,0-9,0	-
Temperature	Increase in 3ºC (2)	-
Colour	Not visible in a 1:20 dilution	-
BOD_5 at 20ºC	25 mg/L O_2	70-90 %
COD	125 mg/L O_2	75 %
TSS	35 mg/L (3)	90 % (3)
Total nitrogen	15 mg/L N	80 %
Total phosphorus	2 mg/L P	70-80

(1)Reduction in effluent load; (2) Temperature in the receptive body after the effluent discharge, measured 30 m downstream the discharge location(3) Optional; BOD_5- Five-day Biochemical oxygen demand; COD – Chemical oxygen demand; ELV – Emission limit values; TSS – Total suspended solids.

The discharge requirements stated in D.L. nº236/98 of 1st of August can be more demanding when the following situations are observed:

a) The receptive environment is classified as a sensitive zone, according to the D.L. nº 152/97 from 19th of June;
b) When the pollution caused by the effluent discharged is prone to cause long range or transboundary effects;

c) The receptive environment is classified as a vulnerable zone according to the D.L. nº 235/97 of 3rd of September;
d) The receptor body is considered a species and habitat protection area to which the maintenance or increase of the water quality is an important conservation factor;
e) The receptor environment is classified as National Agricultural Reserve according to D-L-nº 196/89 from 14th of June.

Microbiological parameters are not contemplated in the reference legislation regarding the wastewater effluents discharges in water masses. However, D.L. nº 236/98 defines some microbiological parameters and their respective recommended maximum value (RMV) and maximum allowable value (MAW), regarding bathing water quality (Annex XV) and irrigation water quality (Annex XVI).

Table 2.7 presents the RMV and MAV for the microbiological parameters (total and faecal coliforms) in bathing waters as set in the D.L. nº 236/98 of 1st of August.

Table 2.7 - RMV and MAV for total and faecal coliforms regarding bathing waters

Parameters	**RMV**	**MAV**
Total coliforms (MPN/100 ml)	500	10 000
Faecal coliforms (MPN/100 ml)	100	2000

RMV - Recommended maximum value; MAV - Maximum allowable value; MPN – Most probable number.

Disinfected wastewater can have several reuse destinations, such as irrigation, car washes, agriculture and industrial reuse (Angelakis & Bontoux, 2001). D.L. nº 119/2019 of 21st of August (Annex I) establishes quality classes and the physical, chemical and microbiological limit values for each class. Table 2.8 describes watering quality classes and the respective minimal level of treatment required.

Table 2.8 - Watering quality classes and respective treatment level required (Source: D.L. nº 119/2019 of 21st of August)

Class	Possible uses	Level of treatment
A	Watering without access restriction (urban and agricultural uses): watering of crops for raw consumption in which the edible part is in direct contact with the water; public and private gardens watering.	More advanced than secondary treatment (disinfection).
B	Watering with access restriction (urban and agricultural uses): watering of crops for raw consumption that grow above ground level in which the edible part is not in direct contact with the water; watering of crops intended for food processing and crops not intended for human consumption, including crops for animal consumption; watering of with access restriction, including leisure and sports areas.	
C	Watering with access restriction (agricultural use): watering of crops for raw consumption , that grow above the soil, in which the edible part is in direct contact with the water; watering of crops intended for food processing and crops not intended for human consumption, including crops for animal consumption.	
D	Watering with access restriction (agricultural use): seed productions, including seeds for industrial use or energy production.	
E	Watering with access restriction (agricultural use): seed production; watering of areas of naturally restricted use. (e.g., hedgerows, containment areas).	

Table 2.9 presents the water quality standards for water reuse for watering purposes according to the respective quality class.

Table 2.9 - Water quality norms for watering reuse (Source: D.L. nº 119/2019 of 21st of August)

Quality class	**BOD ($mg.L^{-1}$)**	**TSS ($mg.L^{-1}$)**	**Turbidity (NTU)**	***E. coli* (cfu/100 mL)**	**Intestinal parasite eggs ($Nº.L^{-1}$)**	**Total N ($mg.L^{-1}$)**	**Total P ($mg.L^{-1}$)**
A	≤10	≤10	≤5	≤10	-		
B	≤25	≤35	-	≤100	-		
C	≤25	≤35	-	≤1000	≤1	15	5
D	≤25	≤35	-	≤10000	≤1		
E	≤40	≤60	-	≤10000	-		

BOD - Biochemical oxygen demand; cfu - Colony forming units; NTU - Nephelometric Turbidity Units; TSS – Total suspended solids.

2.5. Ecotoxicology and ecotoxicological tests

Ecotoxicology can be defined as the science created to solve problems regarding contamination by natural and synthetic compounds, and its effects on the species as well as all the ecosystems, enabling the prediction of biological responses to the toxicity of chemical compounds, and the evaluation of the ecotoxicological disturbance of the environments, through concentration-effect and concentration-response curves (Hoffman *et al.*, 2003). Thus, aquatic toxicology intends to study the effects of toxic agents on aquatic organisms at cellular, individual, populational and community levels (Magalhães & Ferrão-Filho, 2008).

The pollution of aquatic ecosystems has been increasing due to the industrial development in the last decades. The major issue is the increasing load of domestic and industrial wastes poured into rivers and marine ecosystems. Thus, the assessment of biological effects of effluent discharges in water masses is today considered relevant, and bioassays identifying the ecological hazard have been shown to be useful and increasingly used in several jurisdictions, aiming at the management and reduction of water pollution (Mendonca et al., 2012; Power & Boumphrey, 2004). The trend for many countries has been to start with chemical hazard-based systems, and then add effluent bioassays and receiving environment evaluations to predict or measure impacts at the ecosystem level (Garric et al. , 1993).

In the early 80's, environmental agencies worldwide, mostly in the United States and Europe, began to develop and implement standardized toxicity tests using aquatic organisms. The "Clean Water Act" is considered a regulatory mark that granted the United States Environmental Protection Agency (USEPA) the authority to implement pollution control programmes and quality standards for all surface waters contaminants. In parallel, USEPA has also established biological monitoring, or biomonitoring, i.e. the use of organisms in order to monitor water quality parameters. On the other hand, the Organisation for Economic Co-operation and Development (OECD) put forth a series of tests and protocols regarding the use of aquatic organisms in ecotoxicological studies, such as algae, crustaceans and fish (Magalhães & Ferrão-Filho, 2008).

Overall, European Union member states do not apply whole wastewater treatment bioassays on a routine regulatory basis (with Germany as an exception), although many countries aim to take that direction. Therefore, toxicity tests have been implemented in some countries in order to assess the impact of domestic and industrial wastewater discharges and establish ELV for aquatic ecosystems protection (Power & Boumphrey, 2004).

The state of current regulatory use of effluent toxicity tests in some of the EU member states, as well as other countries, are summarized in Table 2.10.

Table 2.10 - Summary of current regulatory use of effluent bioassays in several countries (Source: Power & Boumphrey, 2004; Agriculture and Resource Management Council of Australia and New Zealand, 2000)

Country	Experience of applying effluent bioassays approaches	Bioassay endpoints	Phyla used
Australia	Broad experience	Acute and chronic	Algae, invertebrates, fish (selection depends on site-specific factors)
Belgium	Some specific research studies on industrial effluents	Acute and chronic	Algae, invertebrates, bacteria.
Canada	No information	Acute and sublethal	Algae, macrophyte, invertebrate and fish
Denmark	Characterization survey covering 23 industries and a few specific research studies	Acute and chronic	Algae, plants invertebrates, bacteria
France	Routine monitoring and occasional use in licensing	Acute, chronic and mutagenicity	Algae, fish, invertebrates, bacteria
Germany	Standardised tests routinely used since 1976. Acute fish tests used as basis for taxation. Regulatory and research and development testing undertaken	Acute, chronic, genotoxicity, mutagenotoxicity	Algae, plants, invertebrates, fish, bacteria
New Zealand	Broad experience	Acute, sub-lethal and chronic	Algae, invertebrates, fish (selection depends on site-specific factors)
Northern Ireland	Bioassays commonly used for characterization, licensing and monitorization purposes	Acute and chronic	Algae, plants, invertebrates, fish, bacteria (at least 4 species)
Norway	Bioassays used in characterization and licensing of industrial effluents on case-by-case basis	No information	Algae, plants, invertebrates, fish

Country	Experience of applying effluent bioassays approaches	Bioassay endpoints	Phyla used
Spain	Some specific research studies on industrial wastewater. Experience in regulatory use of Daphnid and *Vibrio fischeri* tests in wastewater control	Acute	Algae (less frequent), invertebrates, bacteria
Sweden	Bioassays are used in licensing effluents and are a part of the Characterisation Industrial Discharges (CID) guidelines since 1989	Acute, chronic, mutagenicity, enzymatic (plus physiological and morphological for field bioassays	Plants, algae, invertebrates, fish, bacteria
The Netherlands	No legislative compulsion for bioassay use. Studies undertaken on industry sectors and frameworks developed	Acute, chronic, mutahenicity, enzymatic, estrogenic effects	Algae, plants, invertebrates, fish
United Kingdom	No regulatory requirement for bioassays use nationally, however some local use for regulatory compliance monitoring; research and development including a coloborative Government/industry demonstration programme. Bioassays for receiving water monitoring of coastal waters	Acute and chronic	Algae, plants, invertebrates, fish, bacteria
United States	No information	Acute and chronic	Algae, invertebrates and fish.

Regarding the management framework, most countries are primarily focused on the source control, although other frameworks have been increasingly studied and developed regarding the receiving environment monitoring with bioassays (Power & Boumphrey, 2004; Australian and New Zealand Environment and Conservation Council, 2000).

In Portugal, like in other EU member states, a legal basis has been implemented regarding the use of toxicity tests in the risk assessment of certain contaminants (e.g., the use of pesticides in agriculture). However, there is no legislation available on bioassays to be used for wastewater treatment effluents monitoring so that most of the existing regulatory framework is merely based on the establishment of ELV for quality control of effluent discharges (Amaro, 2006; Mendonca et al., 2012).

WWTP's are unable to eliminate residues completely – it can only attempt to decrease pollution levels to an acceptable value, therefore it is important to assess if the treatment processes in WWTP are sufficient to provide a satisfactory decrease of environmental impact on the species living in the receiving ecosystem (Gargosova & Urminska, 2017). Physical and chemical analysis can assess many of the pollutants, but it is hardly possible to identify all the compounds that can be present in the wastewater, often in very low concentrations. Additionally, the identification of the compounds does not provide enough information regarding their effects on the environment, specially concerning the possibility of combined effects (additive, synergistic or antagonistic) of the toxicant mixtures. Furthermore, the possible degradation of pollutants to metabolites can lead to an increase or decrease in toxicity, which is also difficult to detect only by chemical analysis. Thus, toxicity assays can be an important supplement to chemical analyses in the evaluation of the negative effects of wastewater discharges to the ecosystems (Bundschuh et al., 2011)

Whole effluent tests include the evaluation of the synergetic, antagonistic and additive effects of all the chemical, physical and biological components which may have a negative effect on the physiological and biological functions of the test organisms. Because the aggregated toxicity of all components of the wastewater effluent is determined, the toxic effect can be limited by only limiting the effluent toxicity, allowing the comparison of effluent toxicity with site specific water quality criteria designed to protect representative and sensitive species, as well as establish discharge limitations that will protect and improve aquatic environments (Metcalf & Eddy, 2003; Silva et al., 2009).

2.5.1. Toxicity tests

Toxicity tests are based on the principle that the response of living organisms to the exposure of toxic agents depends on the dose of the toxic agent. Therefore, aquatic toxicity tests are designed to describe a concentration-response relationship (concentration-response curve). Acute toxicity tests usually evaluate the concentration-response relationship for survival, whereas chronic tests evaluate sublethal effects such as growth, reproduction and behaviour (Hoffman et al., 2003).

The type of test to be performed must be chosen according to the test purpose, the test organism requirements, the available resources and the toxic agent characteristics. Some organizations working on environmental protection, e.g. OECD, USEPA, Environment Canada or the International Organization for Standardization (ISO), have been developing and implementing standardized toxicity tests. The standardization of these tests is advantageous since they allow the comparation of data obtained from different laboratories (Costa et al., 2008).

Acute toxicity tests

Acute toxicity tests evaluate the effects on an organism after a single or multiple exposure during a short period of time, in view of the species life span. Usually, the endpoint of acute toxicity tests is mortality. However, other non-lethal manifestations can also be studied, such as immobility or growth inhibition (Costa et al., 2008).

The main goal of these tests is to determine the median effect concentration (EC_{50}), which represents the concentration of a certain toxic compound where the response (immobility, growth inhibition and inhibition of luminescence, among others) is obtained for 50% of the test population, and generally last from 0 to 96 hours (Magalhães & Ferrão-Filho, 2008).

Acute toxicity tests with aquatic organisms are broadly used to study the potential toxic effect of several pollutants brought by different sources, from pesticides from intensive agricultural to oil spills and domestic and industrial wastewater discharges, allowing the assessment of the efficiency of wastewater treatment plants (Walker et al., 2001; Magalhães & Ferrão-Filho, 2008).

According to Magalhães & Ferrão-Filho (2008), these toxicity tests can present some limitations, such as:

i. They cannot determine the increase in mortality after the exposure, or access the adverse effects when the latency period is longer than the usual duration of the test;
ii. They only evaluate the toxicity to one single species at the time. In a multispecies context as under real-word conditions, the toxic compound can be transferred through the food chain, leading to bioaccumulation and biomagnification. Additionally, species interactions such as competition and predation can increase the levels of stress on the organism, resulting in higher sensibility;
iii. Only one life stage is tested, while the sensitivity of the organism “in other life stages may be greater;
iv. Different species from the same biological community have different levels of sensibility to a certain pollutant;
v. Sublethal effects that can lead to death of the organism due to prolonged exposure are not considered.

Chronic toxicity tests

Chronic toxicity tests are used to measure the effects of chemical substances on aquatic species for a time period that can comprise a larger part to the entire life cycle of the test-organism. The absence of toxic effects on the organism in acute toxicity tests does not mean that the substance is not toxic for the species after prolonged exposure. Thus, chronic toxicity tests allow to access possible toxic effects of certain substances under prolonged exposures and sublethal concentrations that allow the organism´s survival, but compromise some of its biological functions (Costa et al., 2008).

Chronic toxicity tests depend directly from results acquired from acute toxicity tests, once sublethal concentrations are determined from the EC_{50} value. These tests are usually more sensitive to the expected dilution in environmental samples than acute toxicity tests (Magalhães & Filho, 2008).

Chronic intoxication can have two different causes: the accumulation of the toxic pollutant in the organism, when the absorbance rate is higher than the elimination rate, and the sum of the effects caused by repeated exposures, without the accumulation of the toxic substance in the organism.

In aquatic ecosystems, chronic effects can be more frequent due to the dilution of xenobiotics in the water mass, the chemical reaction between different substances, the quick association from most of the solid particles to organic matter and the sedimentation of suspended solids. Thus, the organisms are exposed to low concentrations of certain pollutants during long periods of time, resulting in chronic sublethal or even lethal effects over time. These tests are also used when acute toxicity tests are not enough to characterize a measurable toxic effect (Magalhães & Ferrão-Filho, 2008).

Static tests

In static tests, the test solution is not replaced throughout the entire duration of the test. This kind of exposure system is appropriate when the concentrations are expected to remain within 80-120% of the initial concentration over the exposure period. The minimum requirement for static tests is the chemical analysis of the highest and lowest test concentration and a concentration around the expected test endpoint. In some cases, where it is expected to observe some variability, it is recommended to measure test concentrations midway through the test (OECD, 2018; Walker et al., 2001).

These tests present some advantages, for instance, they are simple and have low costs, requiring few resources such as manpower, space and equipment. They are generally recommended for some test species (e.g. algae), when the organism would easily be lost if static-renewal or flow-through systems would be used. On the other hand, the decrease in dissolved oxygen (DO) is one of the biggest disadvantages of static tests, which can lead to the increase on COD and BOD. It can also cause the loss of toxic substances through e.g. adsorption and/or volatilization, resulting in a decrease in the apparent toxicity (OECD, 2018; USEPA, 2002).

Static-renewal tests

In static renewal tests the test solution is periodically replaced on a batch basis, and the test organisms are exposed to a fresh solution of the sample always with the same concentration every 24h (or any other prescribed interval). The test organisms can either be transferred from one test chamber to another, or by replacing a portion or all the test solution in the test chambers (OECD, 2018; USEPA, 2002).

These tests have several advantages, such as the reduced possibility of DO depletion caused by high COD or BOD or health effects from metabolic wastes from the test organisms in the solution, the reduced possibility of the decreasing concentrations of toxic compounds through absorption and/or volatilization, and the fact that the organisms (that rapidly deplete energy reserves) are fed when the test solutions are renewed, maintaining them in a healthier state. The disadvantages of static-renewal tests are the requirement of a greater volume of test solution (e.g. effluent) and a smaller chance of considering temporal variations that may occur in waste properties (USEPA, 2002).

Continuous flow-through tests

The continuous flow-through tests are characterized by a constant replacement of the test solution throughout the entire duration of the test. These tests are recommended when concentrations are expected to decline by more than 20% over the experimental period in static or static-renewal tests. Continuous flow-through tests present several advantages, for example an easier maintenance of the DO concentrations in the test chambers, the reduction of the possibility of toxicant loss due to volatilization, adsorption, degradation and/or uptake and a more representative evaluation of the toxicity of the test solution. On the other hand, flow-through tests require larger volumes of sample and dilution water, are more complex and require expensive equipment and more space, making it difficult to perform multiple or overlapping sequential tests. (OECD, 2018; USEPA, 2002).

2.5.2. Toxicological endpoints

Toxicological endpoints are values obtained from toxicity tests that result from specific measurements made during and/or at the conclusion of the test, allowing to quantify the effect of a toxic agent on a given individual, population or community. Endpoints can be divided into two broad categories: assessment endpoints and measure of effects. Assessment endpoints are related to the population, community or ecosystem that is to be preserved, for example the population growth rate or the sustainable yield. On the other hand, measures of effects refer to variables measured, commonly at an individual level, that are used to evaluate the assessment endpoints. These can often include descriptions of the effects of toxic agents on survival, growth and reproduction. Other measures of effect include descriptions of effects on a community level (for example, respiration, photosynthesis and diversity) or even on a cellular level such as physiological and histopathological effects (Hoffman et al., 2003).

The parameters typically used to define acute toxicity are the median lethal concentration (LC_{50}) and the median effective concentration (EC_{50}). These parameters aim to determine the dose (or the concentration in food, air or water) which will cause a toxic response to 50% of the test population, for example, LC_{50} is normally used when mortality is the endpoint, whereas EC_{50} is used when a sublethal effect (such as immobilization, fatigue or avoidance) is the endpoint (Metcalf & Eddy, 2003; Walker et al., 2001).

The results of chronic toxicity are often analysed statistically to determine the lowest-observed-effect concentration (LOEC), the no-observed-effect concentration (NOEC) and the chronic value (ChV). The LOEC and NOEC parameters can only be determined when a higher dose or concentration has produced an effect ,and do not provide confidence intervals of its estimates. On the other hand, ChV is usually used interchangeably with the maximum acceptable toxicant concentration (MATC), which is obtained through the geometric mean of the NOEC and the LOEC. The endpoints include all the parameters of interest (eg. egg hatchability, length, weight, behaviour, number of neonates/number of neonates produced per adult, physiological effects and survival). Similar to acute toxicity data, lethal concentration (LC) or effective concentration (EC), usually EC_{10}

or LC_{10}, can also be used with chronic toxicity data to describe chronic tolerance levels (Warne & Dam, 2008; Walker et al. 2001, Murado & Prieto, 2013; Hoffman et al., 2003).

Table 2.11 summarizes the definition and exposure time for some of the toxicity parameters.

Table 2.11 - Definition and exposure time of some toxicity parameters (source: Costa & Olivi, 2008)

Parameter	Definition	Exposure time
LD50	Dose of a sample required to kill 50% of the members of the tested population after being submitted to the test conditions for a certain exposure time.	24 to 96 hours
LC50	Concentration of a sample required to kill 50% of the members of the tested population after being submitted to the test conditions for a certain exposure time.	24 to 96 hours
EC50	Concentration of a sample necessary to induce an acute effect in 50% of the test organisms after submitted to the test conditions for a certain exposure time.	24 to 48 hours
LOEC	Lowest concentration of a sample that causes a statically significant toxic effect on the members of the tested population after being submitted the test conditions for a certain exposure time.	7 days
NOEC	Highest concentration of a sample that does not cause a statically significant toxic effect on the members of the tested population after being submitted the test conditions for a specified exposure duration.	7 days

Even though NOEC and LOEC have broadly been used and reported in literature, the use of these parameters has been criticised in recent years, since it has been discussed that the nomenclature might be misleading, the methods by which they are calculated are not the most appropriate and the statistical methods used are not always valid. Therefore, NOEC and LOEC have been replaced by other parameters, for example the inhibition concentration for specific effect sizes such as 50%, 20% and, most commonly, 10% (Warne & Dam, 2008).

The parameters mentioned above are commonly used in aquatic ecotoxicology and are standardized and regulated according to the environment in which the organisms are exposed. Therefore, they must be expressed in the same units as the concentration of the substance or compound present in the ecosystem, which are usually milligrams per liter ($mg.L^{-1}$) or percentage (%) (Costa et al., 2008).

2.5.3. Toxicity tests organisms

Several species have been used internationally in toxicity tests, allowing a better assessment and characterization of the acute and chronic effects of toxic agents in receptor bodies. The most

commonly used organism groups in laboratory tests are invertebrates, fishes, aquatic plants, algae and some bacteria. The sensibility of organisms to its surrounding environment manifests at a biochemical, physiological, morphological and/or behavioural level, and depends on age, sex, nutritional level, development phase, genetics and intra/interspecies competition, as well as environmental factors such as temperature and luminosity (Magalhães & Ferrão-Filho, 2008; Tothill & Turner, 1996).

The selection of the test organism must consider certain criteria, such as abundance and availability, ecological representativeness, knowledge regarding its biology, physiology and dietary patterns, the population uniformity, constant sensibility, commercial relevance and simplicity regarding laboratory cultivation and handling. Preferably, the organism species should also be native for a better representativeness of the ecosystem (Magalhães & Ferrão-Filho, 2008; Kenaga, 1978). Below, test organisms most commonly used in aquatic toxicity testing based on these criteria are discussed for each of the taxonomic groups (bacteria, algae, aquatic plants, invertebrates and fish).

Bacteria

The use of animals for toxicity testing has been criticized in the past years for ethical reasons. Thus, techniques using bacteria have been developed and proposed as alternatives for some animal toxicity assays (ECETOC, 2005; Costa et al., 2008). The most studied test parameter evaluated in bioassays with bacteria is bioluminescence that can be observed in some bacteria species. All luminescent bacteria tests are conducted using the Microtox Toxicity Analyzer. This test measures the reduction of the luminescence naturally irradiated by the bacteria when in contact with a toxic agent. *Vibrio fischeri* (Figure 2.1.) is a marine gram-negative bacterium with bioluminescent properties, being the most commonly used species in toxicity test with luminescent bacteria. The determination of the inhibitory effect of water samples on the light emission of *V. fischeri* has been standardized (ISO 11348-3:2007). Another endpoint that needs to be considered is the metabolic inhibition. Some of the species used in these tests are *Escherichia coli* and *Pseudomonas putida* (Bulich et al.,1990; Hwang et al., 2008).

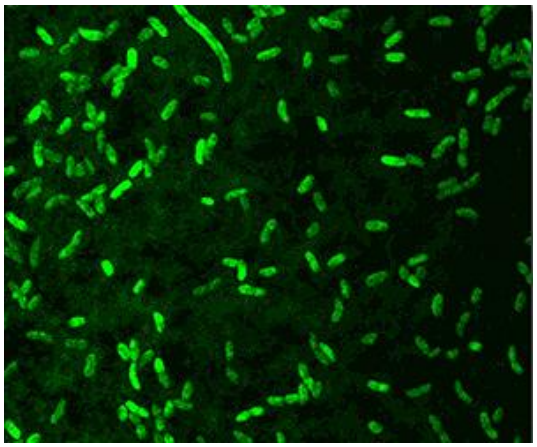

Figure 2.1 – *V. fischeri* (Source: Microbelog, 2012)

Toxicity tests using bacteria are advantageous since they are quick, sensitive, cheap and easy to perform. On the other hand, they have as disadvantages the interference of turbidity in luminescence, the eventual need for pH adjustment, the requirement of sample salinity addition for freshwater samples, which may affect bioavailability and a variation in the range of sensitivity for reference substances depending on the preparation of the bacteria (freshly prepared, freeze or liquid-dried) (Costa et al., 2008; ECETOC, 2005).

Algae

Growth inhibition tests with freshwater algae are recommended by the OECD and have been increasingly used in bioassay test batteries for environmental management of wastewater and leachates discharges, since algae are primary producers, and any change in this trophic level will disrupt the remaining levels of the food chain. Algal toxicity tests are relatively simple and inexpensive, and, although they are short-term tests, they can be considered at the same time both chronic and sublethal, since algae are short-lived, being conducted with several generations of individuals in large numbers. Some of the interferences and limitations of bioassays with algae are the influence of nutrients, which may cause an accelerated growth of the organism, the presence of particles which may interfere with the growth measurement and the presence of EDTA, which is a normal constituent of the test medium, but may interfere with the bioavailability of metals (ECETOC, 2005; Mohan, 1999, Nyhom & Kalqvist, 1989).

Examples of standard freshwater algae used in toxicity tests are the green algae species *Chlorella vulgaris (*Figure 2.2) and *Raphidocelis subcapitata* (Figure 2.3), considering that they have a fast reproduction rate and are easily cultivated in a laboratory (Costa et al., 2008). However, diatoms (e.g. *Navicula pelliculosa*) and cyanobacteria (*Anabaena flos-aquae*) have also frequently been used and recommended (e.g. EFSA, 2013).

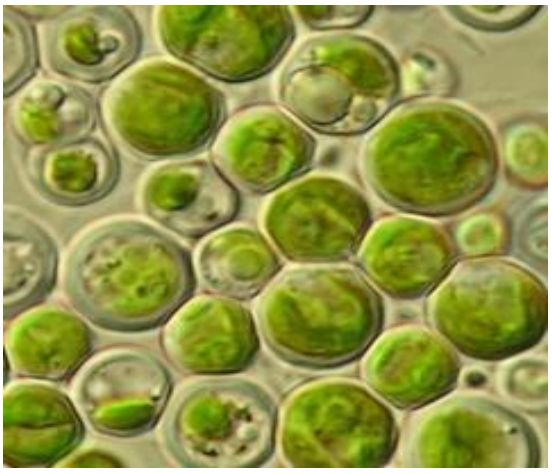

Figure 2.2 – *C. vulgaris* (Algae research and supply, 2019)

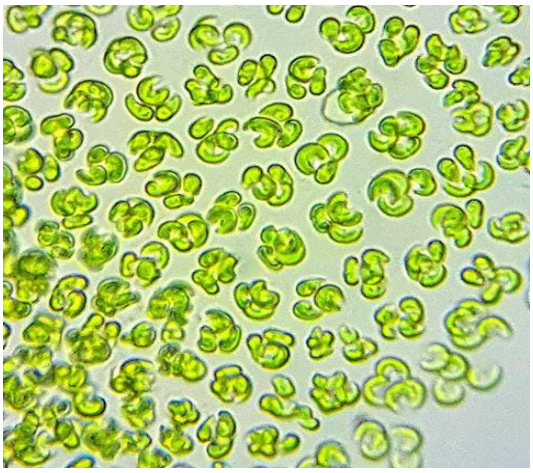

Figure 2.3 – *R. subcapitata* (Source: Göttingen, 2019)

Aquatic plants

The use of aquatic plants (macrophytes) in water quality assessments has been common and shown to be effective in detecting toxic agents in aquatic environments. Plants are primary producers and an essential component of a balanced ecosystem. Additionally, aquatic plants perform important ecological functions such as nutrients removal (Mohan & Hosseti, 1999).

Some of the advantages of the use of aquatic plants as test organisms for toxicity tests are usually their small size and simple structure (in the case of floating plants that are most commonly used, c.f. next paragraph), allowing an easy laboratory handling, fast reproduction rate and genetically homogenous populations, as well as a fast growth rate and high surface area to volume ratio. In similarity with bioassays using algae, a high concentration of nutrients and the presence of EDTA may affect the optimal development of toxicity tests using aquatic plants (ECETOC, 2005; OECD, 2019).

The aquatic floating plants of the genus *Lemna* (commonly called duckweed) are the most frequently used aquatic plant in ecotoxicology, especially *Lemna minor* (Figure 2.4) and *Lemna gibba.* Their main form of reproduction is vegetative propagation, where the frond primordia starts to develop and grow out of the pockets of mother plants. Toxicity tests using the genus *Lemna* have been standardized and aim to quantify substance related effects on vegetative growth over the course of the test (Appenroth et al., 2013; OECD, 2006). In recent years, however, the sole use of *Lemna* has been disputed since sediment-rooting macrophytes may be more sensitive than *Lemna* to certain pollutants, such as herbicides with a specific mode of action (EFSA, 2013).

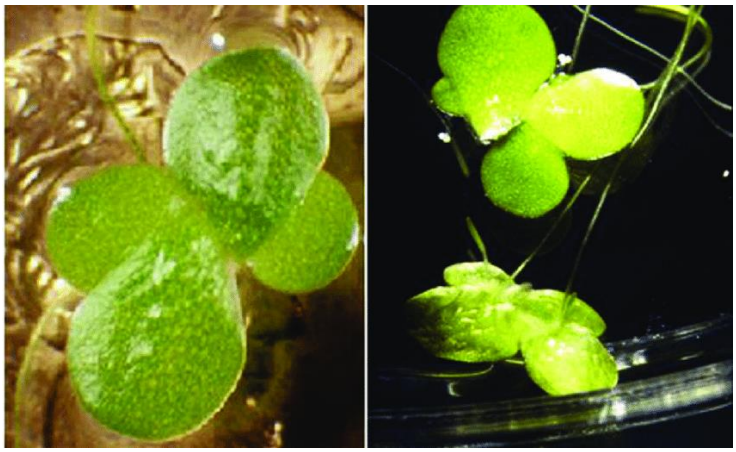

Figure 2.4 – *L. minor* (Source: Everett et al., 2012)

Invertebrates

Invertebrates have been widely used in the assessment of toxic effects of pollutants to aquatic ecosystems. The mostly used organism to evaluate toxicity of water and wastewater treatment effluents is *Daphnia magna*. *D. magna* is a planktonic crustacean that belongs to the Daphniidae family and the Branchiopoda class. It is commonly called water flea and can be found in almost any permanent temperate water body, from big lakes to small temporary ponds. They are usually the dominant zooplankter, being the predominant food for planktivorous fish, which represents an essential part of the food chain in these habitats (Ebert, 2005; Tatarazako & Oda, 2007).

The body-plan of daphnids features a short, segmented body and a compressed carapace that partly encloses the phyllopods (several pairs of flattened limbs). Daphnids are suspension feeders, feeding on small particles suspended in the water. The phyllopods beat in a co-ordinated rhythm, generating a current within the carapace chamber and allowing the food particles to be channelled to the animal's mouth. Even though this feeding system is so efficient that even bacteria can be collected, their food mainly consists of planktonic algae (Ebert, 2005; Lampert, 2011). Figure 2.5 presents a microscopic picture of a Daphnid

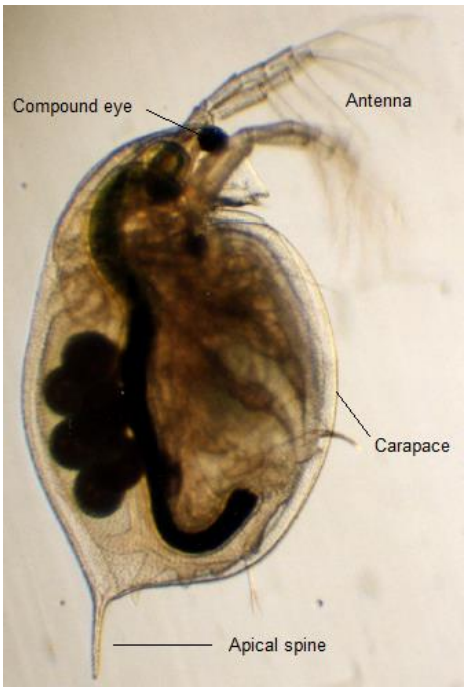

Figure 2.5 - Microscopic picture of a daphnid (Source: Ebert , 2005)

D. magna is a cyclical parthenogen, being able to reproduce both by parthenogenis and sexual reproduction. During the parthenogenic cycle, females produce either diploid eggs that will directly develop into daughters, or diploid asexual eggs that will develop into sons. The same female can produce haploid eggs that require fertilization by males. After the fertilization, these eggs are enclosed in a protective shell (*ephippia*) and undergo a diapause phase that eventually results in female offspring (Ebert, 2005;Tatarazako & Oda, 2007). The induction of sexuality seems to be caused by environmental stress, for example, increased competition and reduced food availability. Abiotic factors such as decreased day length and sharp temperature variations also seem to play a role in the transition from asexual to sexual reproduction in daphnids (Enserink et al., 1990). The life cycle of a parthenogenic daphnid is described in Figure 2.6.

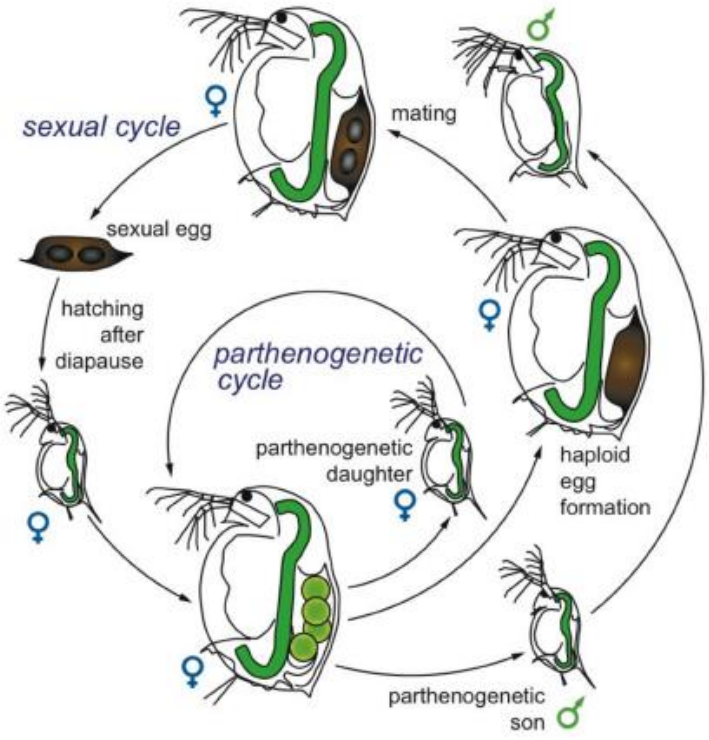

Figure 2.6 - Life cycle of a parthenogenic daphnid (Source: Ebert , 2005)

Daphnids are very sensitive organism and their health is easily affected by changes in diet or environment conditions, showing sensitivity to a variety of contaminants. Additionally, its short doubling time and simplicity regarding laboratory handling make *D. magna* one the mostly used species in ecotoxicity tests (Hoffman et al., 2003; Movahedian & Bina, 2005). On the other hand, the use of daphnids in bioassays, as well as other invertebrates, presents as disadvantages the difficulty in counting the organism in turbid or coloured water, and surfactant effects in effluents containing surface-active materials (e.g. clumping or flotation of organisms), which may cause physical effects and subsequent death (although means exist to minimise this effect) (ECETOC, 2005).

Acute lethality tests with *D. magna* are well established and standardized (DAPHTOXKIT F magna and ISO 6431:2012,OECD, 2004). In these tests, the organism is exposed to certain toxic agents or aqueous matrices under controlled conditions and living and/or mobile individuals are counted after the required incubation period. Generally, *D. magna* is cultured at a temperature of 20 to 25ºC, under a photoperiod of either 16:8 hours light:darkness or continuous illumination, and the tests should be performed with neonates less than 24 hours old. Various water sources may be acceptable for culturing *D. magna*, including the use of natural (uncontaminated) spring water. Chronic toxicity tests (21 days) with daphnids have also been performed (Hoffman et al., 2003; Rizzo, 2011).

Ceriodaphnia dubia is also commonly used in bioassays in both 48-hour acute and 7-day partial life cycle tests. *C. dubia* is much smaller than *D. magna* and therefore more difficult to see.

However, their fast reproduction rate and may have a slightly greater sensitivity compared to *D. magna* makes them a requested test organism (Hemming et al. , 2002). The techniques used in *C. dubia* toxicity tests are the same as those for *D. magna* and the common endpoints are survival, and survival and reproduction for acute and chronic tests, respectively (Hoffman et al., 2003).

Fish

Acute toxicity tests with fish species aim to assess the mortality due to contamination of natural environments. These tests can be both static and flow-through, depending on the objectives and the resources available. Partial or complete life-cycle (i.e. chronic) toxicity tests involving several or all the development stages are also used in order to establish water quality criteria for aquatic ecosystems, since the sensitivity may vary depending on the life stage of the organism. Some of the standard species used in fish bioassays are *Danio rerio* (Figure 2.7) and *Oncorhynchus mykiss* Figure 2.8) (Lammer et al., 2009; McKim, 2011).

Toxicity tests using fish present some advantages, such as good sensibility. On the other hand, they are very time consuming, involve specialized equipment and operators with adequate skills, require a larger volume of effluent and a larger area. Additionally, animal ethics considerations have been encouraging reduction in the number of chordate organism used in ecotoxicity testing (ECETOC, 2005; Farré & Barceló, 2003).

Figure 2.7 – *D. rerio* (Source:Braunbeck & Lammer, 2006)

Figure 2.8 – *O. mykiss* (Source: Sartore, 2019)

The species to be used in ecotoxicological tests regarding, for example, the risk assessment of the use of pesticides is established by the European Food Safety Authority (EFSA PPR, 2013). The same occurs for the establishment of environmental quality standards in the WFD (EC, 2011). However, the most adequate species to be used for effluent toxicity testing are yet to be officially determined and established.

3. Aim of research

This dissertation work was initiated to address some of the issues discusses above. More specifically, it was aimed at increasing our knowledge on the environmental toxicity of alternative disinfection methods and the test species that should be used to this end. Therefore, the general objectives of this dissertation were:

- To study the toxicity of an effluent disinfected with peracetic acid (PAA) on the crustacean *Daphnia magna;*
- To compare the sensitivity of *D. magna* with the other species commonly used in bioassays;
- To evaluate toxicity testing as a complement to emission limit values (ELV) in the risk assessment of wastewater effluents.

4. Methods

4.1. Case study: Wastewater Treatment Plant of Beirolas

The wastewater treatment plant of Beirolas is designed to serve a population equivalent of 213 510,and to receive a flowrate of 54 500 m^3/day ("Águas do Tejo Atlântico", 2019). A picture of the WWTP is presented in Figure 4.1.

Figure 4.1 – WWTP of Beirolas

The liquid phase is responsible to receive the influent wastewater to the WWTP and convert it to dischargeable wastewater to the receiving waterbody (Tejo River estuary) by means of treatment. It consists of a preliminary treatment, primary treatment, secondary biological treatment and tertiary treatment. The liquid treatment phase is described below by sequential order (Fonte, 2017):

1. Preliminary system that consists of screening, sieving and grit removal. The screening includes a set of grids with different sized mesh, being followed by a sieving process by a Step-Screen type sieving with 6mm aperture.
2. Aerated grit chamber that removes grit and sand before the primary treatment.
3. Primary treatment materialized in with two circular conventional clarifiers, each one with a 32 m diameter and a liquid height of 3.15 m.
4. Equalization following the primary treatment materialized in an equalization tank with a usable volume of 10 230 m^3 and a retention time of 4 hours.
5. Secondary treatment materialized in a biological reactor (divided in three compartments: aerobic, anoxic and anaerobic) conceived to remove carbon, nitrogen and phosphorus, and secondary sedimentation materialized in three conventional clarifiers, each one with a usable volume of 11 725 m^3. The sludge produced in the secondary clarifiers is recycled in

the biological reactor and the excess is purged and carried to the sludge thickening process.

6. Tertiary treatment consisting of a filter, from which the service water is obtained, followed by an UV light unit with a 254 nm wavelength (5 modules with 6 lamps each), that provides disinfection of the treated wastewater.

The solid treatment line is responsible for the sludge treatment, including sludge thickening, stabilization through anaerobic digestion and biogas production, dewater and storage. The sludge treatment phase is described below by sequential order (Fonte, 2017):

1. Sludge thickening materialized in a gravity thickener for primary sludge and mechanical thickener (dissolved air flotation) for secondary sludge, which allows an increase of solid concentration of 2g/L to 30 g/L.
2. Stabilization through an anaerobic digestion process where the primary and secondary sludges are mixed. The sludge is kept in the digestor for 20 days at a constant temperature of 35º C, resulting in a decrease of volatile matter content and biogas production. The biogas produced is stored in a biogas holder and is aimed for electricity production.
3. Sludge dewatering materialized in belt-press filters chemically enhanced with polymer addiction;
4. Sludge chemical stabilization using quicklime with a pH increase to values around 11.5 to12.5.

In Figure 4.2 the current treatment line of both the liquid and solid phases of Beirolas WWTP is visualized.

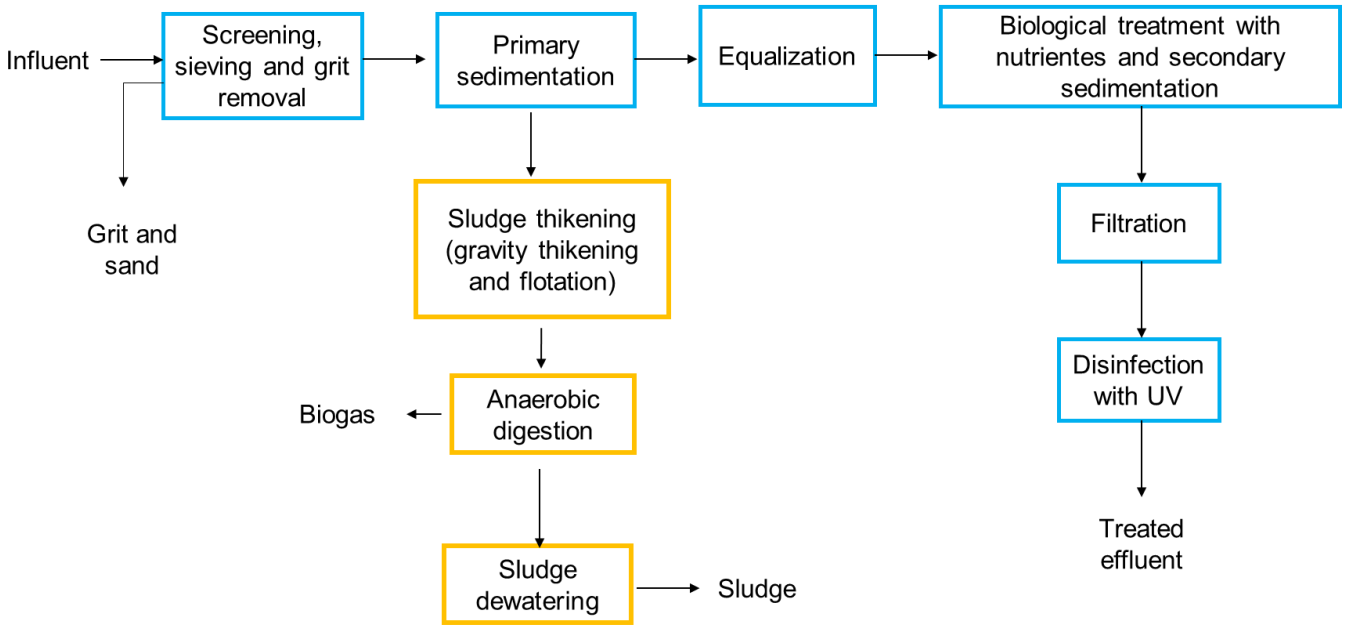

Figure 4.2 - Simplified diagram of Beirolas WWTP treatment system (Source: Fonte, 2017)

4.2. Experimental design of the laboratory test

The laboratory experiment was carried out according to an experimental plan divided in four phases: 1) sampling; 2) secondary effluent characterization; 3) characterization of the effluent treated with peracetic acid; and 4) *D. magna* acute toxicity test. The description of the experimental plan is presented in Table 4.1

Table 4.1 - Experimental plan

Phase	Description
Sampling	Sampling of the secondary effluent at WWTP of Beirolas
Secondary effluent characterization	Physico-chemical analysis: • COD • pH • TSS • Turbidity • Kleldahl nitrogen Microbiological analysis: • Total coliforms • Faecal coliforsms
Characterization of the effluent treated with PAA	Physico-chemical analysis: • COD • pH • TSS • Turbidity • Kleldahl nitrogen Microbiological analysis: • Total coliforms Faecal coliforms
***Daphnia magna* acute toxicity test**	• Reference test with 5 potassium dichromate concentrations and EC_{50} determination • Test with secondary effluent • Test with treated effluent applying 3 peracetic acid concentrations

BOD_5- Five-day Biochemical oxygen demand; COD – Chemical oxygen demand; ELV – Emission limit values; TSS – Total suspended solids.

The physico-chemical and microbiological characterization of both secondary and disinfected effluents was carried out within the framework of another master's dissertation that was conducted in parallel by the student Joana Bettencourt Brito. Her research aimed at evaluating the potential of peracetic acid for disinfection of wastewater effluents (Brito, 2019). After the PAA concentrations with efficacy to disinfect the wastewater were established, the toxicity of these concentrations to *D. magna* was evaluated in the scope of the present dissertation.

4.2.1. Physico-chemical characterization of the wastewater

COD

Chemical oxygen demand (COD) is an indirect method to measure the organic material content in wastewater. The basis for the COD test is that nearly all organic compounds can be fully oxidized to CO_2 with a strong oxidizing agent under acidic conditions.The oxidizing agent is potassium dichromate ($K_2C_r2O_7$) and sulfuric acid (H_2SO_4) is used to achieve the desired acidification conditions. This method is based on the reflux of a sample in strongly acid solution with a known excess of potassium dichromate. After digestion, the remaining unreduced potassium dichromate is titrated with ferrous ammonium sulphate to determine the amount of $K_2C_r2O_7$ consumed and calculate the oxidizable matter in terms of oxygen equivalent (A.P.H.A, 1998).

pH

The principle for electrometric pH measurement is the determination of hydrogen ions activity by potentiometric measurement using a standard hydrogen electrode and a reference electrode (Wtw inoLab pH/ION 735 Manual). The electrode must be calibrated against standard buffer solutions of known pH. Since temperature affects pH results, this parameters must be measured during every pH measurement (A.P.H.A, 1998)

Total Suspended Solids (TSS)

Total suspended solids (TSS) are determined through vacuum filtration of the secondary effluent sample, after which the residue retained at the filter is dried to a constant weight at 105º C for 2 hours. Afterwards, the residue is stretched and weighed, being that the increase in weight of the filter represents the total suspended solids (A.P.H.A, 1998).

Kjeldahl nitrogen

Kjeldahl nitrogen is the sum of organic nitrogen and ammonia nitrogen. In the presence of H_2S0_4, potassium sulphate (K_2SO_4) and a catalyst mixture, amino nitrogen of many organic materials is converted into ammonium, and so is free ammonia. By adding a base, the ammonia may be determined colorimetrically by titration with a standard mineral acid. Thus, the effluent samples were digested and then distilled in a semi-automatic distillation unit (Velp Scientifica UDK 139). Afterwards, the ammonium was measured by titration (A.P.H.A, 1998).

Turbidity

The method used for turbidity measurement is the nephelometric method, which is based on a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions. The higher the intensity of the scattered light, the higher the turbidity. Initially, the sample is shaken to homogenise itafter which the turbidity is measured with the turbidimeter (0-1000 NTU HANNA Fast Tracker HI98703).

4.2.2. Acute toxicity testing with Daphnia magna

The acute toxicity tests with *D. magna* were conducted according to the standard operating procedure 6341 of the International Standardization Organization (ISO 6341:2012) using the Daphtoxkit developed in the Laboratory for Environmental Toxicology and Aquatic Ecology at the Ghent University in Belgium. The acute toxicity test was chosen over a chronic test due to its simplicity, short test duration (c.f. section 2.5.1) and because it was intended to obtain an EC_{50} value.

The Daphtoxkit is used worldwide in toxicity tests and contains *D. magna* dormant eggs (*ephippia*) that are protected with a chitinous capsule (*ephippium*). These eggs can be stored for a long period of time without losing their viability and develop into neonates in about 3 days when placed under specific environmental conditions, which can be immediately used in the toxicity tests.

The Toxkit microbiotests, in comparison with the conventional bioassays, present as a major advantage the incorporation of the test organisms in a dormant form, from which they can be activated at any time according to demand. This eliminates the need for continuous recruitment and stock culturing of test organisms and hence lowers the cost and laboratory handling complexity.

The contents of the Daphtoxkit F *magna* are the following:

- Six 1 ml vials with ephippia, being that each vial contains enough dormant eggs to suffice one full toxicity test;
- Two sets of four small glass bottles with concentrated salt solutions to make up 2 x 2 litres of Standard Freshwater for the preparation of the hatching and toxicant dilution medium.Its composition is:

Vial 1: $NaHCO_3$ (67.75 mg/L)
Vial 2: $CaCl_2.2H_20$ (294mg/L)
Vial 3: $MgSO_4.7H_20$ (123.25 mg/L)
Vial 4: KCl (5.75 mg/L)

- Six polystyrene petri dishes with a 5 cm for the hatching of the *epphipia*;
- Six polycarbonate multiwell test plates composed of 5 rinsing wells and 24 wells for the toxicant solutions;
- Six parafilm strips for sealing the multiwell to minimize evaporation during the tests;

- Six polyethylene micropipettes for transfer of the test organisms;
- A microsieve with 100 µ mesh for the rising of the *ephippia.*

The tests conducted with *D. magna* followed the six steps outlined below.

I. Preparation of Standard Freshwater

The standard freshwater is used both as a hatching medium for the ephippia and as dilution medium for preparation of the toxicant dilution series in the reference test and the test with the effluent. It was prepared with distilled water and a combination of four concentrated salts solutions that come in 4 individual vials (see above).

In order to perform the intended tests, 2 litres of standard freshwater were prepared according to the procedure presented below:

1. A 2000 ml volumetric flask was filled with approximately 1 litre of distilled water;
2. Vial number 1 ($NaHCO_3$) was uncapped and its content poured into the flask;
3. The last operation was repeated for vial 2 ($CaCl_2.2H_20$), vial 3 ($MgSO_4.7H_20$) and vial 4 (KCl)
4. The distilled water was added up to the 2000 mL mark and the flask stoppered and shaken to homogenize the medium.

Once the tests were performed over the course of two weeks, the medium was stored in the refrigerator in darkness to ensure maintenance of its properties.

II. Hatching of the *ephippia*

It takes 3 days in optimal conditions for the embryonic development of *D. magna* eggs to occur. Thus, the hatching of the *ephippia* was initiated 3 days prior to the start of the tests with the effluent. The optimal conditions for this procedure are an illumination of min. 6000 lux and a temperature range of 20-22ºC. The minimum required to perform one complete test is around 120 neonates, and since the organisms should not be older than 24h at the start of the test, the organisms must be collected at the latest 90h after the beginning of the incubation.

The procedure for the hatching is listed below:

1. The content of two vials with *ephippia* were poured into a microsieve;
2. The *ephippia* were thoroughly rinsed with tap water to eliminate all traces of the storage medium.
3. The *ephippia* were transferred into 3 hatching petri dishes with enough volume of pre-aerated standard freshwater to assure that all the *ephippia* were submerged.
4. The hatching petri dishes were covered and incubated in a culture chamber for 72h, at 22,5ºC and a continuous illumination of 6700 lux, 6600 lux and 6400 for the left, middle and right petri dishes, respectively.

This procedure was performed prior to both the reference test and the test with the effluent.

Table 4.2 presents the summary of all the conditions under which the hatching procedure was performed.

Table 4.2 - Summary table with the conditions for the hatching of the ephippia procedure

Parameter	**Value**
Incubation time	72 h
Temperature (mean ± SD)	21.9 ± 1 ºC
Illumination (mean ± SD)	6567 ± 125 lux
Neonates age	<24 h

The hatching petri dishes with the ephippia submerged in the standard freshwater medium are shown in Figure 4.3.

Figure 4.3 - Hatching petri dishes with the ephippia

III. Pre-feeding of the test organisms

The pre-feeding of the neonates prevents mortality by starvation (that could potentially bias the results) in the 48h toxicity test during which the neonates are not fed. Therefore, the organisms are fed a suspension of *Spirulina* for two hours prior to the test.

The pre-feeding of the neonates was performed as follows:

1. Two vials with *Spirulina* powder were filled with standard freshwater;

2. Each vial was thoroughly shaken in order to homogenize the contents;
3. Two hours prior to the collecting of the neonates for the test, the algae suspension was equally poured into the 3 hatching petri dishes and the contents swirled to distribute the food evenly.

This procedure was performed prior to both the reference test and the test with the effluent.

IV. Reference test with potassium dichromate

The performance of a reference test is advised in order to validate the correct execution of the test procedure and sensitivity of the test organisms. The reference toxicant used in this study was potassium dichromate ($K_2Cr_2O_7$). The optimal conditions for this procedure are an illumination of min. 6000 lux and a temperature range of 20-25ºC.

The potassium dichromate reference test was performed as described below:

1. 10 mg of potassium dichromate were weighed on an analytical balance and transferred into a 1000 ml flask which was filled to the mark with distilled water to make up a 10 mg/L toxicant concentration;
2. The following volumes of the potassium dichromate solution from the 1000 ml flask were transferred to five 100 ml flasks:
   - 32 ml to flask 1 (3.2 mg $K_2Cr_2O_7$/L)
   - 18 ml to flask 2 (1.8 mg $K_2Cr_2O_7$/L)
   - 10 ml to flask 3 (1.0 mg $K_2Cr_2O_7$/L)
   - 5.6 ml to flask 4 (0.56 mg $K_2Cr_2O_7$/L)
   - 3.2 ml to flask 5 (0.32 mg $K_2Cr_2O_7$/L)
3. Standard freshwater was added to all flasks up to the mark, the flasks were stoppered and shaken to homogenize the solutions;
4. The multiwell plate was filled with the toxicant solutions with different concentrations and a standard freshwater solution as the control test. Each well was filled with 10 ml of the respective solution;
5. A random amount of pre-fed neonates was transferred from the hatching petri ditches to another dish containing the intended test solution. This step intends to minimize the error due to the increase in dilution because of the medium transferred along with the organisms. Afterwards, five neonates were transferred into each test well.
6. The mobility and mortality of the organisms were monitored and registered after 24h and 48h after the beginning of the test.

Every multiwell plate is provided with 4 test wells for the controls and 4 test wells for each of a maximum of five treatments. Subsequently, in order to obtain statistically acceptable results, each test concentration, as well as the controls must be assayed in 4 replicates. The multiwell plate used in the reference test, as well as the spatial arrangement of the controls and the potassium dichromate solutions with different concentrations can be observed in Figure 4.4.

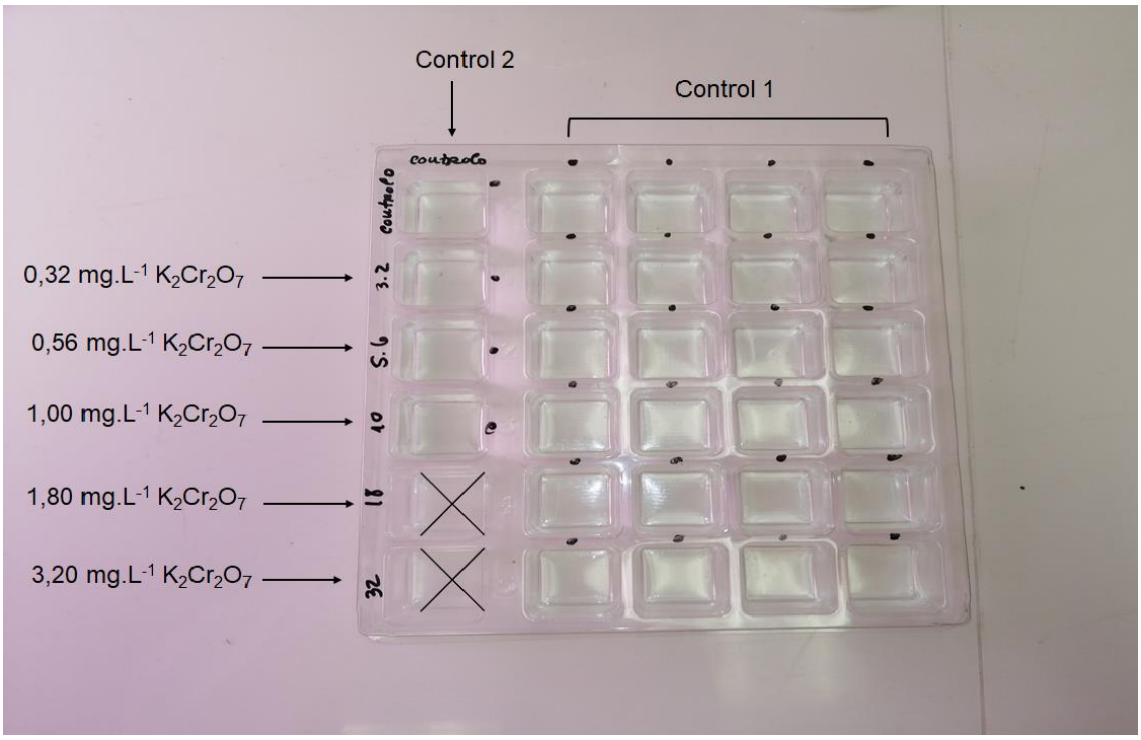

Figure 4.4 - Multiwell plate used in the reference test

The summary of the conditions under which the reference test was performed can be observed in Table 4.3.

Table 4.3 - Summary table with the conditions for the reference test

Parameter	**Test conditions**
Test type	Static
Temperature	22,5 ± 2 ºC
Light intensity	6500 ± 82 lux
Photoperiod	Continuous illumination
Duration	48h
$K_2Cr_2O_7$ concentrations	5 (0.32 mg.L^{-1}, 0.56 mg.L^{-1}, 1.00 mg.L^{-1}, 1.80 mg.L^{-1}, 3,20 mg.L^{-1}) and 2 controls
Number of replicants	4 (control 1, control 2 and each potassium dichromate concentration)
Endpoints	Immobilization and mortality (after 24h and 48h)

V. Effluent test

The effluent test was performed with secondary effluent from the Beirolas WWTP and the same wastewater after disinfection with three different concentrations of PAA (5 mg. L^{-1}, 10 mg. $L^{-1}$and 15 mg. L^{-1}). The concentrations were chosen based on a jar-test made in order to determine the most effective PAA concentrations for disinfection of the wastewater under study (Brito, in prep.). The test conditions in this test were the same as those in the reference test (Table 4.3).

The procedure for the effluent test is listed below:

1. A stock solution of PAA was prepared. The PAA concentration is calculated multiplying the molarity by the molar mass, resulting in 155 903 mg. L^{-1}. This concentration involves very small volumes of PAA, so a dilution was required. Therefore, 10 mL of PAA were diluted in 1000 ml of distilled water, resulting in a PAA stock solution with a concentration of 1559 mg. L^{-1}.
2. In a 1000 ml flask, 3.2 mL PAA stock solution and 1000 mL effluent were added in order to obtain a solution with 5 mg PAA/L. This step was repeated with 6,41 ml PAA to obtain a solution with 10 mg PAA/L and 9.2 ml PAA to get the 15 mg PAA/L test solution.
3. An additional solution with a 15 mg PAA/L was prepared with standard freshwater as dilution solution, with the goal of studying the effect of PAA on *D. magna* without the influence of the effluent. Two control treatments were made with the standard freshwater solution.
4. The filling of the multiwell plate and the transfer of the pre-fed neonates were made in according with the procedure described in points 4 and 5 of the reference test section.
5. The immobility and mortality of the organisms were monitored and registered after 24h and 48h after the beginning of the test. The organism that showed movement after being shaken with a needle were considered immobile, while those that showed no movement after disturbance were considered dead.

The multiwell plate used in the test with the effluent, as well as the spatial arrangement of the controls and the potassium dichromate solutions with different concentrations can be observed in Figure 4.5.

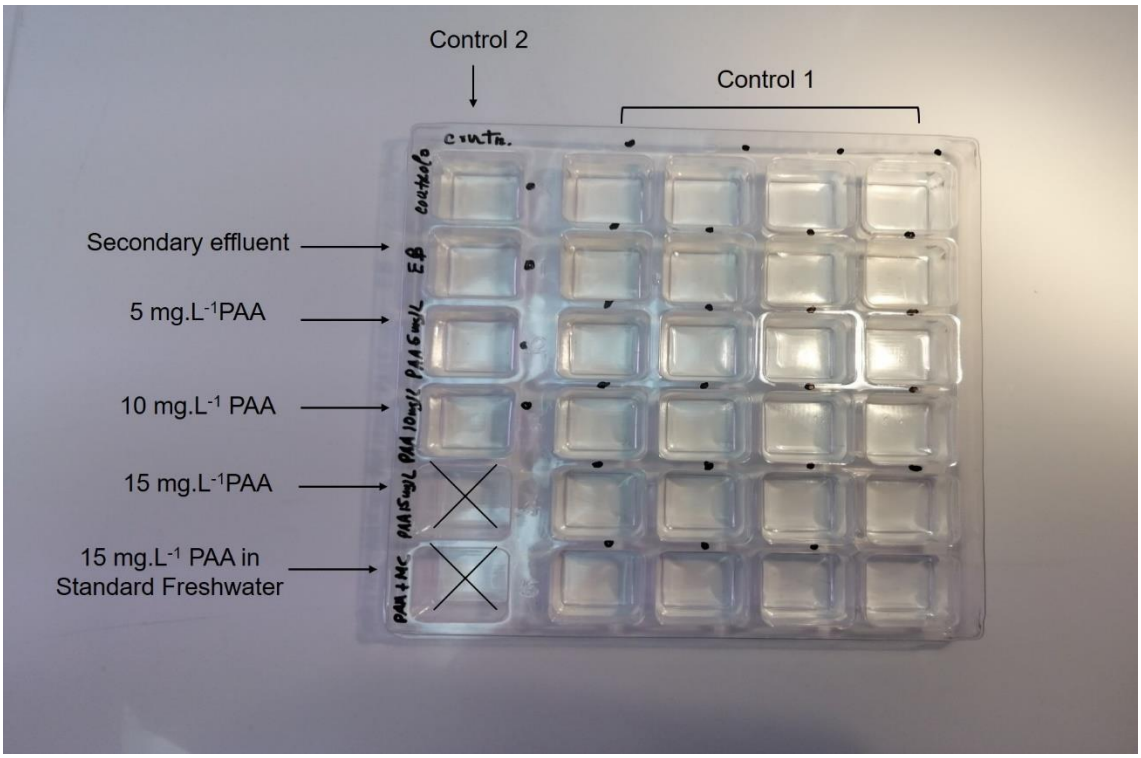

Figure 4.5 - Multiwell used in the test with the effluent

The summary of the conditions under which the test with the effluent was performed is provided in Table 4.4.

Table 4.4 - Summary table with the conditions for the test with the effluent

Parameter	**Test conditions**
Test type	Static
Temperature	24 ± 0.3 ºC
Light intensity	6433 ± 206 lux
Photoperiod	Continuous illumination
Duration	48h
PAA concentrations	3 (5 mg.L^{-1}, 10 mg.L^{-1}, 15 mg.L^{-1}), 1 solution of 15 mg PAA/L in standard freshwater and 2 controls
Number of replicates	4 (control 1, control 2, and PAA concentration)
Endpoints	Immobilization and mortality

VI. EC_{50} calculation

Values of 24h and 48h- EC_{50} for the acute toxicity test were determined according to Moreira et al. (2019) with the three-parameter logistic curves using the Statistica 7.0 software (Statsoft, 2004), with a 95% confidence interval. All statistical tests were considered significantly different when $p<0.05$ (Systat 2008).

4.3. Relative tolerance (Trel) calculation

With the intention of obtaining data to determine the relative tolerance of several species in relation to *D. magna*, studies were procured in Web of Knowledge in which the toxicity of effluents to *D. magna* and at least one other species was evaluated. Thus, the search criteria were the following:

TS = (Effluent OR Wastewater) and TS = (Daphnia OR Daphnid)

From this search, 1734 papers were obtained. Secondly, the abstracts from these papers were evaluated in order to certify if they indeed concerned a study evaluating the toxicity of effluents in *D. magna* and at least another species. This second phase of the research resulted in 279 papers, which were individually analysed in full, being the following information registered in an excel sheet:

- Effluent type (domestic/industrial/pharmaceutical/others)
- Chemical characterization of the effluent;
- Physical and chemical tests performed on the effluent (pH/COD/BOD_5/TSS/DO/others);
- Types of treatment to which the effluent was subjected;
- Localization of the effluent sampling;
- Species used in the toxicity tests;
- Sample characteristics (treated/untreated/WWTP collection site/others);
- Toxicity results and respective units;
- Toxicity assessment parameter and endpoint;
- Toxicity test duration;
- Additional observations.

To assure uniformity of the results, only papers presenting toxicity values in concentrations units were considered, i.e. papers presenting toxicity values such as EC_{50}, LC_{50}, NOEC, LOEC and IC_{50}. Subsequently, papers only indicating an effect percentage of e.g. raw wastewater were omitted. In this way, a total of 46 papers were included in this study.

The relative tolerance was calculated according to Daam & Rico (2018), as is described in equation 2.

$$\text{Trel} = \frac{\text{Toxicity value of a species other than } \textit{D. magna}}{\text{Toxicity value for } \textit{D. magna}} \qquad (2)$$

A Trel of one thus indicates a sensitivity equal to that of *D. magna*, whereas a Trel value lower than one indicates that the other species is more sensitive than *D. magna*, and a Trel value larger than one indicates that *D. magna* shows a lower sensitivity than that of the other species.

5. Results and Discussion

5.1. Wastewater characterization

Wastewater samples from the Beirolas WWTP were collected after the secondary treatment and the physical-chemical and microbiological parameters determined were: COD, pH, TSS, Kjeldahl nitrogen and total and faecal coliforms. Table 5.1 presents the data obtained regarding the physical-chemical parameters as well as their ELV. As may be deducted from this Table, all physical and chemical parameters mentioned in the D.L 152/97, 19th of June (COD, pH and TSS) present values within these ELV.

Table 5.1 - Physico-chemical and characterization of the secondary effluent of Beirolas WWTP (before disinfection) and comparison with the ELV

Parameter	Secondary effluent	ELV
COD (mg.L^{-1})	25.6	125
pH (-)	7.5	6.0-9.0
TSS (mg.L^{-1})	28.0	35.0
Turbidity (NTU)	1.06	-
Kjeldhal nitrogen (mg N-Kj. L^{-1})	17.4	-

ELV - Emission limit value; COD – Chemical Oxygen Demand; TSS – Total suspended solids; NTU - Nephelometric Turbidity Units.

Regarding the microbiological parameters. Table 5.2 presents the total and faecal coliforms as determined in the wastewater without PAA treatment, as well as their maximum allowable values (MAW). As anticipated, the coliform values for the secondary effluent are above MAV stated in D.L. nº 236/98 of 1st of August, confirming the need for a disinfection process.

Table 5.2 – Total and faecal coliforms before disinfection with PAA

Parameter	Secondary effluent	MAV
Total coliforms (MPN/100 mL)	140 000	10 000
Faecal coliforms (MPN/100 mL)	4500	2000

MAV – Maximum available value; MPN – Most probable number

To evaluate the disinfection efficacy of PAA of the wastewater studied, the effluent was disinfected with a range of PAA dosages and different contact times (c.f. Brito, in prep.). The concentrations chosen to be tested in this study for ecotoxicity to *D. magna* were based on the results obtained in that study , i.e. those PAA treatments that were shown to be effective. These dosages and contact times are presented in Table 5.3. According to these results, the 5 mg. L^{-1}, 10 mg. L^{-1} and 15 mg. L^{-1} PAA concentrations were tested in the bioassay.

Table 5.3 - PAA concentrations and contact times

PAA concentration (mg. L^{-1})	Contact time (min)
5	20
10	20
15	15

After the different PAA dosages were applied, parameters such as pH, temperature and turbidity were determined. TSS and Kjeldahl nitrogen were assumed not to be influenced by the presence of PAA, based on the results of a previous study conducted by Inácio (2018). Table 5.4 shows the obtained results.

Table 5.4 – Wastewater characterization after disinfection with different PAA concentrations

PAA dose mg.L^{-1}	COD (mg.L^{-1})	pH	Temperature (ºC)	Turbidity (NTU)
5	57.25	6.4	21.30	1.06
10	110.09	6.9	20.9	1.06
15	44.04	6.5	20.2	1.06

COD – Chemical Oxygen Demand; NTU - Nephelometric Turbidity Units.

Regarding COD, there was an increase compared to the secondary effluent, although the correlation observed between increase in PAA concentration and increase in COD was not linear. Despite the higher COD value, the final values still comply with the ELV. According to Kitis, (2004), for each addition of 5 mg.L^{-1} of PAA, it is expected that 13mg.L^{-1} of acetic acid is formed, considering the PAA decomposition stoichiometry, resulting in a 14 mg.L^{-1} increase in COD. Therefore, observing the results, it is possible to conclude that COD increase for each concentration did not evolve as expected according to the literature. This discrepancy can be explained by the presence of hydrogen peroxide residues that consumes oxidation agents such as potassium dichromate (used in the COD determination) interfering in the estimation of the COD measurements (Lee et al., 2011; Luukkonen et al., 2014).

Respecting pH values, the PAA application resulted in a decrease in pH, which was expected since PAA reacts in water mainly to became acetic acid and oxygen or hydrogen peroxide (Kitis et al., 2004). Nevertheless, this parameter is also still within the ELV. Temperature and turbidity did not show any significant variation due to any of the PAA application in the effluent.

The total and faecal coliforms after disinfection with PAA are presented in Table 5.5. Given the results, it is possible to observe that all the values are below the MAV as set in current legislation.

Table 5.5 – Total and faecal coliforms after disinfection with PAA

PAA concentration ($mg.L^{-1}$)	**Contact time (min)**	**Total coliforms (MPN/100 mL)**	**Faecal coliforms (MPN/100 mL)**
5	20	120	>1
10	20	6	>1
15	15	6	>1

MPN – Most probable number

Based on the above, the physical-chemical and microbiological characteristics of the PAA treated effluent are within the current limits as set in Legislation. This hence indicates that under the current Legislation, the effluent has acceptable quality characteristics to be discharged. In the next section, it is evaluated whether this also implies that no toxic side-effects are to be expected on *D. magna* from this PAA-treated effluent.

5.2. Toxicity tests with *Daphnia magna*

The dead and immobile organisms were counted 24 and 48 hours post start exposure to the secondary effluent with and without treatment by the different PAA concentrations, as well as a solution with PAA and standard freshwater. The raw data with the number of dead and immobile daphnids in each replicate are presented in Annex A.

5.2.1. Reference test

After 24 hours from the start of the reference test, the dead and immobile organism from the 4 wells for the controls and each potassium dichromate treatment were summed and the percentages of mortality and immobility were determined. The results are shown in Figure 5.1.

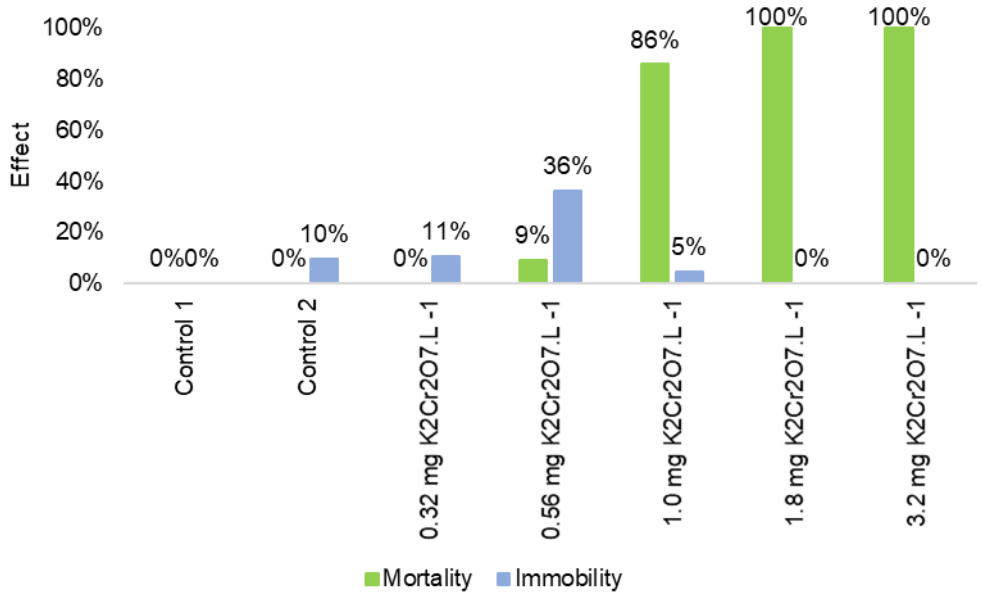

Figure 5.1 - Percentage of dead and immobile daphnids in the reference test after 24h

Regarding the control tests, no mortality was observed in Control 1 while control 2 shows 0% mortality and 10% immobile organisms (i.e., 2 out of 21). Since the number of dead and immobile organisms did not exceed 10% in both control tests, the control treatment adhered to this validity criterium set in OECD (2004). Mortality was only observed in the 0.56 mg/ L^{-1} of potassium dichromate treatment with a 77% increase from 0.56 mg. L^{-1} to 1.0 mg. L^{-1}. This increase coincides with what was expected for a valid test, since the EC_{50} – 24h range according to OECD (2004) is 0.6 - 2.1 mg. L^{-1}. The wells corresponding to 1.8 mg. L^{-1} and 3.2 mg. L^{-1} showed 100% of mortality for the organisms.

The results for the same organisms 48h after the start of the test are presented in Figure 5.2 .

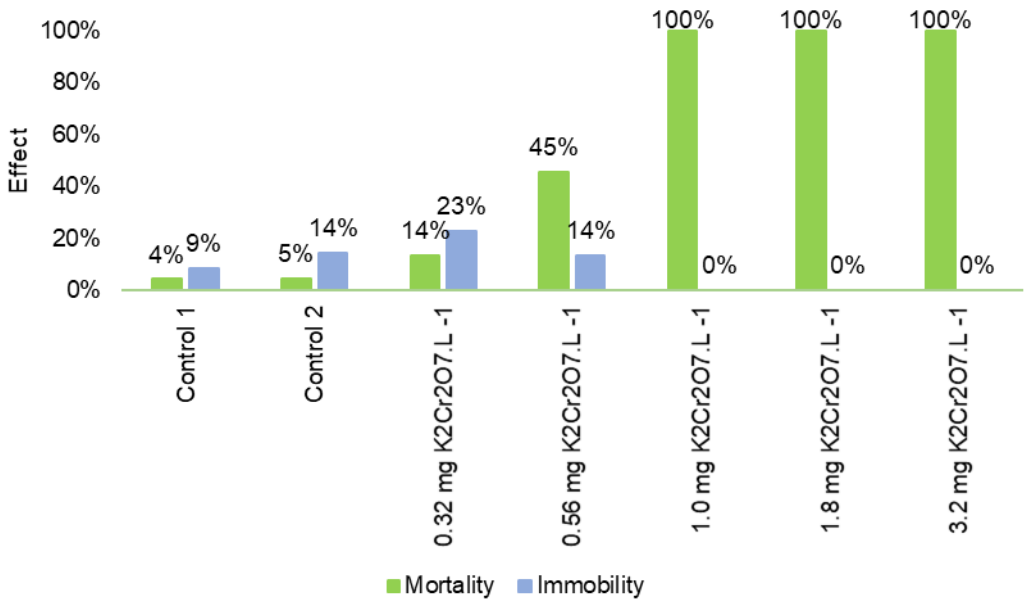

Figure 5.2 - Percentage of dead and immobile daphnids in the reference test after 48h

There was a significant increase in dead and immobile organisms in control 1 and 2, being that the percentages for both combined slightly exceeded the admissible limit of 10% after 48h. This can thus compromise the validity of the reference test. Some test condition limitations may have been responsible for this:

- The Daphtoxkit was used six months after its expiration date (December 2018);
- The hatching of the *ephippia* was delayed by two days due to environmental disturbance in the laboratory, which led to a divergence from the optimal hatching conditions for *D. magna*: The temperature inside the culture chamber was monitored throughout an entire day for two days to assure the temperature would be stable during the test duration, however, an accidental drop in temperature from 23ºC to 13ºC was observed, forcing the hatching chambers to be transferred to another culturing space in the laboratory.

A three times increase in mortality (14%) as compared to the controls was observed in the organisms exposed to 0.32 mg potassium dichromate L^{-1} and immobility was 12% (23%) higher when compared to the previous day (11%; Figure 4.1). In the wells with 0.56 mg potassium dichromate L^{-1}, the death of some of the immobile organism counted in the 24h test was observed. Exposure of the organisms to 1.0 $mg.L^{-1}$, 1.8 $mg.L^{-1}$ and 3.2 $mg.L^{-1}$ concentrations of potassium dichromate resulted in 100% mortality after 48h (Figure 4.2).

The 24h-EC_{50} and 48h-EC_{50} calculated based on the results of the reference test are presented in Table 5.6.

Table 5.6 - EC_{50} for the potassium dichromate reference test on *D. magna*

Test duration	**EC_{50} (mg. L^{-1})**
24 hours	0.55 (0.43 – 0.66)
48 hours	0.51 (0.41 – 0.61)
OECD guideline for 24h	0.6-2.1

The OECD Guideline for Testing of Chemicals – *Daphnia* sp. Acute Immobilisation test (OECD, 2004) only considers the 24h-EC_{50} in order to assure that the test conditions are reliable. The EC_{50}-24h for the potassium dichromate is slightly under the range presented on the OECD guideline. This may be attributed to reasons as discussed above.

5.2.2. Test with the effluent

After 24 hours from the start of the test with the effluent, the dead and immobile organisms from the 4 wells for the controls, untreated secondary effluent, effluent disinfected with the different PAA concentrations and the 15 mg PAA L^{-1} prepared in standard freshwater were counted and the percentages of mortality and immobility were assessed. The results are shown in Figure 5.3.

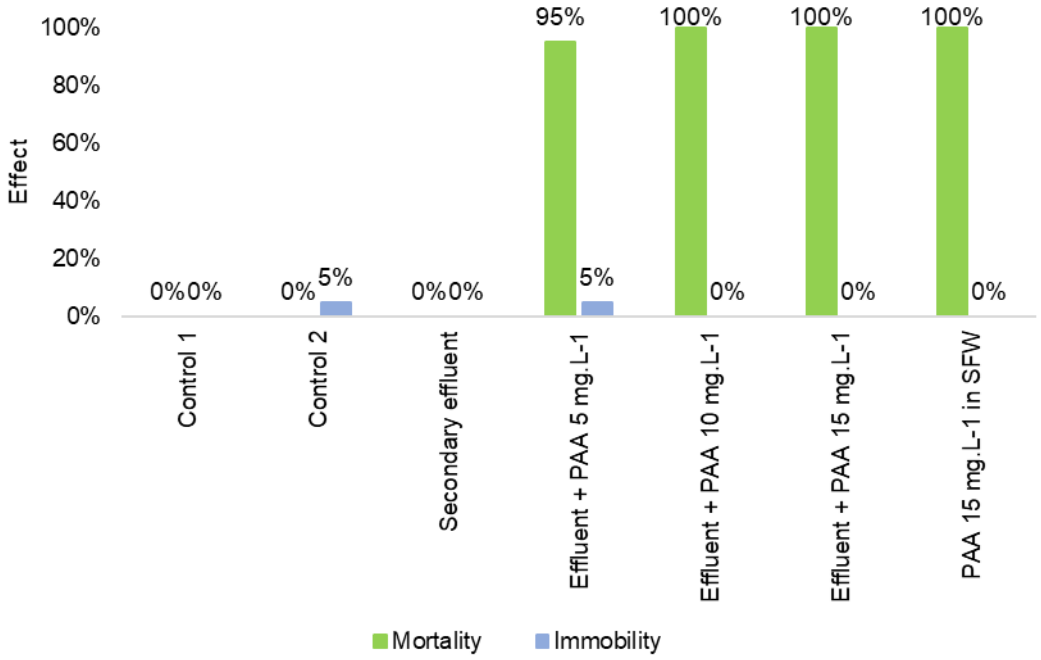

Figure 5.3 - Percentage of dead and immobile daphnids after 24h of exposure to the secondary and disinfected effluent

Regarding the controls, control 1 showed 100% survival and both control 1 and control 2 did not exceed 10% of combined mortality and immobility, which validates the test regarding this condition

(c.f. OECD, 2004). The exposure to secondary effluent caused no visible harm to the organisms, and the survival rate was 100% with no dead or immobile organisms. On the other hand, the wells with organism exposed to any of the PAA concentrations tested showed 0% survival rate, with 95% of immobile organisms and 5% of dead organisms for an exposure to effluent with 5 mg. L^{-1} PAA. The exposure to the effluent with 10 mg. L^{-1} and 15 mg. L^{-1} PAA concentrations resulted in 100% mortality of the organisms. The solution with 15 mg PAA L^{-1} in standard freshwater obtained the same results as the two previous treatments, which indicates that the mortality and immobility in this test are a result of PAA induced toxicity on *D. magna* rather than resulting from any influence of the effluent.

The results for the same organisms 48h after the start of the test are presented in Figure 5.4.

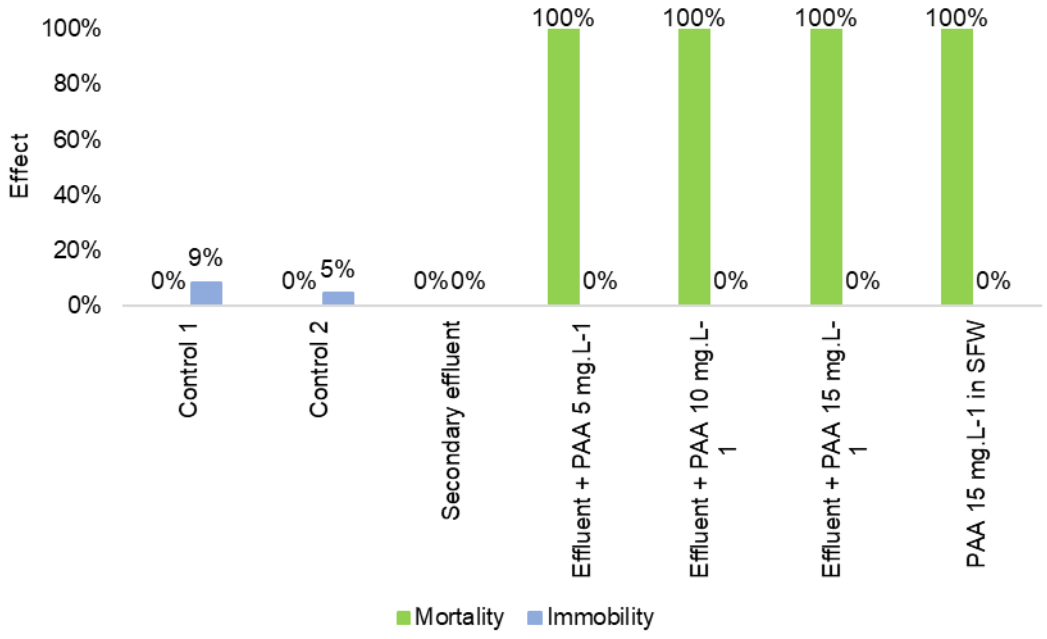

Figure 5.4 - Percentage of dead and immobile daphnids after 48h of exposure to the secondary and disinfected effluent

As compared to 24h post start treatment, the controls after 48h showed an increase in immobile organisms, although no mortality was observed. Subsequently, the test remained valid regarding this criterion. The 100% survival rate of the organisms exposed to the secondary effluent was still observed after 48h, which indicates an absence of toxicity of the secondary effluent without disinfection to *D. magna*. All organisms exposed to any of the PAA concentrations evaluated suffered 100% mortality 48h after the start of the test, including the solution with PAA and standard freshwater. This thus implies that the single individual that had not died (but was already immobile) already in the 5 mg PAA L^{-1} treated wastewater after 24h also died after 48h (c.f. Figures 5.3 and 5.4).

The multiwell plate was left in the culture chamber for an additional seven days after the test with the effluent was finished. During this week, the conditions inside the culture chamber was submitted to accidental changes (the temperature dropped from 23ºC to 12.8ºC, several degrees below

optimal temperature range for *D. magna* culturing). This resulted in death of all the organism in control 1 and 2, but a 100% survival rate of the organism in the secondary effluent well was still observed. This observation not only supports the hypothesis that the secondary effluent was non-toxic to the daphnids, but also implies that the effluent has in its constitution a range of nutrients that helps to sustain the daphnids metabolism.

Due to the almost 100% mortality of the organisms exposed to any of the PAA concentrations tested, even after the first 24 hour of the test, it was not possible to determine the EC_{50} or LC_{50} for PAA to *D. magna*. These results were expected considering that the applied concentrations were significantly higher than the EC_{50} values of PAA reported for *D. magna* in previous studies. Henao et al. (2018) reviewed the results from several studies regarding PAA toxicity to *D. magna* and other species. They reported 48h-EC_{50} of PAA disinfected effluents on *D. magna* ranging from 0.15-1.1 $mg.L^{-1}$, with the exception of the value reported by Licata-Messana (1995). The latter authors observed a much higher toxicity (EC_{50} – 48h 0.035 – 0.35 $mg.L^{-1}$), although this value might not be representative due to the high H_2O_2 fraction in the commercial product used in that study (Licata-Messana, 1995). Liu et al. (2015) assessed the toxicity of different PAA concentrations to *D. magna*, observing 24h-LC_{50} values ranging 0.18 – 2.6 mg. L^{-1}. The maximum EC_{50} value for *D. magna* reported in these studies is below the minimum concentration evaluated in the present study (5 $mgPAA.L^{-1}$), which may thus explain the high level of mortality denoted in the present study at all PAA concentrations tested.

It should be noted, however, that the comparison of the results obtained in this dissertation with the results obtained from other authors cannot be considered a direct comparison, since different effluents with different natures can vary significantly regarding their characteristics. Thus, this comparison only aims to give a better perception of the level of toxicity of the PAA concentrations used in the present study.

Even though PAA shows high toxicity in *D. magna,* studies have found higher toxicity results for chlorine, one of the most common disinfection methods. Costa et al (2014), for example, studied the toxicity of residual chlorine level in disinfected effluent and calculated an EC_{50} of 0.16 mg. L^{-1}. In addition, Negreira et al. (2015) determined the toxicity of major chlorine disinfection byproducts on several species, including *D. magna*, which resulted in LC_{50} – 48h values ranging from 0.008 to 0.260 $mg.L^{-1}$.

5.3. *Daphnia magna* relative tolerance (Trel) study

In the papers used for this literature review study, the taxonomic groups evaluated were bacteria algae, macrophytes, rotifers, crustaceans, insects, and fish. Table 5.7 presents the number of Trel values that could be calculated for each of the taxonomic groups, as well as the species corresponding to each taxonomic group whose toxicity to wastewater effluents was determined. The number of papers in which each taxonomic group was evaluated is also presented (Table 5.7).

Table 5.7 - Studied species for each taxonomic group

Taxonomic group	Species	Number of studies	Number of Trel values
Bacteria	*Vibrio fischeri*	23	112
Algae	*Chlorella vulgaris, Desmodesmus subspicatus, Euglena gracilis, Minutocellus polymorphus, Raphidocelis subcapitata, Scenedesmus subspicatus, Selenastrum capricornutum* (former name of *R. subcapitata*)	17	76
Macrophytes	*Lemna minor*	6	25
Rotifers	*Brachionus calyciflorus; Lecanequadridentata*	5	36
Crustaceans	*Artemia franciscana, Artermia salina, Ceriodaphnia dubia, Chaetocorophium lucasi, Daphnia longispina, Daphnia obtusa, Daphnia pulex, Moina macrocopa, Thamnocephalus playturus*	16	76
Insects	*Chironomus* sp.	1	8
Fish	*Danio rerio, Oncorhynchus mykiss, Pimephales promelas; Poecilia reticulata.*	18	42
Total	-	46	379

From Table 5.7 it may be concluded that there is a large variation in the number of species evaluated, the number of studies that address each taxon and the amount of toxicity data available between the different taxonomic groups. This difference in representativeness can mislead the results regarding a certain taxonomic group or species in comparison with its real sensitivity. Logically, the results regarding a taxonomic group or species that is only represented by a few

studies depends on the specific conditions of those studies. For example, although algae are represented by various species, Trel values for macrophytes could only be calculated for *L. minor* (Table 5.7). It may be questionable, however, whether *L. minor* is representative for the sensitivity of all other macrophyte species.

Based on the 379 toxicity values analysed, the relative tolerance of each species to *D. magna* was calculated. The EC_{50} and the calculated relative tolerance results for all taxonomic groups and respective species considered in this study are presented in Annex C.

Subsequently, a boxplot chart with the Trel of each taxonomic group was generated, which can be observed in Figure 5.5. The minimum, first quartile, median, third quartile and maximum values that support the boxplot chart can be consulted in Annex D.

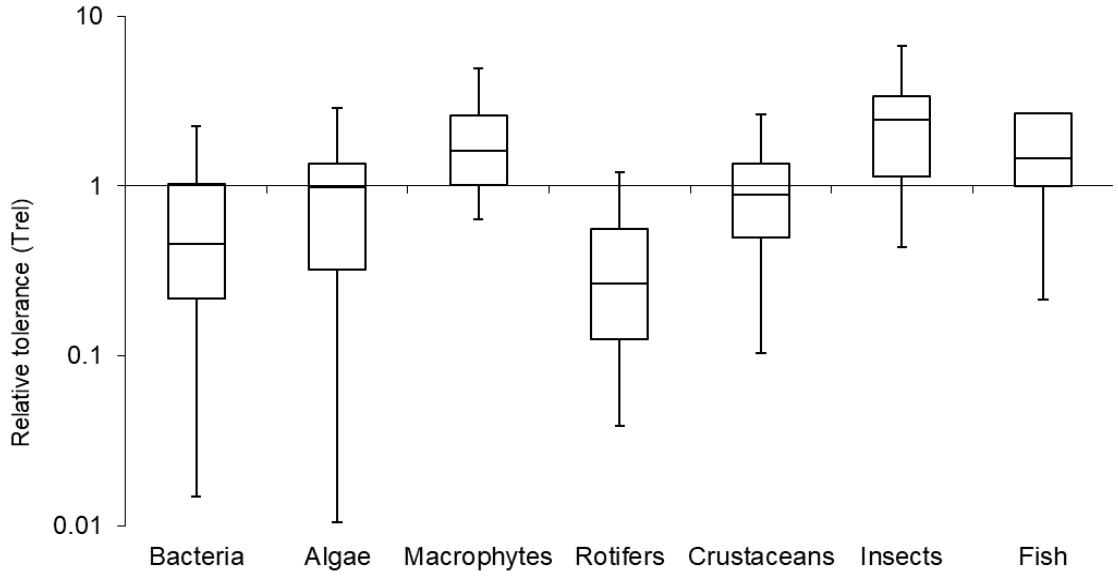

Figure 5.5 - Boxplot with the Trel (relative tolerance) of each taxonomic group compared to *D. magna*

The Trel of the most frequently tested species from each group was also analysed. Thus, the number of cases in which toxicity value for the species was within the considered Trel ranges was enumerated. The most frequently tested species were *Vibrio fischeri* for bacteria*, Raphidocelis subcapitata* for Algae*, Lemna minor* for Macrophytes, *Lecane quadridentata* for Rotifers, *Thamnocephalus platyurus* for Crustaceans, *Chironomus* sp. for Insects and *Danio rerio* for fish. The results are presented in Table 5.8.

Table 5.8 - Number of toxicity values within the Trel ranges for the most frequent species of each taxonomic group

Test species	0.01 > Trel ≤ 0.1	0.1 > Trel ≤ 1	1 > Trel ≤ 10	Trel > 10	Sum
Vibrio fischeri	15	67	23	6	111
Raphidocelis subcapitata	6	32	21	0	59
Lemna minor	0	7	18	0	25
Lecane quadridentata	6	23	0	0	29
Thamnocephalus platyurus	0	37	19	0	56
Chironomus tentans	0	2	5	1	8
Danio rerio	0	6	6	2	14

Bacteria

Regarding the bacteria group, and according to Figure 5.5, it is possible to observe that the 95% CI is 0.22 to 1.03 (Annex D). Once the Trel=1 is still within the interquartile range (IQR), it is considered that bacteria are not significantly more sensitive to effluents than *D. magna* (Daam et al., 2011). However, since the third quartile (Q_3=1.03) is very close to Trel=1, it is still plausible to assume that bacteria are generally more sensitive than *D. magna*.

Within the bacteria group, *V. fischeri* was the most frequently tested species. The bioluminescence inhibition toxicity test is standardized in ISO 11348-3:2007 (ISO, 2007) and, according to Parvez et al. (2006), is one of the first assays chosen in a test battery, based on its speed, cost consideration and sensitivity. *Photobacterium phosphoreum* was only evaluated in Tisler & Zagorc-Koncan (1994). This study assessed the toxicity of effluents from a chemical industry, and *P. phosphoreum* was less sensitive than *D. magna*, with a Trel of 8.80.

V.fischeri was contemplated in 23 studies and corresponds to 111 Trel values. The percentages of Trel values within each interval are presented in Figure 5.6.

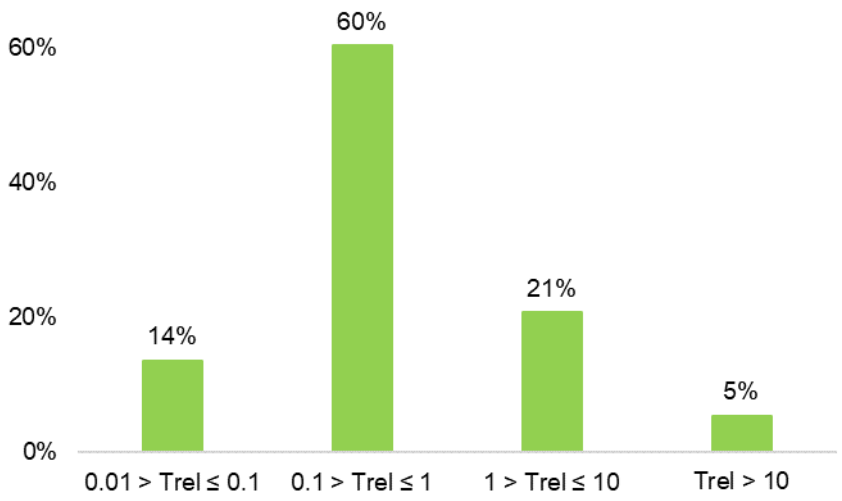

Figure 5.6 - Percentages of Trel (relative tolerance) values within each interval for *V.fischeri*

It is possible to observe that *V. fischeri* is more sensitive than *D. magna* in most of the cases, showing a relative tolerance inferior to 1 in 74% of the cases. According to Tisler & Zagorc-Koncan (2008), who studied the toxicity of several textile and industrial effluents, *D. magna* revealed high sensitivity to the samples of the textile effluent, while *V. fischeri* showed more sensitivity to the raw samples from the chemical industry, indicating the use of daphnids and luminescent bacteria in a complementary way. Similar results were observed in Picado et al. (2008), in which the ecotoxicological effects of the discharge of seventeen industrial effluents in Trancão River basin were assessed over a large period of time. In this study a battery of tests was performed, being that the *V. fischeri* showed the lowest EC_{50} values of the battery in 10 out of 17 cases, followed by *D. magna*, which had the lowest EC_{50} values in three cases.

However, *V. fischeri* showed a very low sensitivity to wastewater effluents in some other studies. In Brienza et al. (2016), for example, this test organism was not sensitive to wastewater spiked with 5 micropollutants (pesticides and pharmaceuticals) representative of the most common organic products widely found in wastewater. According to Munkittrick et al. (1991), Microtox was less sensitive than acute lethality tests with daphnids to insecticides, herbicides, pharmaceutical and textile effluents, as well as highly lipophilic contaminants, and was not as sensitive as *D. magna* for inorganic compounds.

Algae

As can be denoted from Figure 5.5**,** the algae shows the largest range between the lower whisker (0.01) and the upper whisker (2.87). The relative tolerance values between the first quartile and the median corresponds to a lower sensibility than *D. magna*, and the values between the median and the third quartile correspond to a higher sensibility compared to *D. magna*.The median presents a value corresponding to a very similar sensibility to that of *D. magna* (Trel = 0.99). Since the IQR

(0.33 to 1.34) comprises the Trel of 1, it is not statistically significant to admit the algae group to be less sensitive than *D. magna*.

The most frequent species throughout the 17 studies was the green algae *Raphidocelis subcapitata,* which is a standard species included in the OECD guidelines for the testing of chemicals for freshwater algae and cyanobacteria (Test nº 201: Freshwater Alga and Cyanobacteria Growth Inhibition Test, (OECD, 2011b)). The percentages of Trel values within each interval are presented in Figure 5.7.

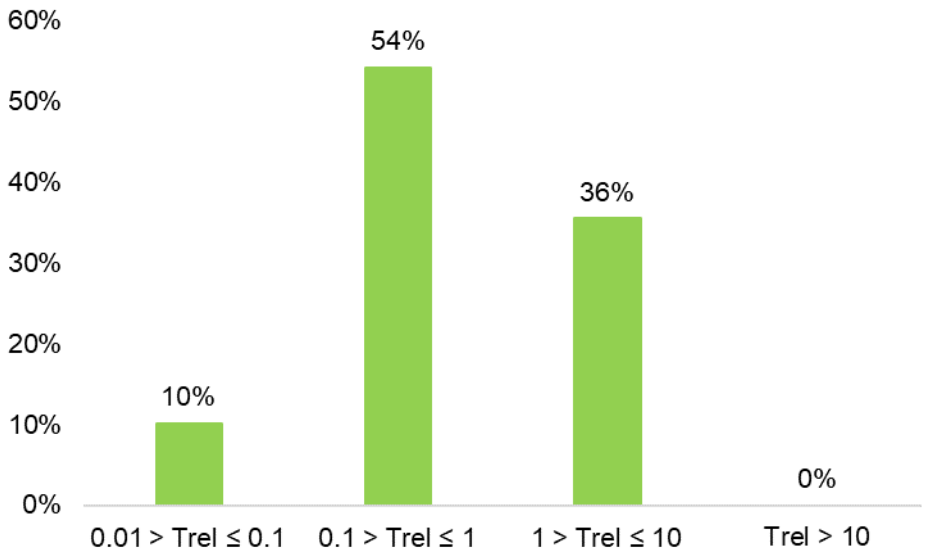

Figure 5.7 - Percentages of Trel (relative tolerance) values within each interval for *R. subcapitata*

According to this figure, in 64% of the cases *R. subcapitata* has a higher to similar sensitivity compared to *D. magna*, and 36% show lower sensitivity, even though there were no occurrences of a relative tolerance superior to 10 (Figure 5.7).

In some studies, *R. subcapitata* was indicated to have the same sensibility as *D. magna*. In Mendonça et al. (2013), a battery of tests was performed in order to evaluate the toxicity of domestic wastewaters from Loures WWTP from different treatment phases. According to this study, *D. magna* and *R. subcapitata* had different variations in the percentage of effect (*D. magna* showed a greater variation, while *R. subcapitata* presented a smaller variation within a range of higher percentage of effect values). However, the Trel between these two species was 1 for every sample, which suggests a similar sensitivity of *D. magna* and *R. subcapitata* to this specific effluent. In Picado et al. (2008), *R. subcapitata* also showed a Trel of 1 in the effluents from 4 out of the 17 industries and a Trel ranging between 0.83 and 0.98 in 3 other industries (paper and recycling industries), also demonstrating an overall similar sensibility between *D. magna* and *R. subcapitata*.

Macrophytes

Observing Figure 5.5, the taxonomic group macrophytes shows the lower range between the lower whisker (0.64) and the upper whisker (1.21). All the Trel values between the first quartile (Q_1=1.01) and third quartile (Q_3=2.58) correspond to a lower sensibility to that of *D. magna.* However, since the Trel=1 is still within the IQR (1.01 to 2.58), it is not statistically significant to consider macrophytes more sensitive to effluents than *D. magna.* On the other hand, and similar to the bacteria group, the first quartile is very close to Trel=1, so it is still plausible to assume that macrophytes may generally be less sensitive than *D. magna*.

Lemna minor was the only macrophyte species tested in the six studies, therefore the obtained results for the macrophytes group are the same as those for *L. minor*. *Lemna* sp. growth inhibition test is standardised in the OECD guidelines for the testing of chemicals (OECD, 2002). The percentages of Trel values within each interval are presented in Figure 5.8.

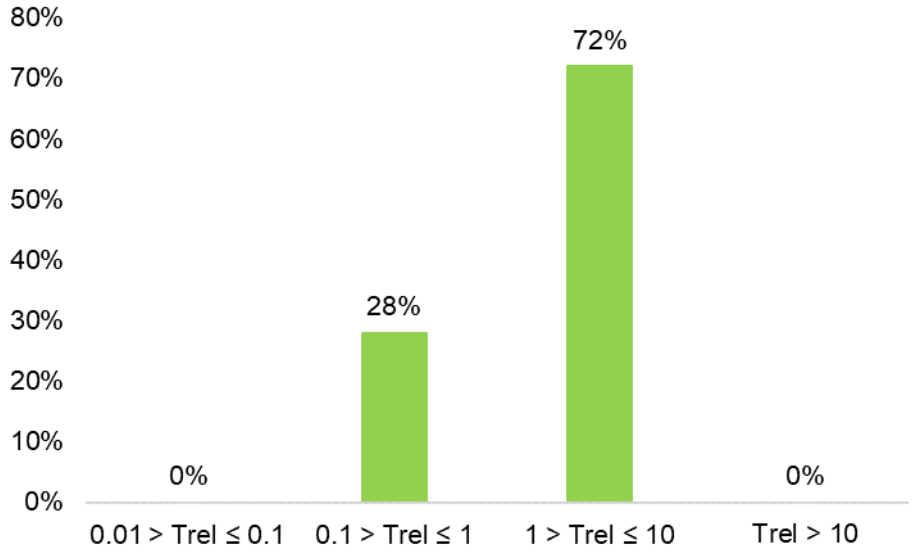

Figure 5.8 - Percentages of Trel (relative tolerance) values within each interval for *L. minor*

In the 6 papers that included macrophytes, *L. minor* appeared to be less sensitive to the effluents than *D. magna* in 72% of the cases, without any toxicity values corresponding to a Trel between 0.01 and 0.1 or superior to 10.

L. minor was included in the Picado et al. (2008) study mentioned above and appeared to be the least sensitive organism to the industrial effluents among *D. magna*, *V. fischeri*, the crustacean *T. platyurus* and *R. subcapitata*. The same occurred in Aydin et al. (2015), where *L. minor* showed the lowest sensitivity to landfill leachates, compared to *D. magna*, *V. fischeri* and *Lepidum sativum*. Rosa et al. (2009) assessed the toxicity of a bleached-kraft pulp mill effluent before and after secondary treatment, in which the secondary effluent caused no toxicity to *L. minor* and *C. dubia*, while *D. magna* and *V. fischeri* suffered toxic effects to the same effluent. In Neculita et al. (2008), acid mine effluents caused acute toxicity to *D. magna* and *C. dubia*, however it only caused a slight growth inhibition in *L. minor*. According to Zaltauskaite et al. (2011), *L. minor* was less sensitive

than *D. magna* to municipal effluents. Finally, Vidal et al. (2012) studied the toxicity of acid mine drainage contaminated with metals in *L. minor*, *Daphnia* sp., *V. fischeri*, and *R. subcapitata*, being that the effluents were shown to be very toxic to all the tested species. Nevertheless, *L. minor* appeared to be considerably less sensitive than *D. magna*, with a Trel of 6.48.

Rotifers

According to Figure 5.5, rotifers present the lowest relative tolerance values, with the lowest values of the first quartile (Q_1 = 0.13), median (0.27) and third quartile (Q_3 = 0.56), with the lower and upper whiskers ranging from 0.04 to 1.21. Additionally, the IQR did not comprise Trel=1, making it statistically significant to admit that rotifers appeared to be more sensitive to effluents than *D. magna*.

This taxonomic group was only represented by two species, *Lecane quadridentata* and *Brachionus calyciflorus.* The test for the acute toxicity to *B. calyciflorus* is standardized by ISO 19827:2016 (ISO, 2016). However, most Trel values for rotifers were obtained for *L. quadridentate*, although it is not a standard species for toxicity tests. On the other hand, *B. calyciflorus* is represented by three studies, while *L. quadridentata* was contemplated in two studies that evaluated several different effluents. The distribution of the percentages of the relative tolerance values over the considered intervals is presented in Figure 5.9.

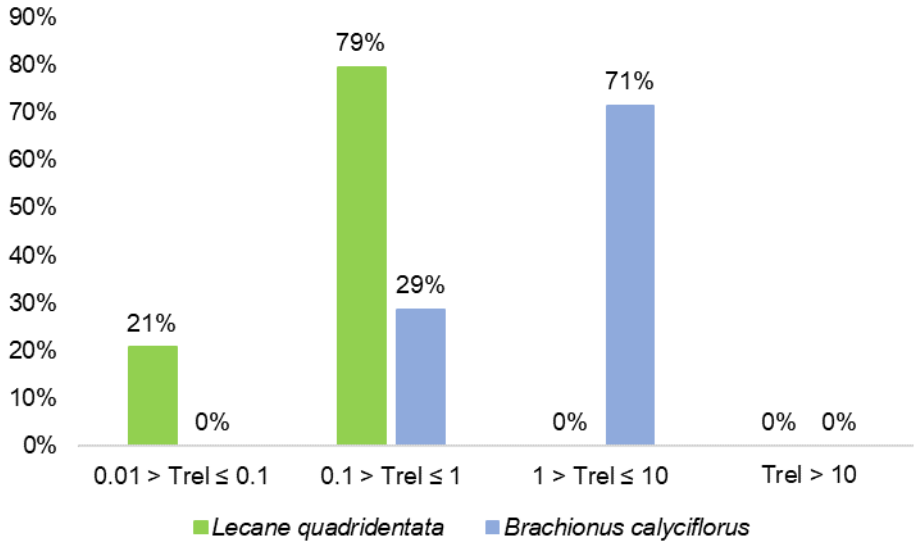

Figure 5.9 - Distribution of the Trel (relative tolerance) over the considered intervals for *L. quadridentata and B. calyciflorus*

Given the results, it is possible to observe that *L. quadridentata* appeared to be more sensitive than *D. magna*, presenting 100% of the toxicity values below a relative tolerance of 1. On the contrary, *B. calyciflorus* was less sensitive than *D. magna* in 71% of the cases. Thus, based on these (limited number of) studies, the standard species did not appear to be the most sensitive. However, this observation may not be the representative of the real sensitivity of these two species, since 28 out

of the 29 toxicity values available for *L. quadridentata* were obtained from Torres-Guzmán et al. (2010), where the organism was used to assess the toxicity of an effluent contaminated with metals at concentrations that exceeded the LC_{50} values for *L. quadridentata* Therefore, in this case, the difference in the amount of toxicity values available in the papers for each species make that the results should be interpreted with caution.

Crustaceans

Regarding the crustaceans taxonomic group, it is possible to observe in Figure 5.5 that the IQR covers the relative tolerance ranges between 0.1 and 1, and between 1 and 10, with a first quartile of 0.50 and a third quartile of 1.36. The lower and upper whiskers range from 0.10 and 2.65, which does not represent a significant variation and indicates that most TreI are relatively close to 1.The median is 0.88, which represents a sensibility close to that of *D. magna*. The IQR ranges from 0.50 and 1.36, comprising Trel=1; therefore, it is not statistically significant to consider the crustacean group more or less sensitive than *D. magna*.

In the studies included in the crustacean dataset, some crustaceans other than *D. magna* but belonging to the Daphnidae family were tested. Ruck (1998), for example, assessed the toxicity of an industrial effluent to *C. dubia*. This organism showed a similar sensitivity to that of *D. magna,* with a relative tolerance of 1.1 (Ruck, 1998). *Daphnia longispina* was mentioned in 3 studies, with Trel values ranging from 0.18 and 0.59 in Pereira et al. (2009) and Antunes et al. (2007), indicating a greater sensitivity of this species than that of *D. magna*. Xavier et al. (2017) studied the toxicity of treated kraft mill effluents to *D. magna* and *Daphnia obtusa*, in which *D. obtusa* showed less sensitivity to the effluent, with a relative tolerance of 4.90.

Daphnia pulex was tested in Vidal et al. (2004), Vidal et al. (2012) and Cooman et al. (2003). Vidal et al. (2012) assessed the toxicity of freshwater contaminated with acid mine effluents to *D. pulex* and *D. magna,* in which *D. pulex* appeared to be more sensitive, with a Trel of 0.45. Cooman et al. (2003) assessed these two species as bioindicators for the toxicity of tannery wastewaters. In this study, *D. pulex* showed less sensitivity than *D.magna* to both treated and untreated 45 days old effluent, with a relative tolerance of 0.85 and 0.88, respectively. However, both species appeared to have a similar sensitivity for the treated effluent with 127 days, with a Trel value for *D. pulex* of 1.0 (Cooman et al., 2003). Finally, Vidal et al. (2004) assessed the toxicity of treated and untreated leather tannery effluents. *D. pulex* showed a very similar sensitivity to that of *D. magna*, with a relative tolerance of 0.90 and 0.99 for a 24-hour exposure to treated and untreated effluents, respectively, and Trel values of 1.03 and 1.10 for the same samples after a 48-hour exposure.

Although there were several Daphnids tested in the papers regarding the crustaceans, the most frequently tested species was *T. platyurus*, being mentioned in 10 studies. The method for the determination of the acute toxicity to *T. platyurus* is standardized by ISO 14380:2011 . The percentages of Trel values within each range are presented in Figure 5.10.

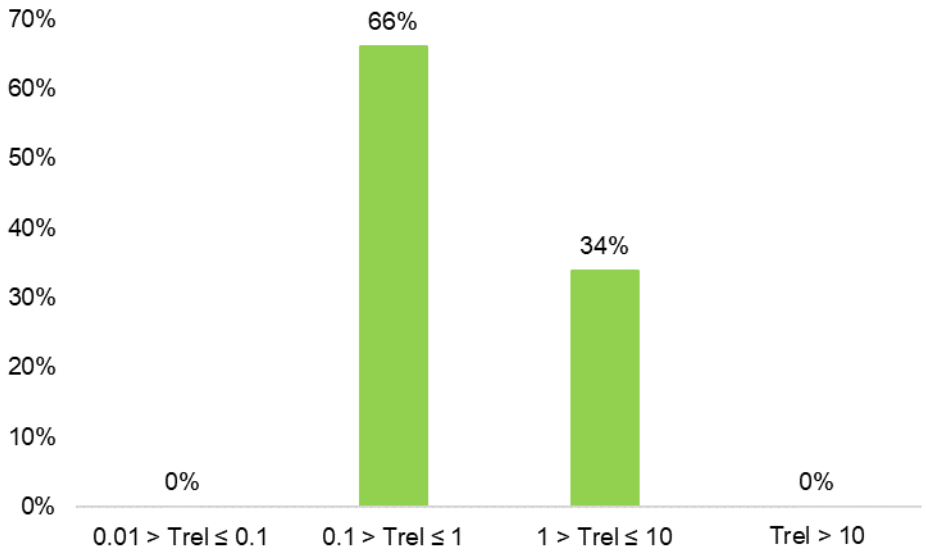

Figure 5.10 - Percentages of Trel (relative tolerance) values within each interval for *T. playturus*

In the considered studies, most of the toxicity values are within the Trel range of 0.1 to 1, indicating lower sensitivity to that of *D. magna*. Although 34% of the toxicity values correspond to a Trel range between 1 and 10, the maximum value recorded was 3.03. Thus, as observed for the whole taxonomic group, the variation in relative tolerance was not very large.

As described in the case of *R. subcapitata, T. playturus* also showed a Trel value of 1 in Picado et al (2008), and therefore the same sensitivity as *D. magna*, for the effluent of 3 industries (laboratory, paper and recycling industries).This can be due to the toxicity of some of the industrial effluents being so high that causes similar effects to species with different levels of sensitivity.

Insects

Similar to macrophytes, and according to Figure 5.5, insects present all the relative tolerance values within the first quartile (Q_1=1.13) and third quartile (Q_3=3.30) superior to 1, which corresponds to a lower sensitivity to the effluents than *D. magna*. It is also the taxonomic group that presents the higher relative tolerance median (2.43). Since the Trel=1 is not comprised by the IQR (1.13 to 3.36), it is considered statistically significant to assume that insects are less sensitive to effluents than *D. magna*.

It should be noted that insects were only represented by one species, i.e. the non-biting midge *Chironomus tentans*, and all Trel values for this species were obtained from a single study. Subsequently, the results of the taxonomic group are the same as for this species. The acute immobilisation test for *Chiromus* sp. is standardised in the OECD test guideline 202 (OECD, 2011a). The percentages of Trel values within each range are presented in Figure 5.11.

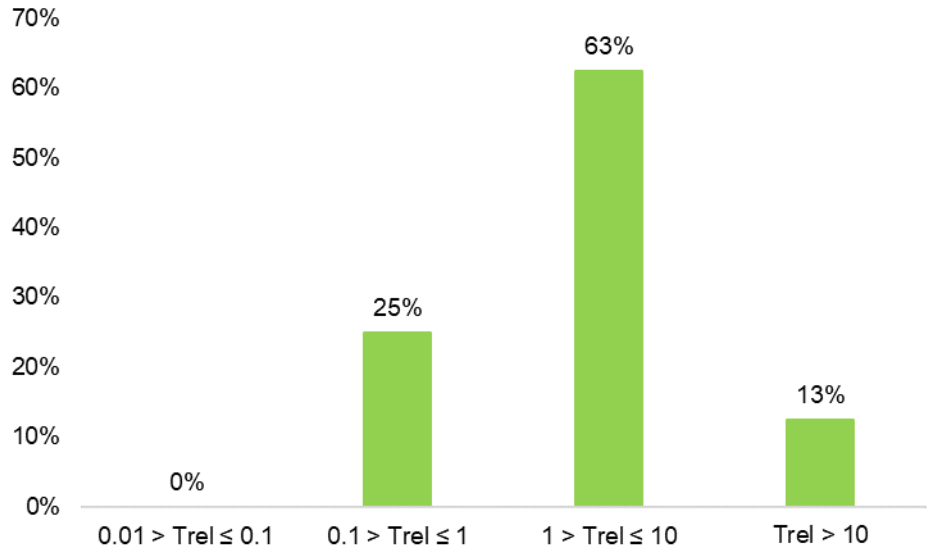

Figure 5.11 - Percentages of Trel (relative tolerance) values within each interval for *C. tentans*

Given the results, it is possible to observe that *C. tentans* was less sensitive than *D. magna* in the majority of the cases (76%). The toxicity of several industrial effluents to *D. magna* and *C. tentans* was assessed in Butarewicz et al. (2019). In this study, *C. tentans* appeared to be more sensitive than *D. magna* in two cases: the effluent from a metallurgical industry and the effluent from a textile industry.The metallurgical effluent showed to have a high acute toxicity for both species, even thought it was more toxic for the insect, resulting in a Trel of 0.44. The high toxicity in this study was attributed to the degreasing process byproducts, such as chlorinated derivatives (trichlorethylene, tetrachlorethylene), aromatic hydrocarbons, corrosion inhibitor, antibacterial agents, among others. These byproducts usually present high toxicity and require purification by neutralization and coagulation. The wastewater from the dye-works from the textile industry were also more toxic to *C. tentans* than for *D. magna*, with a relative tolerance of 0.60. Dyes and detergents are primarily responsible for the toxicity of effluents containing these compounds, since, according to Butarewicz et al. (2019) these substances are resistant do biodegradation.

Contrary to the above, Butarewicz et al. (2019) studied the toxicity of the untreated effluent from an inorganic industry, which appeared to be much more toxic to *D. magna* as compared to *C. tentans*. The EC_{50} of this effluent to the crustacean was 0.02% (v/v), in contrast with the EC_{50} of 1.18% (v/v) for *C. tentans*, resulting in a Trel of 59, which is much higher than the second highest value of Trel of *C. tentans* to *D. magna* (Trel = 3.36 for the effluents of a mining industry). The cause of such a high acute toxicity of the effluent for *D. magna* was linked to post-production liquids containing zinc.

Fish

Similar to what was observed for macrophytes and insects, and according to Figure 5.5, all the fish Trel values within the first quartile (Q1=1.00) and third quartile (Q3=2.68) are equal or superior to 1, which suggest a lower sensitivity to the effluent to that of *D. magna*. It is also the taxonomic

group that presents the second higher median (1.45). Once the Trel=1 is still within the IQR it is not statistically representative to consider fish less sensitive to effluents than *D. magna*. However, since the first quartile is equal to Trel=1, it is still plausible to assume that fish are less sensitive than *D. magna*.

For this taxonomic group, *Pimephales promelas,* commonly known as fathead minnow, was the most frequent species, with 15 Trel values. However, around 70% of the values for this species were obtained from one single study conducted by Choir & Meier (2001). On the other hand, *Danio rerio* presented 14 Trel values from 7 studies. Therefore, for representative reasons, this was considered the most frequent species for the fish taxonomic group. The fish embryo acute toxicity test for *D. rerio* is standardized in the OECD guidelines for testing in chemicals (OECD, 2013). The percentages of Trel values within each range are presented in Figure 5.12.

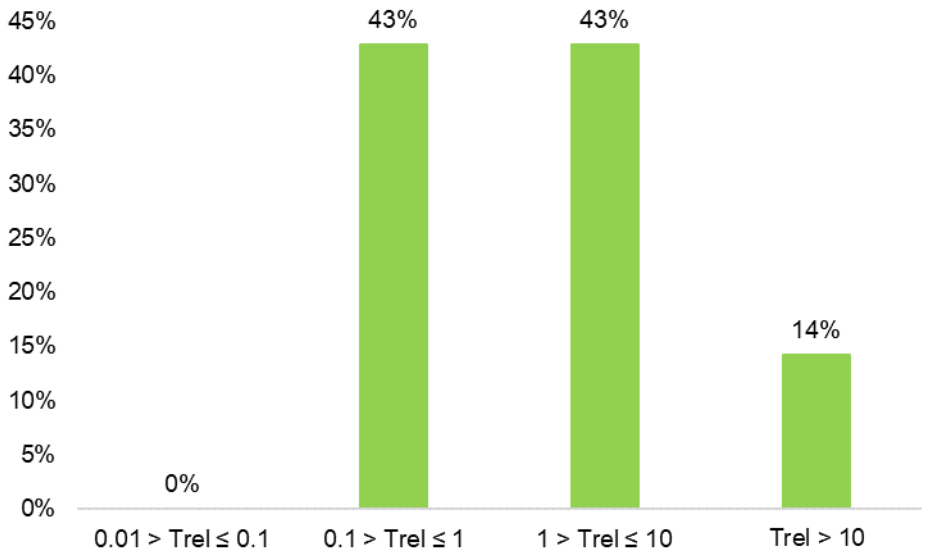

Figure 5.12 - Distribution of the relative tolerance over the considered intervals for *D. rerio*

Given the results, it is possible to observe the interval between 0,1 and 1 comprises 43% of the Trel values for *D. rerio*, being that this percentage corresponds to 6 Trel values from which 5 of them are inferior to 1, showing higher sensitivity to that of *D. magna*.

Karadima et al. (2010) assessed the toxicity of the effluents from a cheese industry to *D. magna*, *D. rerio* and *T. platyurus*. In this study, an assessment of ecological risk in the discharge site was performed in order to evaluate the ecosystem response to the effluent, being that the results indicated a high level of ecological risk. *D. rerio* appeared to be the most sensitive species to the effluent, while *D. magna* showed the highest LC_{50} value, resulting in Trel values of *D. rerio* from 0.66 to 0.75.

On the other hand, Grinevicius et al. (2008) studied the toxicity of textile effluent on *D. magna, D. rerio, V. fischeri* and *Artemia* sp. In this study, *D. rerio* also showed a higher sensitivity to that of *D. magna* with a Trel value of 0.25. The high toxicity of this effluent to the fish might have been caused

by a high concentration of transitional metals (such as Fe, Co, Mn, and Mo), to which the fish were not able to compensate for the oxidative stress associated with these contaminants.

Tišler et al. (2004) assessed the toxicity of a tannery effluent, to which *D. rerio* also appeared to be more sensitive than *D. magna*. The toxicity of this effluent in this study was attributed mainly to the fact that the effluent was overloaded with organic and inorganic compounds, exceeding the permissible limits for the discharge of wastewaters in the receiving streams. Additionally, the high toxicity of this effluent was also attributed to the disinfection agent, which increased significantly the toxicity when applied to the effluent (Tišler et al., 2004).

Rouvalis & Iliopoulou-Georgudaki (2010) studied the toxicity of olive oil mill effluents, in which *D. rerio* also appeared to be more sensitive than *D. magna*. The toxicity in this effluent was due to physicochemical parameters showing different degrees of influence depending on the species, being that *D. rerio* showed to have its toxicity correlated to parameters such as phenols, COD and total phenols (Rouvalis & Iliopoulou-Georgudaki, 2010).

The remaining 57% of the Trel values correspond to a lower sensitivity of *D. rerio* as compared to *D. magna* (Trel superior to 1), with Trel values ranging from 1.0 to 41, which suggest several levels of sensitivity across the different studies assessing *D. magna* and *D. rerio*.

In general, fish showing a lesser sensitivity to wastewater than *D. magna* is a positive result regarding the strategy of decreasing the use of vertebrates in toxicity tests, since replacement alternatives for acute and chronic toxicity to reduce and refine fish testing (such as data-driven approaches and interspecies extrapolations) have been researched and developed in the last years (Halder et al., 2014).

6. Conclusions

The toxicity bioassay performed with *D. magna* and the secondary effluent disinfected with PAA allowed to obtain the following conclusions:

- The PAA concentrations evaluated in the present study were highly toxic to *D. magna*, hampering the EC_{50} determination due to the high mortality rate during the first 24 hours of the test at all concentrations tested. This result is corroborated with EC_{50} values of PAA for *D. magna* reported in literature, which were significantly lower;

- The secondary effluent from Beirolas WWTP before disinfection, did not appear to have toxic effects on *D. magna* and even appeared to be a better medium for the survival of the test organisms, even though the environmental conditions were not optimal. This was discussed to be likely due to the presence of nutrients and particles to serve as food in the effluent;

- Even though the physical, chemical and microbiological parameters determined for the disinfected effluent in this study were within the emission limit values (ELV) as set in current Legislation, the 48h exposure of *D. magna* to the effluent disinfected with PAA resulted in 100% mortality for the organisms, which suggests that toxicity tests are a much needed complement to the physical and chemical tests contemplated in national and international legislation;

The bibliographic review on the relative toxicity tolerance between *D. magna* and other tests species allowed to obtain the following conclusions:

- The taxonomic groups that appeared to be more sensitive than *D. magna* to the several contemplated effluents were bacteria and rotifers. Regarding the bacteria group, *V. fischeri* was the most frequently tested species in all the analysed papers and showed a higher sensitivity than *D. magna* in most of the cases, which corroborated with it being one of the most common species to be used in toxicity tests, Thus, it is plausible to conclude that, when using a test battery, *V. fischeri* should be one of the species to be included;

- Rotifers were the taxonomic group registering a lower relative tolerance median in the boxplot chart and a lower sensitivity in general. However, these results are highly influenced by the small number of studies and hence articles evaluating rotifers, so that additional studies are needed to validate whether this holds true for a wider range of effluents (and rotifer species);

- For crustaceans other than *D. magna*, most species appeared to have a sensitivity to the effluents similar to that of *D. magna*, with a narrow range of relative tolerance values;

- Taxonomic groups with higher biological complexity (i.e. fish, insects and macrophytes) showed less sensitivity than *D. magna*, with some exceptions for *D. rerio, O. mykiss, C. tentans,* and *L. minor* for some industrial effluents;

- No single species nor taxonomic group shows the greatest sensitivity to the wide range of existing effluents. Therefore, for a comprehensive and reliable characterisation of effluents toxicity and impact on the receiving ecosystem, a battery of tests including organisms from different taxonomic groups with different endpoints is recommended.

7. Future developments

Regarding the *D. magna* toxicity test:

- Given that the sensitivity study indicated that species other than *D. magna* may be more sensitive, a battery of acute toxicity tests using other species from different taxonomic groups (especially those that were indicated to be potentially more sensitive) should be conducted with the effluent evaluated in this study as well as other effluents;
- In the present study, only the acute toxicity of the treated and untreated effluent was evaluated, whereas wastewater receiving waterbodies are likely to be prone to a continuous flow of wastewater. Subsequently, it is recommended to perform chronic tests with *D. magna* and other species from different taxonomic groups applying effluent and PAA concentrations similar to the actual conditions measured at the discharge site in the wastewater receiving water body;
- Performing *in situ* toxicity tests and monitoring communities of the various taxonomic groups next to the discharge site of the final effluent may provide valuable information on the actual risks under a real-word setting;
- Carry out further studies on the disinfection potential of PAA, more specifically its efficiency at lower concentrations and/or evaluating whether a longer residual time before discharging may prevent eventual side-effects on beneficial organisms;
- Evaluate the physico-chemical and microbiologic quality of the effluent after the disinfection process (UV light) that is currently used in Beirolas WWTP and the eventual reduction in ecotoxicity from this treatment.

Regarding the bibliographic review study on *D. magna* relative tolerance to wastewater effluents:

- In the present study, only papers presenting (mostly acute) toxicity values (i.e. EC50 and LC50) were included for further analysis, which was especially due to the low availability of studies evaluating chronic wastewater ecotoxicity. This may be complemented with articles measuring toxicity through other endpoints (more specifically Toxic Units – which are obtained from EC_{50} values) in other to consolidate the present results and broaden the studyoutcome.
- Study the species sensitivity for more specific effluent types found in the analysed papers, i.e. calculate the TreI separately for domestic, industrial, hospital/pharmaceutical effluents, among others. This may provide insights whether specific effluents especially require the inclusion of particular species in test batteries of these effluents.
- Based on the outcome of these additional analyses, indicate specific research gaps and provide recommendations for how to include ecotoxicological testing in risk assessment procedures of wastewater. Ultimately, this should lead to a toxicity assessment scheme to be implemented in Legislation for setting rules for wastewater ecotoxicity evaluation.

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9. Annexes

Annex A

Number of counted organisms in the reference test for 24 hours.

Sample	Organisms	A	B	C	D
Control 1	Alive	6	7	5	5
	Immobilized	0	0	0	0
	Dead	0	0	0	0
	Total	6	7	5	5
Control 2	Alive	6	5	4	4
	Immobilized	0	0	1	1
	Dead	0	0	0	0
	Total	6	5	5	5
0,32 mg/L $K_2Cr_2O_7$	Alive	5	5	3	4
	Immobilized	0	0	1	1
	Dead	0	0	0	0
	Total	5	5	4	5
0,56 mg/L $K_2Cr_2O_7$	Alive	3	2	4	3
	Immobilized	2	2	2	2
	Dead	0	1	0	1
	Total	5	5	6	6
1,00 mg/L $K_2Cr_2O_7$	Alive	1	0	0	1
	Immobilized	0	0	0	1
	Dead	4	5	5	4
	Total	5	5	5	6
1,80 mg/L $K_2Cr_2O_7$	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
3.2 mg/L $K_2Cr_2O_7$	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	6	8	7
	Total	5	6	8	7

Number of counted organisms in the reference test for 48 hours.

	Organisms	**A**	**B**	**C**	**D**
Control 1	Alive	6	6	4	4
	Immobilized	0	1	1	0
	Dead	0	0	0	1
	Total	6	7	5	5
Control 2	Alive	4	4	5	4
	Immobilized	2	1	0	0
	Dead	0	0	0	1
	Total	6	5	5	5
0,32 mg/L $K_2Cr_2O_7$	Alive	5	3	2	4
	Immobilized	1	1	2	1
	Dead	1	1	1	0
	Total	7	5	5	5
0,56 mg/L $K_2Cr_2O_7$	Alive	3	1	3	2
	Immobilized	2	1	0	0
	Dead	0	3	3	4
	Total	5	5	6	6
1,00 mg/L $K_2Cr_2O_7$	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	6
	Total	5	5	5	6
1,80 mg/L $K_2Cr_2O_7$	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
3.2 mg/L $K_2Cr_2O_7$	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	6	8	7
	Total	5	6	8	7

Number of counted organisms in the test with the effluent for 24 hours.

Sample	Organisms	A	B	C	D
Control 1	Alive	5	7	6	5
	Immobilized	0	0	0	0
	Dead	0	0	0	0
	Total	5	7	6	5
Control 2	Alive	5	5	5	5
	Immobilized	0	0	1	0
	Dead	0	0	0	0
	Total	5	5	6	5
Secondary effluent	Alive	6	5	5	5
	Immobilized	0	0	0	0
	Dead	0	0	0	0
	Total	6	5	5	5
5 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	1	0
	Dead	5	5	4	5
	Total	5	5	5	5
10 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
15 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
15 mg/L PAA in standard freshwater Medium	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	6	6	5	5
	Total	6	6	5	5

Number of counted organisms in the test with the effluent for 48 hours.

Sample	Organisms	A	B	C	D
Control 1	Alive	4	7	6	4
	Immobilized	1	0	0	1
	Dead	0	0	0	0
	Total	5	7	6	5
Control 2	Alive	6	5	5	5
	Immobilized	0	0	1	0
	Dead	0	0	0	0
	Total	6	5	6	5
Secondary effluent	Alive	6	5	5	5
	Immobilized	0	0	0	0
	Dead	0	0	0	0
	Total	6	5	5	5
5 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
10 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
15 mg/L PAA	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	5	5	5	5
	Total	5	5	5	5
15 mg/L PAA in standard freshwater Medium	Alive	0	0	0	0
	Immobilized	0	0	0	0
	Dead	6	6	5	5
	Total	6	6	5	5

Annex B

Statistical output of the EC_{50} – 48h determination.

Nonlinear Regression **segunda-feira, julho 01, 2019, 16:04:32**

Data Source: Data 1 in Notebook2
Equation: Sigmoidal; Logistic, 3 Parameter
f = if(x<=0; if(b<0;0;a); if(b>0; a/(1+abs(x/x0)^b); a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b)))))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0,9451	0,8932	0,8846	0,7924

	Coefficient	Std. Error	t	P
a	5,1600	0,2763	18,6754	<0,0001
b	2,7028	0,6367	4,2453	0,0003
x0	0,5486	0,0556	9,8763	<0,0001

Analysis of Variance:

	DF	SS	MS
Regression	3	311,3021	103,7674
Residual	25	15,6979	0,6279
Total	28	327,0000	11,6786

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	2	131,2663	65,6332	104,5252	<0,0001
Residual	25	15,6979	0,6279		
Total	27	146,9643	5,4431		

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0,0617)

W Statistic= 0,9300 Significance Level = <0,0001

Constant Variance Test Passed (P = 0,0027)

Fit Equation Description:
[Variables]
x = col(1)
y = col(2)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2
reciprocal_pred = 1/abs(f)
reciprocal_predsqr = 1/f^2
[Parameters]
a = max(y) "Auto {{previous: 5,16}}
b = 1 ' {{previous: 2,70282}}
x0 = if(x50(x;y;,5)<>0; x50(x;y;,5); if(mean(x)<>0; mean(x); 1)) "Auto {{previous: 0,548635}}
[Equation]
f = if(x<=0; if(b<0;0;a); if(b>0; a/(1+abs(x/x0)^b); a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b)))))
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare

"fit f to y with weight reciprocal_pred
"fit f to y with weight reciprocal_predsqr
[Constraints]
a>0
x0>0
[Options]
tolerance=0,0000000001
stepsize=1
iterations=200

Number of Iterations Performed = 11

Statistical output of the EC_{50} – 48h determination.

Nonlinear Regression **segunda-feira, julho 01, 2019, 16:46:17**

Data Source: Data 1 in Notebook3
Equation: Sigmoidal; Logistic, 3 Parameter
f = if(x<=0; if(b<0;0;a); if(b>0; a/(1+abs(x/x0)^b); a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b)))))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0,9377	0,8793	0,8697	0,7781

	Coefficient	Std. Error	t	P
a	4,5542	0,2713	16,7880	<0,0001
b	3,3824	0,9799	3,4516	0,0020
x0	0,5119	0,0502	10,2019	<0,0001

Analysis of Variance:

	DF	SS	MS
Regression	3	238,8659	79,6220
Residual	25	15,1341	0,6054
Total	28	254,0000	9,0714

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	2	110,2944	55,1472	91,0973	<0,0001
Residual	25	15,1341	0,6054		
Total	27	125,4286	4,6455		

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0,0267)

W Statistic= 0,9154 Significance Level = <0,0001

Constant Variance Test Passed (P = <0,0001)

Fit Equation Description:
[Variables]
x = col(1)
y = col(2)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2
reciprocal_pred = 1/abs(f)
reciprocal_predsqr = 1/f^2
[Parameters]
a = max(y) "Auto {{previous: 4,5542}}
b = 1 ' {{previous: 3,38239}}
x0 = if(x50(x;y;,5)<>0; x50(x;y;,5); if(mean(x)<>0; mean(x); 1)) "Auto {{previous: 0,511937}}
[Equation]
f = if(x<=0; if(b<0;0;a); if(b>0; a/(1+abs(x/x0)^b); a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b)))))
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare

"fit f to y with weight reciprocal_pred
"fit f to y with weight reciprocal_predsqr
[Constraints]
a>0
x0>0
[Options]
tolerance=0,0000000001
stepsize=1
iterations=200

Number of Iterations Performed = 11

Annex C

Summary table with the Trel calculation and relevant parameters.

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
1	Aguayo et al., 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 9	99.0	% of dilution	1.00	0.0105	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 8	98.0	% of dilution	2.00	0.0278	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 4	84.0	% of dilution	16.00	0.1684	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 3	85.0	% of dilution	15.00	0.2206	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 7	90.0	% of dilution	10.00	0.2222	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 1	80.0	% of dilution	20.00	0.2500	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 2	70.0	% of dilution	30.00	0.4286	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 5	70.0	% of dilution	30.00	0.4762	EC50 (growth inhibition)	48h
1	Aguayo et al. 2004	D & I[1]	Estrogenic substance	No information	*Chlorella vulgaris,*	Algae	WWTP 6	78.0	% of dilution	22.00	0.7333	EC50 (growth inhibition)	48h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Desmodesmus subspicatus*	Algae	Chemical raw	0.79	% (v/v)	0.79	0.3160	EC50 (Growth inhibition)	72h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Desmodesmus subspicatus*	Algae	Textile 2	not detected due to low toxicity	% (v/v)	100.00	2.0964	EC50 (Growth inhibition)	72h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Desmodesmus subspicatus*	Algae	Chemical treatment	not detected due to low toxicity	% (v/v)	100.00	2.8329	EC50 (Growth inhibition)	72h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Desmodesmus subspicatus*	Algae	Textile 1	58.9	% (v/v)	58.90	196.3333	EC50 (Growth inhibition)	72h
136	Machado et al. 2014	Hospital	Hemodialysis effluent	Raw hemodialysis effluent	*Euglena gracilis*	Algae	Mean value	70.9	%	76.90	0.8848	EC50 (mobility)	30 min
220	Ruck 1998	D & I[1]	No information	No information	*Minutocellus polymorphus*	Algae	Industrial effluent	0.83	%	0.83	0.3074	EC50 (Growth inhibition)	72h
186	Pereira et al. 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Pseudokirchneriella subcapitata*	Algae	Untreated 1	0.6	%	0.60	0.0210	EC50 (Cell density)	96h
202	Ribé et al. 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 2 treated	33	%	33.00	0.0330	EC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 5 treated	34	%	34.00	0.0340	EC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 3 treated	48	%	48.00	0.0480	EC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 5 untreated	50	%	50.00	0.0554	EC50 (Growth inhibition)	72h
137	Machado et al 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pseudokirchneriella subcapitata*	Algae	Conventional phsyco-chemical treatment	0.51	%	0.51	0.0793	IC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 3 untreated	135	%	135.00	0.1350	EC50 (Growth inhibition)	72h
137	Machado et al. 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pseudokirchneriella subcapitata*	Algae	GAC	3.63	%	3.63	0.2242	IC50 (Growth inhibition)	72h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Pseudokirchneriella subcapitata*	Algae	Treated with R. oryzae 2	19.7	%	19.70	0.2329	EC50 (Cell density)	96h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Pseudokirchneriella subcapitata*	Algae	Untreated 2	31.4	%	31.40	0.3201	EC50 (Cell density)	96h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment	*Pseudokirchneriella subcapitata*	Algae	Treated with photo-Fenton 2	19.3	%	19.30	0.3522	EC50 (Cell density)	96h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
				with Rhizopus oryzae/ photo-Fenton oxidation)									
215	Rosa et al., 2009	Industrial	Bleach-kraft pulp mill	Secondary treatment	*Pseudokirchneriella subcapitata*	Algae	Before secondary treatmenr	25.4	%	25.40	0.4593	EC50 (Growth inhibition)	72h
137	Machado et al 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pseudokirchneriella subcapitata*	Algae	CER	8.34	%	8.34	0.5151	IC50 (Growth inhibition)	72h
164	Neculita et al. 2008	Industrial	Acid mine drainage wastewater	Passive bioreactors	*Pseudokirchneriella subcapitata*	Algae	10 days hydraulic retention time	24.2	%(v/v)	24.20	0.5500	LC50 (Mortality)	48h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 2 untreated	71	%	71.00	0.5591	EC50 (Growth inhibition)	72h
71	Gallego et al., 2009	Industrial	o-cresol, m-cresol, p-cresol	Biological treatment using Pseudomonas putida	*Pseudokirchneriella subcapitata*	Algae	Batch reactor after treatment	5	% (v/v)	5.00	0.5682	EC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 1 untreated	16	%	16.00	0.6400	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 15	31-nd	% effluent (v/v)	65.50	0.6550	EC50 (Growth inhibition)	72h
6	Antunes et al., 2007b	Industrial	water from mine pit ponds (heavy metals - uranium)	Raw uranium mine wastewater	*Pseudokirchneriella subcapitata*	Algae	Spring	41.6<60.4<82.8	%	60.40	0.7225	EC50 (Immobolization)	96h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Pseudokirchneriella subcapitata*	Algae	Treated with R. oryzae 1	46.9	%	46.90	0.7397	EC50 (Cell density)	96h
6	Antunes et al. 2007b	Industrial	water from mine pit ponds (heavy metals - uranium)	Raw uranium mine wastewater	*Pseudokirchneriella subcapitata*	Algae	Autumn	25.49<27.0<28.42	%	27.00	0.7542	EC50 (Immobolization)	96h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 10	10-nd	% effluent (v/v)	55.00	0.8271	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 1	9.9-nd	% effluent (v/v)	54.95	0.8863	EC50 (Growth inhibition)	72h
276	Zgorska, Arendarczyk, & Grabinska-Sota, 2011	Hospital	No information	No information	*Pseudokirchneriella subcapitata*	Algae	N.A	18.77	%	18.77	0.9041	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 17	5.6-nd	% effluent (v/v)	52.80	0.9760	EC50 (Growth inhibition)	72h
147	Mendonça et al., 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Mon 10h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Mon 14h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Tues 10h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Tues 14h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Mon 14h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Mon 23h	90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Tues 10h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Tues 14h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Fri 14h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Fri 23h	>90	%	90.00	1.0000	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 8	nd	% effluent (v/v)	100.00	1.0000	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 11	46-nd	% effluent (v/v)	73.00	1.0000	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 14	nd	% effluent (v/v)	100.00	1.0000	EC50 (Growth inhibition)	72h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 12	5.6-nd	% effluent (v/v)	52.80	1.0272	EC50 (Growth inhibition)	72h
24	Brienza et al 2016	Domestic	Terciary treatment with AOP (advanced oxidation processses - hidroxil and sulfate radicals)	No information	*Pseudokirchneriella subcapitata*	Algae	Raw wastewater	98	%(v/v)	98.00	1.0889	EC50 (Growth inhinition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Fri 23h	>90	%	90.00	1.2162	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 6	nd	% effluent (v/v)	100.00	1.2195	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Mon 23h	>90	%	90.00	1.3433	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Tues 23h	>90	%	90.00	1.3433	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample B Fri 10h	>90	%	90.00	1.3433	EC50 (Growth inhibition)	72h
202	Ribé et al 2012	Domestic	Landfill leachates	Pine bark biosorbent	*Pseudokirchneriella subcapitata*	Algae	Sample 4 untreated	49	%	49.00	1.4000	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 2	13-nd	% effluent (v/v)	56.50	1.4752	EC50 (Growth inhibition)	72h
253	Tønning et al., 2005	Industrial	Chemi-thermo-mechanical pulp mill wastewater	No information	*Pseudokirchneriella subcapitata*	Algae	Untreated wastewater	43	ml/L	4.30	1.4828	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 7	5.6-nd	% effluent (v/v)	52.80	1.5172	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Fri 14h	>90	%	90.00	1.6981	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Tues 23h	>90	%	90.00	1.7308	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 16	7.0-nd	% effluent (v/v)	53.50	2.2863	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 13	59-nd	% effluent (v/v)	79.50	2.4961	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 4	5.6-nd	% effluent (v/v)	52.80	2.8085	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 5	nd	% effluent (v/v)	100.00	2.9851	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 3	13-nd	% effluent (v/v)	56.50	3.0053	EC50 (Growth inhibition)	72h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Pseudokirchneriella subcapitata*	Algae	Sample A Fri 10h	>90	%	90.00	3.2143	EC50 (Growth inhibition)	72h
193	Picado et al 2008	Industrial	No information	No information	*Pseudokirchneriella subcapitata*	Algae	Industry 9	3.1-nd	% effluent (v/v)	51.55	4.3138	EC50 (Growth inhibition)	72h
262	Vidal et al., 2012	Industrial	Acid mine drainage	No information	*Pseudokirchneriella subcapitata*	Algae	N.A	6.65	mg/L	15.64	5.3746	EC50 (Yield)	72h
213	Rosa et al 2001	Industrial	Textile	No information	*Scenedesmus subspicatus*	Algae	Untreated	6.2	%	6.20	0.2480	EC50 (Mortality)	72h
220	Ruck 1998	D & I[1]	No information	No information	*Selenastrum capricornutum*	Algae	Industrial effluent	0.21	%	0.21	0.0778	EC50 (Growth inhibition)	72h
249	Tisler & Zagorc-Koncan, 1994	Industrial	Effluent from white pigment production	No information	*Photobacterium phosphoreum*	Bacteria	N.A	18.26	% vol	18.26	8.7788	LC50 (Mortality)	30 min
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Fri 23h	1.1	%	1.10	0.0149	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 12	0.1-2.2	% effluent (v/v)	1.15	0.0224	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Fri 23h	2.8	%	2.80	0.0311	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Tues 23h	2.2	%	2.20	0.0423	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Fri 14h	2.3	%	2.30	0.0434	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Mon 23h	3.1	%	3.10	0.0463	EC50 (Inhibition of luminescence)	15 min

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Mon 14h	5.2	%	5.20	0.0578	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al. 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 1	0.45	%	0.45	0.0659	EC50 (Inhibition of luminescence)	15 min
71	Gallego et al 2008	Industrial	o-cresol, m-cresol, p-cresol	Biological treatment using Pseudomonas putida	*Vibrio fischeri*	Bacteria	Batch reactor after treatment	0.7	% (v/v)	0.70	0.0795	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Tues 10h	7.2	%	7.20	0.0800	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Tues 23h	5.6	%	5.60	0.0836	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Tues 14h	7.9	%	7.90	0.0878	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Fri 10h	6	%	6.00	0.0896	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Fri 14h	8.8	%	8.80	0.0978	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Mon 23h	9	%	9.00	0.1000	EC50 (Inhibition of luminescence)	15 min
253	Tønning et al., 2005	Industrial	Chemi-thermo-mechanical pulp mill wastewater	No information	*Vibrio fischeri*	Bacteria	Untreated wastewater	3	ml/L	0.30	0.1034	EC50 (Inhibition of luminescense)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 11	0.13	%	0.13	0.1204	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 2 treated	133	%	133.00	0.1330	EC50 (Inhibition of luminescence)	30 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 4	4	%	4.00	0.1379	EC50 (Inhibition of luminescence)	8 min
252	Tišler et al. 2004	Industrial	Tannery wastewater	Desinfection with bactericide	*Vibrio fischeri*	Bacteria	N.A.	3.9	% (v/v)	3.90	0.1523	EC50 (Inhibition of luminescense)	30 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 7	0.23	%	0.23	0.1608	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 10	0.52	%	0.52	0.1640	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 4	0.28	%	0.28	0.1647	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Mon 10h	17.2	%	17.20	0.1911	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 9	0.35	%	0.35	0.1989	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample A Fri 10h	5.6	%	5.60	0.2000	EC50 (Inhibition of luminescence)	15 min
146	Mendonlça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 9	4	%	4.00	0.2105	EC50 (Inhibition of luminescence)	13 min
213	Rosa et al 2001	Industrial	Textile	No information	*Vibrio fischeri*	Bacteria	Untreated	5.4	%	5.40	0.2160	EC50 (Inhibition of luminescence)	15 min
82	Grinevicius et al 2009	Industrial	Textile effluents	remediation by a pulverized chitosan system/remediation with biologic and physico-chemical effluent)	*Vibrio fischeri*	Bacteria	Non remediated textile effluent	10.64	% (v/v)	10.64	0.2172	EC50 (Inhibition of luminescence)	5 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 6	0.27	%	0.27	0.2231	EC50 (Inhibition of luminescence)	15 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 2	5	%	5.00	0.2273	EC50 (Inhibition of luminescence)	6 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 13	0.57-14	% effluent (v/v)	7.29	0.2287	EC50 (Inhibition of luminescence)	15 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Tues 14h	20.8	%	20.80	0.2311	EC50 (Inhibition of luminescence)	15 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 3	4	%	4.00	0.2353	EC50 (Inhibition of luminescence)	7 min

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 3	0.22	%	0.22	0.2391	EC50 (Inhibition of luminescence)	15 min
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Vibrio fischeri*	Bacteria	Treated with photo-Fenton 2	13.4	%	13.40	0.2445	EC50 (Inhibition of luminescence)	5 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 8	0.42	%	0.42	0.2485	EC50 (Inhibition of luminescence)	15 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 5	5	%	5.00	0.2500	EC50 (Inhibition of luminescence)	9 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 6	11.0-31	% effluent (v/v)	21.00	0.2561	EC50 (Inhibition of luminescence)	15 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 1	8	%	8.00	0.2667	EC50 (Inhibition of luminescence)	5 min
259	Vasseur et al. 1984	Industrial	Pharmaceuticals/ artificial textilles sewage/silicones/organic dyes	No information	*Vibrio fischeri*	Bacteria	Effluent nº 2	0.36	%	0.36	0.2903	EC50 (Inhibition of luminescence)	10 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 16	1.2-13	% effluent (v/v)	7.10	0.3034	EC50 (Inhibition of luminescence)	15 min
40	Cotman et al 2004	Industrial	Tanney wastewater	No information	*Vibrio fischeri*	Bacteria	no information	6.08	%	6.08	0.3055	EC50 Inhibition of luminescence	30 min
259	Vasseur et al 1984	Industrial	Pharmaceuticals/ artificial textilles sewage/silicones/organic dyes	No information	*Vibrio fischeri*	Bacteria	Effluent nº 1	0.18	%	0.18	0.3103	EC50 (Inhibition of luminescence)	10 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 1 untreated	8	%	8.00	0.3200	EC50 (Inhibition of luminescence)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 10	3.1-43	% effluent (v/v)	23.05	0.3466	EC50 (Inhibition of luminescence)	15 min
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Vibrio fischeri*	Bacteria	Treated with R. oryzae 1	22.8	%	22.80	0.3596	EC50 (Inhibition of luminescence)	5 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 2	0.58	%	0.58	0.3602	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 12	0.7	%	0.70	0.3646	EC50 (Inhibition of luminescence)	15 min
146	Mendonça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 6	3	%	3.00	0.3750	EC50 (Inhibition of luminescence)	10 min
10	Aydin et al. 2015b	Industrial	Landfill leachattes	raw landfill leachate	*Vibrio fischeri*	Bacteria	February	0.623	%	0.63	0.3765	EC50 (Inhibition of luminescence)	30 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Tues 10h	34.9	%	34.90	0.3878	EC50 (Inhibition of luminescence)	15 min
10	Aydin et al. 2015	Industrial	Landfill leachattes	raw landfill leachate	*Vibrio fischeri*	Bacteria	April	0.625	%	0.63	0.4058	EC50 (Inhibition of luminescence)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 5	7.9-21	% effluent (v/v)	14.45	0.4313	EC50 (Inhibition of luminescence)	15 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 5	0.51	%	0.51	0.4435	EC50 (Inhibition of luminescence)	15 min
7	Arias-barreiro et al. 2010	Industrial	Tannery wastewater (chromium contamination)	Raw wastewater	*Vibrio fischeri*	Bacteria	N.A	14.16 (9.4-21.3)	%	14.16	0.4484	EC50 (95% confidence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 5 untreated	411	%	411.00	0.4557	EC50 (Inhibition of luminescence)	30 min
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 13	1.05	%	1.05	0.4688	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 5 treated	470	%	470.00	0.4700	EC50 (Inhibition of luminescence)	30 min
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Vibrio fischeri*	Bacteria	Sample B Mon 14h	42.6	%	42.60	0.4733	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 2 untreated	62	%	62.00	0.4882	EC50 (Inhibition of luminescence)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 14	27-nd	% effluent (v/v)	63.50	0.6350	EC50 (Inhibition of luminescence)	15 min

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
81	Gotvajn et al. 2009	Industrial	Industrial landfill leachate	no treatment	*Vibrio fischeri*	Bacteria	Sample 2	0.7	%(v/v)	0.70	0.6364	EC50 (Inhibition of luminescence)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 8	28-nd	% effluent (v/v)	64.00	0.6400	EC50 (Inhibition of luminescence)	15 min
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Vibrio fischeri*	Bacteria	Chemical raw	1.6	% (v/v)	1.60	0.6400	EC50 (Inhibition of luminescense)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 11	3.4-nd	% effluent (v/v)	51.70	0.7082	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 3 treated	749	%	749.00	0.7490	EC50 (Inhibition of luminescence)	30 min
146	Mendonlça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 8	7	%	7.00	0.8750	EC50 (Inhibition of luminescence)	12 min
215	Rosa et al 2009	Industrial	Bleach-kraft pulp mill	Secondary treatment	*Vibrio fischeri*	Bacteria	Before secondary treatmenr	48.9	%	48.90	0.8843	EC50 (Inhibition of luminescence)	5 min
24	Brienza et al 2016	Domesti c	Terciary treatment with AOP (advanced oxidation processses - hidroxil and sulfate radicals)	No information	*Vibrio fischeri*	Bacteria	Raw wastewater	80	%(v/v)	80.00	0.8889	EC50 (Inhibition of luminescence)	30 min
201	Restrepo et al., 2017	Domesti c	Municipal solid waste leachate	No information	*Vibrio fischeri*	Bacteria	PR3 a	2.62	%	16.08	0.8971	EC50 (Inhibition of luminescence)	15 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 4	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 5	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 6	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 7	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 8	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Vibrio fischeri*	Bacteria	Sample 11	90	% (in %of metal plating wastewater)	90.00	0.9000	EC50 (Inhibiton of luminescence)	30 min
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Vibrio fischeri*	Bacteria	May	2.52	%	2.52	0.9618	EC50 (Inhibition of luminescence)	30 min
83	Guerra (2001)	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Vibrio fischeri*	Bacteria	Sample 4	35.5	% (v/v)	0.52	0.9630	LC50 (mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 15	nd	% effluent (v/v)	100.00	1.0000	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 3 untreated	1000	%	1000.00	1.0000	EC50 (Inhibition of luminescence)	30 min
146	Mendonlça et al 2007	Industrial	Cork boiling wastewaters	Raw cork wastewater	*Vibrio fischeri*	Bacteria	Sample 7	5	%	5.00	1.0000	EC50 (Inhibition of luminescence)	11 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 2	12.0-66	% effluent (v/v)	39.00	1.0183	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 1	27-nd	% effluent (v/v)	63.50	1.0242	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 17	11-nd	% effluent (v/v)	55.50	1.0259	EC50 (Inhibition of luminescence)	15 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 4 treated	67	%	67.00	1.2885	EC50 (Inhibition of luminescence)	30 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 7	1.7-90	% effluent (v/v)	45.95	1.3204	EC50 (Inhibition of luminescence)	15 min
262	Vidal et al 2012	Industrial	Acid mine drainage	No information	*Vibrio fischeri*	Bacteria	N.A	3.99	mg/L	3.99	1.3711	EC50 (Inhibition of luminescence)	15 min

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
259	Vasseur et al 1984	Industrial	Pharmaceuticals/ artificial textilles sewage/silicones/organic dyes	No information	*Vibrio fischeri*	Bacteria	Effluent nº 3	41.4	%	41.40	1.6235	EC50 (Inhibition of luminescence)	10 min
202	Ribé et al 2012	Domesti c	Landfill leachates	Pine bark biosorbent	*Vibrio fischeri*	Bacteria	Sample 4 untreated	66	%	66.00	1.8857	EC50 (Inhibition of luminescence)	30 min
38	Choir & Meier 2001	Industrial	Metal plating waste water	No information	*Vibrio fischeri*	Bacteria	Sample 10	90	% (in %of metal plating wastewater)	90.00	2.0930	EC50 (Inhibiton of luminescence)	30 min
248	Tisler & Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Vibrio fischeri*	Bacteria	Textile 2	not detected due to low toxicity	% (v/v)	100.00	2.0964	EC50 (Inhibition of luminescense)	30 min
276	Zgorska et al 2011	Hospital	No information	No information	*Vibrio fischeri*	Bacteria	N.A	46.17	%	46.17	2.2240	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 4	0.64-nd	% effluent (v/v)	50.32	2.6766	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 3	0.99-nd	% effluent (v/v)	50.50	2.6859	EC50 (Inhibition of luminescence)	15 min
193	Picado et al 2008	Industrial	No information	No information	*Vibrio fischeri*	Bacteria	Industry 9	5.6-60	% effluent (v/v)	32.80	2.7448	EC50 (Inhibition of luminescence)	15 min
248	Tisler & Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Vibrio fischeri*	Bacteria	Chemical treatment	not detected due to low toxicity	% (v/v)	100.00	2.8329	EC50 (Inhibition of luminescense)	30 min
259	Vasseur et al 1984	Industrial	Pharmaceuticals/ artificial textilles sewage/silicones/organic dyes	No information	*Vibrio fischeri*	Bacteria	Effluent nº 4	2.64	%	2.64	3.0000	EC50 (Inhibition of luminescence)	10 min
81	Gotvajn et al 2009	Industrial	Industrial landfill leachate	no treatment	*Vibrio fischeri*	Bacteria	Sample 4	4.5	%(v/v)	4.50	3.2143	EC50 (Inhibition of luminescence)	30 min
81	Gotvajn et al 2009	Industrial	Industrial landfill leachate	no treatment	*Vibrio fischeri*	Bacteria	Sample 1	11.3	%(v/v)	11.30	3.5313	EC50 (Inhibition of luminescence)	30 min
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Vibrio fischeri*	Bacteria	Sample 1	3.97	% (v/v)	1.63	4.7941	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Vibrio fischeri*	Bacteria	Sample 2	2.73	% (v/v)	0.80	5.0000	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Vibrio fischeri*	Bacteria	Sample 3	23.4	% (v/v)	1.06	5.0476	LC50 (mortality)	24h
38	Choir & Meier 2001	Industrial	Metal plating waste water	No information	*Vibrio fischeri*	Bacteria	Sample 2	51.83	% (in %of metal plating wastewater)	51.83	7.1886	EC50 (Inhibiton of luminescence)	30 min
248	Tisler & Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Vibrio fischeri*	Bacteria	Textile 1	2.5	% (v/v)	2.50	8.3333	EC50 (Inhibition of luminescense)	30 min
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Vibrio fischeri*	Bacteria	March	24.34	%	24.34	31.2051	EC50 (Inhibition of luminescence)	30 min
201	Restrepo et al 2017	Domesti c	Municipal solid waste leachate	No information	*Vibrio fischeri*	Bacteria	PR1 a	8.38	%	8.65	31.4455	EC50 (Inhibition of luminescence)	15 min
201	Restrepo et al 2017	Domesti c	Municipal solid waste leachate	No information	*Vibrio fischeri*	Bacteria	PR2 a	8.7	%	8.40	40.6290	EC50 (Inhibition of luminescence)	15 min
38	Choir & Meier 2001	Industrial	Metal plating waste water	No information	*Vibrio fischeri*	Bacteria	Sample 1	0.93	% (in %of metal plating wastewater)	0.93	155.0000	EC50 (Inhibiton of luminescence)	30 min
38	Choir & Meier 2001	Industrial	Metal plating waste water	No information	*Vibrio fischeri*	Bacteria	Sample 3	30.96	% (in %of metal plating wastewater)	30.96	2064.0000	EC50 (Inhibiton of luminescence)	30 min
38	Choir & Meier 2001	Industrial	Metal plating waste water	No information	*Vibrio fischeri*	Bacteria	Sample 9	77.19	% (in %of metal plating wastewater)	77.19	5937.6923	EC50 (Inhibiton of luminescence)	30 min
220	Ruck 1998	D & I[1]	No information	No information	*Artemia franciscana*	Crustacea	Industrial effluent	36.8	%	36.80	13.6296	EC50 (Growth inhibition)	72h
276	Zgorska et al 2011	Hospital	No information	No information	*Artemia salina*	Crustacea	N.A	59.87	%	59.87	2.8839	LC50 (Mortality)	24H
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Artenia salina*	Crustacea	Sample 1	3.97	% (v/v)	3.97	11.6765	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Artenia salina*	Crustacea	Sample 2	2.73	% (v/v)	2.73	17.0625	LC50 (mortality)	24h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Artenia salina*	Crustacea	Sample 4	35.5	% (v/v)	35.50	65.7407	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Artenia salina*	Crustacea	Sample 3	23.4	% (v/v)	23.40	111.4286	LC50 (mortality)	24h
220	Ruck 1998	D & I[1]	No information	No information	*Ceriodaphnia dubia*	Crustacea	Industrial effluent	3	%	3.00	1.1111	EC50 (Mortality)	48h
220	Ruck 1998	D & I[1]	No information	No information	*Chaetocorophium cf lucasi*	Crustacea	Industrial effluent	0.64	%	0.64	0.2370	EC50 (Growth inhibition)	72h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Daphnia longispina*	Crustacea	Treated with photo-Fenton 2	10	%	10.00	0.1825	EC50 (Immobilization)	48h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Daphnia longispina*	Crustacea	Treated with R. oryzae 2	36.1	%	36.10	0.4267	EC50 (Immobilization)	48h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Daphnia longispina*	Crustacea	Untreated 1	14.2	%	14.20	0.4965	EC50 (Immobilization)	48h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Daphnia longispina*	Crustacea	Control 2	62.1	%	62.10	0.6330	EC50 (Immobilization)	48h
186	Pereira et al 2009	Industrial	Kraft pulp mill effluent	Secondary and tertiary treatment (treatment with Rhizopus oryzae/ photo-Fenton oxidation)	*Daphnia longispina*	Crustacea	Treated with R. oryzae 1	52	%	52.00	0.8202	EC50 (Immobilization)	48h
6	Antunes et al., 2007b	Industrial	water from mine pit ponds (heavy metals - uranium)	Raw uranium mine wastewater	*Daphnia longispina*	Crustacea	Autumn	16.95<20.5<24.25	%	20.50	0.5726	EC50 (Immobolization)	48h
6	Antunes et al. 2007b	Industrial	water from mine pit ponds (heavy metals - uranium)	Raw uranium mine wastewater	*Daphnia longispina*	Crustacea	Spring	41.6<49.3<60.2	%	49.30	0.5897	EC50 (Immobolization)	48h
270	Xavier et al 2017	Industrial	Kraft mill effluents	Primary and secondary treatment (activated sludge)	*Daphnia obtusa*	Crustacea	Influent	>100	%	100.00	4.8591	LC50 (Mortality)	48h
262	Vidal et al 2012	Industrial	Acid mine drainage	No information	*Daphnia pulex*	Crustacea	N.A	1.31	mg/L	1.31	0.4502	EC10 (Growth)	72h
39	Cooman et al., 2003	Industrial	Tanney wastewater	No information	*Daphnia pulex*	Crustacea	Influent 45 days	12.25	%	12.25	0.8472	LC50 (mortality)	48h
39	Cooman et al., 2003	Industrial	Tanney wastewater	No information	*Daphnia pulex*	Crustacea	Effluent 45 days	53.66	%	53.66	0.8816	LC50 (mortality)	48h
261	Vidal et al., 2004	Industrial	Leather tannery effluents	Activated sludge	*Daphnia pulex*	Crustacea	Influent	36	%	36.00	0.9000	LC50 (Mortality)	24h
261	Vidal et al 2004	Industrial	Leather tannery effluents	Activated sludge	*Daphnia pulex*	Crustacea	Aerobic effluent	68	%	68.00	0.9855	LC50 (Mortality)	48h
39	Cooman et al., 2003	Industrial	Tanney wastewater	No information	*Daphnia pulex*	Crustacea	Effluent 127 days	67.65	%	67.65	1.0253	LC50 (mortality)	48h
261	Vidal et al 2004	Industrial	Leather tannery effluents	Activated sludge	*Daphnia pulex*	Crustacea	Influent	35	%	35.00	1.0294	LC50 (Mortality)	48h
261	Vidal et al 2004	Industrial	Leather tannery effluents	Activated sludge	*Daphnia pulex*	Crustacea	Aerobic effluent	68	%	68.00	1.0968	LC50 (Mortality)	24h
39	Cooman et al 2003	Industrial	Tanney wastewater	No information	*Daphnia pulex*	Crustacea	Influent 127 days	8.66	%	8.66	1.5035	LC50 (mortality)	48h
273	Yi et al. 2010)	Industrial	Textile and dyeing effluents	Primary treatment, flotation, denitrification, secondary treatment and fenton treatment	*Moina macrocopa*	Crustacea	Final effluent	95.2	Toxic units	95.20	1.6850	EC50 (Immobilization)	48h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 17	5.6-nd	% effluent (v/v)	5.60	0.1035	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Mon 14h	33	%	33.00	0.3667	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Mon 23h	33	%	33.00	0.3667	EC50 (Mortality)	24h
102	Karadima et al. 2009	Industrial	Cheese manufacturing effluent	aerobic fermentation	*Thamnocephalus platyurus*	Crustacea	Average value	0.69	%	0.69	0.3791	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Mon 10h	35.4	%	35.40	0.3933	EC50 (Mortality)	24h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Tues 14h	35.4	%	35.40	0.3933	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Mon 14h	36.2	%	36.20	0.4022	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Tues 14h	36.2	%	36.20	0.4022	EC50 (Mortality)	24h
217	Rouvalis & Iliopoulou-Georgudaki, 2010	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	OMW average	1.77	% (v/v)	1.77	0.4041	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Tues 10h	37	%	37.00	0.4111	EC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 7	2.8-26	% effluent (v/v)	14.40	0.4138	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 9	0.73	%	0.73	0.4148	LC50 (Mortality)	24h
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Thamnocephalus platyurus*	Crustacea	May	1.09	%	1.09	0.4160	LC50 (mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Mon 23h	28.1	%	28.10	0.4194	EC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 2	1.3-31	% effluent (v/v)	16.15	0.4217	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Fri 23h	43.5	%	43.50	0.4833	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Fri 14h	44.5	%	44.50	0.4944	EC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 11	15-59	% effluent (v/v)	37.00	0.5068	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Fri 23h	41.1	%	41.10	0.5554	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Tues 23h	29.4	%	29.40	0.5654	EC50 (Mortality)	24h
103	Karadima et al 2010	Industrial	Cheese manufacturing effluent	aerobic fermentation	*Thamnocephalus platyurus*	Crustacea	Pure cheese whey effleunt	1.56	%	1.56	0.5843	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Tues 10h	54.8	%	54.80	0.6089	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Tues 23h	42.5	%	42.50	0.6343	EC50 (Mortality)	24h
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Thamnocephalus platyurus*	Crustacea	February	1.12	%	1.12	0.6747	LC50 (mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample B Fri 10h	46.6	%	46.60	0.6955	EC50 (Mortality)	24h
147	Mendonça et al 2013	Domestic	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Fri 14h	37.7	%	37.70	0.7113	EC50 (Mortality)	24h
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Thamnocephalus platyurus*	Crustacea	April	1.12	%	1.12	0.7273	LC50 (mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 5	0.9	%	0.90	0.7826	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 6	40-nd	% effluent (v/v)	70.00	0.8537	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 3	1.7-31	% effluent (v/v)	16.35	0.8697	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 9	1.1-20	% effluent (v/v)	10.55	0.8828	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 16	9.9-33	% effluent (v/v)	21.45	0.9167	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 10	30-nd	% effluent (v/v)	65.00	0.9774	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 13	5.6-57	% effluent (v/v)	31.30	0.9827	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 8	nd	% effluent (v/v)	100.00	1.0000	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 14	nd	% effluent (v/v)	100.00	1.0000	LC50 (Mortality)	24h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 15	nd	% effluent (v/v)	100.00	1.0000	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 12	3.9-nd	% effluent (v/v)	51.95	1.0107	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 4	5.6-33	% effluent (v/v)	19.30	1.0266	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 4	1.85	%	1.85	1.0882	LC50 (Mortality)	24h
276	Zgorska et al 2011	Hospital	No information	No information	*Thamnocephalus platyurus*	Crustacea	N.A	22.62	%	22.62	1.0896	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 12	2.11	%	2.11	1.0990	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 13	2.48	%	2.48	1.1071	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 1	41-nd	% effluent (v/v)	70.50	1.1371	LC50 (Mortality)	24h
147	Mendonça et al 2013	Domesti c	No information	Secondary and tertiary (desinfection treatment)	*Thamnocephalus platyurus*	Crustacea	Sample A Fri 10h	37.9	%	37.90	1.3536	EC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 10	4.32	%	4.32	1.3628	LC50 (Mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industry 5	25-71	% effluent (v/v)	48.00	1.4328	LC50 (Mortality)	24h
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Thamnocephalus platyurus*	Crustacea	March	1.16	%	1.16	1.4872	LC50 (mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 6	1.9	%	1.90	1.5702	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 8	2.89	%	2.89	1.7101	LC50 (Mortality)	24h
220	Ruck 1998	D & I	No information	No information	*Thamnocephalus platyurus*	Crustacea	Industrial effluent	4.8	%	4.80	1.7778	EC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 7	2.62	%	2.62	1.8322	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 1	12.54	%	12.54	1.8360	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 2	3.74	%	3.74	2.3230	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 11	3.14	%	3.14	2.9074	LC50 (Mortality)	24h
176	Paixão et al 1999	Industrial	olive oil mill wastewater	No information	*Thamnocephalus platyurus*	Crustacea	Sample 3	2.79	%	2.79	3.0326	LC50 (Mortality)	24h
103	Karadima et al 2010	Industrial	Cheese manufacturing effluent	aerobic fermentation	*Danio rerio*	Fish	Pure cheese whey effleunt	0.66	%	0.66	0.2472	LC50 (Mortality)	7d
82	Grinevicius et al., 2008	Industrial	Textile effluents	remediation by a pulverized chitosan system/remediation with biologic and physico-chemical effluent)	*Danio rerio*	Fish	Non remediated textile effluent	12.22	% (v/v)	12.22	0.2495	EC50 (Mortality)	7 days
252	Tisler et al 2004	Industrial	Tannery wastewater	Desinfection with bactericide	*Danio rerio*	Fish	N.A.	7.3	% (v/v)	7.30	0.2852	LC50 (Mortality)	96h
217	Rouvalis & Iliopoulou-Georgudaki 2010	Industrial	olive oil mill wastewater	No information	*Danio rerio*	Fish	OMW average	1.52	% (v/v)	1.52	0.3470	LC50 (Mortality)	48h
102	Karadima et al 2009	Industrial	Cheese manufacturing effluent	aerobic fermentation	*Danio rerio*	Fish	Average value	1.55	%	0.75	0.4121	LC50 (Mortality)	48h
278	Zhou et al., 2015	Industrial	Coking wastewater	No information	*Danio rerio*	Fish	Influent	3	%	3.00	1.0000	LC50 (Mortality)	96h
278	Zhou et al 2015	Industrial	Coking wastewater	No information	*Danio rerio*	Fish	Effluent	100	%	100.00	1.2987	LC50 (Mortality)	96h
158	Na et al., 2017	Industrial	Coking wastewater	Anaerobic-anoxic-oxic and advanced oxidation process	*Danio rerio*	Fish	Untreated effluent WWTP 1	1.91	%	1.91	1.2993	EC50 (Mortality)	96h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
158	Na et al 2017	Industrial	Coking wastewater	Anaerobic-anoxic-oxic and advanced oxidation process	*Danio rerio*	Fish	Untreated effluent WWTP 2	2.93	%	2.93	1.5181	EC50 (Mortality)	96h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Danio rerio*	Fish	Chemical treatment	69.6	% (v/v)	69.60	1.9717	LC50 (Mortality)	96h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Danio rerio*	Fish	Textile 2	not detected due to low toxicity	% (v/v)	100.00	2.0964	LC50 (Mortality)	96h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Danio rerio*	Fish	Chemical raw	15.5	% (v/v)	15.50	6.2000	LC50 (Mortality)	96h
105	Kern et al., 2015	Hospital	Hospital laundry wastewaters	Untreated laudry wastewaters	*Danio rerio*	Fish	Average value	29.25	%	29.25	14.5522	EC50 (Mortality)	48h
248	Tisler and Zagorc-Koncan 2008	Industrial	Textile industry	No information	*Danio rerio*	Fish	Textile 1	12.2	% (v/v)	12.20	40.6667	LC50 (Mortality)	96h
209	Rodgers, 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Add filter 18 March 92	100	%	100.00	1.0000	LC50 (Mortality)	96h
209	Rodgers 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Add CaCl2 18 March 92	100	%	100.00	1.4514	LC50 (Mortality)	96h
209	Rodgers 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Untreated 18 March 92	89	%	89.00	1.5724	LC50 (Mortality)	96h
209	Rodgers 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Add filter 25 March 92	not letal	%	100.00	1.6949	LC50 (Mortality)	96h
164	Neculita et al 2008	Industrial	Acid mine drainage wastewater	Passive bioreactors	*Oncorhynchus mykiss*	Fish	10 days hydraulic retention time	100	%(v/v)	100.00	2.2727	LC50 (Mortality)	96h
209	Rodgers 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Untreated 25 March 92	89.4	%	89.40	2.9701	LC50 (Mortality)	96h
209	Rodgers 1994	Industrial	Boiler blowdwon effluent	Addition of calcium/ humic acid	*Oncorhynchus mykiss*	Fish	Add CaCl2 25 March 92	not letal	%	100.00	4.0486	LC50 (Mortality)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 4	1.49	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 5	100	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 6	not performed	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 7	not performed	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 8	not performed	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating waste water	No information	*Pimephales promelas*	Fish	Sample 11	not performed	% (in %of metal plating wastewater)	100.00	1.0000	IC25 (fathead minnows)	96h
106	Khan et al. 1992	Industrial	no information	secondary impoundment system	*Pimephales promelas*	Fish	Year 2 round III	>100	%	75.20	1.1899	LC50 (Mortality)	7d
137	Machado et al 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pimephales promelas*	Fish	CER	39.9	%	39.90	1.3394	LC50 (Mortality)	48h
38	Choi & Meier 2001	Industrial	Metal plating waste water	No information	*Pimephales promelas*	Fish	Sample 10	not performed	% (in %of metal plating wastewater)	75.16	1.7479	IC25 (fathead minnows)	96h
137	Machado et al 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pimephales promelas*	Fish	GAC	38.62	%	38.62	2.3854	LC50 (Mortality)	48h
137	Machado et al 2017	Industrial	metal finishing effluents	Bach reactor, decanter, filter bed column, GAC, column bed with cationic exchange resin	*Pimephales promelas*	Fish	Conventional phsyco-chemical treatment	21.4	%	21.40	3.3281	LC50 (Mortality)	48h

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 2	3.16	% (in %of metal plating wastewater)	56.76	7.8724	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 1	0.16	% (in %of metal plating wastewater)	2.99	498.3333	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 3	0.007	% (in %of metal plating wastewater)	12.79	852.6667	IC25 (fathead minnows)	96h
38	Choi & Meier 2001	Industrial	Metal plating wastewater	No information	*Pimephales promelas*	Fish	Sample 9	not performed	% (in %of metal plating wastewater)	88.86	6835.3846	IC25 (fathead minnows)	96h
214	Rosa et al., 2007	Industrial	Textile sludge	No information	*Poecila reticulata*	Fish	Fresh sludge leachate	100	%	100.00	2.0000	LOEC (Mortality)	24h
213	Rosa et al 2001	Industrial	Textile	No information	*Poecilia reticulata*	Fish	Untreated	50	%	50.00	2.0000	EC50 (Mortality)	48h
117	Laliberte, 2018	Industrial	gold-mine effluent	Biological reactors (with nitrogen removal) and precipitation	*Rainbow trout*	Fish	Untreated	35.4	%	7.59	0.2144	LC 50 (Mortality)	96h
117	Laliberte, 2018	Industrial	gold-mine effluent	Biological reactors (with nitrogen removal) and precipitation	*Rainbow trout*	Fish	Treated	65.9	%	33.00	0.5008	LC 50 (Mortality)	96h
14	Bellemare et al. 2006	Industrial	mining industry	No information	*Trout*	Fish	Treated	23	%(v/v)	23.00	0.2300	EC50 (mortality)	96h
14	Bellemare et al 2006	Industrial	mining industry	No information	*Trout*	Fish	Untreated	19	%(v/v)	19.00	0.5000	EC50 (mortality)	96h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Metallurgical industry wastewater	1.46	%	1.46	0.4384	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp..	Insecta	Textile industry	37.19	%	37.19	0.5990	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Metallurgical industry wastewater	6.6	%	6.60	1.1321	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Mining drilling fluids	4.65	%	4.65	1.9295	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	wate from processing of waste paper	22.93	%	22.93	2.4316	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Mechanical industry	8.84	%	8.84	3.2620	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Mining drilling fluids	34.1	%	34.10	3.3596	EC50 (mortality)	24h
25	Butarewicz et al., 2019	Industrial	heavy metals, PAH's, PCB	No information	*Chironomus* sp.	Insecta	Inorganic industry	1.18	%	1.18	59.0000	EC50 (mortality)	24h
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 15	28-nd	% effluent (v/v)	64.00	0.6400	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 7	5.1-44	% effluent (v/v)	24.55	0.7055	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 8	49-nd	% effluent (v/v)	74.50	0.7450	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 14	65-nd	% effluent (v/v)	82.50	0.8250	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 2	6.3-60	% effluent (v/v)	33.15	0.8655	EC50 (Growth inhibition)	7 days
275	Zaltauskaite et al., 2011	Domestic	No information	Secondary treatment	*Lemna minor*	Macrophyte	N.A	55.3	%	55.30	0.9702	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 6	62-nd	% effluent (v/v)	81.00	0.9878	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 17	18-nd	% effluent (v/v)	59.00	1.0906	EC50 (Growth inhibition)	7 days
10	Aydin et al. 2015b	Industrial	Landfill leachattes	raw landfill leachate	*Lemna minor*	Macrophyte	May	3.05	%	3.05	1.1641	EC50 (Growth rate)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 11	73-nd	% effluent (v/v)	86.50	1.1849	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 1	52-nd	% effluent (v/v)	76.00	1.2258	EC50 (Growth inhibition)	7 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lemna minor*	Macrophyte	February	2.24	%	2.24	1.3494	EC50 (Growth rate)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 10	90-nd	% effluent (v/v)	95.00	1.4286	EC50 (Growth inhibition)	7 days

Study number	Study citation	Effluent type	Effluent chemical characterization	Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
215	Rosa et al 2009	Industrial	Bleach-kraft pulp mill	Secondary treatment	*Lemna minor*	Macrophyte	Before secondary treatmenr	no effect	%	100.00	1.8083	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 12	88-nd	% effluent (v/v)	94.00	1.8288	EC50 (Growth inhibition)	7 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lemna minor*	Macrophyte	April	2.93	%	2.93	1.9026	EC50 (Growth rate)	7 days
164	Neculita et al 2008	Industrial	Acid mine drainage wastewater	Passive bioreactors	*Lemna minor*	Macrophyte	10 days hydraulic retention time	90.5	%(v/v)	90.50	2.0568	LC50 (Mortality)	96h
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 3	27-60	% effluent (v/v)	43.50	2.3138	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 4	30-63	% effluent (v/v)	46.50	2.4734	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 5	75-nd	% effluent (v/v)	87.50	2.6119	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 13	90-nd	% effluent (v/v)	95.00	2.9827	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 16	49-nd	% effluent (v/v)	74.50	3.1838	EC50 (Growth inhibition)	7 days
193	Picado et al 2008	Industrial	No information	No information	*Lemna minor*	Macrophyte	Industry 9	6.3-nd	% effluent (v/v)	53.15	4.4477	EC50 (Growth inhibition)	7 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lemna minor*	Macrophyte	March	4.83	%	4.83	6.1923	EC50 (Growth rate)	7 days
262	Vidal et al 2012	Industrial	Acid mine drainage	No information	*Lemna minor*	Macrophyte	N.A	19.45	mg/L	18.86	6.4811	EC50 (Yield)	7 days
275	Zaltauskaite et al 2011	Domestic	No information	Secondary treatment	*Lactuca sativa*	Other (not aquatic spp)	N.A	0.472	%	47.20	0.8281	EC50 (Growth inhibition)	120h
7	Arias-barreiro et al., 2010	Industrial	Tannery wastewater (chromium contamination)	Raw wastewater	*Latuca sativa*	Other (not aquatic spp)	N.A	31.58(21.4-46.6)	%	9.76	0.3091	EC50 (95% confidence)	5 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lepidum sativum*	Other (not aquatic spp)	May	2.61	%	2.61	0.9962	EC50 (Root lenght)	3 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lepidum sativum*	Other (not aquatic spp)	April	1.87	%	1.87	1.2143	EC50 (Root lenght)	3 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lepidum sativum*	Other (not aquatic spp)	February	4.6	%	4.60	2.7711	EC50 (Root lenght)	3 days
10	Aydin et al. 2015b	Industrial	Landfill leachettes	raw landfill leachate	*Lepidum sativum*	Other (not aquatic spp)	March	3.32	%	3.32	4.2564	EC50 (Root lenght)	3 days
220	Ruck 1998	D & I[1]	No information	No information	*Fellaster zelandiae*	Other (sea urchin)	Industrial effluent	0.69	%	0.69	0.2556	EC50 (Growth inhibition)	72h
24	Brienza et al 2016	Domestic	Terciary treatment with AOP (advanced oxidation processses - hidroxil and sulfate radicals)	No information	*Brachionus calyciflorus*	Rotifera	Raw wastewater	90	%(v/v)	90.00	1.0000	LC50 (mortality)	48h
220	Ruck 1998	D & I[1]	No information	No information	*Brachionus calyciflorus*	Rotifera	Industrial effluent	6.9	%	6.90	2.5556	EC50 (Mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Brachionus plicatilis*	Rotifera	Sample 1	0.19	% (v/v)	0.19	0.5588	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Brachionus plicatilis*	Rotifera	Sample 2	0.24	% (v/v)	0.24	1.5000	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Brachionus plicatilis*	Rotifera	Sample 3	0.42	% (v/v)	0.42	2.0000	LC50 (mortality)	24h
83	Guerra 2001	Industrial	Effluents containing penolic compounds	Raw phenolic effluent	*Brachionus plicatilis*	Rotifera	Sample 4	3.84	% (v/v)	3.84	7.1111	LC50 (mortality)	24h
220	Ruck 1998	D & I[1]	No information	No information	*Brachionus plicatilis*	Rotifera	Industrial effluent	4.4	%	4.40	1.6296	EC50 (Growth inhibition)	72h
254	Torres-Guzmán et al., 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 5 effluent dry season	2.6	Toxic units	38.46	0.0385	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	ww 7 effluent dry season	2.1	Toxic units	47.62	0.0476	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 4 effluent rainy season	2.1	Toxic units	47.62	0.0476	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 6 effluent rainy season	2.6	Toxic units	38.46	0.0769	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 5 effluent rainy season	2.2	Toxic units	45.45	0.0909	LC50 (Mortality)	48h

Study number	Study citation	Effluent type	Effluent characterization	chemical Types of treatment	Test species	Tax group	Effluent	Results	Units	RESULTS (% effluent)	Trel	Parameter	Duration
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 7 effluent rainy season	2.1	Toxic units	47.62	0.0952	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 1 effluent dry season	1.7	Toxic units	58.82	0.1176	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 2 effluent dry season	1.7	Toxic units	58.82	0.1176	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 4 effluent dry season	1.6	Toxic units	62.50	0.1250	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 1 effluent rainy season	1.6	Toxic units	62.50	0.1250	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 2 effluent rainy season	2.1	Toxic units	47.62	0.1429	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 5 influent dry season	2.0	Toxic units	50.00	0.1500	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 6 effluent dry season	2.0	Toxic units	50.00	0.1500	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 4 influent dry season	2.6	Toxic units	38.46	0.1538	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 2 influent dry season	3.7	Toxic units	27.03	0.1892	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 3 effluent dry season	2.1	Toxic units	47.62	0.1905	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 3 effluent rainy season	1.9	Toxic units	52.63	0.2105	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 3 influent rainy season	7.6	Toxic units	13.16	0.2632	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 7 influent rainy season	7.5	Toxic units	13.33	0.2667	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 6 influent rainy season	5.7	Toxic units	17.54	0.3333	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 5 influent rainy season	8.3	Toxic units	12.05	0.3373	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 2 influent rainy season	5.6	Toxic units	17.86	0.3929	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 6 influent dry season	3.8	Toxic units	26.32	0.4211	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 3 influent dry season	4.2	Toxic units	23.81	0.4286	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 4 influent rainy season	4.2	Toxic units	23.81	0.4762	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 1 influent rainy season	3.9	Toxic units	25.64	0.5128	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 7 influent dry season	8.3	Toxic units	12.05	0.5422	LC50 (Mortality)	48h
254	Torres-Gúzman et al 2010	Domestic	No information	No information	*Lecame quadridentata*	Rotifera	WWTP 1 influent dry season	4.9	Toxic units	20.41	0.5714	LC50 (Mortality)	48h
84	Guerrero-jiménez & Silva-briano, 2017	Domestic	no information	No information	*Lecane quadridentata*	Rotifera	Average value	14.36	% (v/v)	25.71	0.8159	EC50 (Mortality)	48h

1 - Domestic and industrial

Annex D

Support table for the boxplot chart.

Labels	Bacteria	Algae	Macrophytes	Rotifers	Crustaceans	Insects	Fish
Min	0.0149	0.0105	0.6400	0.0385	0.1035	0.4384	0.2144
Q_1	0.2172	0.3281	1.0135	0.1250	0.4955	1.1321	1.0000
Median	0.4557	0.9880	1.6184	0.2667	0.8828	2.4316	1.4514
Q_3	1.0259	1.3433	2.5773	0.5588	1.3582	3.3596	2.6778
Max	5938	196.3	25.00	36.00	111.4	59.00	6835
IQR	0.8086	1.0152	1.5638	0.4338	0.8627	2.2275	1.6778
Upper Outliers	20.00	8.00	3.00	6.00	10.0	2.00	8.00
Lower Outliers	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
For the Box (IQR and Median)							
Q2-Q1	0.2384	0.6599	0.6049	0.1417	0.3874	1.2995	0.4514
Q3-Q2	0.5702	0.3553	0.9589	0.2922	0.4753	0.9280	1.2264
For the Whiskers							
Q_3+1.5*IQR	2.2388	2.8660	4.9230	1.2096	2.6522	6.7009	5.1944
Q_1-1.5*IQR	-0.9957	-1.1947	-1.3322	-0.5257	-0.7986	-2.2092	-1.5166
Upper Whisker	2.2388	2.8660	4.9230	1.2096	2.6522	6.7009	5.1944
Lower Whisker	0.0149	0.0105	0.6400	0.0385	0.1035	0.4384	0.2144
W_{upper}-Q_3	1.2130	1.5228	2.3457	0.6507	1.2940	3.3413	2.5166
Q_1-W_{lower}	0.2024	0.3176	0.3735	0.0865	0.3920	0.6936	0.7856
For the Outliers							
Max	5938	196.33	25.00	36.00	111.4	59.00	6835
Min	#N/D	#N/D	#N/D	#N/D	#N/D	#N/D	#N/D