

# Contribution of ecotoxicological approach for environmental assessment of urban Wastewater Treatment Plant under rehabilitation

**Bárbara Salazar Diogo**

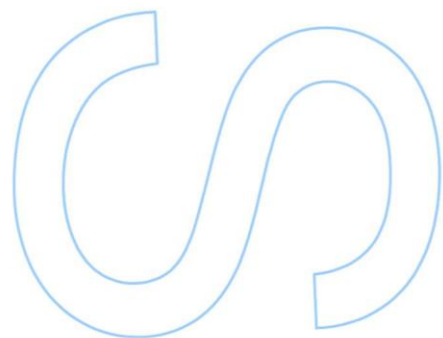
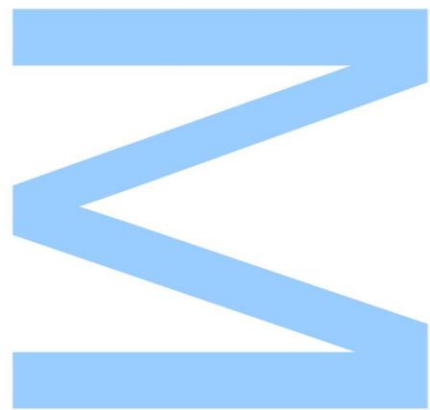
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## **Orientador**

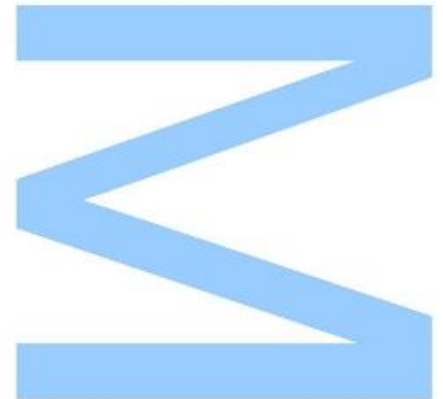
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas. O Presidente do Júri,

Porto, \_\_\_\_/\_\_\_\_/\_\_\_\_



Dissertação submetida a Faculdade de Ciências da Universidade do Porto, para a obtenção do grau de Mestre em Recursos Biológicos Aquáticos, da responsabilidade do Departamento de Biologia.

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# Resumo

Os tratamentos utilizados nas Estações de Tratamento de Águas Residuais Urbanas (ETAR) apresentam baixa eficiência na eliminação de contaminantes de preocupação emergente. As avaliações de ecotoxicidade de efluentes podem ser usadas como ferramenta complementar para entender os possíveis impactos das descargas de efluentes de ETAR nos corpos de água e na biota do ecossistema recetor. Desta forma, ferramentas ecotoxicológicas provaram ser sensíveis e adequadas na avaliação da eficiência dos tratamentos utilizados em ETAR. Assim, o objetivo principal deste estudo foi avaliar a toxicidade das diferentes amostras, ao longo de uma ETAR em reabilitação [águas residuais brutas (A), efluente preliminar (B), efluente final (C) e do ambiente ecorecetor (D)]. A avaliação foi realizada sazonalmente (durante 1 ano), recorrendo a ensaios ecotoxicológicos multi-espécies: ensaios de viabilidade celular com *Escherichia coli* (duas estirpes diferentes), *Rhodospirillum rubrum* (estirpe LF2), *Arthrobacter* sp. (estirpe FF13) e *Pseudomonas putida* (estirpe N3BL); ensaios de inibição de crescimento usando a microalga *Raphidocelis subcapitata* e a macrófita *Lemna minor*; e ensaios agudos e de taxa de alimentação usando o microcrustáceo *Daphnia magna*. Foram avaliados parâmetros adicionais após a exposição aos efluentes para *L. minor* (conteúdo de clorofila total, MDA e prolina) e *D. magna* (atividade de CAT e GSTs e níveis de TBARS). No geral, as diferentes espécies apresentaram sensibilidades às amostras testadas. O crescimento de *R. rubrum*, *Arthrobacter* sp. e *P. putida* não foi afetado por nenhuma das amostras, enquanto que a viabilidade celular das duas estirpes de *E. coli* diminuiu na presença das amostras A, B e D. As amostras C e D não revelaram toxicidade aguda para *D. magna*, mas causaram a diminuição da taxa de alimentação, e em alguns casos, peroxidação lipídica. Em todas as estações, as amostras A e B foram ligeiramente tóxicas ( $10 < EC_{50} < 100$ ) para *L. minor*, enquanto a C e D foram ligeiramente tóxicas apenas no inverno ( $EC_{50} = 93.5\%$  and  $58.8\%$ , respetivamente). Os resultados obtidos para *D. magna* e *L. minor* demonstraram que estes organismos podem ser utilizados como bioindicadores de toxicidade, se incorporados em programas de monitorização e avaliação ambiental. O oposto aconteceu com a *R. subcapitata*, que neste estudo não foi considerada uma boa espécie modelo para estudar a toxicidade de águas residuais. Este estudo demonstrou a utilidade de ferramentas ecotoxicológicas para avaliar o impacto das descargas das ETAR, maximizando a proteção ambiental e visando a boa qualidade ecológica das águas recetoras.

**Palavras-chave:** Ecotoxicologia; ETAR; Tratamentos; Multi-espécies.

## Abstract

The treatments used in urban wastewater treatments plants (WWTPs) have low efficiency when it comes to eliminating a large number of contaminants of emerging concern. Ecotoxicity assessments of the effluents can be used as a complementary tool to understand the possible impacts of discharges of WWTP effluents in water bodies and the receptor ecosystem biota. In this way, ecotoxicological tools have been proven to be sensitive and appropriate approaches in evaluating the effectiveness of treatments used in WWTPs. So, the main objective of this study was to evaluate the toxicity of different samples, throughout a WWTP in rehabilitation [raw wastewaters (A), preliminary effluents (B), final effluents (C), and the ecoreceiving environment (D)]. The evaluation was conducted seasonally (during 1year), where multispecies ecotoxicological assays were performed, such as bacteria cell viability assays using *Escherichia coli* (two different strains), *Rhodospirillum rubra* (strain LF2), *Arthrobacter* sp. (strain FF13), and *Pseudomonas putida* (strain N3BL); growth inhibition assays using the microalga *Raphidocelis subcapitata* and the macrophyte *Lemna minor*; and acute and feeding rate assays using the microcrustacean *Daphnia magna*. Additional parameters were also evaluated after exposure to the different effluent samples for *L. minor* (the content of total chlorophyll, MDA, and proline) and *D. magna* (CAT and GSTs activities, and TBARS levels). Overall, the different species showed different sensitivities to the samples tested. *R. rubra*, *Arthrobacter* sp., and *P. putida* growth were not affected by any of the samples, while cell viability of *E. coli* strains decreased in the presence of samples A, B, and D. Sample C and D did not reveal acute toxicity for *D. magna* but caused a decrease in the feeding rate and, in some cases, lipid peroxidation. In all seasons, A and B were slightly toxic ( $10 < EC_{50} < 100$ ) to *L. minor*, whereas C and D were slightly toxic only in winter ( $EC_{50} = 93.5 \%$  and  $58.8 \%$ , respectively). The results obtained with *D. magna* and *L. minor* showed that these organisms can be used as bioindicator species of toxicity if incorporated into programs for monitoring and environmental assessment. The opposite occurs with *R. subcapitata*, which was not considered a good model species to study the toxicity of wastewaters, in this study. The present study demonstrated the usefulness of ecotoxicological tools to assess the impact of discharges of WWTPs, maximizing environmental protection and aiming at the good ecological quality of receiving waters.

**Keywords:** Ecotoxicology; WWTP; Treatments; Multispecies.

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## List of abbreviations

|                        |                                                       |
|------------------------|-------------------------------------------------------|
| <b>Aut</b>             | Autumn                                                |
| <b>ASTM</b>            | American Society for Testing and Materials            |
| <b>BOD<sub>5</sub></b> | Biochemical Oxygen Demand                             |
| <b>CAT</b>             | Catalase                                              |
| <b>CFU</b>             | Colony-forming Unit                                   |
| <b>DOC</b>             | Dissolved Organic Carbon                              |
| <b>EC<sub>50</sub></b> | Effective Concentration for 50% organisms             |
| <b>GST<sub>s</sub></b> | Glutathione S-transferases                            |
| <b>ISO</b>             | International Organization for Standardization        |
| <b>MBR</b>             | Membrane Bioreactor                                   |
| <b>MDA</b>             | Malondialdehyde                                       |
| <b>MUG</b>             | 4-methyl-umbelliferyl-beta-D-glucuronide              |
| <b>NA</b>              | Nutrient Agar                                         |
| <b>OECD</b>            | Organization for Economic Cooperation and Development |
| <b>PhACs</b>           | Pharmaceutically active compounds                     |
| <b>Spr</b>             | Spring                                                |
| <b>Sum</b>             | Summer                                                |
| <b>TBARS</b>           | Thiobarbituric Acid Reactive Substances               |
| <b>TSS</b>             | Total Suspended Solids                                |
| <b>UV</b>              | Ultraviolet                                           |
| <b>VSS</b>             | Volatile Suspended Solids                             |
| <b>WFD</b>             | Water Framework Directive                             |
| <b>Win</b>             | Winter                                                |
| <b>WWTP</b>            | Wastewater Treatment Plant                            |

# 1. Introduction

The growth of the human population, associated with the technological and industrial revolution in the second half of the 20<sup>th</sup> century, led to a significant change in the quality of life in society. Today's generation is driven by the high need for consumption, which is reflected in the increase of the amount of waste produced and released into the environment (Gargosova and Urmanska, 2017). These residues include several contaminants of emerging concern (Golovko et al., 2021), such as synthetic chemicals, pharmaceutical products, and personal care products, that have been increasingly detected in wastewater systems and natural water (Besse and Garric, 2009; García et al., 2014; Halling-Sørensen et al., 1998; Ministério do Ambiente, 1997; Pablos et al., 2015; Reungoat et al., 2011). In this context wastewater treatment plants (WWTP) play a fundamental role in the removal of organic matter and reduction of contaminants of emerging concern, minimizing their potential adverse effects in aquatic ecosystems. Even though many of these effects are still unknown, it is known that the permanence and the possible bioaccumulation of these compounds in aquatic systems can compromise all the biota that depend on these ecosystems (Caliman and Gavrilescu, 2009; Halling-Sørensen et al., 1998). Therefore, the main objective of WWTPs is to treat wastewater from domestic and industrial activities, before it is released into the environment (e.g., rivers) (Hreiz et al., 2015). Wastewaters are complex mixtures of compounds, which in addition to organic and inorganic residues, include a huge amount of macronutrients and chemicals that may release in the aquatic ecosystems (Bundschuh, 2014; Gargosova and Urmanska, 2017).

According to the sensitivity of the ecoreceiving environment, the treatments along the WWTP can be more or less complex, depending on the characteristics of the affluent wastewater and the hypothesis of the reuse of the treated effluent (Monte et al., 2016). In a wastewater treatment plant, the affluent can be subjected to four treatment phases: i) the preliminary treatment (to avoid obstructions of the treatment circuits removing coarse solids, sands, oils, and fats; ii) the primary treatment for the removal of suspended solid particles; iii) the secondary treatment, often a biological treatment, consists of reducing organic matter and nutrients in the effluent through the work development of specific microbiological communities; and iv) the tertiary treatment, used if the ecoreceiving environment of the effluents is considered a sensitive area or the objective is to reuse the treated effluents (e.g., for irrigation or washing) (Monte et al., 2016; Oliveira, 2017). This latter treatment aims to improve the quality of the final effluent, through the elimination of the remaining nutrients (e.g., nitrogen and phosphorus,

responsible for eutrophication of water) and parasites, using disinfection by ultraviolet radiation, ozonation, or treatment with chlorine (Moreira, 2014; Sonune and Ghate, 2004). Despite the increase in knowledge and technology of several treatments, great amounts of compounds (in the order of ng/L and µg/L) of anthropogenic origin are found in wastewater and the recipient discharge ecosystems (Fatta et al., 2007; Halling-Sørensen et al., 1998; Kümmerer, 2009). To eliminate these pollutants it is necessary to apply more advanced treatments and find more effective treatment strategies since conventional treatments used in WWTPs have low efficiency in the elimination of these compounds, namely pharmaceutically active compounds (PhACs), industrial and household chemicals, and personal care products (García et al., 2013; Knopp et al., 2016). An example of a technology that can replace conventional treatment processes and provide a safer supply of reusable water, is the Membrane Bioreactor (MBR) (Arévalo et al., 2012). This treatment combines conventional biological treatment with a physical separation based on membranes. Depending on the pore size of the membrane, it can be classified into microfiltration or ultrafiltration. MBR technology is considered a green technology, as it allows the discharge of high-quality treated effluents, with a reduced number of treatment steps and without the use of disinfection chemicals (Bertanza and Pedrazzani, 2012; Melin et al., 2006; Zularisam et al., 2006).

Since the 1970s and 1980s, several countries such as Germany, Belgium, Denmark, Sweden, and Spain have used ecotoxicological tools to monitor the efficiency of the treatments used in WWTP (Cebere et al., 2009; Power and Boumphrey, 2004). This approach allows the assessment of possible impacts of discharges of WWTP effluents on water bodies and ecosystem dynamics, namely the effects on aquatic organisms of different trophic levels (Mendonça et al., 2013, 2009). Vasquez and Fatta-Kassinos, (2013) demonstrated that freshwater species such as *Daphnia magna* and *Raphidocelis subcapitata* were more sensitive to wastewater effluents than marine species (*Artemia salina* and *Vibrio fischeri*). Mendonça et al. (2013) concluded that *Vibrio fischeri* and *Daphnia magna* tests are suitable to distinguish and evaluate the effectiveness of treatments used in WWTP. Thus, ecotoxicological studies have proved to be great tools for assessing the effectiveness of the instruments used in WWTPs for the treatment of wastewater, as well as unmasking the effects (synergism, antagonism, potentiation, or summation) of complex mixtures that may occur (Gargosova and Urmanska, 2017; Mendonça et al., 2011b; Villegas-Navarro et al., 1997).

This study aims to evaluate the ecotoxicity of effluents from a Wastewater Treatment Plant in rehabilitation and the respective ecoreceiving environment, through

multispecies ecotoxicological tools. To achieve this goal, organisms of different trophic levels and parameters were assessed: bacteria cell viability assays using *Escherichia coli* (two different strains), *Rhodospirillum rubrum* (strain LF2), *Arthrobacter* sp. (strain FF13), and *Pseudomonas putida* (strain N3BL); growth inhibition assays using the microalgae *Raphidocelis subcapitata* and the floating plant *Lemna minor*; and acute and feeding rate assays using the microcrustacean *Daphnia magna*. Sub-individual parameters were also evaluated after exposure to the different effluent samples for *L. minor* (the content of total chlorophyll, malondialdehyde, and proline) and *D. magna* [catalase (CAT) and glutathione S-transferases activities (GSTs), and thiobarbituric acid reactive substances (TBARS) levels].

## 2. Materials and methods

### 2.1. Characterization of WWTP in study

The Arreigada Wastewater Treatment Plant is located in Paços de Ferreira (41° 14' 56.74"N, 8° 23' 57.27" W), in the Porto district (north of Portugal), treating urban effluents using preliminary and biological treatment with Membrane Bioreactor (MBR). For the last years, this WWTP has operated above its capacity (5800 m<sup>3</sup>/day) (PEV, 2020). This leads to the discharge of effluents with inadequate treatment, or even without treatment, to the Ferreira River (a small northern river and ecoreceiving environment of the effluents from this WWTP). The spring of Ferreira River is in the municipality of Paços de Ferreira, pouring into the Sousa River in Gondomar. It runs about 43 km along several municipalities: Paços de Ferreira, Paredes, Valongo and Gondomar (Figure 1).

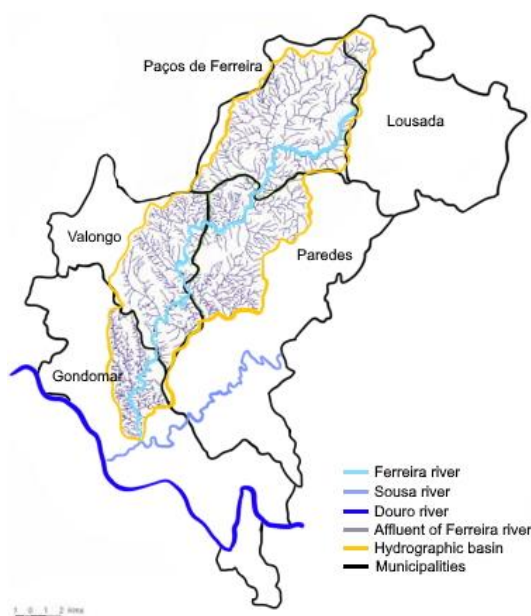


**Fig. 1** - Hydrographic basin of Ferreira River, adapted from (Rodrigues et al., 2006).

To increase the efficiency of wastewater treatments and consequently reduce the pollution of the Ferreira River, the WWTP has undergone an expansion and rehabilitation in 2020. This intervention resulted in the installation of a MBR treatment with an ultrafiltration process. With this improvement, it is expected to increase the water treatment capacity of the WWTP by 70 %, through the treatment of 10.000 m<sup>3</sup> water/day (Hidrofunção, n.d.). Since the samples were collected in year-zero, the affluent that arrives at the WWTP has abnormal characteristics when compared to a typical urban affluent (Table 1).

According to decree-law nº 198/2008, a section of the Ferreira River, from the source to the confluence with the Ermida stream (Sobrado, Valongo), is considered a sensitive river area since it does not comply with a parameter indicated in Directive 75/440/EEC (quantity of *Escherichia coli*) (Conselho da União Europeia, 1975; Ministério do Ambiente, 2008). The discharge of the WWTP is located upstream from the Ermida stream, therefore the effluent is released into a sensitive area. Since the last sampling period, a new decree-law (nº 77/2021) was established. According to decree-law nº 77/2021 (Ministério do Ambiente, 2021a) and Ordinance nr.188/2021 (Ministério do Ambiente, 2021b) a new section of the Ferreira River, corresponding to fishing waters, is considered a sensitive river area since it does not comply with the quantity of ammonium (NH<sub>4</sub><sup>+</sup>) indicated in Directive 2000/60/EEC (União Europeia, 2000). This new classification does not compromise the results obtained in this study, considering the fact that the discharge of the WWTP is performed in a sensitive area, as the entire watercourse of the Ferreira River corresponds to fishing waters (Agência Portuguesa do Ambiente, 2014; Ministério da Agricultura, 2021).

## 2.2. Sampling procedure

To conduct the present study, four effluent samples (A, B, C, and D) were collected along the treatments of the WWTP (Figure 2). Effluent samples collected in site A corresponds to the reception of the gross affluent wastewater (raw wastewater). Effluent samples from site B stand for the effluent after going through the preliminary treatment (after removal of coarse solids, sands, oils, and fats). Site C stands for the effluent after biological treatment (after undergoing MBR treatment) and site D corresponds to the Ferreira River sampling point after the discharge of the effluent from the WWTP.

Effluent samples were collected in each season, along the year 2020/2021 (4 sampling periods): autumn (Aut), winter (Win), spring (Spr), and summer (Sum). Several physical and chemical parameters were measured *in situ* [pH, oxygen (mg/L and %),

conductivity ( $\mu\text{S}/\text{cm}$ ), and temperature ( $^{\circ}\text{C}$ ), with a multiparametric probe (Multi 3630 IDS SET F). Additionally, 6 L of each effluent sample (A, B, C, and D) were collected with plastic bottles, and transported in the dark at  $4^{\circ}\text{C}$  to the laboratory, to conduct a physical and chemical characterization of effluents and laboratory bioassays. Physical and chemical analyses were performed on the sampling day, and bioassays were started within a maximum period of 24 h after the collection of the samples.

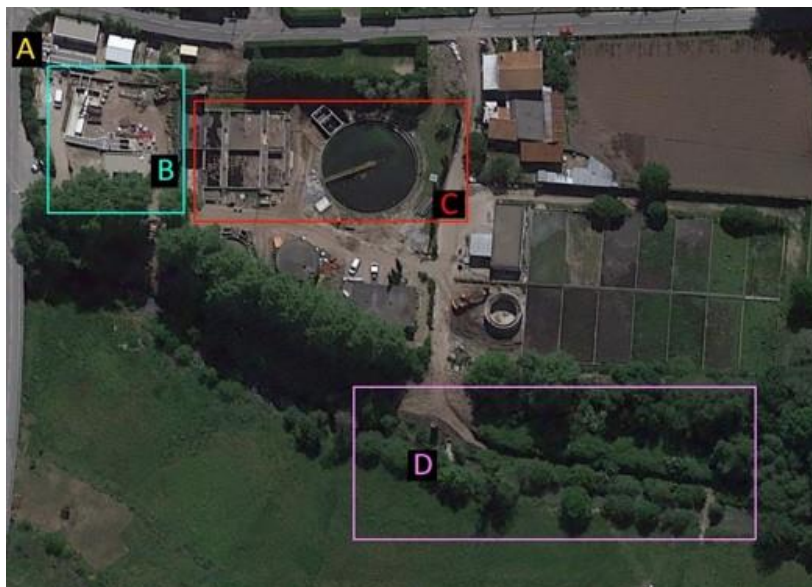


Fig. 2 - Sampling points along the WWTP (image from Google Earth).

### 2.3. Water physical and chemical analysis

In the laboratory, the effluent samples were used to determine the concentration of nitrites ( $\text{mg NO}_2^-/\text{L}$ ), nitrates ( $\text{mg NO}_3^-/\text{L}$ ), ammonium ( $\text{mg NH}_4^+/\text{L}$ ), total phosphorus ( $\text{mg P}_{\text{total}}/\text{L}$ ), using a bench photometer (Spectroquant Multy Colimeter), and the respective test procedure: 1.14776, 1.14752 and 1.14848. The five-day biochemical oxygen demand ( $\text{BOD}_5$ ), as well as the turbidity level, were also quantified (APHA, 1989). To determine the dissolved organic carbon (DOC), an aliquot of each sample was filtered through a Whatman GF/C filter (47 mm diameter and  $1.2\ \mu\text{m}$  pore) and the absorbance of the filtrate was measured at  $\lambda=320\ \text{nm}$  (Williamson et al., 1999). The total suspended solids (TSS) and the volatile suspended solids (VSS) contents were quantified using three filters obtained from the filtration of each sample (APHA, 1989).

### 2.4 Microbiological monitoring

The microbiological quality of the effluent samples was determined regarding the quantification of *Escherichia coli* (EN ISO 9308-3, 1998) and *Enterococcus* (EN ISO 7899-2, 2000), the count of total microorganisms at  $22^{\circ}\text{C}$  and  $36^{\circ}\text{C}$  (EN ISO 6222, 1999) and total coliforms (EN ISO 9308-1, 2014).

All laboratory procedures for microbiological monitoring were performed under aseptic conditions. For the *Escherichia coli* counting, the miniaturized method (MUG / EC method) was used (EN ISO 9308-3, 1998). This method consists of inoculating 96-well plates with a dehydrated culture medium and the 4-methyl-umbelliferyl-beta-D-glucuronide (MUG) substrate, which when hydrolyzed allows the detection of a specific enzyme that produces a blue fluorescence when observed with UV radiation at 366 nm. The count of the total microorganisms (aerobic mesophilic microorganisms) was performed, according to the technique of incorporating 1 mL of each effluent sample, and respective dilutions, in Nutrient Agar culture medium (NA), and the incubation occurred at 22 °C and 36 °C, for  $68 \pm 4$  h and  $44 \pm 4$  h, respectively (EN ISO 6222, 1999). The count of the total coliforms and Enterococcus was performed using the membrane filtration technique (Millipore filtration system with 0.45  $\mu$ m porosity filtering membranes), 100 mL of each sample was filtered and, if necessary, respective decimal dilutions (EN ISO 7899-2, 2000; EN ISO 9308-1, 2014). The filtering membranes were placed on the surface of selective culture media (Membrane Lauryl and Slanetz-Bartley Agar, respectively) and incubated for 24 h at the appropriate temperature.

## 2.5. Test organisms and maintenance conditions

The organisms selected to perform this study belong to several biological levels, that play different key functions in the aquatic food web. A set of bacteria – *Arthrobacter* sp., *Pseudomonas putida*, *Rhodopirellulla rubra*, and *Escherichia coli* (two different strains), a microalgae *Raphidocelis subcapitata*, a macrophyte *Lemna minor*, and the Cladocera water flea *Daphnia magna* were the species selected to conduct this study. The last three species are considered standard organisms in aquatic ecotoxicology tests (OECD, 2011, 2008, 2006), while bacteria represent the basis of the trophic web playing different roles in the environment. *Escherichia coli*, an indicator of water quality, is commonly used in molecular and ecotoxicological studies (Botelho et al., 2012), while *Pseudomonas* sp and *Arthrobacter* sp. have already been shown to be able to degrade several compounds (e.g., pharmaceutical products and pesticides) (Scheublin et al., 2013; Wasi et al., 2013). Although there have not been many studies with *R. rubra* it is known that it plays an important role in the carbon and nitrogen cycles and that it belongs to a ubiquitous group (Marinho et al., 2020).

### 2.5.1 Bacterial strains

*Arthrobacter* sp. (strain FF13) is a gram-positive bacterium commonly found in several environments. This aerobic soil bacterium (Hagedorn and Holt, 1975), belongs to the

phylum *Actinobacteria* and was isolated from *Fucus spiralis*, collected on a beach in Porto vicinity, Portugal (41°09'N; 8°40'W). *Escherichia coli* is a gram-negative bacterium that belongs to the phylum *Proteobacteria*, considered a good indicator of faecal pollution in water environmental samples (Leclerc et al., 2001). In this study, two different strains were used, *Escherichia coli* ATCC® 25922™ (reference strain) and a strain isolated from an environmental water sample from the river Febros (high ecological relevance) in Avintes, Portugal (Cabral and Marques, 2006). *Pseudomonas putida* (strain N3BL) is a gram-negative bacterium, easily found in soil and aquatic habitats, that belongs to the phylum *Proteobacteria*. This bacterium occurs in high oxygenated environments and was isolated from a marine sponge (Flores et al., 2014). *Rhodopirellula rubra* (strain LF2) is a marine gram-negative bacterium belonging to the phylum *Planctomycetes*. This bacterium was isolated on the north coast of Portugal, from a biofilm community on the surface of marine macroalgae *Laminaria* sp. (Lage and Bondoso, 2011).

*Escherichia coli* strains and *Pseudomonas putida* were cultivated in Nutrient Agar (NA) (3 g/L yeast extract, 5 g/L peptone, 12 g/L agar), while *Arthrobacter* sp. and *Rhodopirellula rubra* were cultivated in modified M13 medium (Lage and Bondoso, 2011). The growth incubations conditions were performed at 26 °C, except for *E. coli* strains that were at 37 °C (Flores et al., 2014; Marinho et al., 2020).

### 2.5.2 *Raphidocelis subcapitata*

*Raphidocelis subcapitata* is a freshwater green microalga used as a bioindicator species in several aquatic ecotoxicological studies, due to its high growth and reproduction rate, and its sensitivity to different toxicants (Cedergreen and Streibig, 2005; OECD, 2011). Previously known as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*, this standard microalga is normally cultured in the Woods Hole MBL medium (Schwartz, 1975), under controlled conditions of temperature ( $23 \pm 2$  °C) and continuous light. The culture maintenance was performed in the exponential growth phase, where the medium was renewed approximately every 7 days (Environment Canada, 1993; Marinho et al., 2020; OECD, 2011; Pinto et al., 2020).

### 2.5.3 *Lemna minor*

*Lemna minor* is a floating macrophyte generally used in ecotoxicology (Cedergreen and Streibig, 2005; Nunes et al., 2014; OECD, 2006). The well-known duckweed is typical of water bodies with a reduced flow or standing water, such as lakes and lagoons (Hillman, 1961). Its size, easy handling, high reproductive capacity, and sensitivity to pollutants and other compounds, are some of the characteristics that make this species an

outstanding test organism (Appenroth et al., 2013). *L. minor* was cultivated in Steinberg medium and maintained at a temperature of  $23 \pm 2$  °C and continuous light, according to the standard guideline OECD 221 (OECD, 2006).

#### 2.5.4 *Daphnia magna*

*Daphnia magna* is a freshwater microcrustacean widely used as a model organism for ecotoxicological tests due to its great sensitivity to different stress factors (Gonçalves et al., 2007; Lari et al., 2017; OECD, 2008, 2004). This Cladocera reproduces asexually, through parthenogenesis and plays an important role as a primary consumer in the aquatic food webs (Pablos et al., 2015; Queirós et al., 2019). Monoclonal cultures of *D. magna* were maintained in synthetic water medium "ASTM hard water" (ASTM, 1988), supplemented with a standard organic additive, *Ascomphyllum nodosum* extract (Baird et al., 1989) at a temperature of  $20 \pm 2$  °C and photoperiod of 16 h<sup>L</sup>:8 h<sup>D</sup>. The microalga *Raphidocelis subcapitata* was used to feed the *D. magna* cultures, in a ratio of  $1.5 \times 10^5$  cells mL<sup>-1</sup>.day<sup>-1</sup>.

### 2.6 Bioassays

#### 2.6.1 Acute bacterial cell viability assay

All the bacteria were initially grown in a solid culture medium and transferred into liquid cultures. The exposure of the bacteria to the effluent samples was performed in the exponential phase of liquid cultures, which corresponds to 0.5 AU for *E. coli* and 0.2 to 0.3 AU for the other bacteria, at  $\lambda = 600$  nm (Flores et al., 2014; Marinho et al., 2020). Absorbance measurements were performed in a spectrophotometer Genesys™ 10Series (Thermo Spectronic).

For the cell viability assays, one milliliter of liquid bacteria culture was centrifuged at 13,400 rpm for 60 seconds (MiniSpin, Eppendorf). In a flow chamber, the cell pellets were resuspended in 1.5 mL of each effluent sample and different exposure periods were evaluated (30, 60, 120, 180, 240, 300 min, and 24 h). After each exposure period, the cell suspensions were centrifuged, and the cells-pellet was resuspended in 50 µL Milli-Q water, to guarantee the stop of the exposure. After that, three drops of 10 µL sample were inoculated in an appropriate solid medium for each strain and the plates were incubated in a culture chamber with optimal conditions (see section 2.4.1). A set of controls were performed with Milli-Q water using the same procedure described for effluent samples. Cell viability was checked after 24 h incubation for *Arthrobacter* sp., *E. coli*, and *P. putida*, and after 3 days for *R. rubra* according to a growth level scale using

a classification ranging from 0 to 4 (Figure 3), in which 4 represents the maximum growth and 0 stands for the absence of growth (Flores et al., 2014; Marinho et al., 2020).

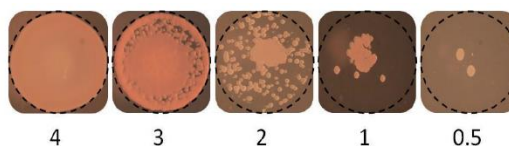


**Fig. 3** - Growth classification range, from a 10  $\mu$ L droplet method showing the different growth levels, 4, 3, 2, 1, and 0.5 (Flores et al., 2014; Marinho et al., 2020).

### 2.6.2 *Raphidocelis subcapitata* growth inhibition test

To evaluate the toxicity of the effluent samples on the microalga *R. subcapitata*, a growth inhibition test was carried out according to standard guidelines (OECD, 2011) with a microplate adaptation (Rodrigues et al., 2021a). To guarantee the exponential growth phase of the inoculum at the beginning of the assay a preculture of microalgae was performed in a test medium 2-4 days before the test. A 24-well microplate was used to conduct the assays, using three replicates per dilution. In each well was added 900  $\mu$ L of each effluent sample dilution and 100  $\mu$ L of the microalgae inoculum with a concentration  $10^4$  cell  $\text{mL}^{-1}$  (corresponding to an initial absorbance of the assay of 0.01 at a 440 nm). A range of dilutions of each effluent sample [20 %, 40 %, 60 %, 80 %, 100 % (raw effluent – without dilution)] were prepared with MBL medium. MBL medium was used as a negative control, and a blank (effluent sample) without algae addition was also used in the assay. The assays were conducted for 72 hours, and the microplates are maintained under controlled conditions of continuous light (24 h,  $\sim 6000$  lux), and temperature ( $23 \pm 2$  °C). The microplates were stirred every day to avoid microalgae sedimentation. After the exposure period, the absorbance of each replicate was measured, at 440nm, in a spectrophotometer (UV-1600PC Spectrophotometer). The results were expressed in yield and used to obtain  $\text{EC}_{50}$  (72 h) and corresponding confidence intervals, according to the protocol of OECD, (2011).

### 2.6.3 *Lemna minor* inhibition test

*Lemna minor* growth inhibition assays were conducted according to standard guidelines (OECD, 2006) with microplate adaptation. This assay was conducted in a static regimen where the microplates were incubated in a climatic chamber, in conditions of continuous light ( $\sim 7000$  lux), and temperature ( $23 \pm 2$  °C) for 7 days. Initially, five effluent samples dilutions [20 %, 40 %, 60 %, 80 %, 100 % (raw effluent – without dilution)] were prepared using Steinberg medium. Six-well microplates were used to conduct this assay, using four replicates per dilution, each one of them containing 10 mL of testing dilutions and 4 fronds of *L. minor*. The control group was conducted with fronds exposed to Steinberg medium. After the exposure period (7 days), the final number of fronds was counted. The

results were expressed in yield and used to obtain EC<sub>50</sub> (7 days) and corresponding confidence intervals (OECD, 2006). At the end of the assay, the fronds were washed with distilled water, dried with absorbent paper, weighted, and stored in Eppendorf microtubes at -80 °C for quantification of the total chlorophyll content and determination of specific biochemical endpoints: malondialdehyde and proline contents.

#### 2.6.3.1. *Photosynthetic pigments (chlorophylls), malondialdehyde, and proline contents*

The total chlorophyll content was determined according to the method described by Lichtenthaler (1987), and adapted by Pinto et al., (2020). Pigments were extracted in 1 mL of 96 % ethanol overnight, at 4 °C, and the absorbance was measured spectrophotometry at the wavelengths of 644.2 nm (Genesys™ 10Series Thermo Spectronic).

The malondialdehyde (MDA), expressed as  $\mu\text{M}$  MDA equivalents/mg fresh weight, is a final product of the lipid peroxidation in plant cells, and its content was determined by the thiobarbituric acid method as described by Elkahoui et al., (2005). The specific and the non-specific absorbance of the supernatant were measured at 532 and 600 nm, respectively. Subtracting the non-specific absorbance (measured at 600 nm) and using the molar extinction coefficient  $\epsilon=155 \text{ mM}^{-1} \text{ cm}^{-1}$ , the concentration of MDA was calculated.

The proline content was determined according to the method described by Bates et al., (1973) and was expressed as mg/mg fresh weight. The absorbance of the chromophore solution was measured at 520 nm (Genesys™ 10Series Thermo Spectronic). A calibration curve obtained by measuring the absorbance of proline standard solutions of known concentration (0.2, 0.1, 0.05, 0.025 e 0.0125 mg/mL) was used to extrapolate the proline content in the *L. minor*.

#### 2.6.4 *Daphnia magna* assays

##### 2.6.4.1 *Acute immobilization test*

*D. magna* acute immobilization assay was performed according to the standard guideline OECD 202 (OECD, 2004). A control group (ASTM) and a range of effluent sample dilutions [20 %, 40 %, 60 %, 80 %, 100 % (raw effluent – without dilution)] were prepared. For each condition, four replicates were prepared in glass vessels with 25 mL of effluent sample dilution or ASTM. In each replicate was added 5 organisms (24 h old, born

between the 3<sup>rd</sup> and the 5<sup>th</sup> broods), and the assay was performed under controlled conditions of temperature ( $20 \pm 2$  °C) and photoperiod (16 h<sup>L</sup>:8 h<sup>D</sup>). The organisms were observed after 48 h of exposure and dead or immobilized organisms were counted for further determination of EC<sub>50</sub> (48 h) values and corresponding confidence intervals.

#### 2.6.4.2 Feeding rate inhibition assay

The feeding rate assay was conducted according to McWilliam and Baird, (2002) with the same adaptation described in Queirós et al., (2019). The assay was conducted in 6-well microplates, and in each replicate was added  $1.5 \times 10^5$  cells mL<sup>-1</sup> of *R. subcapitata* and 12.5 mL of the effluent sample: 100 % of the effluent sample was tested if no acute toxicity was detected (EC<sub>50</sub> not determined), otherwise, a range of concentrations of that effluent sample was tested to guarantee that the value of the inferior confidence interval of EC<sub>50</sub> was not exceeded. Five replicate per treatment were used and five neonates, with 4 or 5 days old and born between the 3<sup>rd</sup> to 5<sup>th</sup> broods, were added to each replicate. A blank with the effluent sample and microalgae, without organisms, was made to account for the potential algae growth during the test period. Before the neonates were added, the absorbance of each replicate was measured at 440 nm (Abs FI<sub>0</sub>) with a spectrophotometer (Genesys™ 10Series Thermo Spectronic). After the organisms were added, the microplates were incubated in a climatic chamber with controlled conditions of temperature ( $20 \pm 2$  °C) in total darkness to prevent algal growth. After 24 h of exposure, the organisms were collected and stored for further analysis, the absorbance of each replicate was measured (Abs FI<sub>24</sub>) at  $\lambda = 440$  nm, and the results were expressed in feeding rate, according to the following equation:

$$F = [(V * (AbsFI_0 - AbsFI_{24})) / t] / n$$

V corresponds to the assay volume used, t stands for the assay period (24 h), and n to the number of organisms per vessel/replicate (n=5) (Allen et al., 1995).

Pools of 15 organisms from each treatment were stored in Eppendorf microtubes at -80 °C for posterior biochemical determinations (oxidative stress and lipid peroxidation biomarkers).

#### 2.6.4.3 Biochemical determinations

Specific oxidative stress and metabolism biomarkers, including catalase (CAT), glutathione S-transferases (GSTs) activities, and levels of lipid peroxidation (levels of thiobarbituric acid reactive substances (TBARS)) were determined in *D. magna* after

feeding assay exposure. Samples were sonicated in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1 % of Triton X-100) using a sonicator (Misonix Microson Ultrasonic Cell Disruptor XL) and centrifuged at 14,000 rpm for 10 min at 4 °C, according to Rodrigues et al., (2021b). Different aliquots were prepared for the different biomarkers quantifications.

All biochemical biomarkers were adapted to microplates and determined in quadruplicate, except for TBARS that were in triplicate. Spectrophotometric readings were performed using a microplate reader (Thermo Scientific, model Multiskan GO, version 1.00.40), with the SkanIt Software 3.2.). All the enzymatic activities were expressed in function of protein content, which was quantified spectrophotometrically (wavelength 595 nm) and adapted to microplates using  $\gamma$ -globulin as a standard (Bradford, 1976).

Catalase (CAT) activity was expressed as nmoles of  $H_2O_2$  consumed per minute, per milligram of protein, and was quantified according to Aebi, (1984) with slight modifications, as described in Rodrigues et al., (2021b). Glutathione-S-transferases (GSTs) activity, expressed in millimoles of thioether produced per minute, per milligram of protein, was determined by the procedure of Habig et al., (1974) and Nunes et al., (2017). Lipid peroxidation was measured by the quantification of TBARS, according to Buege and Aust, (1978), with some adaptations, as described in Nunes et al. (2017). TBARS levels were expressed as malondialdehyde (MDA) equivalents (mmoles) per milligram of protein.

## 2.7. Statistical analysis

The estimation of  $EC_{50}$  values for *D. magna* was performed by modeling immobilization as binomial data [using the R package “drc”; (Ritz and Streibig, 2005)], with a special case of the log-logistic dose-response model, where the asymptotes of the curve are fixed to be 1 (all organisms are immobilized) and 0 (none are immobile), following the rationale of Ritz, (2010).

$EC_{50}$  values, and respective CIs (using delta method), were determined by fitting a non-linear concentration-response toxicity model (LL3) to the *R. subcapitata* and *L. minor* yield data using the drc package (Ritz and Streibig, 2005) for R software. Yield [the difference between the biomass (cell densities, cells/mL) at the beginning and the end of the assay for each replicate of controls and treatments (OECD, 2011)] was modeled as a continuous variable using a three-parameter logistic model, where the lower asymptotes of the curve were fixed to 0, following the rationale of Ritz, (2010). According to the classification proposed by Tonkes et al., (1999), wastewater samples can be classified as: very toxic ( $EC_{50} < 1\%$ ), moderately toxic ( $1\% < EC_{50} < 10\%$ ) or slightly toxic ( $10\% < EC_{50} < 100\%$ ).

The data from bioassays were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene test. For all bioassays and biomarkers results, a one-way ANOVA was conducted, followed by the Dunnett test which was carried out to determine differences between the dilutions of effluents and the control treatment. The adopted level of significance was 0.05. All the statistical analysis was done using SPSS Statistics v26.

## 3. Results and Discussion

### 3.1. Physical and chemical parameters

The results from the physical and chemical analysis of the effluent samples are presented in Table 1. In this case, the WWTP in study is in its starting year (year-zero) after the rehabilitation and implementation of the membrane bioreactor treatment, there are no applicable decree-laws or discharge requirements. According to Monte and Albuquerque (2010), the typical composition of untreated urban sewage in Portugal may have a range of variations in certain parameters, such as TSS (90-430 mg/L), VSS (34-109 mg/L),  $BOD_5$  (444-1338 mg/L), ammonium (32-81 mg/L) and  $P_{total}$  (3.5-13 mg/L). It is noteworthy that the WWTP is in its starting year after the rehabilitation and, therefore, the raw affluent (sample A) did not reveal a typical composition of an untreated urban affluent (less than 40 % of the volume of a typical affluent). The results obtained here

show values below expectations for BOD<sub>5</sub> (all seasons), TSS and VSS (Win), ammonium (Aut and Win), and P<sub>total</sub> (Aut, Win, and Spr). However, in a typical case, according to the decree-law nº 152/97, the discharges from the wastewater treatment plants must satisfy treatment requirements taking into account the sensitivity of the receiving medium. In the case of the Arreigada WWTP, since its effluent is released into a sensitive receiving environment (Ministério do Ambiente, 2008) it would be necessary to guarantee that the parameters are within the limit that the legislation considers acceptable (BOD<sub>5</sub> < 25 mg/L; TSS < 35 mg/L; P<sub>total</sub> < 2 mg/L; Table 1) or that a minimum percentage reduction in relation to the affluent is guaranteed (70-90 % BOD<sub>5</sub>; 90 % TSS; 80 % P<sub>total</sub>) (Ministério do Ambiente, 1997). Comparing the values obtained with the limit values, we verified that the final effluent (C) would only exceed the value of phosphorus released in the summer (P<sub>total</sub> = 2.82 mg/L). However, if we take into account the minimum percentage reduction of the parameters listed above, it is possible to verify that in most samples the requirements would not be met. As for the minimum percentage of P<sub>total</sub> reduction, this reduction was never higher than 72.97 % [reduction percentage obtained in spring, between the values obtained in sample A (2.22 mg/L) and sample C (0.60 mg/L)] which shows that they would not comply with the minimum reduction imposed by decree-law nº 152/97. The same happened with the BOD<sub>5</sub> value, which was only efficiently reduced in the winter [reduction percentage 75.8 %, between the values obtained in sample A (9.23 mg/L) and sample C (2.23 mg/L)], with an increase being visible in the remaining samples. TSS removal was not effective in winter, exhibiting a reduction of 30.7 % [reduction percentage between the values obtained in sample A (36.1 mg/L) and sample C (25.0 mg/L)].

**Table 1** - Results of the physical and chemical parameters: temperature (Temp), conductivity (Cond), turbidity (Turb), dissolved oxygen (O<sub>2</sub>), pH, nitrites (NO<sub>2</sub><sup>-</sup>), nitrates (NO<sub>3</sub><sup>-</sup>) ammonium (NH<sub>4</sub><sup>+</sup>), total phosphorus (P<sub>total</sub>), biochemical oxygen demand (BOD<sub>5</sub>), dissolved organic carbon (DOC), total suspended solids (TSS) and the volatile suspended solids (VSS). Sampling sites: A (raw wastewaters), B (preliminary effluents), C (final effluents), and D (ecoreceiving environment). Sampling periods: Aut - autumn, Win - winter, Spr - spring, and Sum - summer). BDL – below detection limit.

|                    |                                      | Temp<br>(°C) | Cond<br>(µS/cm) | Turb<br>(m <sup>-1</sup> ) | O <sub>2</sub><br>(mg/L) | O <sub>2</sub><br>(%) | pH  | NO <sub>2</sub> <sup>-</sup><br>(mg/L) | NO <sub>3</sub> <sup>-</sup><br>(mg/L) | NH <sub>4</sub> <sup>+</sup><br>(mg/L) | P <sub>total</sub><br>(mg/L) | BOD <sub>5</sub><br>(mg/L) | DOC<br>(m <sup>-1</sup> ) | TSS<br>(mg/L) | VSS<br>(mg/L) |
|--------------------|--------------------------------------|--------------|-----------------|----------------------------|--------------------------|-----------------------|-----|----------------------------------------|----------------------------------------|----------------------------------------|------------------------------|----------------------------|---------------------------|---------------|---------------|
| EQS <sup>(1)</sup> |                                      |              |                 |                            | ≥5                       | 60-120%               |     |                                        | ≤25                                    | ≤1                                     | ≤0.1                         | ≤6                         |                           |               |               |
| Samples            | Established limited <sup>(2)</sup> . |              |                 |                            |                          |                       |     |                                        |                                        |                                        |                              | <2<br>-80%                 | <25<br>-70/<br>90%        | <35<br>-90%   |               |
|                    |                                      |              |                 |                            |                          |                       |     |                                        |                                        |                                        |                              |                            |                           |               |               |
| A                  | Aut                                  | 19.5         | 462             | 1.428                      | 1.17                     | 13.0                  | 7.7 | 1.048                                  | 11.2                                   | 6.8                                    | 1.6                          | 1.14                       | 0.909                     | 108.3         | 95.0          |
|                    | Win                                  | 12.6         | 24              | 0.163                      | 9.84                     | 95.2                  | 7.4 | 0.024                                  | 20.9                                   | 11.0                                   | 1.34                         | 9.23                       | 0.596                     | 36.1          | 29.4          |
|                    | Spr                                  | 17.7         | 817             | 0.695                      | 0.06                     | 0.7                   | 7.9 | 2.360                                  | 20.0                                   | 55.2                                   | 2.22                         | 0.03                       | 0.607                     | 284.7         | 73.6          |
|                    | Sum                                  | 19.3         | 829             | 0.938                      | 0.005                    | 0.1                   | 7.8 | 0.501                                  | 18.9                                   | 87.0                                   | 5.47                         | 0.004                      | 0.610                     | 341.7         | 101.7         |
| B                  | Aut                                  | 19.6         | 1170            | 1.366                      | 0.55                     | 5.7                   | 7.8 | 1.128                                  | 11.2                                   | 12.4                                   | 2.32                         | 0.52                       | 1.159                     | 121.7         | 115.0         |
|                    | Win                                  | 12.5         | 23              | 0.191                      | 9.75                     | 93.7                  | 7.4 | 0.025                                  | 21.9                                   | 9.6                                    | 1.41                         | 9.27                       | 0.623                     | 40.1          | 18.3          |
|                    | Spr                                  | 17.4         | 836             | 0.757                      | 0.08                     | 0.7                   | 7.8 | 1.320                                  | 57.0                                   | 53.6                                   | 0.93                         | 0.05                       | 0.552                     | 329.2         | 73.6          |
|                    | Sum                                  | 19.4         | 840             | 0.973                      | 0.008                    | 0.1                   | 7.9 | 0.354                                  | 24.3                                   | 84.5                                   | 5.16                         | 0.01                       | 0.361                     | 395.0         | 121.7         |
| C                  | Aut                                  | 20.8         | 693             | 0.059                      | 5.39                     | 61.4                  | 6.1 | 1.464                                  | 4.0                                    | 1.92                                   | 1.92**                       | 2.36**                     | 0.593                     | 3.8           | 2.8           |
|                    | Win                                  | 13.4         | 30              | 0.009                      | 8.63                     | 75.0                  | 5.6 | 0.003                                  | 64.8                                   | 0.78                                   | 0.69**                       | 2.23                       | 0.596                     | 25.0**        | 16.1          |
|                    | Spr                                  | 19.0         | 472             | 0.032                      | 4.50                     | 50.8                  | 5.6 | 0.371                                  | 64.0                                   | 4.2                                    | 0.60**                       | 4.47**                     | 0.124                     | 5.8           | 3.4           |
|                    | Sum                                  | 20.7         | 414             | 0.044                      | 4.25                     | 48.5                  | 6.4 | 0.249                                  | 80.1                                   | 0.18                                   | 2.82*                        | 1.72**                     | 0.152                     | 14.4          | 9.4           |
| D                  | Aut                                  | 16.6         | 389             | 0.262                      | 7.40                     | 76.4                  | 7.3 | 0.328                                  | 3.3                                    | <b>8.8</b>                             | <b>1.21</b>                  | <b>7.39</b>                | 0.382                     | 76.5          | 19.8          |
|                    | Win                                  | 12.2         | 84              | 0.064                      | 10.52                    | 100.7                 | 6.6 | 0.006                                  | 15.8                                   | 0.59                                   | <b>0.11</b>                  | 5.62                       | 0.637                     | 33.9          | 7.2           |
|                    | Spr                                  | 16.5         | 18              | 0.094                      | 9.35                     | 99.2                  | 7.4 | BLD                                    | 18.4                                   | <b>4.4</b>                             | BLD                          | <b>9.33</b>                | 0.014                     | 27.4          | 9.5           |
|                    | Sum                                  | 17.8         | 195             | 0.055                      | 8.65                     | 93.4                  | 7.4 | 0.197                                  | <b>25.6</b>                            | <b>6.64</b>                            | <b>0.65</b>                  | <b>8.62</b>                | 0.071                     | 30.2          | 12.7          |

\*Above the acceptable limit according to DL n° 152/97.

\*\* Does not meet the minimum percentage reduction in relation to the respective sample A (affluent sample) according to DL n° 152/97.

Bold values stand for values outside the environmental quality standards (EQS) established by WFD<sup>(1)</sup>.

According to the limits established by the Water Framework Directive (WFD) (União Europeia, 2000), the ecological status of the Ferreira River (sample D) remained moderate throughout all sampling periods, due to the non-compliance with parameters such as nutrient content (high values of ammonium and total phosphorus) and BOD<sub>5</sub> (Table 1). It is important to emphasize that the final effluent (sample C) contained high amounts of nutrients (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and P<sub>total</sub>) in most seasons, which can contribute to the degradation of the ecological status of the receiving environment.

After the Arreigada WWTP rehabilitation, with the implementation of the MBR treatment, it will be expected that the final effluent had better quality since several studies already demonstrated that, after MBR treatment, it is possible to obtain an effluent with low values of suspended solids, turbidity, BOD<sub>5</sub>, and microorganisms (Daigger et al., 2005; Yang, 2013). When the MBR treatment is used it is possible to effectively remove the expected percentages of TSS (>99 %), BOD<sub>5</sub> (>97 %), ammonium (80-90 %), total phosphorus (>62-97 %) (Melin et al., 2006; Yang, 2013). Regarding the TSS, the maximum efficiency reached in this study was 97.8 % [efficiency obtained in spring, between the values obtained in sample A (284.7 mg/L) and sample C (5.8 mg/L)]. As for BOD<sub>5</sub>, it is even possible to verify an increase in most samplings (Aut, Spr, and Sum). As for ammonium, a lesser removal percentage was only achieved in the autumn

[effectiveness of 71.8 %, between sample A (6.8 mg/L) and sample C (1.92 mg/L)], while the total phosphorus reached a removal percentage of 72.97 % in the spring [72.97 %, between sample A (2.22 mg/L) and sample C (0.60 mg/L)].

### 3.2. Microbiological monitoring

The results obtained in the microbiological monitoring of the samples collected during the different seasons are shown in Table 2. Overall, the number of indicators of fecal contamination (*Escherichia coli*, fecal Enterococci, and total coliforms) was high, except for the number of total coliforms in sample C, in the winter. According to the literature, on average, a raw affluent of a WWTP before any treatment contains from  $10^7$  to  $10^9$  total coliforms and  $10^6$  to  $10^8$  fecal coliforms, and depending on the treatments, it can decrease 1 to 3 orders of magnitude (George et al., 2002). In this study, when applied the biological treatment with the membrane bioreactor (MBR), there is a reduction of 2 to 3 orders of magnitude ( $10^7$  to  $10^4$  in Aut, Spr, and Summer;  $10^5$  to  $10^3$  in Winter) (Table 2). However, if the WWTP was working normally it would be expected that most of these organisms would not be found after the membrane bioreactor, as described by Hai et al., (2014), Shang et al., (2005), and Zhang and Farahbakhsh, (2007). Moreover, coliform bacteria would be greatly reduced in the final effluent (sample C), since the removal capacity of the MBR process varies from  $10^5$  and  $10^8$ . However, this is not verified in the here-presented study, and a high number of microorganisms was detected in samples C (Table 2). The values of the total coliforms for the raw affluent (sample A) and the effluent after preliminary treatment (B), in all seasons, were countless, due to the high load recorded. Regarding the final effluent (C) (except for the winter sample) and the Ferreira River sample (D) the total coliforms are also countless. The total coliforms in the Ferreira River was higher than sample C but lower than samples A and B (Table 2). The latter was already predictable since this river has been characterized with high bacterial concentrations, namely total and fecal coliforms, for several years (Ministério do Ambiente, 2001).

**Table 2** - Results of microbiological monitoring for each sample: A (raw wastewaters), B (preliminary effluents), C (final effluents), and D (ecoreceiving environment), in different sampling periods (Aut - autumn, Win - winter, Spr - spring and Sum -summer).

|   |     | Count of <i>Escherichia coli</i> | Count of total <i>Enterococcus</i> | Count of total microorganisms |             | Count of total coliforms |
|---|-----|----------------------------------|------------------------------------|-------------------------------|-------------|--------------------------|
|   |     | CFU/100 mL                       | CFU/100 mL                         | 22°C CFU/mL                   | 36°C CFU/mL | Nº coliforms/100 mL      |
| A | Aut | 6.4x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Win | 2.5x10 <sup>5</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Spr | 8.6x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Sum | 8.6x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
| B | Aut | 7.1x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Win | 3.2x10 <sup>5</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Spr | 8.5x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Sum | 8.0x10 <sup>7</sup>              | Countless                          | Countless                     | Countless   | Countless                |
| C | Aut | 7.0x10 <sup>4</sup>              | 160                                | 15                            | 54          | Countless                |
|   | Win | 5.3x10 <sup>3</sup>              | 100                                | 310                           | 693         | 100                      |
|   | Spr | 6.0x10 <sup>4</sup>              | 100                                | 13                            | 51          | Countless                |
|   | Sum | 8.4x10 <sup>4</sup>              | 200                                | Countless                     | Countless   | Countless                |
| D | Aut | 9.2x10 <sup>4</sup>              | Countless                          | Countless                     | Countless   | Countless                |
|   | Win | 3.6x10 <sup>4</sup>              | 200                                | Countless                     | Countless   | Countless                |
|   | Spr | 9.0x10 <sup>4</sup>              | 400                                | Countless                     | Countless   | Countless                |
|   | Sum | 9.4x10 <sup>4</sup>              | Countless                          | Countless                     | Countless   | Countless                |

Over the last years, WWTPs with the MBR treatment have revealed some problems in the membranes, such as membrane fouling (Özkan and Uyanık, 2017). This phenomenon occurs when the pores of the membranes are totally or partially blocked due to the retention/accumulation of microorganisms, particles, and colloids (Meng et al., 2009). When the ultrafiltration process used at the WWTP is efficient, all particles and microorganisms larger than 0.1-0.01 µm (including bacteria, yeasts, some colloidal particles, and organic macromolecules) are removed. (Hai and Yamamoto, 2011; Jegatheesan and Visvanathan, 2014). The obtained results for the total coliforms (high microbiological level of the final effluent) can be explained due to changes in the performance of the membranes, fouling or even loss of membrane integrity, compromising the entire process and effectiveness of the treatments, as demonstrated by Hai and Yamamoto (2011) and Chae et al., (2014). In this case, this phenomenon is intensified since the WWTP was in year-zero (starting-year after rehabilitation), and the treatments were not fully working.

### 3.3. Bioassays

#### 3.3.1 Acute bacterial viability

Table 3 summarizes the effects of the effluent samples on the cell viability of *Arthrobacter* sp., *Pseudomonas putida*, and *Rhodopirellula rubra*. No effects were observed after effluent samples in the cell viability of *Arthrobacter* sp., *Pseudomonas*

*putida*, and *Rhodopirellula rubra* for all sampling seasons (Table 3). Indeed, several studies already demonstrated that *Arthrobacter* sp. and *Pseudomonas* sp. had the ability to degrade a wide variety of compounds (Kumar et al., 2019; Marinho et al., 2020), and reduce the physical and chemical properties of the effluents, such as BOD, DOC, and TSS (Atagana et al., 1999; Shruthi et al., 2012; Smitha et al., 2012), potentially using the compounds present in samples as energy sources. Moreover, biological treatment with *Arthrobacter* sp. and *Pseudomonas* sp. showed that these bacteria degrade organic and inorganic constituents and reduce ammonium and phosphates levels in wastewater effluents (Smitha et al., 2012). Indeed, *Pseudomonas* sp. is one of the most common bacteria used in pollutant degradation and bioremediation processes (Akpör and Muchie, 2010; Aragaw, 2020; Sonune and Garode, 2015), such as *Pseudomonas putida* commonly used for bioremediation of industrial effluents (Safitri et al., 2015). Unlike the previous species, there is not much data in the literature relating *Rhodopirellula rubra* with wastewater. However, it is known that this species can use nitrite, nitrate, and ammonium (nutrients that exist in considerable amounts in wastewater) as a source of carbon (Flores et al., 2014). It should be noted that *Planctomycetes* (the phylum of this bacterium), have already been detected in wastewater samples and wastewater treatment bioreactors (Chouari et al., 2003). Fuerst, (2017) described the unique ability, of one group of this phylum, to anaerobically oxidize ammonium (anammox), thus having the possibility to be used as a remediator for nitrogen-rich wastewater.

**Table 3** - Results of growth levels of *Rhodopirellula rubra*, *Arthrobacter* sp., and *Pseudomonas putida* after effluent samples exposure periods (30, 60, 120, 180, 240, 300min and 24 h) (see Figure 2 to growth classification range).

|                             |     |   | CTL | 30min | 60min | 120min | 180min | 240min | 300min | 24h |
|-----------------------------|-----|---|-----|-------|-------|--------|--------|--------|--------|-----|
| <i>Rhodopirellula rubra</i> | Aut | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Win | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Spr | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Sum | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
| <i>Pseudomonas putida</i>   | Aut | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Win | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Spr | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Sum | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
| <i>Arthrobacter</i> sp.     | Aut | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Win | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Spr | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             | Sum | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                             |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |

Table 4 summarizes the effects of the effluent samples on the cell viability of the *Escherichia coli* strains. The two strains of *Escherichia coli* were differently sensitive to the tested samples, revealing higher sensibility compared to the other three bacteria (Table 3). In autumn, the growth of *E. coli* (from the Febros river) was not affected by any sample while *E. coli* ATCC 25922 cell viability was affected after 30 min of exposure to samples A and B, and after 2 h of sample D. In winter, the growth of ATCC 25922 strain was inhibited after being exposed for 240 min and 120 min to samples B and D, respectively. However, the growth of ATCC 25922 strain was much more affected by sample A after 30 min (growth level 0.5), and 100% bacterium death was recorded after 1 h. The same pattern was observed in spring, for both strains, without any growth recorded only after 5 h (300 min) of exposure. In the case of sample B on spring, was observed a decrease in cell viability of both strains (growth level 0.5) after 30 min of

exposure, and death after 60 and 120 min for *E. coli* ATCC and *E. coli* (from the Febros River), respectively. In the presence of the sample from the Ferreira River (D), the ATCC 25922 strain was affected after 30 min and ends up dying after 180 min of exposure and the other *E. coli* (from Febros River) shows a gradual decrease of growth until no growth was recorded after 300 min of exposure.

**Table 4** - Results of growth levels of each *Escherichia coli* strains after effluent samples exposure periods (30, 60,120, 180, 240, 300 min, and 24 h) (see Figure 2 for growth classification range).

|                                         |     |   | CTL | 30min | 60min | 120min | 180min | 240min | 300min | 24h |
|-----------------------------------------|-----|---|-----|-------|-------|--------|--------|--------|--------|-----|
| <i>Escherichia coli</i><br>ATCC 25922   | Aut | A | 4   | 3     | 3     | 3      | 2      | 2      | 2      | 2   |
|                                         |     | B | 4   | 3     | 2     | 2      | 2      | 2      | 2      | 2   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 4     | 4     | 3      | 3      | 2      | 2      | 2   |
|                                         | Win | A | 4   | 0.5   | 0     | 0      | 0      | 0      | 0      | 0   |
|                                         |     | B | 4   | 4     | 4     | 4      | 4      | 3      | 3      | 3   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 4     | 4     | 3      | 3      | 2      | 2      | 2   |
|                                         | Spr | A | 4   | 0.5   | 0.5   | 0.5    | 0.5    | 0.5    | 0      | 0   |
|                                         |     | B | 4   | 0.5   | 0     | 0      | 0      | 0      | 0      | 0   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 1     | 0.5   | 0.5    | 0      | 0      | 0      | 0   |
|                                         | Sum | A | 4   | 0.5   | 0.5   | 0.5    | 0.5    | 0.5    | 0.5    | 0   |
|                                         |     | B | 4   | 3     | 3     | 2      | 2      | 2      | 2      | 2   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 1     | 0.5   | 0.5    | 0.5    | 0.5    | 0.5    | 0   |
| <i>Escherichia coli</i><br>Febros river | Aut | A | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | B | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         | Win | A | 4   | 0.5   | 0.5   | 0.5    | 0.5    | 0.5    | 0.5    | 0.5 |
|                                         |     | B | 4   | 4     | 4     | 4      | 4      | 3      | 3      | 3   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         | Spr | A | 4   | 0.5   | 0.5   | 0.5    | 0.5    | 0.5    | 0      | 0   |
|                                         |     | B | 4   | 0.5   | 0.5   | 0      | 0      | 0      | 0      | 0   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 4     | 3     | 2      | 1      | 0.5    | 0      | 0   |
|                                         | Sum | A | 4   | 2     | 2     | 2      | 2      | 2      | 2      | 2   |
|                                         |     | B | 4   | 3     | 3     | 2      | 2      | 2      | 2      | 2   |
|                                         |     | C | 4   | 4     | 4     | 4      | 4      | 4      | 4      | 4   |
|                                         |     | D | 4   | 3     | 3     | 2      | 2      | 2      | 2      | 2   |

Regarding the summer sampling, growth inhibition of *E. coli* strains was observed after 30 min of exposure to samples A and D (Table 4). Moreover, no growth of *E. coli* ATCC 25922 and a reduction of growth (growth level 2) of *E. coli* (from the Febros River) was recorded after 24 h of exposure. Sample B causes a growth decrease of both strains, until the growth level 2 (Table 4). No negative effects were recorded for the two strains of *E. coli* for sample C in all seasons, which may indicate that the negative effects on the growth of strains in samples A and B ends up being eliminated in the MBR treatment. Furthermore, it appears that *E. coli* (from Febros River) is more resistant than *Escherichia coli* ATCC 25922, since its growth was either not affected by the presence of the samples, or it is generally less and later affected by them. *Escherichia coli* ATCC 25922 is used as a reference strain for antibiotic susceptibility testing, known to be sensitive to various compounds, such as antibiotics (ATCC, n.d.). On the other hand, the other strain of *E. coli* was isolated from an environmental and contaminated water

sample (Febros River) and probably is better adapted to polluted environments. Flores et al., (2014) demonstrated that the growth of this strain was not affected by pollutants (arsenic, copper, fungicide Zidomil and dish detergent), but was sensitive to phenol and sodium azide. Marinho et al., (2020), found that the growth of this strain was affected only by some compounds, such as Myclobutanil and Azoxystrobin, and only after a long exposure period (180 and 240min, respectively). Thus, according to the results obtained, it was found that after the MBR treatment, samples C and D were not toxic for *E. coli* strains. This cell viability assay revealed that, depending on the species used, it can be quick, simple, and applicable in evaluating of toxicity of wastewater samples.

### 3.3.2 *Raphidocelis subcapitata*

Figure 4 represents the EC<sub>50</sub> values and the respective confidence intervals for the acute toxicity of the effluent samples, regarding the different sampling periods for *Raphidocelis subcapitata*. Based on the ecotoxicological categories proposed by Tonkes et al., (1999), the Ferreira River sample (D) of autumn and the final effluent (C) of winter were considered slightly toxic for *R. subcapitata*. The same happens with samples A, B, and C in spring, and A and B in summer (Figure 4). Thus, it is noteworthy that the results do not allow us to establish a toxicity gradient since it was possible to observe different responses, without any clear pattern/trend, for the same sample in different sampling periods.

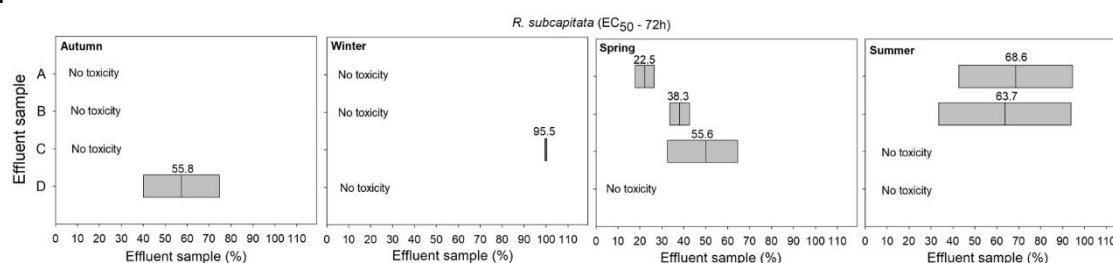


**Fig. 4** - EC<sub>50</sub> values (% of effluent samples) for *Raphidocelis subcapitata* after exposure to effluent samples [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] collected in the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer). The EC<sub>50</sub> value is depicted above the median of its respective boxplot and the breadth of the boxes represents the confidence intervals at 95 %.

The growth rate of algae is influenced by physical and chemical factors, such as light, temperature, pH, and nutrients, as well as by the presence of certain substances such as heavy metals, pesticides, and personal care products (Larsdotter, 2006). In the present study, it was verified that samples A and B had a high amount of ammonium (between 53.6 and 87 mg/L) which according to Larsdotter, (2006) it can affect the growth of *R. subcapitata* when it exceeds 20 mg/L. In the case of sample C, the low pH values may have influenced its growth since the pH in the standard test medium is higher than 7.2 (Schwartz, 1975).

Microalgae belong to the first level of the aquatic trophic chain and are known as sensitive to various compounds and environmental changes (Silva et al., 2009; Xin et al., 2021). This means that if their dynamics are disturbed, the higher levels of the ecosystems can be altered (Lewis, 1995). However, microalgae growth depends on several factors, such as the chemical composition of the sample, and the presence or absence of certain substances (nutrients and organic compounds) that may not only affect this growth but also alter its sensitivity to several toxic substances (Blinova, 2004).

Although microalgae are organisms widely used to assess the toxicity of pollutants, in recent years, some studies have revealed that they are not the most suitable model species to use in ecotoxicological studies with complex mixtures, such as nutrient-rich effluents (Mendonça et al., 2011b). Moreover, colored water affects algae growth by reducing the photosynthetic efficiency due to the degree of water transparency (Gartiser et al., 2010; Mendonça et al., 2011b).

Tišler and Zagorc-Končan (1997) showed that the growth of *Scenedesmus quadricauda* was affected when exposed to industrial wastewater ( $EC_{50}$  24 h = 57.5 %), even though it was the least sensitive organism when compared to other tested organisms (such as *Daphnia pulex* and *Oncorhynchus mykiss*). On the other hand, Berrebaan et al., (2020) found that some municipal effluents were minor toxic ( $EC_{50}$  72 h = 55.4 % and 61.83 %) for *Raphidocelis subcapitata*, due to the complex biological effects-pollutants relationship. Mendonça et al., (2011a) demonstrated that when using acute assays with different species (*Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, *Daphnia magna*, and *Lemna minor*), only the test carried out with the microalgae did not allow the assessment of the toxicity removal efficiency nor to distinguish the different treatments used. The here-presented results are in agreement with Mendonça et al., (2011a) since it was found that despite being sensitive to some samples in certain sampling periods, the microalgae *Raphidocelis subcapitata* did not prove to be the most suitable organism to evaluate the toxicity of the different samples obtained from the WWTP.

### 3.3.3. *Lemna minor*

The results obtained in the growth inhibition assay ( $EC_{50}$  values and the respective confidence intervals) with *Lemna minor* are shown in Figure 5. According to Tonkes et al., (1999), samples A and B were slightly toxic ( $10\% < EC_{50} < 100\%$ ) to this organism in all seasons, while samples C and D only showed slight toxicity in winter ( $EC_{50} = 93.5\%$  and  $EC_{50} = 58.8\%$ , respectively).

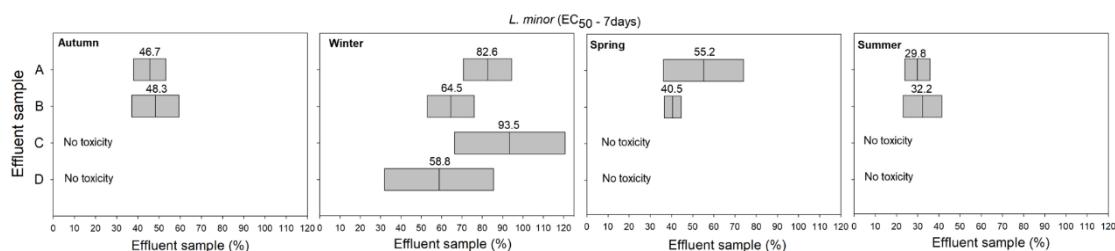


**Fig. 5** -  $EC_{50}$  values (%) of effluent samples for *Lemna minor* after exposure to effluent samples [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] collected in the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer). The  $EC_{50}$  value is depicted above the median of its respective boxplot and the breadth of the boxes represents the confidence intervals at 95 %.

The physical and chemical parameters, by themselves, were not enough to explain the toxic effect recorded on *L. minor*. Several studies describe wastewater as a complex mixture, which may contain several compounds (impossible to quantify chemically) that can inhibit the growth of macrophytes, namely steroids, hormones, heavy metals, pesticides, and others (Fekete-Kertész et al., 2015; Radić et al., 2011). Despite *L. minor* showing the capacity of removing toxic compounds and nutrients in wastewaters, often used as a phytoremediator (Alkimin et al., 2020), they also demonstrated to be one of the most sensitive organisms used in a battery of ecotoxicological assays involving wastewaters (Blinova, 2004, 2000). Indeed, the here-obtained results showed that *L. minor* was the most sensitive species, where acute toxicity was observed in all effluent samples before the biological treatment with MBR, and in winter also samples C and D showed slightly acute toxicity. Zaltauskaite et al., (2014) tested the phytotoxicity of municipal and industrial wastewater effluents in *L. minor*, obtaining also slight acute toxicity with an  $EC_{50}$  (7 days) value of 57.13% for untreated wastewater and 47.20 % for biologically treated wastewater. Previously, Zaltauskaite et al., (2011) verified that the municipal effluents had no significant adverse effect on the growth of *L. minor*, after the biological treatment, while the raw affluent showed toxicity ( $EC_{50}$  7days = 55.3 %).

Many plants are considered less sensitive than animals to contaminants (Lewis and Thursby, 2018; Lytle and Lytle, 2001). However, the macrophyte *Lemna minor* has proven to be a useful organism to assess the toxicity of industrial (Alkimin et al., 2020;

Taraldsen and Norberg-King, 1990) and municipal effluents (Zaltauskaite et al., 2011). Beyond the acute toxicity, the physiologic parameters of *L. minor* have also been used as a biomarker (e.g. total chlorophyll content, MDA, and Proline) to assess the toxicity of wastewater and river samples (Radić et al., 2011, 2010). Figure 6 shows the total chlorophyll content when exposed to the effluent samples throughout the different seasons.

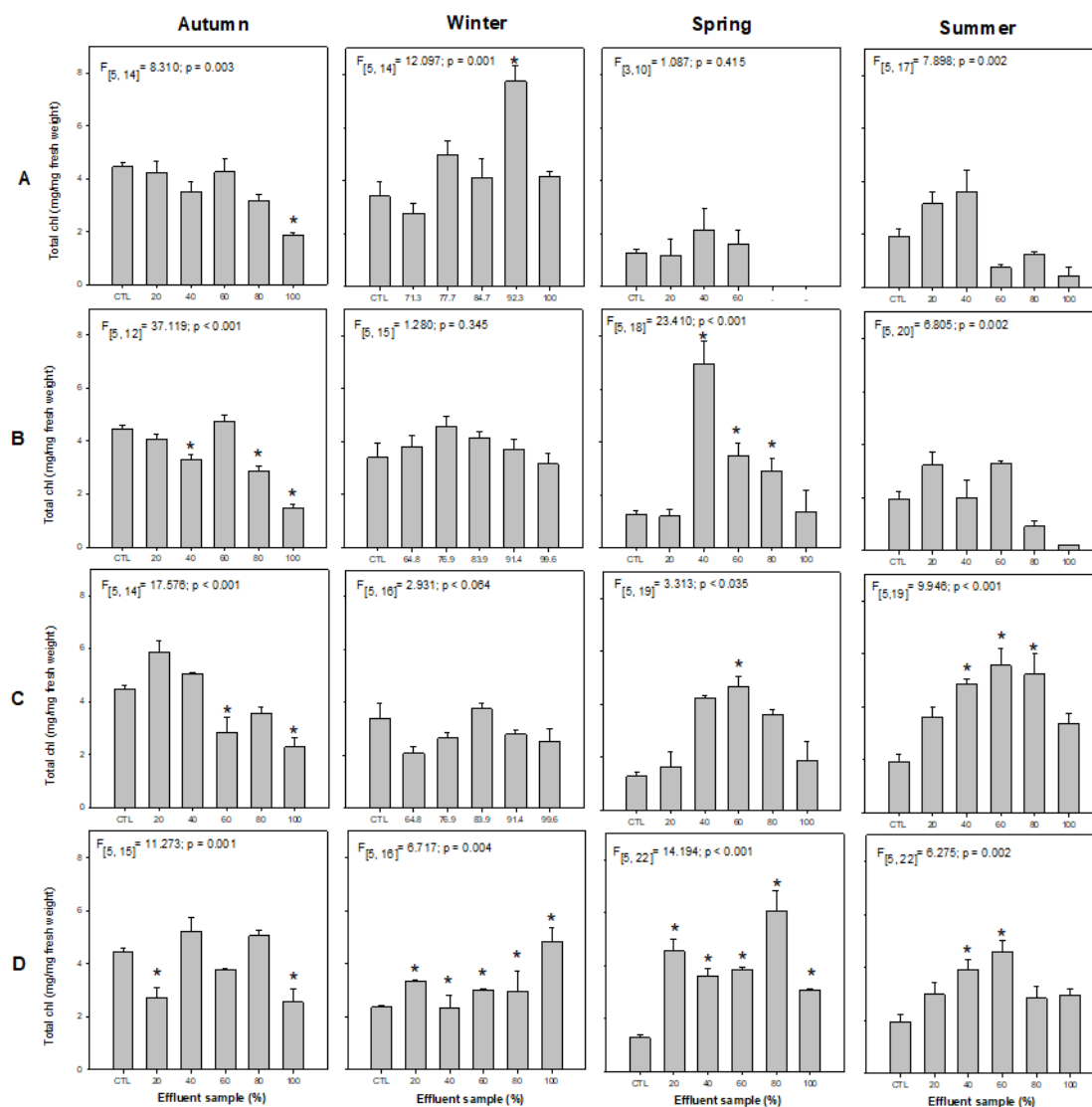


**Fig. 6** - Effects of effluent samples (%) [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] from the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer) on the content of total chlorophyll (Chl) in *Lemna minor*. Data are expressed as mean  $\pm$  standard error (SE). \*Stand for significant differences between treatments and the CTL group (Dunnett test,  $p < 0.05$ ).

Overall, no pattern was observed in chlorophyll content when *L. minor* was exposed to the different dilutions of effluent samples. However, it was generally found that in the presence of some autumn dilutions the chlorophyll content decreased significantly (100 % of sample A; 40 %, 80 %, and 100 % of sample B; 60 % and 100 % of sample C; 20 % and 100 % of sample D). The opposite was observed in the remaining samplings where a significant increase was registered (92.3 % of sample A of winter; 40

%, 60 %, and 80 % of sample B of spring; 60 % of sample C of spring, and 40 %, 60 % and 80 % of summer). When exposed to the dilutions of sample D, a significant increase in total chlorophyll content in *L. minor* was recorded, except for 20 % and 100% of autumn.

The determination of chlorophyll content in plants is a parameter rather sensitive to pollution (Alkimin et al., 2019; Fekete-Kertész et al., 2015) and therefore, a sensitive indicator of toxicity (Taraldsen and Norberg-King, 1990). Despite that, this parameter is not commonly used for toxicity studies of wastewater. Any alteration in the levels of chlorophylls contents can be used as an indicator of the physiological state of the plant (Nunes et al., 2014) since they may affect the metabolic pathways and physiological functions of the species (Brkanac et al., 2010) which can lead to lower yields of photosynthesis and growth inhibition (Myśliwa-Kurczel and Strzałka, 2002). One of the typical symptoms of oxidative stress in plants is a result of the changes in the synthesis/degradation of chlorophylls (Khaleghi et al., 2012). Overall, in the here-presented study, the total chlorophyll of *L. minor* was generally affected when exposed to the autumn samples, where a significant decrease was recorded. These results are in accordance with the results obtained by other authors, that reported a reduction of the chlorophyll content when *L. minor* was exposed to industrial and sewage wastewater (Singh and Singh, 2006). The increase of total chlorophyll content, when exposed to the D samples, can occur due to a protective mechanism of the plant in response to the stress caused by the samples. García-Valenzuela et al., (2005) explained that the increase of total chlorophyll occurred due to the development of photosynthetic machinery components (an increase in the number of thylakoids/chloroplasts). However, this parameter was not considered relevant, since it was not possible to establish a direct relationship with the toxic effect of the samples.

Figure 7 shows the contents of MDA and proline in *L. minor* after effluent samples exposure. No significant differences were recorded for the concentration of MDA levels in *L. minor* after the exposure to sample A, except for the highest percentages of effluent tested at winter (92.3 %). After exposure of sample B, a significant increase of MDA concentration in *L. minor* was detected in autumn, and in the highest effluent percentage tested at winter. A significant increase in the concentration of MDA levels was also recorded of samples C and D of autumn and summer, as well as in 100 % of sample C of spring, and 100 % of sample D of winter and spring. It is noteworthy that despite no acute toxicity has been detected in samples C and D in autumn, spring, and summer (Figure 5), the results obtained regarding the MDA content indicate an effect on the metabolic pathways, physiological functions, and potential development and growth of

this macrophyte since membrane damage (lipid peroxidation) was detected. These results show that the quantification of MDA concentration is a sensitive parameter that can be an important indicator of physiological stress (Xia et al., 2021). Radić et al., (2011) described MDA as a product of the decomposition of polyunsaturated fatty acids (PUFA) of the membranes, and, therefore, an endproduct of lipid peroxidation. Throughout a study with industrial wastewater, the levels of MDA in *L. minor* increased due to the number of heavy metals found in these effluents (Radić et al., 2011). Zaltauskaite et al., (2014) also described the induction of lipid peroxidation (an increase of MDA content) in *L. minor* when exposed to untreated and biologically treated wastewaters.

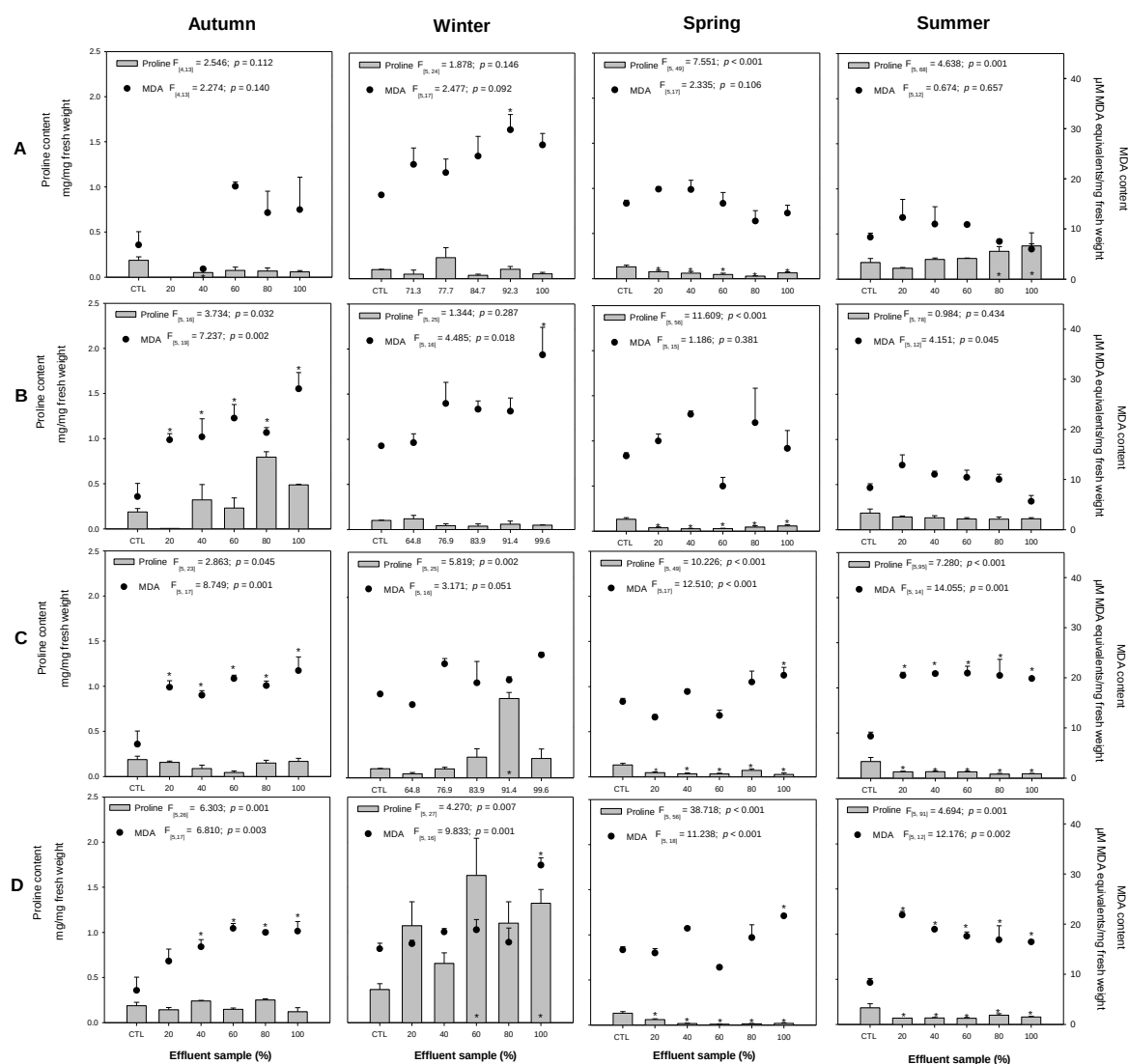


**Fig. 7** - Effects of effluent samples (%) [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] from the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer) on lipid peroxidation (MDA content) and proline content in *Lemna minor*. Data are expressed as mean  $\pm$  standard error (SE). \*Stand for significant differences between treatments and the CTL group (Dunnett test,  $p < 0.05$ ).

Regarding the proline content results in Figure 7. A significant decrease was observed for all the dilutions of all the effluents in spring, and in C and D in summer. A significant increase in proline content was occasionally observed in some samples with

higher effluent concentrations (e.g., A in summer, C and D in winter). Proline is an amino acid involved in the regulation of oxidative stress which plays an important role in the redox balance and cell homeostasis (Nunes et al., 2014; Szabados and Savouré, 2010). The most frequent pattern of response to oxidative stress involves the increase of proline content (Nunes et al., 2014), as observed with samples C and D of winter and sample A of summer. However, the decrease of proline content in *L. minor* after exposure of some effluent samples (all in spring, and C and D in summer) can also be explained by the effects of proline in the direct scavenging of free radicals, and not only in the intermediate process of oxidative stress regulation, as shown by Bohnert and Jensen (1996) and Nunes et al., (2014).

*L. minor* was revealed to be a good model organism for studies involving wastewaters, although some parameters, namely the proline content, did not prove to be the best indicator of sensitivity. MDA content proved to be sensitive and revealed that the majority of samples C and D (final effluents and samples from the receiving media) caused oxidative damage in this organism, even without presenting acute toxicity. It was also verified that the acute assays with *Lemna minor*, complemented with the biochemical assays, allowed the evaluation of the toxicity of the effluents.

### 3.3.4 *Daphnia magna*

The results of the *D. magna* acute immobilization assay are shown in Figure 8. No acute toxicity was recorded in winter effluent samples. Effluent sample A showed to be slightly toxic to *D. magna*, in autumn and summer, while sample B showed slight toxicity in autumn, spring, and summer seasons. No acute toxicity was recorded for C and D effluent samples in all sampling seasons, which indicates the effectiveness of the treatments of the WWTP in the elimination of the compounds that are toxic to *D. magna*.

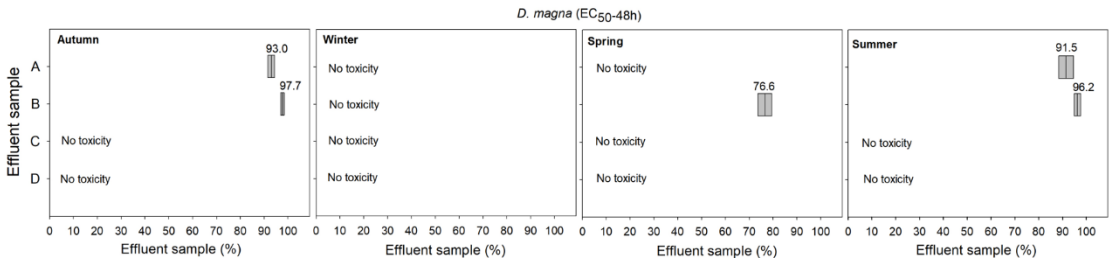


**Fig. 8** - EC<sub>50</sub> values (% of effluent samples) for *Daphnia magna* after exposure to effluent samples [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] collected in the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer). The EC<sub>50</sub> value is depicted above the median of its respective boxplot and the breadth of the boxes represents the confidence intervals at 95 %.

Movahedian et al., (2005) recorded when assessing the *D. magna* toxicity of the different effluents of a WWTP (from the raw effluent to the final effluent), a decrease of toxicity was found following a reduction of organic and inorganic compounds over the different WWTP treatments. Extensive literature relates the effect of physical and chemical properties of different samples with toxicity for *D. magna*, stating that large amounts of total suspended solids (TSS > 98mg/L), high turbidity and reduced dissolved oxygen (< 3 mg/L) can negatively affect this organism (Chapman et al., 2017; Chen et al., 2012). In the present study, it was found that, in general, *D. magna* may have been adversely affected by samples with high TSS ( $\geq 108$  mg/L), turbidity ( $\geq 0.757$  m<sup>-1</sup>), and reduced dissolved oxygen concentrations (< 5 mg/L).

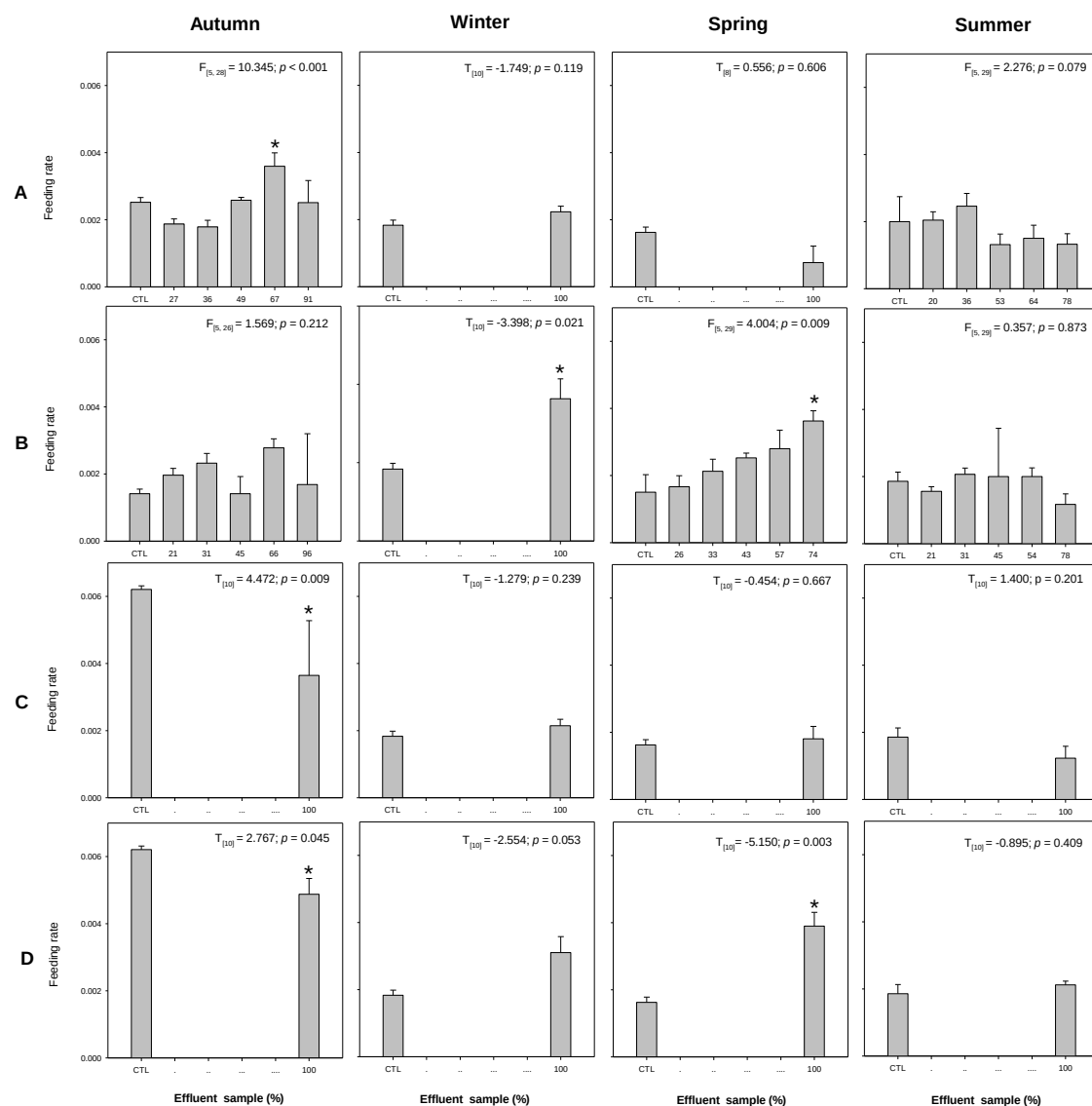


**Fig. 9** - Results of feeding rate assay of *Daphnia magna* when exposed to the different effluents (%) [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)], from the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer). Data are expressed as mean  $\pm$  standard error (SE). \*Stand for significant differences between treatments and the CTL group (Dunnett test,  $p < 0.05$ ).

*Daphnia magna* is the standard excellence species used to assess the toxicity effects of individual substances or complex mixtures, like domestic and industrial effluents (Celente et al., 2020; Ra et al., 2008; Verma, 2008). Despite being considered more tolerant than smaller cladocerans and other zooplankton species (e.g., *Ceriodaphnia* sp.) (Blinova, 2000; Koivisto, 1995), this organism is also considered an adequate species model to monitor the different treatments of the WWTPs (Mendonça et al., 2013). Besides acute toxicity assessment, a feeding rate assay was carried out to improve the knowledge of toxicity effects of effluent samples (Figure 9), since food ingestion (feeding) is one of the most essential biological processes of all organisms. This assay has been applied to study the physiological response of *Daphnia magna* when exposed to various toxic chemicals and is considered a sensitive tool in comparison to other standardized tests, for example, toxicity tests involving bioluminescent bacteria and algae growth (Barata et al., 2008; Yi et al., 2010).

Regarding sample A, only in autumn, a significant increase of feeding rate was recorded after exposure at 67 % of the effluent sample. In the winter and spring seasons, a significant increase in the feeding rate of *D. magna* was registered after the exposure to 100 % and 74 % of sample B, respectively. A significant decrease in the feeding rate was verified when *Daphnia magna* was exposed to 100% of sample C in autumn and 100 % of sample D in autumn and spring. The increase in the feeding rate in the presence of samples A and B can be explained by the fact that zooplanktonic cladocerans, such as *Daphnia magna*, are non-selective filter-feeders organisms that feed on suspended particles. Indeed, these organisms can, therefore, use colloidal particles, bacteria (Gellis and Clarke, 1935), algae, flagellates, and detritus as nutrient sources (Marinho et al., 2018).

Serra et al., (2019), considered that organisms such as *D. magna* can be used as an alternative method to tertiary treatments in WWTPs, as they are capable to remove suspended particles (Serra et al., 2014; Shiny et al., 2005) and emerging pollutants (Matamoros et al., 2012). In this case, during a short exposure period (24 h – feeding test time) these organisms may be able to remove small particles, reduce nutrients and improve effluent quality. Nevertheless, with the increase in the exposure time (acute exposure of 48 h) and the dilution of effluents, we verified that these could become toxic, causing death or immobilization (Figure 8). In the presence of samples C and D of autumn the opposite happens, although they do not cause any lethal acute toxicity, the feeding rate decreased showing that the sublethal feeding response is more sensitive than lethal acute toxicity. These results are in agreement with the results obtained by Yi et al., (2010) which verified that textile and dye effluents inhibited *D. magna* feeding rate,

despite not being acutely toxic. Currently, the combined study of toxicological tests of post-exposure feeding of *D. magna* with biochemical determinations has increased, since they allow the evaluation of the effects of complex mixtures and may complement studies on ecological monitoring of water quality (Barata et al., 2007; Damásio et al., 2008).

To perceive the effects of occurred at the cellular level, different specific oxidative stress and metabolism biomarkers were determined in *D. magna* after WWTP samples exposure (Figures 10 and 11). One of the primary enzymes responsible for the catalysis of hydrogen peroxide ( $H_2O_2$ ) into  $H_2O$  and  $O_2$  is catalase (Rodrigues et al., 2021c). When the organisms are exposed to compounds that increase the formation of reactive oxygen species (ROS) the activity of this antioxidant defense enzyme can be altered resulting in oxidative stress (Rodrigues et al., 2021c). When exposed to raw WWTP affluent (sample A), the activity of CAT (Figure 10) significantly decreased in the presence of most percentages tested in autumn and the presence of 36 % and 78 % of the summer sample. A significant increase of CAT activity was observed only at 100% of the spring sample and the lowest percentage tested in the summer (20 %). Regarding sample B, a significant decrease of CAT activity was recorded in spring and most autumn samples, except for the highest effluent percentage of autumn (96 %) and all percentages tested in summer. *D. magna* exposure to 100 % of the effluent sample C showed a significant increase of CAT activity in all sampling periods, except for winter season. Organisms exposed to samples from the Ferreira River (sample D) showed an increase in CAT activity in all sampling periods, except for autumn samples where a significant decrease was recorded.

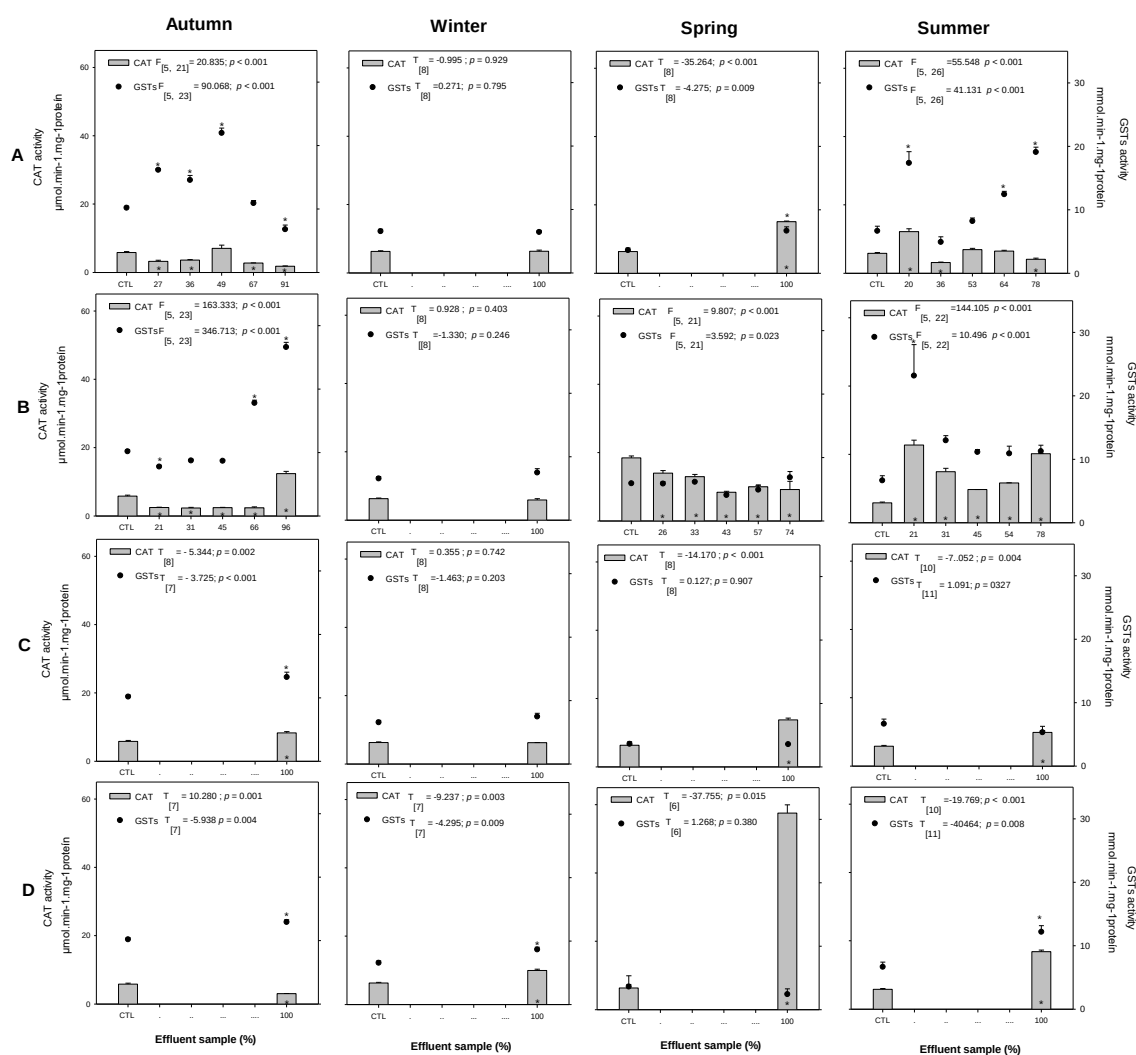


**Fig. 10** - Biochemical results of GSTs and CAT activities of *Daphnia magna*, after exposure to the different effluents (%) [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)], from the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum - summer). Data are expressed as mean  $\pm$  standard error (SE). \*Stand for significant differences between treatments and the CTL group (Dunnett test,  $p < 0.05$ ).

Glutathione-S-transferases (GSTs) are a group of isoenzymes capable of turning toxic compounds more easily excretable by catalyzing their conjugation with reduced glutathione (GSH), playing an important role in detoxification and antioxidant defense (Rodrigues et al., 2021c). The activity of GSTs (Figure 10) significantly increased in sample A in spring and summer, as well as in the lowest effluent percentages tested in autumn. As for sample B, a significant decrease was detected in the lowest percentage (21 %) of autumn, while the opposite occurred in the highest percentages tested (66 % and 96 %) of autumn and the lowest (21 %) of summer. No significant differences to the control in GSTs activity were observed in any sampling period when the organisms were exposed to the final effluent (sample C), except for the autumn sample where a significant increase was observed at 100 % of the effluent sample. The Ferreira River

samples caused an increase in GSTs activity in all sampling periods, except for spring (no significant differences to the control).

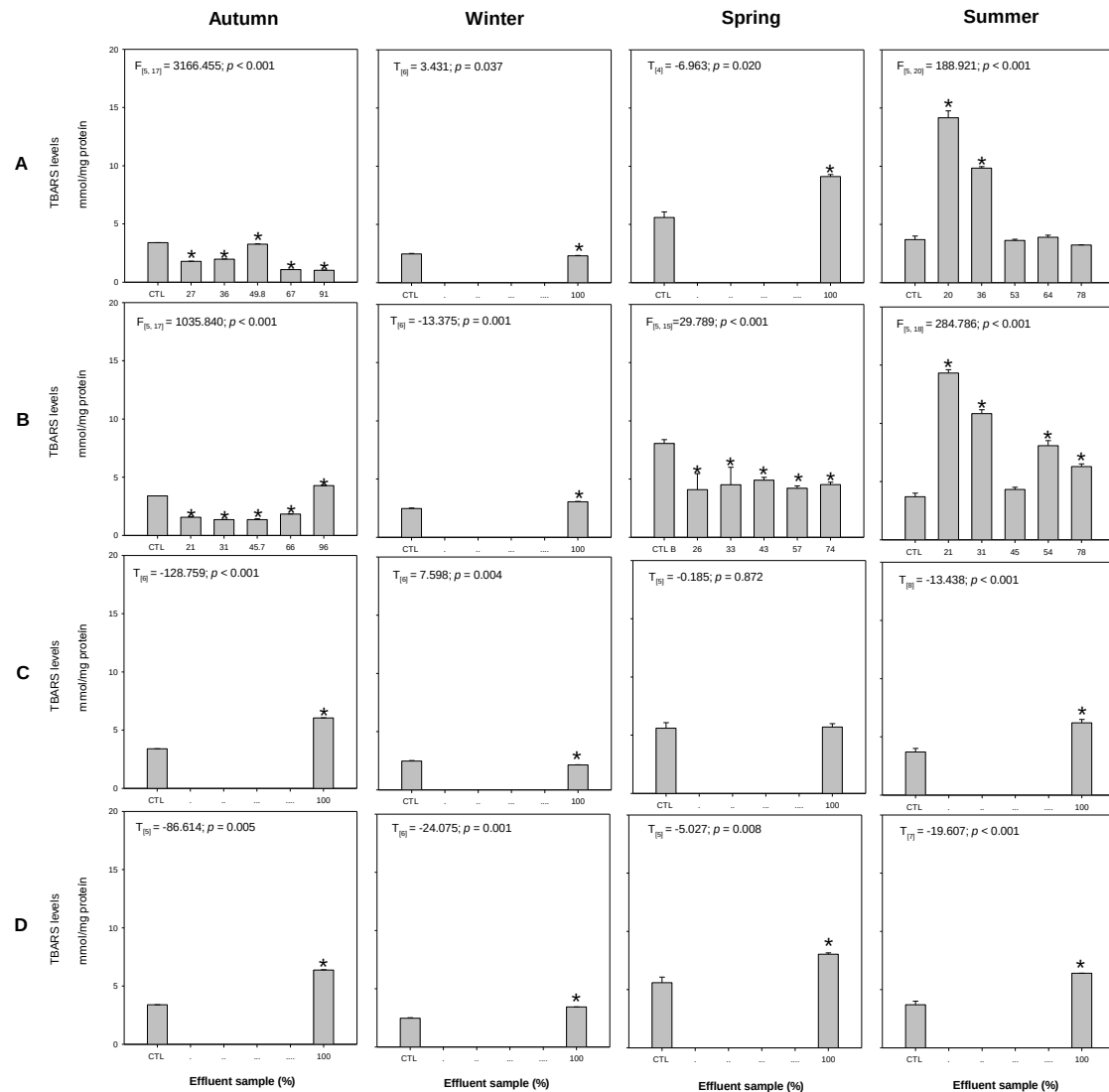


**Fig. 11** - Biochemical results of TBARS levels of *Daphnia magna*, after exposure to the different effluents (%) [raw wastewaters (A), preliminary effluents (B), final effluents (C), and ecoreceiving environment (D)] from the different seasons (Aut - autumn, Win - winter, Spr - spring and Sum-summer). Data are expressed as mean  $\pm$  standard error (SE). \*Stand for significant differences between treatments and the CTL group (Dunnett test,  $p < 0.05$ ).

Figure 11 shows the results of lipid peroxidation, in *Daphnia magna* after 24h exposure to the effluent samples. Higher levels of TBARS indicate a higher cell/tissue toxicity, through the induction of lipid peroxidation (Rodrigues et al., 2021c). According to the here-obtained results, a decrease in TBARS levels in the presence of sample A during autumn and winter was recorded, while in the spring and summer samples, a significant increase was observed. Sample B caused a decrease of TBARS level in all seasons, apart from winter and summer samples, as well as the highest percentage (96 %) of autumn. Regarding sample C, a significant increase in TBARS levels was observed at 100 % of autumn and summer samples, while a significant decrease was observed in winter. A

significant increase in the TBARS levels of *D. magna* after being exposed at sample D was observed in all the seasons.

Kim et al., (2012) determined the activity of antioxidant enzymes such as CAT, SOD (superoxide dismutase), and GPx (glutathione peroxidase) to assess the oxidative stress caused by industrial effluents in *Daphnia magna* and *Moina macroscopa*, after a 24 h exposure. In this study, it was verified that despite the increased activity of these enzymes, oxidative stress was detected even in samples where acute toxicity was not observed, as it happened in the present study with samples C (Aut and Sum) and D (all seasons). According to the results here-obtained, the activity of CAT and GSTs, and potentially other non-studied forms of antioxidant defense, might have been crucial to prevent oxidative damage in the cell membranes of organisms exposed to effluent sample A (Aut and Win), B (Aut and Spr) and C (Win and Spr). In the case of the remaining samples, where oxidative damage was verified, despite the mobilization of detoxification and antioxidant defense pathways, these mechanisms were not enough to neutralize and prevent lipid peroxidation. Although there are not many studies about the evaluation of the biochemical effects of wastewater to *D. magna*, other studies reported oxidative stress and metabolic disturbances in other aquatic organisms, that despite being phylogenetically different species, have similar biochemical mechanisms and metabolic pathways. Cazenave et al., (2014) also demonstrated that exposure to untreated domestic wastewater induced oxidative stress in vital organs (brain, liver, and kidney) but not in gills of the fish *Prochilodus lineatus* (freshwater fish). Despite the increased activity of GSTs, oxidative damage in the brain, liver, and kidney was not prevented (Cazenave et al., 2014). On the other hand, the increase of glutathione reductase and catalase activities prevented lipid peroxidation and consequently the oxidative stress in gills of *P. lineatus*. High levels of antioxidant activities enzymes [Glutathione reductase (GR), catalase (CAT)] were also reported in rainbow trout after exposure to municipal wastewater (Sturve et al., 2008) and in *Lasmigona costata* (freshwater mussel) when exposed to municipal effluents rich in pharmaceutical and personal care products (PPCPs) (Gillis et al., 2014).

In this study, the use of sensitive biochemical parameters (CAT, GSTs activities, and TBARS levels) allowed the evaluation of the toxicity of the effluents. However, the feeding assay did not reveal as much sensitivity as the others, despite having punctual effects at certain dilutions. Regarding the acute exposure tests, the MBR treatment showed its effectiveness in eliminating the toxicity for *D. magna*. If these results are

complemented with biochemical parameters, it is verified that the final effluents and samples from the ecoreceiving environment cause oxidative damage in this organism.

## 4. Conclusion

The use of ecotoxicological tests together with physical and chemical characterization, as well as microbiological, can be an important approach for a complete evaluation of wastewater effluents from a WWTP in year-zero. Since the WWTP was going through rehabilitation, the microbiological characterization revealed a high number of fecal contamination indicators, such as *Escherichia coli*, fecal Enterococci, and total coliforms in the final effluent.

The here-obtained results showed that the toxicity of effluent samples depends on the treatments at the WWTP and the species tested. While *Arthrobacter* sp., *Pseudomonas putida*, and *Rhodopirellulla rubra* were not sensitive to the tested samples, the two strains of *Escherichia coli* showed some sensitivity, and *Raphidocelis subcapitata* did not reveal to be a good species model for this type of study. On the other hand, the ecotoxicological assays with *Daphnia magna* and *Lemna minor* showed sensitivity to the different effluent samples of WWTP (e.g., affluent sample A and preliminary effluent B). Despite, the final effluent from the WWTP and the sample of Ferreira River (samples C and D) did not present acute toxicity to *D. magna* and *L. minor*, in almost all samples, biochemical disturbances were detected in the presence of these effluents (e.g., increase of CAT and GST activity and increase of concentration of MDA, respectively). These results demonstrate the sensibility of these parameters evaluated with *Daphnia magna* and *Lemna minor* and how they can be used as an early warning of toxicity must be incorporated into programs for monitoring and environmental assessment of wastewater effluents.

The results obtained from the battery of multispecies assays showed that the effluents from the Arreigada WWTP and the ecoreceiving environment exhibited toxicity. However, these results were expected since this WWTP is still undergoing adjustments after the rehabilitation works (year-zero). In the future, it is important to continue with a monitoring program of treatments, to ensure that no further discharge of effluents that can compromise the environment occurs. These environmental problems can be transversal to many other WWTP and the present study showed that the use of an ecotoxicological approach can be a great tool to assess the impact of discharges, contributing to a more precise establishment of discharge conditions, while maximizing environmental protection, aiming at the good ecological quality of the receiving waters.

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