Microbiological quality of grass irrigated with different water sources

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Abstract

In the present study the microbiological quality of grass from different locations in the city of Lisbon was assessed. The green areas presented different accessibilities and were irrigated with different water sources (groundwater, potable water and reclaimed water). Grass samples were collected between October 2020 and January 2021 and were analyzed for microbiological indicators (*Escherichia coli* and intestinal enterococci) and enteric viruses (Norovirus (Genogroups I and II) and Hepatitis A virus), using real-time quantitative Polymerase Chain Reaction. The presence of fecal contamination from dogs was also tested for one location, through the use of mitochondrial DNA markers, as well as, the effects of environmental variables on the survival of indicator microorganism on the grass.

All the grass samples showed a high degree of bacterial contamination, the majority presenting higher concentrations of enterococci compared to *Escherichia coli* concentrations, suggesting the presence of fecal contamination from animal origin. The locations with high accessibility also showed the presence of fecal contamination from human origin, indicated by occurrences of enteric viruses (human Norovirus Genogroups I and II). Contamination from animals and use of the green spaces by people are the main sources of microbiological contamination present in the grass.

Keywords: Reclaimed water, landscape irrigation, fecal contamination, pathogenic microorganisms.

Resumo

No presente estudo foi avaliada a qualidade microbiológica de diferentes relvados na cidade de Lisboa. Os relvados apresentam diferentes acessibilidades e são irrigados com diferentes fontes de água (água subterrânea, água potável e água residual tratada para reutilização). As amostras de relva foram recolhidas entre Outubro de 2020 e Janeiro de 2021 e foram analisadas para indicadores microbiológicos (*Escherichia coli* e enterococcus intestinais) e vírus entéricos (Norovírus (Genogrupos I e II) e vírus da Hepatite A), através de reação em cadeia de polimerase em tempo real. A presença de contaminação fecal de cães foi também testada para um dos relvados, através de marcadores de DNA mitocondrial, bem como, os efeitos das variáveis ambientais na sobrevivência dos indicadores microbiológicos na relva.

Todas as amostras de relva apresentaram um alto grau de contaminação bacteriana, a maioria apresentando maiores concentrações de enterococcus comparativamente às concentrações de *Escherichia coli*, sugerindo a presença de contaminação fecal de origem animal. Os relvados com acessibilidade pública também apresentaram presença de contaminação fecal de origem humana, indicada pela ocorrência de vírus entéricos (Norovírus humano Genogrupos I e II). A contaminação por animais e o uso de áreas verdes por pessoas são as principais fontes de contaminação microbiológica presente nos relvados.

Palavras chave: Água para reutilização, rega paisagística, contaminação fecal, microrganismos patogénicos.

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1. Introduction

Globally, the need for freshwater is increasing, often exceeding availability. Recently, there has been an increase in water stress, which is an indicator of water scarcity and deterioration of water quality (saline intrusion, eutrophication, pollution, etc) (Bixio et al., 2006). Over 2 billion people live in countries experiencing high water stress, whereas 4 billion people live in conditions of severe water scarcity during at least one month of the year (United Nations, 2018).

Water scarcity is mainly driven by two factors: climate conditions, which influences the seasonal water supply and the availability of freshwater resources; and water demand, which is influenced by population trends and socioeconomic developments. In Europe, countries located primarily in the Mediterranean region are considered a "hot spot" for water stress conditions, especially during the summer months, since there is an increase of water demand for agriculture, public water supply and tourism (Amec Foster Wheeler et al., 2016; EEA, 2017). However, water scarcity also affects western, eastern and northern areas, usually due to urbanization combined with high abstractions from the energy and industrial sectors for cooling purposes and from the public water supply sector. With human populations increasing on a global scale, conflicts over available freshwater are likely to be exacerbated (Raso, 2013).

Treated wastewater represents one of the most readily available sources of water to meet the increasing demands of water for non-potable uses. Large volumes of wastewater are produced daily, which after undergoing proper treatment in the wastewater treatment plant (WWTP) may have different applications, from agricultural and landscape irrigation, industrial uses, non-potable urban uses, among others (Marecos do Monte & Albuquerque, 2010).

Emergent reuse of wastewater for irrigation is paramount for arid climate countries. However, it is important to consider the potential risks of water reuse for the environment and public health. If water recycling is the final purpose, important issues arise, such as microbiological levels present in wastewater treated effluents. The use of treated wastewater for irrigation depends on three interrelated variables: the level of treatment, the method of irrigation and the type of culture to be irrigated, making it possible to control the risks to public health.

Reutilization of water for irrigation can only take a step forward if proven safe for human health on chemical and microbiological levels. For this purpose, both the water used for irrigation and the culture to be irrigated must be assessed for a better evaluation of the potential risks of microbiological contamination of different microbiological groups (i.e. bacteria, enteric viruses).

Being Portugal a country with numerous agricultural areas and green spaces, the use of treated wastewater is undoubtedly a benefit especially due to scarcity of water resources for irrigation.

1.1. Background

In May 2017, Lisbon became a member of the Urban Water Agenda 2030 network of cities. The main objective of this initiative is to encourage, support and enable local governments and water utilities to take action in the promotion of treated wastewater in urban centers (European Commission, 2017). As a result, the Strategic Plan for the Reutilization of Water in Lisbon (PLERAL 2020) was created to respond to the accepted compromises. With the implementation of the plan it is estimated that, by 2030, 25% of water for irrigation of green spaces and street washing is treated wastewater (*Câmara Municipal de Lisboa - MUNICÍPIO de LISBOA*, n.d.).

"Parque Tejo" is one of the parks intended to be irrigated with treated wastewater. Currently, the park is being irrigated with water from two wells, which is intended to be substituted with treated wastewater. The captured water from the wells is stored in a tank and from there the infrastructures of the irrigation system of "Parque Tejo" are supplied.

Figure 1 – General aerial view of "Parque Tejo".

The park totals an area of about 90 hectares, being the biggest green area of "Parque das Nações" (Figure 1) (*Parque Tejo - JF Parque Das Nações*, n.d.). It is also a metropolitan park open to the practice of various sports, leisure and educational activities. The irrigation of green spaces is done through sprinklers and only a small fraction is irrigated with drip irrigation, which are divided in different irrigation sectors. "Parque Tejo" has the greatest expression of sprinkler irrigation and volume of water required, with about 90% of total volume of water consumed for irrigation in the North Zone of "Parque das Nações". The irrigation system is controlled through a software,

which allows the control of the time and duration that each sector is irrigated, based on the irrigation schedule defined. During the summer months each sector is irrigated 20 minutes and in autumn and spring for about 10 minutes (depending on meteorological conditions).

Decree-Law (DL) nº 119/2019 establishes that license applicants for projects of production and/or utilization of treated wastewater are obliged to conduct a risk assessment. In this DL quality requirements and monitorization criteria are also defined, as well as risk management tasks. Minimization of risks can be achieved through the application of multiple barriers adjusted to each specific project, that consist in the imposition of safety barriers in terms of water treatment and physical barriers to minimize direct contact with the treated wastewater, namely by ingestion, and risk of leaching, percolation or run-off of contaminants and pollutants that can be present in the water.

Lisbon City Hall (CML) assessed the risk to human health with the use of treated wastewater for irrigation in the different zones of the park by applying the methodology defined in DL nº 119/2019. The risk assessment conducted by the CML took into account factors such as the identification of hazards, routes of exposure and receptors, as well as the exposure scenarios, in order to be able to calculate the vulnerability, damage and respective global risk in different zones. Conclusions of the risk assessment consider the overall risk to human health negligible for the different levels of danger analyzed, taking into account the quality of the RW (class A) and the applied barriers.

1.2. Objectives

The main objective of the present study was to develop a methodology to assess the microbiological quality of grass from different green spaces irrigated with different water sources (groundwater, potable water and reclaimed water). Fecal contamination of the grass areas was assessed, through several microorganisms chosen, namely indicator microorganisms (*Escherichia coli* and intestinal enterococci) and enteric viruses (Norovirus (Genogroups I and II) and Hepatitis A virus). To determine the origin of pollution, a Microbial Source Tracking method was also performed, through the use of mitochondrial DNA markers specifically for dogs, since it is very common for people to frequent the park with their dogs. The effects of environmental variables (precipitation, temperature and solar radiation) on the survival of indicator microorganisms, on grass surface, was also assessed.

1.3. Thesis Structure

The present dissertation is divided in 5 chapters. This chapter outlines the background, definition of the objectives and scope of the thesis.

In the second chapter, Literature Review, the state of the art on the subject under study is presented, being divided into 4 subchapters: General considerations, Microbiological indicators and pathogen microorganisms, Water reutilization in Europe and Water reuse in Portugal.

This is followed by the third chapter, Methods, where a brief description of the study area, sampling and analysis is made.

The fourth chapter, Results and Discussion, contains the description and analysis of the results.

Finally, in the last chapter, Conclusions, the most important aspects to be considered are highlighted, as well as the recommendations and limitations of the present work.

2. Literature Review

2.1. General considerations

Water is a fundamental resource that affects populations living conditions and public health. Nowadays factors such as population growth and climate change increase the pressure on the world´s freshwater sources. Throughout the world, particularly in semiarid regions, there have been extensive water withdrawals leading to reduced flows in rivers and declining water tables (Raso, 2013). Moreover, due to climate change the periods of drought are becoming more frequent and lasting longer (Kovats et al., 2015).

In Europe the Mediterranean region is particularly impacted by climate change, experiencing more extreme events like heat waves and droughts (Kovats et al., 2015), leading to water scarcity and deterioration of water resources. Water scarcity is, however, no longer just a problem for southern Europe. Despite the colder climate of northern Europe, these countries also face seasonal water stress. According to data from the European Environmental Agency (EEA) 15% to 25% of the total European territory has been affected by water scarcity (EEA, 2017).

Figure 2 shows the water use in Europe by sector, where agriculture in Europe remains the sector that exerts more pressure on water sources, representing more than half (59%) of total water uses in 2017 (EEA, 2017).

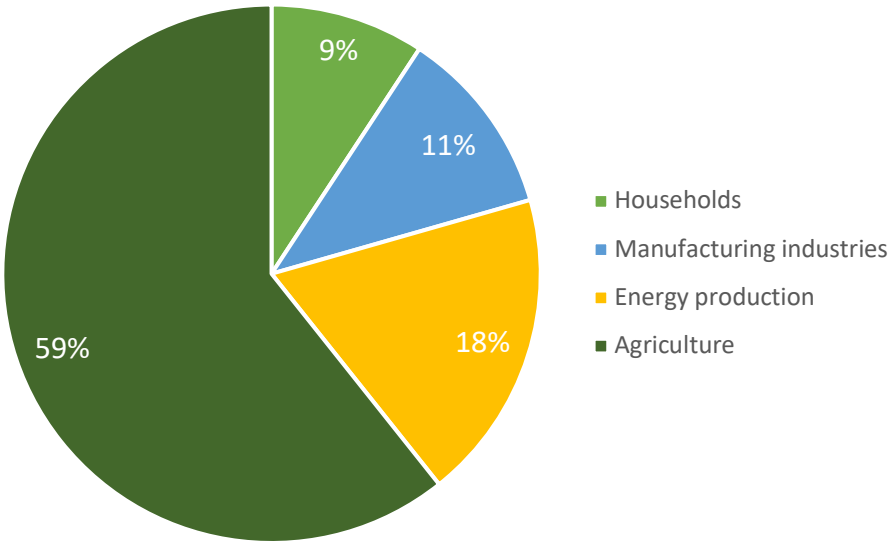


Figure 2 - Water use in Europe, 2017. Source: (EEA, 2017).

Southern European countries are the major consumers of water for irrigation purposes, mainly due to their drier climates, using around 95% of the total volume of irrigation

water at the European level. Figure 3 displays the volume of water used for irrigation (10^9 m^3/year) in each country in the European Union (EU) in 2017, which shows that Spain, Italy, Portugal and Greece are the countries that consume higher quantities of water for irrigation purposes (FAO, 2017). The water needs vary seasonally, with an increase in water consumption in the summer months, due to the subsequent increase in irrigation needs and tourist activity. It is estimated that during the summer months the consumption of water for agriculture increases 60% (Amec Foster Wheeler et al., 2016). Water scarcity affects not only the environment, but also results in serious social and economic consequences. The energy sector is particularly vulnerable to water scarcity and drought situations, since it heavily depends on water availability (BIO, 2015; Lehner et al., 2005).

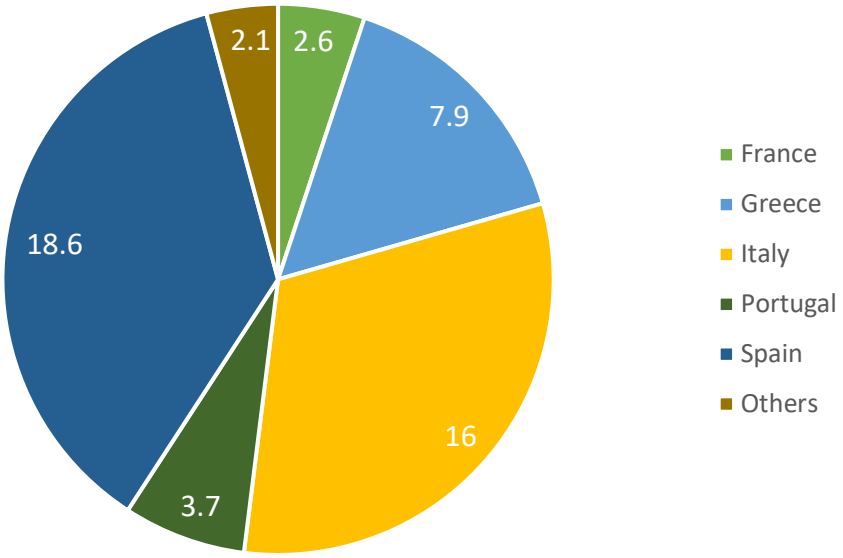


Figure 3 – Volume of water withdrawn for irrigation purposes in the EU in 2017 (10^9 m^3/year). Source: (FAO, 2017).

According to information reported in the 2009 River Basin Management Plans, the quality of more than half of surface water bodies in Europe is in less than good ecological status, being the implementation of measures necessary in order to achieve the objectives delineated by the Water Framework Directive (WFD) (Alcalde Sanza & Gawlik, 2014; European Commission, 2012). The WFD identifies over abstraction of surface and groundwater bodies as a significant pressure in some areas of Europe and a driver to water scarcity. In order to achieve good status of surface and groundwater bodies it is necessary that the member states identify the pressures over the resources and adopt appropriate measures.

In 2007, the Communication on water scarcity and droughts set a water hierarchy to tackle water scarcity, under which water saving is the preferred management method, followed by alternatives such as water reuse (Bourguignon, 2018). The 2012 Communication, "The Blueprint for Safeguarding European Waters" acknowledged the potential for water reuse and the importance for the EU to implement measures to

encourage the use of treated wastewater (Alcalde Sanza & Gawlik, 2014; European Commission, 2012). This document proposes ways to implement water policies in the EU, to achieve a better integration of politics and complete the European Legal Framework. Furthermore, the 2012 fitness check on EU freshwater policy also identifies water scarcity as one of the main issues, identifying the importance of alternative water supply options with low environmental impact (Bourguignon, 2018).

With increasing water needs, particularly resulting from public supply, agriculture irrigation, industrial use, recreational and sports use, etc., it becomes imperative to consider alternative water sources such as the use of Reclaimed Water (RW).

RW, as defined by the Environmental Protection Agency (EPA), is municipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for a range of purposes (US Environmental Protection Agency, 2012). In addition to the use of this terminology, terms such as RW, water reuse and recycled water are all used within the Member States and globally to define wastewater reuse.

In the EU the use of RW still remains below its potential. For this reason, in recent years, the European Commission (EC) put forward new measures to allow and encourage the use of RW (Bourguignon, 2018).

In 2018, the EC put forward a proposal with the aim of promoting the use of RW in order to alleviate the water scarcity issues in the EU. The aim of this proposal was only for the use for RW for agricultural irrigation purposes. With this in mind, the proposal sets requirements for RW quality.

According to the 91/271/EEC European Council directive, urban wastewater is the result of domestic wastewater or the combination of domestic, industrial and stormwater that are collected in the public drainage network. Wastewater that results from industrial activity often contains high levels of metals, metalloids and volatile or semi volatile compounds (Qadir et al., 2010), while domestic wastewater can be particularly harmful due to microbiological contamination (Mara, 1976).

According to Monte and Albuquerque (2010) the quality of the RW is the most important factor regarding the possible reuse applications. The main applications of RW, in descending order of volume of use, are: agricultural irrigation, landscape irrigation, industrial recycling and reuse, groundwater recharge, some recreational/environment uses (such as lakes and ponds), non-potable urban uses and also as a potable use, where the RW is blended with raw water to produce water for consumption.

Besides the importance of the water quality, other important factors also determine the selection of which applications can be implemented (Marecos do Monte & Albuquerque, 2010):

- The type of technology used to treat the wastewater, i.e. the type of treatment the wastewater undergoes;

- The equilibrium between demand and supply of the RW, i.e. the balance between the volume of water needed for a certain use and the available volume of RW;
- The infrastructures required for the realization of the reuse, such as reservoirs and transport and distribution systems;
- Economic sustainability of the project;
- Mitigation of environmental impacts and public health risks associated to the use of RW.

RW is preferably used for practices that register a greater demand for water in quantitative terms and that are compatible with the quality from treated WWTP effluents.

2.1.1. Reclaimed water for irrigation purposes

Agriculture is the sector with the greatest demand for water, requiring massive amounts of potable water for its maintenance, contributing to depletion of natural potable water sources. Therefore, the possibility of using RW in this sector can contribute to increase the savings on consumption-safe water. Additionally, the type of applications requiring alternative water sources increases as water scarcity increases, such as recreational/environmental uses and landscape irrigation, among others. Landscape irrigation comprises the irrigation of parks and golf courses.

In 2016, the total irrigatable area in the EU was 15,5 million hectares (ha) (Eurostat, 2016). There is variation regarding irrigation needs among the Member States, with southern countries having the largest irrigatable areas, mainly due to regional climate. In countries with drier climate, where precipitation is not sufficient for plant growth, irrigation becomes essential. Supplemental irrigation is vital to produce high quantities of crops with good quality in semi-arid climates especially during dry seasonal periods. This leads to irrigation being the major driving force behind water abstraction globally (Eurostat, 2016).

In terms of volume of water, landscape irrigation is the second largest application for RW in developed countries. The USA is a great example of the use of RW for recreational and landscape irrigation. Other regions are also following this trend, such as Europe and the Far East (Marecos do Monte & Albuquerque, 2010).

A wastewater reuse system for landscape irrigation is analogous to a reuse system for agricultural irrigation, with the difference that the irrigated plants are, in this case, ornamental. Irrigation water must satisfy the plant water needs, promoting a good vegetative development, while at the same time making sure the quality of these waters meets the requirements to avoid public health risks. One motive for this type of reuse application is economic, especially in places with high water demand for recreational purposes and landscape irrigation, therefore leading to high irrigation costs. In Portugal,

Algarve has become a main focus of interest for water reuse, not only due to the climate, but also due to the importance of the tourism sector, leading to an increase of the demand of water for recreational purposes and landscape irrigation, namely irrigation of golf courses (Marecos do Monte & Albuquerque, 2010).

Other motives for this use include environmental protection. Additionally, the production of RW in a WWTP is independent of the time of the year, being relatively constant. However, in tourist cities fluctuations are more pronounced. This creates a compatibility between demand of water for irrigation and the supply of RW produced in the WWTP. The storage of RW can constitute a solution to maximize the benefit of the use of RW for irrigation.

The use of RW for irrigation purposes leads to positive and negative impacts, depending on the project planning and management (WHO, 2006). In addition to health concerns, environmental risks must be considered. There can also be environmental impacts resulting from the use of RW, therefore it is important to take into account the soil characteristics, the topography and presence of aquifers. Consequently, when using non-conventional water sources for irrigation it is necessary to pay attention to aspects of physical and chemical nature, saline and microbiological, which can condition the use of the RW. Table 1 describes the characteristics of the wastewater, with the most significant impact on the soil-plant biosystem and on the equipment.

Table 1 - Wastewater characteristics and their impact. Source: (Marecos do Monte & Albuquerque, 2010).

Characteristics	**Parameters**	**Effects**
Salinity	Total dissolved solids (TDS), conductivity, specific ions (Na, Ca, Mg, Cl, B)	High salinity affects plant development. Ions, such as Na, B, Cl, can be toxic to plants. Na can induce permeability problems in the soil.
Suspended solids	Total Suspended Solids (TSS)	Can lead to clogging of equipment.
Biodegradable organic matter	Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD)	In treated domestic effluents, the organic matter content, in general does not lead to problems and can be beneficial for the biosystem.
Refractory organic compounds	Specific compounds (phenols, pesticides, halogenated hydrocarbons)	Resistant to conventional treatment processes. Some can be toxic. The presence of these compounds can limit the use of the effluent for irrigation.
Nutrients	Nitrogen, phosphorous, potassium	Essential nutrients for plant development. When in excess can lead to pollution of groundwater.
Hydrogen ionic activity	pH	The pH of wastewater can affect the solubility of metals and the soil alkalinity.
Heavy metals	Cd, Cr, Cu, Fe, Hg, Ni, Zn	Accumulation of heavy metals in the soil. Toxic to plants. Limiting factor in the use of RW.
Residual chlorine	Chlorine free and combined	Excessive amounts of free chlorine can lead to leaf damage. Combined chlorine does not cause problems.
Pathogen microorganisms	Fecal coliforms, helminths, microbiological indicators	Disease transmission.

The use of RW for agriculture is considered a source of water and nutrients. Using it for irrigation can be an environmental benefit, since the nutrients are used by the crops instead of being discharged into water bodies, reducing the risk of eutrophication. However, there is also a risk of run-off into water bodies, which will also contribute to eutrophication (Amec Foster Wheeler et al., 2016; Maurer & Davies, 1993). Additionally,

the use of RW can reduce significantly the application of fertilizers and its associated costs (Maurer & Davies, 1993; Pedrero et al., 2010, 2013, 2014).

Some studies have proved the agronomical advantages of reusing RW, however RW composition and chemical properties need to be periodically monitored to avoid an imbalance in nutrient supply, which can lead to problems related to excessive vegetative growth, delayed or uneven maturity or reduced quality (Pedrero et al., 2010).

Besides the advantage of supplying the nutrients, the use of RW can pose a risk of accumulation of salts in the soil and an accumulation of heavy metals in plants and soils, affecting crops quality and safety, and also cause damage to the soil structure due to high levels of sodium (Intriago et al., 2018; Pedrero et al., 2010). There are, however, some studies on the effects of RW on soil that demonstrated there were no significant impacts in regards to salinity or heavy metal accumulation in the soil (Abedi-Koupai et al., 2006; Pedrero et al., 2013, 2014). A study done by Pedredo et al. (2014), where saline RW combined or not with a regulated deficit irrigation (RDI) strategy were used to irrigate a commercial young grapefruit orchard for 3 years. Results of this study show that the use of RW or RDI does not contribute to increase the salinity of the soil, due to the salts being washed away by rainfall. There was an increase in the levels of Na, Cl and B on the leaf mineral content, but their concentrations did not exceed the toxic threshold (Pedrero et al., 2014). Long term studies (more than 5 years), found an increase in soil salinity after the application of RW to an agricultural soil (Nicolás et al., 2016; Pereira et al., 2011).

Based on the results from these studies, it is necessary to implement intensive monitoring and management strategies to avoid salinization. Measures such as the type of irrigation method, soil drainage and selection of cultures can contribute to minimize impacts on the soil and plants (Marecos do Monte & Albuquerque, 2010).

The risks to health and the environment from pollutants such as bacteria, viruses and emerging pollutants and priority substances, such as those detected occasionally in discharges from WWTP described in Table 1, are also perceived as an obstacle (Amec Foster Wheeler et al., 2016; Estévez et al., 2012). Although still limited, there already exists knowledge on the impact of using RW for agricultural practices, regarding microbiological consequences. Christou et al. (2014) reported that there was no microbiological contamination (total coliforms, fecal coliforms, *E. coli* , *Salmonella* spp., *Listeria* spp.) in tomato crops irrigated with two qualitatively distinct RW, treated with different depuration technologies (Slow Sand Filtration and Chlorination; Membrane Bioreactor and UV radiation) (Christou et al., 2014).

Intriago et al. (2018) assessed the microbiological quality of baby romaine lettuces irrigated with different types of effluents, including a secondary effluent of a WWTP and a conventional irrigation water. Although the irrigation water analysis of *E. coli* concentrations slightly surpassed the legislation threshold for secondary effluent water, conventional water irrigated plants presented the highest *E. coli* concentrations, showing that the concentration of microorganisms in the irrigation water is not necessarily the main factor in the contamination of crops (Intriago et al., 2018; Pachepsky et al., 2011). A study done by Holvoet et al. (2014) showed that there was a

much higher prevalence of *E. coli* in the irrigation water compared to the irrigated lettuces, with *E. coli* testing positive for 5% of lettuce samples, while 75% of the irrigation water showed to be positive for *E. coli* (Holvoet et al., 2014). A similar result was observed in a study where pepper fruits were irrigated with both surface water and tertiary treatment effluent. The levels of *E. coli* were assessed, and no correlation could be established between the prevalence of *E. coli* in irrigation water and presence in fruit, since contamination of the fruits seems negligible (Lopez-Galvez et al., 2016).

Forslund et al. (2013), assessed the contamination present on tomatoes irrigated with wastewater treated with different depuration technologies. Bacteria were detected in low concentration on crops, suggesting that contamination with fecal derived bacteria (*E. coli*) was negligible, despite the higher concentration of *E. coli* in the irrigation waters. The authors also note that wild animals or birds seem to be a likely source of *E. coli* contamination of the soil (Forslund et al., 2013). Land and Smith (2007) also argued that background *E. coli* populations present in the soil may be due to fecal contamination sources, like wild animals and birds (Lang & Smith, 2007). Similarly, results from a study by Vergine et al. (2015) note the existence of a source of microbiological contamination (animal feces) different from the irrigation (with effluent of a pilot scale membrane bioreactor), with more pronounced effects on grass than topsoil (Vergine et al., 2015).

The irrigation method is another variable to consider in order to control public health risks, since some methods can promote direct contact with the cultures, namely sprinkler irrigation (Marecos do Monte & Albuquerque, 2010). Forslund et al. (2013) notes that contamination of crops with pathogens can occur with sprinkler irrigation, as the edible part of the plant or fruit is directly exposed to the applied water or to soil splashing. Drip irrigation is less likely to contaminate crops since it applies water at the soil surface, while subsurface irrigation is safer since the water is applied directly to the roots with minimal transfer of pathogens to the crop surface. However, results from this study show that it is impossible to state whether subsurface drip irrigation is safer than surface irrigation or sprinkler irrigation, since almost all crop samples were free of *E. coli* and there were no significant differences in the level of contamination in the soil samples between the irrigation methods.

Sprinkler irrigation can also lead to the formation of aerosols, which can pose a public health risk, since the aerosols may contain pathogen microorganisms (Adegoke et al., 2018). This method of irrigation can cause not only the contamination of the watered plants and objects that come into contact with the RW, but also the aerosols formed can be inhaled by both people and animals (Marecos do Monte & Albuquerque, 2010).

Additionally, the cost of the necessary infra-structures to supply the area to be irrigated with RW, that is determined mainly by the distance between the area to supply and the WWTP that produces the RW, may be a decisive factor in the implementation of a project for the reuse of wastewater for irrigation (Marecos do Monte & Albuquerque, 2010).

2.1.2. Wastewater treatment and water quality requirements

In the WWTP the wastewater undergoes a series of treatments in order to remove pollutants and contaminants present in the water by physical, chemical and biological methods. The type of treatments the wastewater goes through in a WWTP have the primary objective of removing pollutants, quantified in terms of TSS, BOD, COD, nitrogen and phosphorous and fecal microorganisms (Marecos do Monte & Albuquerque, 2010).

The levels of treatment in a WWTP are classified as: preliminary, primary, secondary and tertiary or advanced treatment.

Preliminary treatment consists in the removal of coarse solids and other materials, such as sand, oils, fat, etc., that can affect the treatment operation. The removal of these materials is done through racks and coarse screens and grit chambers (Metcalf & Eddie, 2003).

The objective of primary treatment is the removal of settleable solids and floating materials (Metcalf & Eddie, 2003), i.e., the removal of organic and inorganic suspended solids through physical and chemical processes via, for example, primary sedimentation tanks. According to the DL n. º 152/97 primary treatment is any physical and chemical process in which the value of BOD_5 present in the wastewater has to be reduced, at least, 20% before discharge and the TSS at least 50%.

Secondary treatment consists in the removal of biodegradable organic matter through the application of biological treatments (Metcalf & Eddie, 2003). This treatment can be divided in the following processes: attached growth treatment processes, where the microorganisms responsible for the conversion of the organic matter are attached to an inert medium; suspended-growth treatment processes in which the microorganisms are maintained in suspension within the liquid; pond processes where the biological treatment is achieved by natural processes, involving the use or bacteria and/or algae. According to the DL n. º 152/97 after secondary treatment the treated wastewater needs to respect the following values:

- [BOD_5 without nitrification] = 25 mg/l O_2 minimum reduction of 70-90 %;
- [COD] = 125 mg/l O_2 or minimum reduction of 75%;
- [TSS] = 35 mg/l O_2 or minimum reduction of 90%.

The objective of tertiary treatment is the removal of remaining suspended solids, nutrients, namely nitrogen and phosphorous, and microorganisms. The level of treatment that the water is submitted depends on the final use of the RW. The secondary treatment produces effluents that, in general, still have a high concentration of microorganism and the presence of some chemical constituents, which is compatible for some applications (Marecos do Monte & Albuquerque, 2010). For applications where there is the possibility of higher exposure to the pathogens, the effluent needs to undergo tertiary treatment, namely disinfection (Metcalf & Eddie, 2003).

For wastewater disinfection, there are methods that have a higher efficiency in the removal of different microorganisms. Both ultraviolet (UV) irradiation and chemical compounds, such as chloride or ozone, are generally efficient methods for bacteria inactivation (Mihelcic & Zimmerman, 2010).

Results from various studies (Ebdon et al., 2012; V. J. Harwood et al., 2005; Lucena et al., 2004; Saleem et al., 2000) show that treatment practices in WWTP are generally effective on bacteria, however there is usually a lack of decrease in viral and protozoan levels. Fecal indicator bacteria (FIB) are used to monitor fecal contamination in water, however the low resistance of FIB to disinfection do not make them effective indicators for microbiological water quality and numerous studies show that there is no correlation between FIB and pathogen occurrences (Baggi et al., 2001; Ebdon et al., 2012; V. J. Harwood et al., 2005; Lucena et al., 2004).

Disinfection processes can be improved through the application of filtration systems. The filtration allows the removal of suspended solids still present in the wastewater that were not removed previously in the treatment line. Consequently, the application of filtration before disinfection allows an increase of the efficiency of the disinfection processes as it decreases water turbidity, which will allow the removal of harmful chemicals and metals (Metcalf & Eddie, 2003).

The application of a final sand filtration treatment contributes to a significant reduction of enteric virus (Baggi et al., 2001). Baggi et al. reported that treatment plants without a fourth treatment stage (sand filtration) were more contaminated with enteric viruses.

Technological development registered in the field of wastewater treatment, namely Membrane Technologies (MBR), allow the removal of microorganisms and chemical pollutants with very high efficiencies (Metcalf & Eddy et al., 2007). Besides the ability of this type of treatment to achieve the desired water quality, it is important to ensure the viability of this kind of project.

However, it is usually uneconomic to treat wastewater to the extent where complete pathogen removal is achieved prior to reuse for certain applications. In general, the treatment processes in a WWTP with the intent to reuse the treated wastewater do not differ from those applied to a WWTP whose goal is to safeguard the receiving water bodies, where the type of treatment that is necessary to implement takes into account factors such as the characteristics of the receiving water body. However, there are norms and legislation that set minimum quality standards for each use typology, using a fit-for-purpose approach. These norms also mention the level of treatment the wastewater needs to be subjected to in order to achieve these values (APA, 2018).

The Regulation (EU) 2020/741 of the European Parliament and of the Council of May 2020 lays down minimum requirements for water quality and monitoring on risk management in order to guarantee a safe use of RW in the context of integrated water management. This regulation aims for a safe use of RW, mainly for agricultural irrigation, ensuring the protection of public health, animal health and of the environment,

contributing to the objectives of Directive 2000/60/EC by addressing water scarcity. The Regulation applies whenever treated urban wastewater is reused, in accordance with article 12(1) of Directive 91/271/EEC, for agricultural irrigation (*Regulation (EU) 2020/741 on Minimum Requirements for Water Reuse*, 2020).

The Regulation sets out minimum requirements applicable to RW intended for agricultural irrigation and indicates preventative measures, i.e. barriers, in order to manage identified risks. Barriers include the adequate level of treatment for each RW quality class, as described in Table 2, and minimum requirements for monitoring and monitoring frequencies to verify the compliance of the RW with the minimum water quality requirements. Crops belonging to a given category shall be irrigated with RW of the corresponding minimum RW quality class.

Table 2 - Indicative technology target according to RW quality class for agricultural irrigation. Source: (*Regulation (EU) 2020/741 on Minimum Requirements for Water Reuse*, 2020).

RW quality class	Indicative technology target
A	Secondary treatment, filtration and disinfection
B	Secondary treatment and disinfection
C	Secondary treatment and disinfection
D	Secondary treatment and disinfection

Regarding Portuguese legislation, Article 57 of the DL n. º 226-A/2007, mentions that RW should be used, whenever possible or appropriate, in particular for irrigation of gardens, public spaces and golf courses ("Decreto-Lei N.º 226-A/2007," 2007).

In 2019, the DL n. º 119/2019 defined specific quality requirements for each type of application for the RW, following a fit-for-purpose approach. This legislation follows the same principles as the International Standardization Organization (ISO) 16075, which consists in the production of RW quality that meets the needs for the intended use. The DL also mentions the type of treatment the RW needs to undergo for each use, monitoring of the RW and risk management, in order to guarantee a safe use of RW, as well as the associated licensing regime. In the quality norms present in the DL, the RW for irrigation purposes needs to undergo advanced treatment (disinfection) ("Decreto-Lei n.º 119/2019," 2019). This type of complementary treatment guarantees the production of RW with adequate quality in order to avoid significant public health risks. Table 3 shows the RW quality requirements for irrigation for each quality class.

Table 3 -Water quality requirements for reuse for irrigation purposes. Source: ("Decreto-Lei n.º 119/2019," 2019).

Quality class	BOD_5 (mg/L O_2)	TSS (mg/L)	Turbidity (NTU)	*E. coli* (CFU/100mL)	Intestinal nematodes eggs (Nº/L)	Ammonia nitrogen (mg NH_4^+/L)	Total nitrogen (mg N/L)	Total phosphorus (mg P/L)
A	≤10	≤10	≤5	≤10		10	15	5
B	≤25	≤35		≤100				
C	≤25	≤35		≤1000	≤1			
D	≤25	≤35		≤10000	≤1			
E	≤40	≤60		≤10000				

2.2. Microbiological indicators and pathogen microorganisms

Indicator microorganisms, such as *E. coli* and *Enterococcus spp.*, are used to assess water quality and monitor fecal contamination (USEPA, 2000). *E. coli* and intestinal enterococci are present in the intestinal tracts of warm-blooded animals (Ahmed et al., 2019; Ishii et al., 2006). One of the most common bacterial pathogenic found in wastewater throughout the world is *E. coli.* Most *E. coli* strains are harmless, but some strains are associated with the occurrence of gastrointestinal symptoms or disease, causing gastrointestinal irregularities, such as hemorrhaging and possible hemolytic-uremic syndrome (USEPA, 2000).

Ideally, indicator microorganisms are non-pathogenic, rapidly detected and easily enumerated, do not reproduce outside the host organism, have similar survival characteristics and can be strongly associated to the presence of pathogens (Scott et al., 2002). Total and fecal coliforms have been, for many years, used as the traditional microbial indicators throughout the world for determining the quality of waters. (USEPA, 2000).

These microorganisms present, however, limitations as indicators, such as the poor correlation with pathogen occurrences (V. J. Harwood et al., 2005), and their inability to provide the specific source of fecal contamination (Carson et al., 2001; Stoeckel & Harwood, 2007). It is important to know the origin of fecal pollution, since it allows to assess associated health risks, as well as the actions necessary to prevent and mitigate them.

The ratio of fecal streptococci to fecal coliforms can determine whether the contamination was of human or nonhuman origin. According to some studies humans and animals contain different numbers and ratios of coliforms and streptococci (Scott et al., 2002, 2005). Observations show that animal feces contain higher levels of fecal streptococci compared to humans' feces, which contain higher levels of fecal coliforms

(Geldreich & Kenner, 1969; Scott et al., 2002). This method provides the advantage of obtaining rapid results, however some studies state that this approach is not reliable and data can be contradictory (Fogarty et al., 2003; Geldreich, 1978; Geldreich & Litsky, 1976; Leclerc et al., 2001; Sinton et al., 1998; Weaver et al., 2005).

Microbial source tracking (MST) tools have been used to obtain information on the source of fecal contamination, whether it came from humans, animals or both (Ahmed et al., 2019; Field & Samadpour, 2007; Scott et al., 2002; Stoeckel & Harwood, 2007). Methods for fecal source identification can be divided into two major categories: culture-based and culture-independent methods. Cultivation-independent techniques use Polymerase Chain Reaction (PCR), that consists in the amplification of a DNA strain (Field & Samadpour, 2007; Stoeckel & Harwood, 2007). Some methods use the presence of virus or bacteria specific for a given host, while other MST methods are based on the detection of mitochondrial DNA (mtDNA), through PCR primers targeting host mitochondrial gene sequences (Field & Samadpour, 2007). The use of mtDNA also has its limitations, since the shedding of mtDNA is not exclusively through feces, but also through urine, blood, skin and saliva (Roslev & Bukh, 2011).

Other microorganisms are reported as major health concerns associated with RW use. These microorganisms include viruses, protozoa and helminths, that are responsible for potentially dangerous pathologies (Adegoke et al., 2018). Table 4 presents the microorganisms that are commonly found in untreated wastewater and their effect on human health.

Table 4 – Infectious agents potentially present in untreated domestic wastewater. Source: (Metcalf & Eddie, 2003)

Microbial group	Organism	Disease
Bacteria	*E. coli*	Gastroenteritis
	Campylobacter jejuni	Gastroenteritis
	Legionella pneumophila	Legionnaires' disease
	Leptospira	Leptospirosis
	Salmonella	Salmonellosis
	Salmonella typhi	Typhoid fever
	Shigella	Shigellosis (bacillary dysentery)
	Vibrio cholerae	Cholera
	Yersinia enterocolitica	Yersinosis (diarrhea)
Protozoa	*Balanditium coli*	Diarreah, dysentery
	Cryptosporidium parvum	Diarreah
	Cyclospora cayetanensis	Severe diarrhea, nausea and vomiting
	Entamoeba histolytica	Diarreah, abcess of the liver and small intestine
	Giardia lamblia	Diarreah, nausea, indigestion
Helminths	*Ascaris lumbricoides*	Ascariasis (Ringworm)
	Enterobius vermicularis	Enterobiasis (Pinworm)
	Taenia saginata	Taeniasis (Beef tapeworm)
	Taenia solium	Taeniasis (Pork tapeworm)
	Trichuris trichiura	Trichuriasis (Whipworm)
Virus	Norovirus	Gastroenteritis
	Hepatitis A virus	Infectious hepatitis
	Adenovirus	Respiratory disease
	Enterovirus	Gastroenteritis, hearth anomalies, meningitis
	Parvovirus	Gastroenteritis
	Rotavirus	Gastroenteritis

Enteric viruses, including norovirus (NoV) and hepatitis A virus (HAV), among others, can be present in human and animal feces, which can lead to the contamination of recreational and drinking water sources (USEPA, 2014). NoV and HAV are included in the United States EPA Contaminant Candidate List Number 4 (USEPA, 2016). The contaminants present in the list are currently not subject to any proposed or promulgated national primary drinking water regulations but are known or anticipated to occur in public water systems.

HAV virus transmission occurs mainly by the fecal-oral route, person to person contact or ingestion of contaminated food and drink. The virus infects the liver, causing symptoms such as, nausea, vomiting, anorexia (loss of appetite), fatigue and fever. The higher risk is usually exposed to young children and older adults with underlying chronic liver disease. HAV is present in a worldwide distribution, the highest prevalence of infection occurring in regions where low standards of sanitation promote transmission

of the virus (Xagoraraki et al., 2014; Yong & Son, 2009). HAV is found only in humans and some monkeys, with no known zoonotic transmission (Lanford et al., 2019).

NoV are the major cause of viral gastroenteritis in humans worldwide. Transmission of the virus occurs primarily via fecal-oral route, direct contact with an infected individual, and contaminated water or food consumption (Thornton et al., 2004). Research suggests that enteric viruses, and in particular NoV, are responsible for a large portion of recreational water illness in freshwater and marine waters impacted by treated wastewater effluent and by urban stormwater runoff and may also be an important pathogen with respect to health risks associated with exposure to RW (Eftim et al., 2017; Thornton et al., 2004). NoV comprise at least five genogroups, which can be further subdivided into more than forty genotypes. Only a few genotypes of the genogroups GI, GII and GIV infect humans. However, NoV have been detected in different mammalian species, including pigs (GII), bovine and ovine species (GIII), rodents (GV) and canines and felines (GVI) (Bodnar et al., 2017; Eftim et al., 2017; Parra, 2019). Seasonal patterns of outbreaks have been reports, with NoV caused gastroenteritis being most common in winter (Parra, 2019).

Also, there have been reports of NoV susceptibility to chemical-based disinfection, due to high mutation rate of NoV causing increasing emergence of NoV strains less susceptible to chemical disinfectants that can survive even after wastewater treatment (Keswick et al., 1985; Rachmadi et al., 2018).

The survival of pathogens in environments is influenced by environmental variables, namely, temperature, moisture content, solar radiation, variations in soil texture, organic matter, rainfall, nutrients and predation (Badawy et al., 1990; Brettar & Hofle, 1992; Byappanahalli et al., 2006; Byappanahalli & Fujioka, 1998; Desmarais et al., 2002; Ishii et al., 2006). Ishii et al. (2006) noted the survival of soilborne *E. coli* strains during the winter months and growth during summer. Some studies report a better survival of bacteriophage, enteric virus and bacteria at low temperatures on plant surfaces (Badawy et al., 1990; Dawson et al., 2005). Avery et al. (2004) reported the survival of *E. coli* from animal feces on grass surface up to 6 months during winter (Avery et al., 2004).

The regrowth of *E. coli* and enterococci may be possible once they are introduced into the environment (V. Harwood et al., 2000). Several studies suggest the ability of *E. coli* to replicate in contaminated soils (Byappanahalli & Fujioka, 1998; Desmarais et al., 2002; Ishii et al., 2006; NandaKafle et al., 2018; Xing et al., 2019) and recent studies report the presence of *E. coli* in temperate forest, watershed soils and pastures (Byappanahalli et al., 2006; Ishii et al., 2006; Nandakafle et al., 2017).

Sidhu et al. (2008) tested the survival of indicator and pathogenic microorganisms *(Salmonella enterica serotype typhimurium, E. coli, Enterococcus faecalis, Staphylococcus aureus* and MS2 (used as an enteric virus surrogate)) under different climatic conditions on grass surface, irrigated with treated effluent. Results showed the rapid inactivation of enteric bacteria at a higher temperature in direct sunlight and where grass has low moisture content (Sidhu et al., 2008). The authors also noted that enteric bacteria can be expected to survive for longer periods of time under shaded

conditions and lower temperature on grass surface. There was not a significant seasonal variation in the inactivation of MS2 (Sidhu et al., 2008).

Another relevant factor refers to microorganisms being washed away via precipitation (Bolton et al., 1999; Brown et al., 1980; Gagliardi & Karns, 2000; Kauppinen et al., 2017; Sjogren, 1995). Several studies state that there is a correlation between high precipitation events and an increase of microorganisms in the run-off waters (Bradbury et al., 2013; Curriero et al., 2001; Gotkowitz et al., 2016; Kauppinen et al., 2017). A study by Sjogren (1995) assessed the survival of *E. coli* applied to ryegrass under different field conditions. Results showed the rate of decline on grass could be influenced by rainfall and radiation, but data did not suggest a significant impact by rainfall. Brown et al. (1980) noted that rainfall had only a slight effect on die-off of fecal coliforms on grass.

2.3. Water reutilization in Europe

Despite the potential advantages of wastewater reuse solutions, RW is still a largely underused resource at EU level. Reports by TYPSA (2013) on wastewater reuse in the EU have been commissioned in order to evaluate the current reuse practices in Europe (Raso, 2013). However, these reports refer to data from 2006-2007. In 2006, it was estimated that the total volume of used RW in the EU amounted 964 million cubic meters per year (Mm^3/year), accounting for 2,4% of the treated urban wastewater effluents in European countries (BIO, 2015; Hochstrat et al., 2006).

Spain and Italy jointly accounted for 60% of the total volume of EU water reuse, in 2006, with Spain accounting for a third of the total EU treated wastewater reuse volume (347 Mm^3/year). Agriculture is the main application of RW for both countries. The use of RW was also significant in Cyprus and Malta, where 89% and 60% of treated effluents are being reused, respectively. In Greece, Spain and Italy the effluents being reused constitute only between 5% and 12% of the total treated effluents from WWTP (BIO, 2015). It is important to note that estimates of water reuse volumes are associated with high uncertainties, as EU Member States seem to have different interpretations of what should be considered and officially reported as water reuse. Volumes corresponding to internal water recycling in the industry, or to planned indirect reuse, may or may not be included in the reported data (BIO, 2015). For example, in Spain discharge of treated wastewater into a river followed by water abstraction is considered water reuse, whereas in Portugal water reuse implies the transport between the WWTP and RW use. In Cyprus, a significant part of treated effluents are also used for agricultural irrigation followed by landscape irrigation (Amec Foster Wheeler et al., 2016). As such, the use of RW to support agricultural and landscape irrigation has significant potential to reduce pressure on freshwater resources, that are under water stress.

In Europe, Mediterranean countries, namely Spain, France, Italy, Malta, Greece, Cyprus and Portugal have the greatest interest in implementing reuse projects mainly for agricultural irrigation (Marecos do Monte & Albuquerque, 2010).

Several Member States already implemented projects involving RW. One encouraging example of water reuse innovation is the Milano Nosedo WWTP in Italy. The WWTP, located in an agriculture region, was established in 2000 and is the largest of the city of Milano, treating approximately 150 Mm^3/year of wastewater (Dantin, 2016). The treatment levels in the WWTP include preliminary treatment (screening and grit and grease removal phase), secondary (activated sludge) and tertiary treatment (disinfection with peracetic acid) with sand filtration preceding disinfection. Energy production is used for heating and cooling systems of the WWTP facility. The RW is used for agricultural irrigation, namely for the production of corn, rice, grass and grain. This allows nutrient recovery, in particular phosphorus which is not renewable and is considered an essential nutrient for crops and plants, and also allows farmers to reduce their needs for fertilizers (Amec Foster Wheeler et al., 2016). In order to encourage acceptance by the general public and farmers, the WWTP organizes open days to present its activities (Dantin, 2016).

Another example is in Spain, where agriculture is the sector with the highest potential. Spain has developed a National Plan for Water Reuse, which aims to develop the legal framework for water reuse, recognizing that RW projects are often driven and encouraged by local authorities. For instance, local regulations in the region of Murcia strongly support the development of water reuse systems for irrigation purposes in this region (BIO, 2015).

The Segura Basin (Murcia) is located in southeast Spain, characterized by semi-arid Mediterranean climate, with mild winters, hot summers and low annual rainfall (CARM, 2012). In this region the overexploitation of many aquifers has resulted in a widespread deterioration of water quality, especially in the coastal zone. The continued overexploitation of these aquifers for irrigation can lead to salinization, soil compaction and undesirable ions toxicity. According to the EEA, due to very intense irrigation, Segura experiences severe water stress almost all year round (EEA, 2017).

Agriculture in the Region of Murcia has been of great socio-economic importance throughout the years, this is evidenced by 31% of the total area of the region being used for agriculture (ESAMUR, 2019), with the annual volume necessary to cover the agricultural water needs of the area exceeding 880 hm^3 (Alcon et al., 2013). The total volume of treated wastewater in 2019 by the 99 WWTP has exceeded 109 hm^3, which represents approximately 10% of the total net demand of the agrarian demand units of the Segura river basin district (ESAMUR, 2019). There has been a gradual implementation of tertiary treatment in Murcia aimed at reducing TSS and disinfecting the effluents to improve the sanitary guarantee of RW. The most commonly used disinfection process is UV radiation, although an additional chlorination tank is used downstream to guarantee more security. MBR have also been implemented in some WWTP, due to specificities of certain locations (CARM, 2012).

The reuse of treated wastewater in Murcia has risen significantly in the last decades. In 2019, 95% of the volume of treated wastewater was used for agricultural irrigation, approximately 2% for indirect infiltration reuse and the remaining 3% being effluents from some coastal WWTP, that have high salinity levels for reuse in irrigation and are

discharged into the Mediterranean Sea (ESAMUR, 2019). In Spain there has been an increasing interest in aquifer recharge with RW since its use is included within Royal Decree 1620/2007, which regulates the reuse of treated wastewater (BOE, 2007).

Given the water shortage in the region, the use of RW, mainly for irrigation, has important economic and environmental implications. The use of RW in this region allowed an increase of 13% in availability of water resources if compared to the natural resources of the basin. Also, there has been a progressive restoration of the Segura Basin, which recovered its fauna and flora, and allowed citizens from Murcia to use the river to practice water sports (CARM, 2012). Figure 4 shows the improvement of the water quality along the length of the Segura Basin from 1987 to 2010.

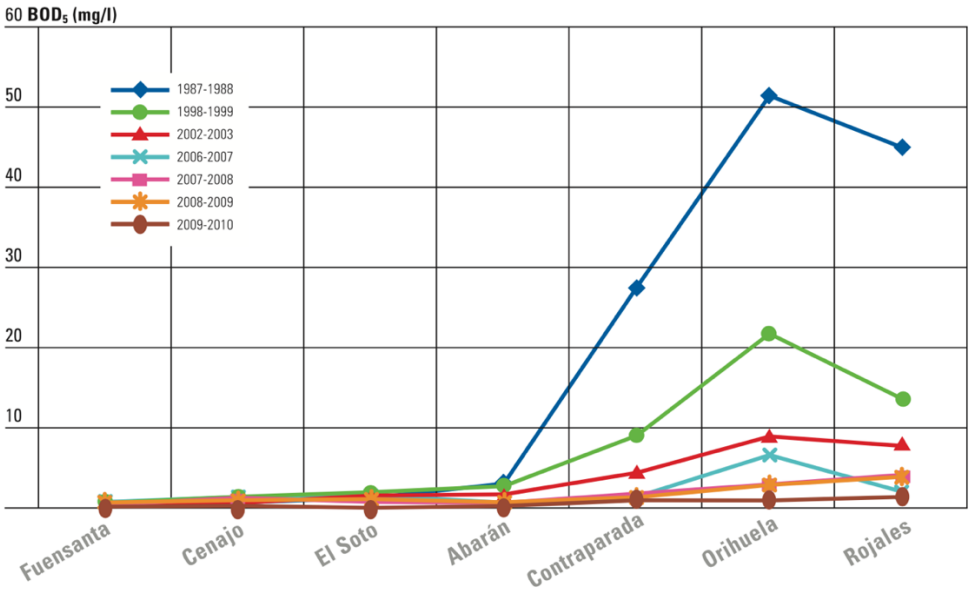


Figure 4 - Water quality along the length of the Segura river from 1987 to 2010. Source: (CARM, 2012).

The potential of water reuse in the EU is growing. Spain and Italy are expected to have the most conspicuous developments at short-term. The AQUAREC project estimated the potential of water reuse in the EU by 2025, through the development of a model in 2006. Overall, the estimate predicts a wastewater reuse volume of 3,222 Mm^3/year in Europe, by 2025, with Spain showing the greatest reuse potential. The project also identifies Italy, Germany, France, Portugal and Greece with high reuse potential (Amec Foster Wheeler et al., 2016; BIO, 2015; Hochstrat et al., 2006; Raso, 2013).

2.4. Water reuse in Portugal

Portuguese climate presents features of Mediterranean climate, particularly regions south of the Tagus river. The climatic variability of Portugal leads to accentuated spatial differences regarding the availability of water resources (Beltrão et al., 2005). Particularly in the south of Portugal, there is a deficit in the water balance of this region, since evapotranspiration exceeds the sum of infiltration and run-off (Marecos do Monte & Albuquerque, 2010). Recurrent droughts severely affect southern Portugal.

The National Plan for the Efficient Use of Water (PNEUEA) is a national instrument, that aims to reduce water losses and optimize the use of water, contributing to minimize water stress and improve the quality of water resources, especially in a country where the climatic variability generates frequent situations of water scarcity (APA & MAMAOT, 2012). The plan was implemented for a period between the years 2012 and 2020.

According to PNUEA, in Portugal the agricultural sector is the sector that consumes more water (>80%). Between the years 2000 and 2009 there was a significant decrease in the total water demand (approximately 43%). This decrease was more significant in the agricultural sector, mainly due to improvement of the global efficiency of water use at national level. This was achieved through the implementation of measures, such as modernization of collective and traditional irrigation systems and rehabilitation and modernization of dams and hydroelectric plants (APA & MAMAOT, 2012).

Despite the reduction in the total water demand, the need to implement systems for the production of treated wastewater for reuse has become increasingly important. In 2019, only 32 management entities produced treated wastewater for reuse, corresponding to only about 1,2% of the wastewater treated in WWTP (ERSAR, 2020). In the majority of new WWTP, the RW is used inside the water treatment companies as service water, for washing, irrigation of green spaces and preparation of reagent solutions. Figure 5 shows that only a small portion of RW is supplied to other entities to be reused.

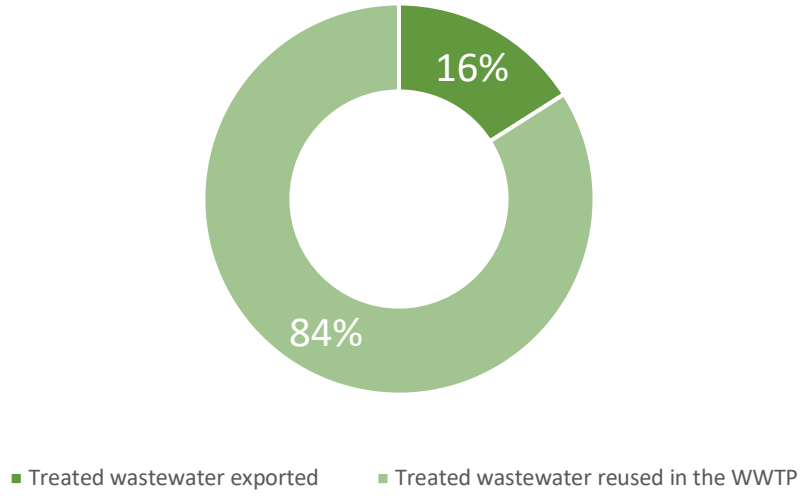


Figure 5 - Extern and intern use of RW. Source: (ERSAR, 2020).

The southern region of the country, namely Alentejo and Algarve, have a high potential for the use of RW for compatible uses, especially due to scarcity of water resources for human consumption and irrigation and the existence of favorable geographical characteristics (MAMAOT, 2007). In the last decades, the importance of the tourism sector has been increasing in Portugal, leading to increases in local consumptions of water, especially for landscape irrigation (golf courses) and recreational uses (swimming pools, aquatic parks, etc.) (Marecos do Monte & Albuquerque, 2010; Martins et al., 2005).

According to the National Strategic Plan for water supply and wastewater sanitation (PEAASAR II), the volume of RW used is much lower than the established goal. PEAASAR II set a minimum target of 10 % of treated wastewater reused by 2013, however this target has not yet been reached (MAMAOT, 2007). In 2011 only 1% of treated wastewater was reused and in 2018 was reused 1,2% of treated effluents (APA et al., 2015; ERSAR, 2020). According to the latest National Strategic Plan for water supply and sanitation (PEENSAAR 2020), the motives to not reach the PEAASAR II goal for RW are (APA et al., 2015):

- lack of economic incentives;
- insufficient public awareness and acceptance;
- high administrative burden to obtain permits;
- high costs associated with transport and assurance of the quality of RW;
- the availability of other water sources at lower prices;
- the lack of adequate legislation.

3. Methods

3.1. General Strategy

To assess the microbiological quality of grass, samples from 4 green areas irrigated with different water sources (groundwater, potable water and RW) and different accessibilities were collected for comparison purposes. Samples were collected between October 2020 and January 2021, however, during this period none of the areas was being irrigated due to meteorological conditions. The case study includes 4 green spaces in Lisbon:

- Children's playground "Parque Aranha" in "Parque Tejo", irrigated with groundwater;
- Football field in "Parque Tejo", irrigated with groundwater;
- Grass inside a WWTP[1], irrigated with treated wastewater;
- Green roof in Instituto Superior Técnico, irrigated with potable water.

In order to standardize and facilitate uniform sampling, the methodology for the microbiological analysis of surfaces was adapted to define a sampling area.

Initially, cultivation methods were used for the enumeration of FIB, as described in 3.3.3., however due to high concentration values for bacteria, it was necessary to perform multiple dilutions in order to be able to quantify bacteria and in some cases, it was impossible to quantify colonies. Therefore, only qPCR, as described in 3.3.5., was performed for the rest of the samples.

In "Parque Aranha" the origin of fecal pollution was also assessed through Microbial Source Tracking.

3.2. Sampling Locations

"Parque Aranha"

"Parque Aranha" is a playground located in "Parque Tejo". Results from the risk assessment conducted by the CML, show that the green zone that includes the playground has the highest risk to human health with the use of RW for irrigation. In Figure 6 it is possible to observe the access and entrance to the playground.

[1] For confidential reasons the WWTP could not be identified.

Figure 6 – Pathway used to access "Parque Aranha".

To collect the grass samples, one sprinkler located in front of the playground was chosen, and four samples were collected at different distances from the sprinkler (Figure 7a). Initially, the effect of irrigation at different distances from the sprinkler on the level of contamination was going to be analyzed at "Parque Aranha", with points 1, 2, 3 and 4 at 2, 4, 6 and 8 meters from the sprinkler, respectively. However, during the sampling period the grass was not being irrigated, due to meteorological conditions, making it impossible to carry out this analysis.

Two soil samples were also collected in the pathway used to access "Parque Aranha". Another green area near "Parque Aranha", not used as a pathway, was selected to collect two grass samples (Figure 7b). This area was especially used by dogs.

Figure 7 – "Parque Aranha": Sampling area near the sprinklers (samples 1 to 4) (Figure 7a, shown on the left), sampling area not used as a pathway (samples 5 and 6) (Figure 7b, shown on the right).

Figure 8 illustrates the sampling distribution, where the blue circle represents the sprinkler and the yellow circles the location of the samples collected.

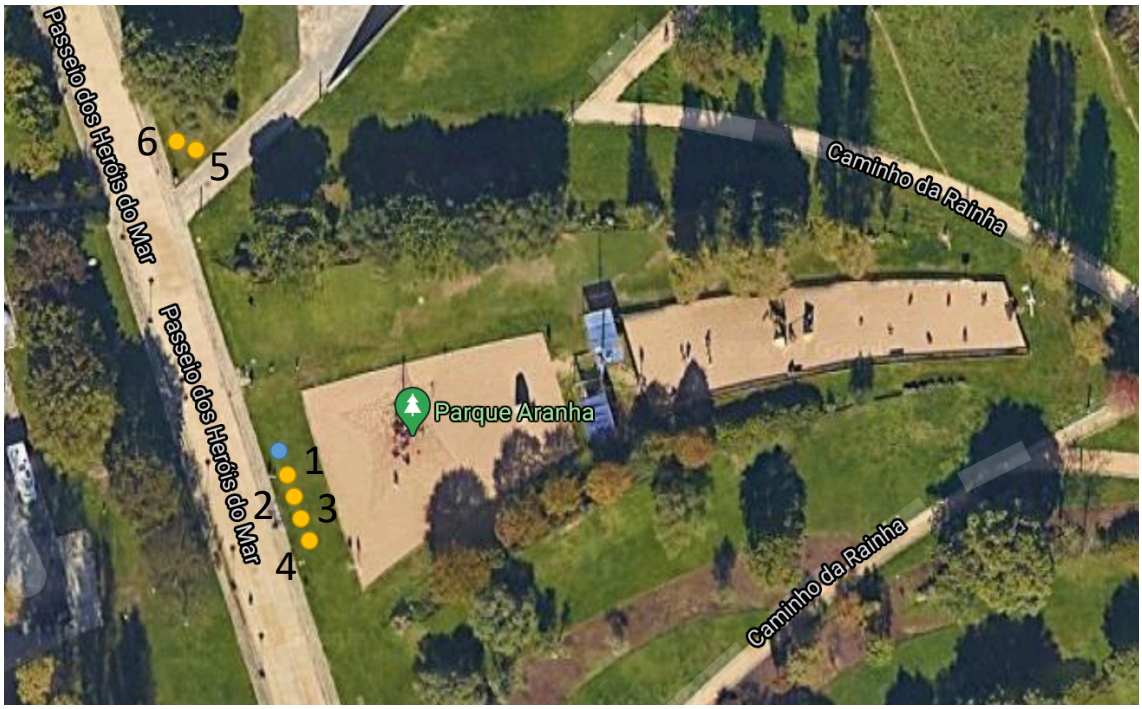


Figure 8 – Location of sampling sites in "Parque Aranha". Blue circle represents the sprinkler and yellow circles the sampling points.

WWTP

Grass samples were also collected from a WWTP in Lisbon. The station has an internal reuse policy, for non-potable purposes, such as washing equipment and streets, preparation of reagents and irrigation of green spaces. The wastewater that is reused inside the WWTP is subjected to complementary treatment, through UV irradiation and addition of sodium hypochlorite.

Four samples (E1, E2, E3 and E4) were collected in one of the green spaces irrigated with RW inside the WWTP installations. Sample E3 was collected from a pathway area used by the WWTP workers to access a shed.

Football field "Parque Tejo"

In the South Zone of "Parque Tejo" there are several areas for the practice of sports, namely football fields, tennis and paddle tennis courts, surrounded by a vast green area. This area was also identified by the CML as one of the areas with high risk for human health with the use of RW for irrigation of the green spaces. In this zone, one sample was collected in an area near the courts (point F1) and another sample in a green area near the benches (point F2), as illustrated in Figure 9.

Figure 9 - Location of sampling sites near the football fields in "Parque Tejo".

IST green roof

Two samples of grass were collected in a green roof located in the Instituto Superior Técnico (IST) campus, which is not easily accessible. This area is irrigated with potable water.

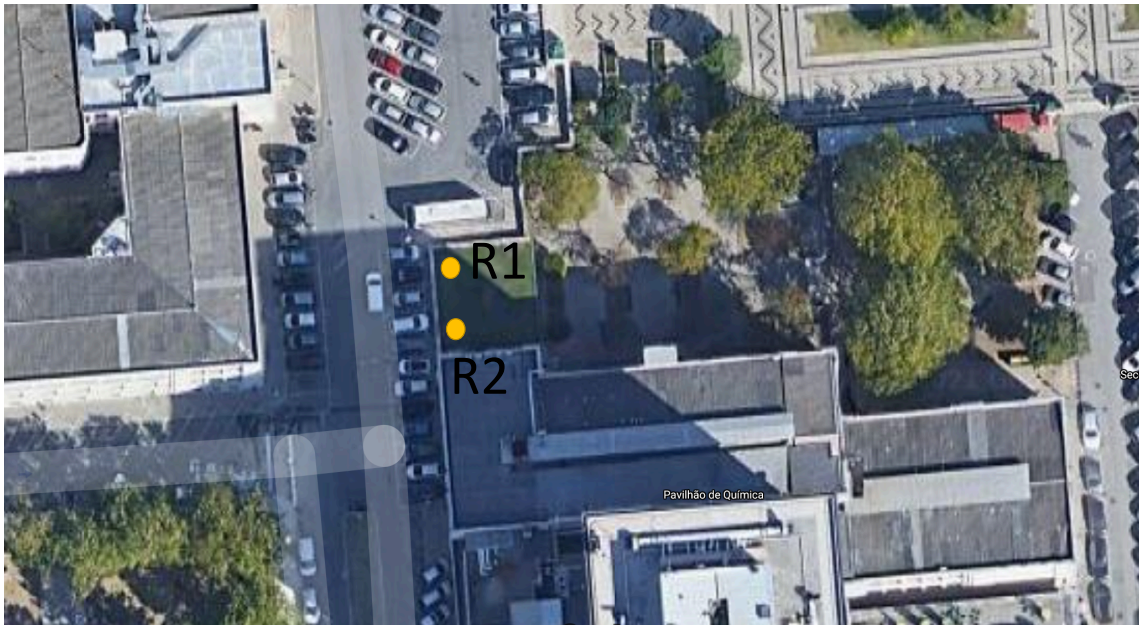


Figure 10 - Location of sampling sites in IST.

3.3. Grass samples collection and processing

3.3.1. Sampling

Grass was collected using previous disinfected scissors to cut the top leaves from a 30cmx30cm area (between 3g and 6g) delimited by a metal frame grid (Figure 11). The grass samples were collected into sterile zip bags and immediately carried to the laboratory to be analyzed.

Samples from "Parque Aranha" were collected between October 2020 and January 2021 as follows: 26/10/2020, 10/12/2020, 21/12/2020, 28/12/2020, 07/01/2021 and 18/01/2021. Grass samples from the green roof in IST, football field in "Parque Tejo" and the WWTP were collected on 19/11/2020, 03/12/2020 and 14/12/2020, respectively.

Figure 11 – Metal frame grid (30cmx30 cm) to facilitate uniform sampling.

3.3.2. Concentration and elution of grass

The grass samples were transferred into sterile containers and weighted. Phosphate buffered saline (PBS) buffer with sodium tripolyphosphate (NaPP) Tween 80 was added in a proportion of 1:30 (w/v). The samples were eluted, followed by agitation at 100 rpm during 120 min at (5±3) ºC. After elution, the samples were centrifuged at 5.445 xg for 10 min at (5±3) ºC, the supernatant was recovered, and the pellet was discarded. Secondary concentration with Polyethylene glycol (PEG) 8000 (final concentration of 20% (w/v)), 1.33% (w/v) of meat extract and 2.17% NaCl (w/v), was performed in the supernatant. The samples were incubated overnight. After this period, the samples were transferred to sterile 50 ml centrifuge tubes and centrifuged at 5500 xg for 30 min at (5±3) ºC, the supernatant was then carefully discarded. Finally, the pellet was resuspended in 2 ml of PBS and the suspension was kept at (-30±3) ºC until further processing.

3.3.3. Enumeration of Fecal Indicator Bacteria

A portion of the concentrated samples was filtered under vacuum through sterile membranes, and the membranes were placed on petri dishes with specific agar nutrient medium. *E. coli* was detected on Tryptone Bile X-glucuronide (TBX) agar (Thermo Fisher Scientific) and enterococci on Slanetz and Bartley agar (Thermo Fisher Scientific) and incubated at 37 ºC. *E. coli* samples were incubated overnight and enterococci for 48 hours. After the incubation period, colonies were quantified. Results were given in CFU/g of grass. To verify enterococci colonies, the membranes were transferred to Esculin medium (Thermo Fisher Scientific) and incubated at 44 ºC for 2 hours.

3.3.4. Nucleic Acid Extraction

After elution and concentration of enteric viruses and bacteria, the extraction of nucleic acid was performed using a commercial kit. The Quick-RNA Viral Kit was used for viral RNA extraction and bacteria extraction was performed using Instagene accordingly to manufacturer's instructions.

Quick-RNA Viral Kit contains silica-based columns that allow an efficient purification of DNA and/or RNA, through the capture of nucleic acids in the silica-membrane, combined with a buffer system that facilitates complete viral particle lysis for efficient nucleic acid isolation. The extraction process begins by adding 800 µL of pre-prepared Viral RNA Buffer (solution was prepared by adding beta-mercaptoethanol to 0.5% (v/v), i.e., 250 µL beta-mercaptoethanol per 50 ml of Viral RNA Buffer) to 400 µL of each sample. Mixture was vortexed and transferred into a Zymo-Spin™ IC Column in a collection tube and centrifuged at 12500 rpm for 2 minutes, after which the column was placed in a new collection tube and the tube containing the filtrate was discarded. 500 µL of the Viral Wash Buffer, previously prepared by adding 24 ml of 100% ethanol to the 6 ml Viral Wash Buffer concentrate, was added to each sample and centrifuged for 30 seconds. After centrifugation the flow-through was discarded. The process was repeated until all of the lysate had passed through the column. Following the addition of 500 µL of ethanol (95-100%) to the columns, the samples were centrifuged for 1 minute to ensure complete removal of the wash buffer. The collection tube was discarded, and the columns were carefully transferred to nuclease-free tubes. 80 µL of DNA/RNA-Free Water was added directly to the columns matrix and centrifuged for 30 seconds. The final eluate (80 µL) was stored at (-30±5) ºC until further analysis.

Extraction of nucleic acids from bacteria for all samples was performed by centrifuging 400 µL of sample at 12000 rpm for 10 minutes. After centrifugation the supernatant was carefully discarded and 100 µL of InstaGene matrix was added to each sample. The samples were then incubated at 56 ºC for 15 min, followed by additional incubation at 95 ºC for 8 minutes. The samples were kept at (-30±5) ºC, prior to further processing.

3.3.5. Microbial Detection and Quantification by Real-Time Polymerase Chain Reaction

Detection and quantification of enteric viruses and bacteria was carried out by molecular biology techniques, namely qPCR.

For the amplifications of bacteria, the qPCR reactions were performed using the Luna Universal Probe qPCR Master Mix (New England Biolabs). The reaction was performed for a final volume of 25 µL of reaction mixture. The master mix is provided in a 2x concentration containing Hot Start Taq DNA Polymerase, uracil-N glycosylase (UNG), dNTP mixture (with dUTP), a passive reference dye and an optimized buffer solution.

The master mix was mixed with each primer, the corresponding probe and sterile DNA and RNA-free water, which was used to adjust the volume to 20 µL.

For detection and quantification of enteric viruses the Luna Universal Probe One-Step RT-qPCR kit (New England Biolabs) was used. The reaction was performed for a volume of 20 µL, containing 2x Luna Universal Probe Reaction Mix One-Step, Luna RT Enzyme Mix, each primer, the corresponding probe and sterile DNA and RNA-free water, in order to adjust the volume to 15 µL. Information regarding primers and probes can be found in Table 5.

Table 5 - Primers and probes used for qPCR.

Microorganism	Primer	Sequence
E. coli	784F	GTGTGATATCTACCCGCTTCGC
	866R	AGAACGGTTTGTGGTTAATCAGGA
	EC807	JOE-TCGGCATCCGGTCAGTGGCAGT-BHQ
Enterococci	ECST784F	AGAAATTCCAAACGAACTTG
	ENC854R	CAGTGCTCTACCTCCATCATT
	GPL813TQ	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA
HAV	HAV68	TCA CCG CCG TTT GCC TAG
	HAV240	GGAGAG CCC TGG AAG AAA G
	HAV150P	CCT-GAA-CCT-GCA-GGA-ATT-AA
NoVGI	F	CGC TGG ATG CGN TTC CA
	R	CCTTAGACGCCA TCATCATTTACTCG
	NVGG1p	FAM TGG ACA GGA GAY CGC RAT CT TAMRA
NoVGII	F	ATG TTC AGR TGG ATG AGR TTC TCW GA
	R	TCG ACG CCA TCT TCA TTC ACA
	P	AGC ACG TGG GAG GGC GAT CG

Master mix reaction mixtures were distributed in a 96-well qPCR microplate (Thermo Scientific, US), each sample and dilutions were added to the respective well and a negative control was added (sterile DNA and RNA-free water). After sealing the plate, it was inserted in the 7300 Real-Time PCR System (Applied Biosystems, US) and set to run. Reaction conditions are displayed in Table 6. Quantification of concentrations in each sample were performed by comparison to the standard curve, with the results given in genome units (GU), and the final concentration was adjusted and expressed as GU g^{-1} of grass.

Table 6 - Temperature profiles used for qPCR.

Microbial group	Temperature (ºC)	Time (min:sec)	Number of cycles	Phases
Bacteria	50	02:00	1	Preincubation
	95	10:00	1	Initial Denaturation
	95	00:15	40	Denaturation
	60	01:00	40	Annealing/Extension
Virus	55	10:00	1	Reverse Transcription
	95	10:00	1	Initial Denaturation
	95	00:15	40	Denaturation
	60	01:00	40	Annealing/Extension

3.3.6. Microbial Source Tracking

In order to assess if the origin of the fecal pollution of the collected samples was mainly dogs, mtDNA present in the samples was analyzed through nested PCR using specific primers for dog.

The mtDNA sequences in study were aligned using the ClustalW program and the specific primers were obtained using the Primer Express software. Primers specificity was confirmed using BLAST. Primers were provided by Thermo Fisher Scientific. The primers sequences are shown in Table 7.

Table 7 -Primers used for both single and nested PCR.

	Primer	Sequence	Amplicon length (bp)
Single PCR primers	Dogmito1-F	5'-ATGGCTCTAGCCGTTCGATTAAC-3'	638
	Dogmito1-R	5'-GGCTAGGAGGACTGAGGTGTTGAG-3'	
Nested PCR primers	Dogmito2-F	5'-CATTAGGATTCACAACCAACCTGTTA-3'	236
	Dogmito2-R	5'-CATTAGGATTCACAACCAACCTGTTA-3'	

PCR was performed in a Veriti 96 well thermal cycler (Applied Biosciences) using ilustra puReTaq ready-to-go PCR beads (GE Healthcare). Single PCR was performed in 25 µL volume using 0.4 pmol/µL of each primer, 5 µL of extracted DNA diluted to 10^{-1} and one

PCR bead. Nested PCR was performed in the same conditions except that 1 µL of the single PCR reaction was used as template DNA and internal primers were used. PCR cycle conditions are shown in Table 8.

Table 8 - PCR steps and cycle conditions.

Temperature (ºC)	Hold Time (s)	Number of cycles	Phases	
94	300	1	Preincubation	
59	300	1	Pre-annealing	
72	120	35	Amplification	Elongation
94	40	35		Denaturation
59	60	35		Annealing
72	600	1	Cooling	

PCR products were observed by agarose gel electrophoresis in 2.5% SeaKem LE agarose (Lonza) gels. 10 µL of PCR product were loaded with 1 µL of 10x DNA loading buffer. 2 µL of 100 bp DNA ladder (New England Biolabs) were also loaded. Gels were run at 60 V using TAE buffer (1x). The DNA was stained by immersion in ethidium bromide solution. The resulting gel was visualized with the G: BOX (Syngene).

3.4. Soil Samples

3.4.1. Sampling

Two soil samples were collected from “Parque Aranha” using 100 ml sterile containers. Both samples were collected in December on 21/12/2020 and 28/12/2020.

3.4.1. Elution

For soil samples the microorganisms chosen for assessment were only bacteria (*E. coli*, enterococci). Twenty-gram of soil were mixed with 40 ml of Ringer 1:1 (w/v). The samples were eluted at 100 rpm for 3 min, after which the samples were left for 20 min to rest in order to allow sedimentation of the soil.

3.4.1. Nucleic Acid Extraction and qPCR

Extraction of nucleic acids was performed by centrifuging 400 µL of sample at 12000 rpm for 10 minutes. Then the supernatant was carefully discarded and 100 µL of InstaGene matrix was added to each sample. Samples were then incubated at 56 ºC for 15 min, followed by additional incubation at 95 ºC for 8 minutes. The samples were kept at (-30±5) ºC, prior to further processing.

For detection and quantification of *E. coli* and enterococci the method described in 3.3.5. for bacteria was performed.

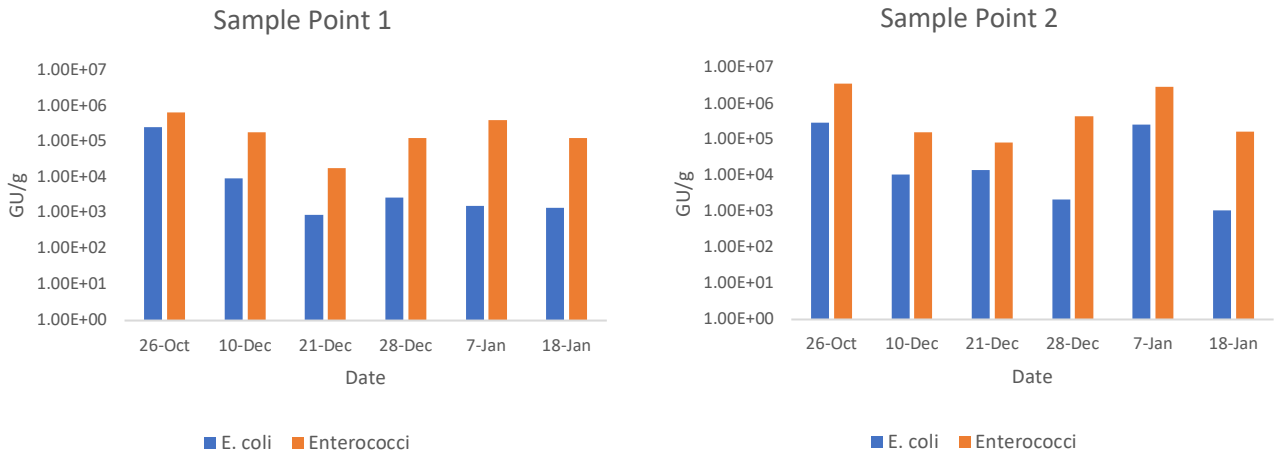
4. Results and Discussion

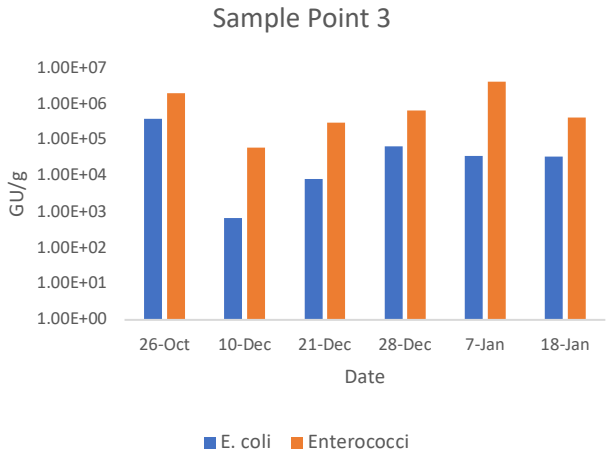
4.1. *E. coli* and Enterococci

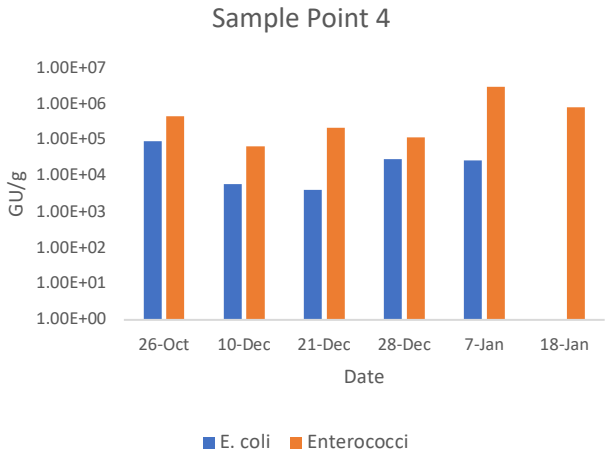
4.1.1. Grass Samples

"Parque Aranha"

Results of the analysis for the presence of bacteria (*E. coli* and enterococci) in the grass samples for each sampling point throughout the several campaigns for "Parque Aranha" are displayed in Figure 12.







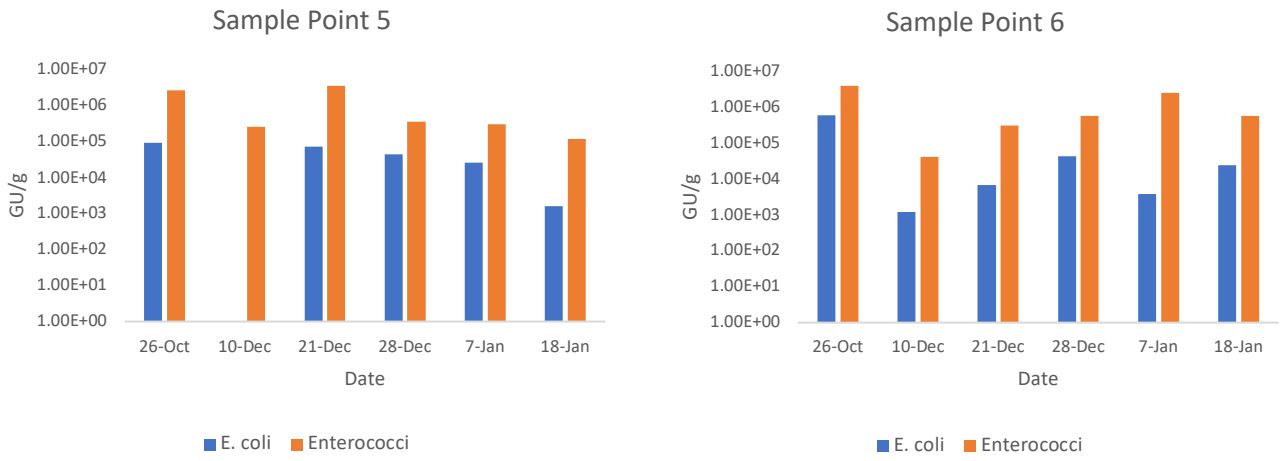


Figure 12 – FIB results for the 6 sampling points in "Parque Aranha" for each campaign. Absent values = Not Detected.

The information of Figure 12, reveals a significant fecal contamination for all sampling points, based on the high levels of both FIB (*E. coli* and enterococci), with all samples displaying higher incidence of enterococci than *E. coli*. A higher number of enterococci compared to *E. coli* suggests a fecal contamination from animal origin (Geldreich & Kenner, 1969; Scott et al., 2002, 2005).

No samples were found to be negative for enterococci and *E. coli* was not detected in two grass samples. Results for "Parque Aranha" seem to display similar variation between points throughout the sampling period, which could indicate that FIB concentrations may be influenced by environmental variables. *E. coli* concentration ranged from 6.73×10^2 GU/g to 6.14×10^5 GU/g with a mean value of 6.97×10^4 GU/g. Enterococci concentration ranged from 1.81×10^4 GU/g to 4.27×10^6 GU/g with mean value of 1.04×10^6 GU/g.

Other locations

For the other locations, it was only possible to make a campaign in each location. Results showed prominent occurrences of enterococci and *E. coli*. Only three samples tested negative for *E. coli*, all of which from the WWTP. Figure 13 shows results for all grass samples from the football field in "Parque Tejo", WWTP and the green roof in IST. Detailed results for each sampling day for all locations can be found in Table A 2 and Table A 3 in the Annexes.

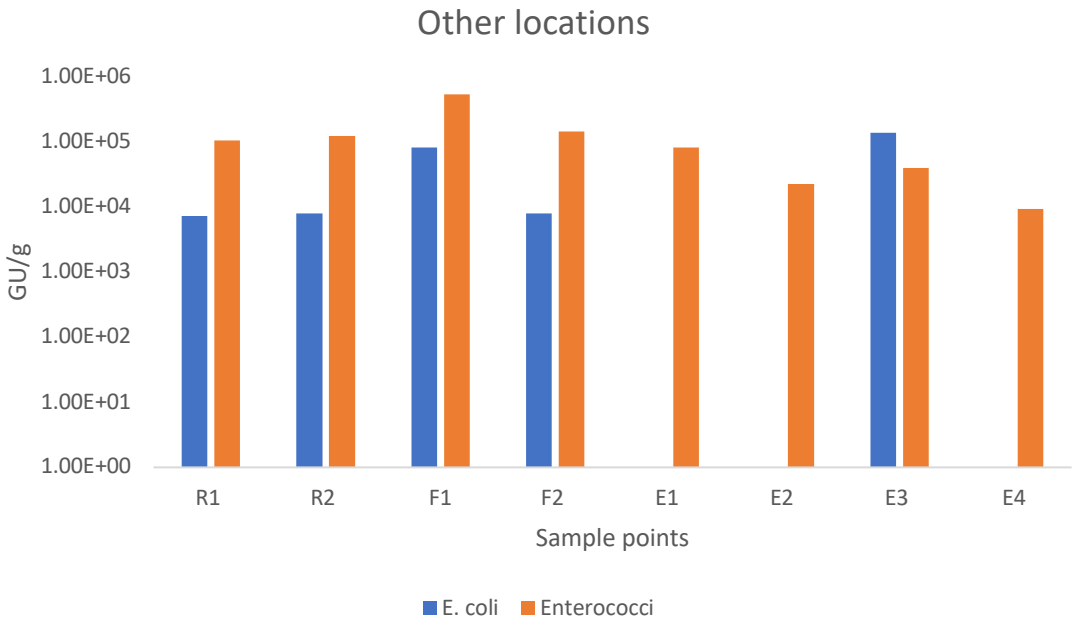


Figure 13 - FIB results for the Green Roof (R1 and R2), Football Field (F1 and F2) and WWTP (E1, E2, E3 and E4). Absent values = Not Detected.

The sample that tested positive for *E. coli* in the WWTP (sample E3) was the only sample that displayed higher concentration of *E. coli* compared to enterococci values. This sample was collected from a pathway area, where the grass was visibly stepped on. The higher number of *E. coli* compared to enterococci values, suggests a fecal contamination from human source. Since all the other samples collected in the WWTP showed higher concentration values for enterococci, the high concentration values for *E. coli* in that point, could be due to contamination being transferred to the grass through the workers' boots. It is also possible for some contamination to remain from the irrigation with RW. Analyzes performed at the RW were provided by the WWTP. Bacterial quality results of the WWTP effluent, between October 2020 and January 2021, are shown in Figure 14. Mean concentration for fecal coliform bacteria present in the RW was 3.25 MPN/100 ml. The results obtained for the WWTP effluent show the presence of fecal contamination, although in low concentrations and in 55% of samples fecal coliforms were not detected. Therefore, irrigation with RW is not the main source of contamination.

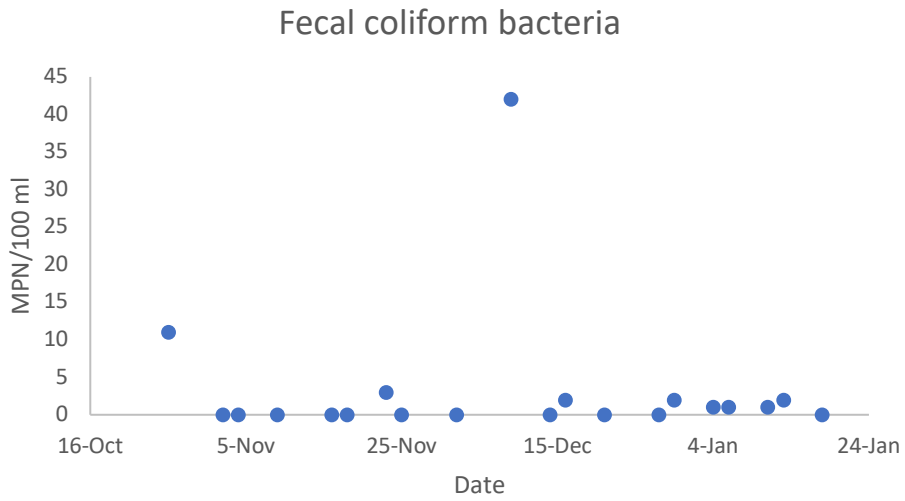


Figure 14 – Fecal coliforms results for RW at the exit of the WWTP (after disinfection), between October 2020 and January 2021, provided by the WWTP.

For the other samples from the WWTP *E. coli* was not detected, which is not expected due to the high concentration values obtained for enterococci. There is no obvious explanation for the absence of *E. coli* in samples from both the WWTP and some from "Parque Aranha" with the enterococci concentration values obtained. There may have been a laboratory error or an environmental factor that led to the die-off of *E. coli*, since *E. coli* is more sensitive than enterococci. However, there is no direct explanation for these results.

All samples from the other locations displayed higher concentration values for enterococci, suggesting fecal contamination from an animal source (Geldreich & Kenner, 1969; Scott et al., 2002, 2005).

For the green roof, football field and WWTP mean concentrations for *E. coli* were 7.61×10^3, 4.52×10^4 and 3.50×10^4 GU/g and for enteroccoci 1.15×10^5, 3.44×10^5 and 3.86×10^4 GU/g, respectively. Mean concentration values for *E. coli* and enterococci in "Parque Aranha" were higher than the concentrations obtained for the other locations, however there was a higher number of samples collected in "Parque Aranha" compared to the other green spaces. Nonetheless, comparing the results from the green roof, WWTP and football field in "Parque Tejo", the football field obtained slightly higher mean concentrations for both *E. coli* and enterococci. In general, results for "Parque Aranha" are similar to the other locations, with overall prominent occurrences of bacteria. Even contamination values for the green roof, which is irrigated with potable water and is not accessible by people or dogs, registered high contamination levels. Analyzes performed at the water from the well that irrigates "Parque Tejo" were provided by CML. Total Coliforms results from July 2020 and March 2021 were <1 MPN/100 ml, which suggests the existence of an exogenous source of fecal contamination.

Several studies from different authors, state that FIB concentration in irrigation waters does not influence FIB concentrations on soil and plants (Holvoet et al., 2014; Lopez-Galvez et al., 2016). Forslund et al. (2013) and Intriago et al. (2018), observed the presence of *E. coli* in samples of control soil (soil that was not exposed to irrigation water or amendment containing fecal contamination) and argued that other environmental fecal contamination sources, like wild animals and birds, could have caused it. Vergine et al. (2015), also noted the existence of another source of microbiological contamination present on the grass different from the irrigation, namely animal feces. Results of the present study confirm that animals, such as birds and dogs (in the case of public parks), seem to be a likely source of contamination.

Treatment processes in WWTP are generally effective on bacteria and *E. coli* and enterococci are generally eliminated after tertiary treatment (Montemayor et al., 2008; Ottoson et al., 2006). Nonetheless, the studies mentioned previously note that no correlation could be established between the prevalence of FIB in irrigation water and presence in plants.

4.1.2. Soil Samples

To assess if the contamination present on the soil could influence the contamination of the grass, two soil samples were collected from "Parque Aranha" near the location of the grass samples collected (points 1 to 4). *E. coli* and enterococci were not detected in any sample. These results are consistent with another study, where effect of microbiological contamination from animal feces was more pronounced in the grass than topsoil (Vergine et al., 2015).

4.2. Microbial Source Tracking

Table 9 presents the results of mtDNA testing performed on the samples from "Parque Aranha". It shows whether samples were positive or negative for dog DNA presence, allowing to pinpoint if the source of contamination in "Parque Aranha" is mainly dogs.

Results show that 28% of total samples contained fecal contamination from dogs. Considering the two green areas in "Parque Aranha" (Figure 8) separately, points 5 and 6 (Figure 7b) tested positive for 58% of the samples and points 1 to 4 (Figure 7a) only tested positive for 13% of samples. Points 5 and 6 location was especially used by dogs, therefore the higher presence of dog mtDNA was expected. Despite the small amount of testing, positives for dog mtDNA should be considered relevant.

Table 9 – Results for the presence of dog mtDNA in grass samples from "Parque Aranha". '+' and '-' for dog DNA presence.

Date	Sampling Points	Dog mtDNA
26/10/2020	1	-
	2	-
	3	-
	4	-
	5	-
	6	-
10/12/2020	1	-
	2	-
	3	-
	4	-
	5	-
	6	+
21/12/020	1	-
	2	-
	3	-
	4	-
	5	-
	6	+
28/12/2020	1	-
	2	-
	3	+
	4	-
	5	-
	6	+
07/01/2021	1	+
	2	-
	3	-
	4	-
	5	+
	6	+
18/01/2021	1	-
	2	+
	3	-
	4	-
	5	+
	6	+

4.3. Environmental variables

After the assessment of *E. coli* and enterococci, the mean concentrations of all sample points in each sampling day for "Parque Aranha" were crossed with rain and temperature data to assess the effect of these environmental factors on the survival of indicator microorganisms on the grass surface (Figure 15 and Figure 16). Data for the month of October was obtained from IPMA from the Gago Coutinho meteorological station and data for the months of November to January were obtained from the IST meteorological station, since there was a failure in the IST meteorological station during October.

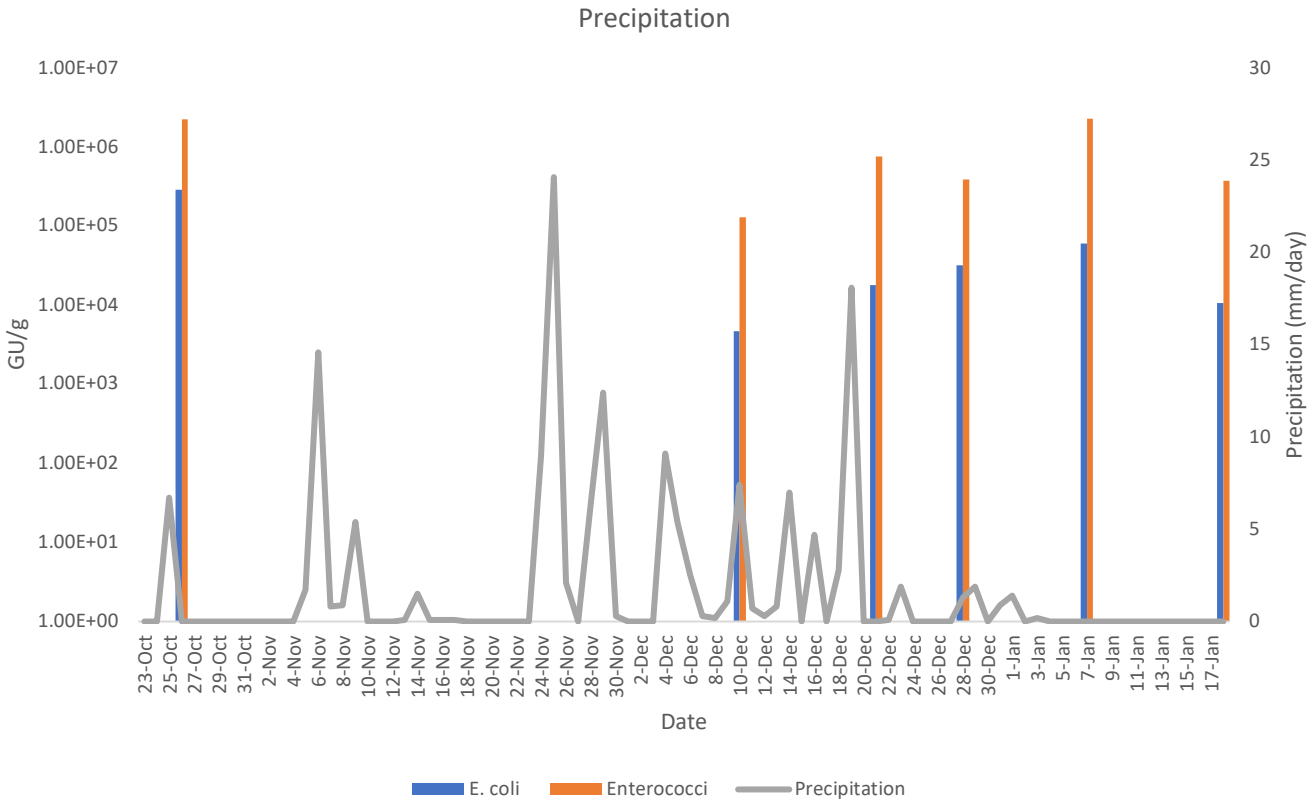


Figure 15 - Mean concentrations of E. coli and enterococci for the 6 campaigns from "Parque Aranha" and daily precipitation data between October 2020 and January 2021.

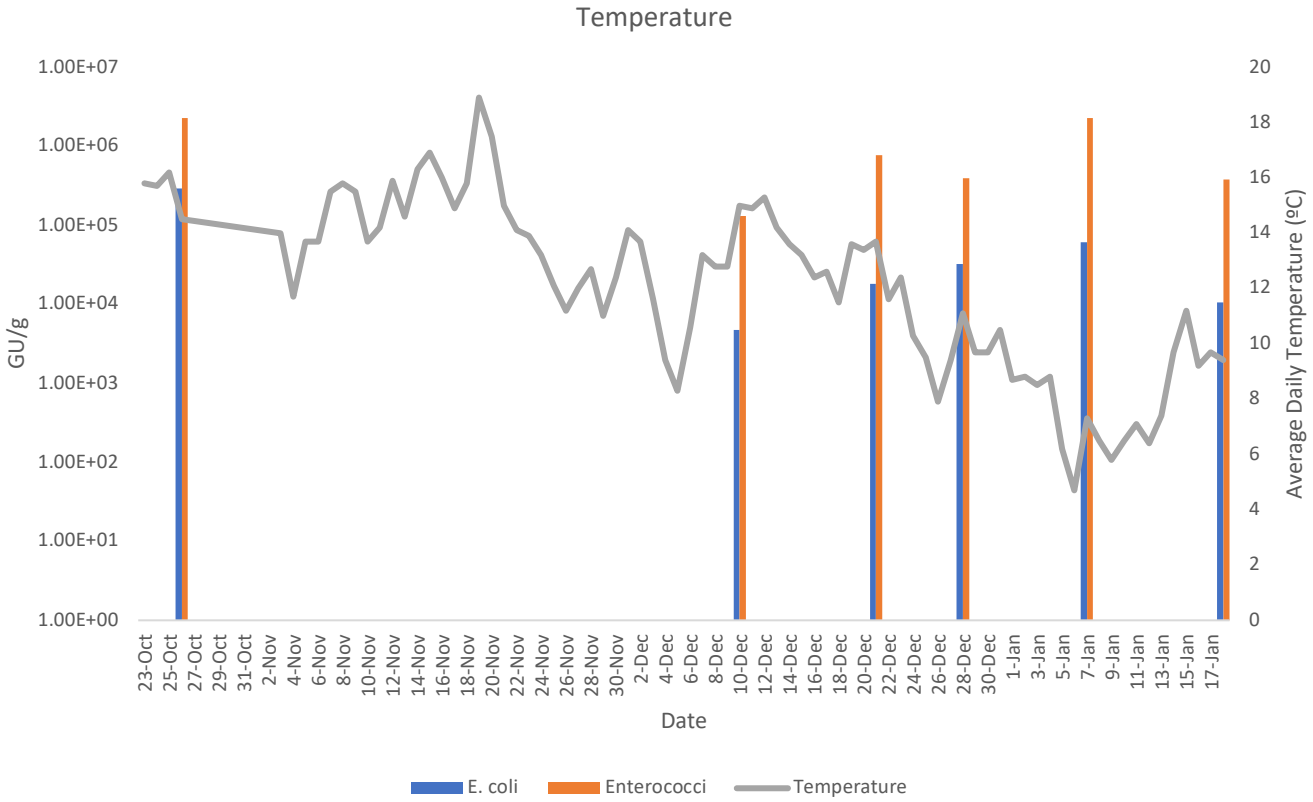


Figure 16 - Mean concentrations of E. coli and enterococci for the 6 campaigns from "Parque Aranha" and average daily temperature data between October 2020 and January 2021.

A Pearson correlation analysis was performed to better assess the effect of precipitation, temperature and solar radiation on contamination present on the grass (Table 10 and Table 11). Solar radiation data was obtained from the IST meteorological station, however it was not possible to obtain data for the month of October. The cumulative precipitation corresponds to the sum of daily precipitations of the 3 days before each campaign and dry weather is the number of days without rain before a campaign.

Table 10 - Correlation analysis between environmental variables and E. coli concentrations.

E. coli						
	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6
n	6	6	6	6	6	6
Cumulative precipitation	-0.04	-0.08	-0.39	0.20	0.02	-0.19
Precipitation day before sampling	**0.97**	**0.58**	**0.51**	**0.40**	0.18	**0.73**
Dry weather period	-0.46	-0.61	0.19	-0.87	0.03	0.06
Average daily temperature sampling day	**0.56**	0.04	-0.28	**0.31**	-0.30	0.14
Average daily temperature day before sampling	**0.65**	0.07	0.02	0.15	-0.07	**0.44**
Average daily temperature previous 3 days	**0.67**	0.15	0.01	0.20	-0.07	**0.41**
Max solar radiation day before sampling	**0.94**	-0.25	-0.80	0.01	-0.95	-0.52
Mean solar radiation day before sampling	-0.63	-0.68	**0.71**	-0.53	**0.61**	**0.94**

Table 11 - Correlation analysis between environmental variables and enterococci concentrations.

Enterococci						
	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6
n	6	6	6	6	6	6
Cumulative precipitation	-0.68	-0.50	-0.44	-0.44	**0.75**	-0.38
Precipitation day before sampling	**0.59**	**0.58**	**0.28**	0.00	**0.52**	**0.45**
Dry weather period	-0.07	-0.21	0.07	**0.38**	-0.67	-0.10
Average daily temperature sampling day	-0.17	-0.29	-0.59	-0.77	**0.57**	-0.44
Average daily temperature day before sampling	-0.12	-0.18	-0.39	-0.57	**0.65**	-0.17
Average daily temperature previous 3 days	-0.09	-0.14	-0.36	-0.53	**0.69**	-0.15
Max solar radiation day before sampling	**0.23**	-0.35	-0.80	-0.67	-0.35	-0.88
Mean solar radiation day before sampling	-0.43	-0.27	0.19	0.08	0.02	**0.37**

The highest correlation was found between precipitation from the day prior to campaigns and concentration of microbiological indicators. In general, higher concentrations of *E. coli* and enterococci were found when it rained the day prior to sampling. The grass in "Parque Tejo" is composed of green surface grass blades and a denser thatch underneath. With precipitation it is possible that microorganisms present in fecal pollution are washed onto the grass and get retained, due to the dense grass underneath, leading to higher concentrations of microorganisms during rain events, which is consistent with results obtained for the soil samples.

Previous research suggests that rainfall does not have a significant impact on the die-off of bacteria on grass and several studies state that there is a correlation between high precipitation events and an increase of microorganisms in the run-off waters, with microorganisms being washed away via precipitation (Brown et al., 1980; Kauppinen et al., 2017; Sjogren, 1995).

Regarding temperature, there seems to be no significant correlation between temperature and concentration of microorganisms on the grass surface, however all samples were collected during winter months. According to another study that assessed the survival of enteric microorganisms on grass surface, higher temperatures have a significant impact on the die-off of bacteria on grass (Sidhu et al., 2008). Therefore, it is expected that during the summer months higher temperatures could have an impact on the inactivation of bacteria present on the grass surface.

In general, maximum and mean radiation from the day prior to sampling also did not show significant correlation, which is unexpected since radiation has a significant impact in the inactivation of microorganisms in water (Gameson & Saxon, 1967; McCambridge & McMeekin, 1981). It is possible that lower parts of the grass leaves are blocked from the solar radiation, therefore leading to less bacteria die-off. Sindhu et al. (2018) noted that green grass leaves absorb more than 90% of radiation which shades the thatch from the influence of sunlight. It was also noted that inactivation of microorganisms was slower during winter with reduced maximum air temperature and solar radiation. Although, some sampling points showed correlation between solar radiation and concentration of *E. coli* (point 1 showed correlation with maximum solar radiation and points 3, 5 and 6 with mean solar radiation). According to previous research *E. coli* is more susceptible to solar radiation decay than enterococci (McCambridge & McMeekin, 1981).

4.4. Enteric viruses

In terms of enteric viruses, all tested samples for HAV were found to be negative. Samples from the WWTP and IST green roof tested negative for all viruses, however, only one campaign was carried out for each of these locations. NoVGII occurred the most often, 27% of all samples (12/44), which represents approximately 32% of positive samples from “Parque Tejo” (12/38), including the football field.

NoVGI was the virus that presented the highest concentration. Samples that tested positive for NoVGI also tested positive for NoVGII, with samples containing one type of enteric virus representing the big majority of samples (83%). NoVGI only tested positive for 2 samples of 38 from “Parque Tejo” (5%), both of which in “Parque Aranha”. Table 12 shows the individual concentrations for each sample and campaign dates. Samples from “Parque Tejo” (including the football field) displayed at least one occurrence of NoVGII in all sampling dates and demonstrated a high presence of genetic material from enteric viruses, from what was expected. The presence of NoV suggests the presence of fecal contamination from human origin.

Table 12 – Concentrations of enteric viruses for each sampling point quantified by qPCR. N.D. – Not Detected.

Sampling Location	Date	Sampling point	HAV (GU/g)	NoVGI (GU/g)	NoVGII (GU/g)
"Parque Aranha"	26/10/2020	1	N.D.	N.D.	2.81×10^3
		2	N.D.	N.D.	N.D.
		3	N.D.	N.D.	2.13×10^3
		4	N.D.	9.90×10^3	1.61×10^3
		5	N.D.	N.D.	N.D.
		6	N.D.	N.D.	N.D.
	10/12/2020	1	N.D.	N.D.	N.D.
		2	N.D.	N.D.	N.D.
		3	N.D.	N.D.	2.25×10^2
		4	N.D.	N.D.	N.D.
		5	N.D.	N.D.	N.D.
		6	N.D.	N.D.	N.D.
	21/12/2020	1	N.D.	N.D.	N.D.
		2	N.D.	N.D.	N.D.
		3	N.D.	N.D.	2.66×10^3
		4	N.D.	N.D.	N.D.
		5	N.D.	N.D.	N.D.
		6	N.D.	N.D.	N.D.
	28/12/2020	1	N.D.	N.D.	N.D.
		2	N.D.	N.D.	N.D.
		3	N.D.	N.D.	N.D.
		4	N.D.	N.D.	N.D.
		5	N.D.	N.D.	N.D.
		6	N.D.	N.D.	4.98×10^3
	07/01/2021	1	N.D.	1.20×10^3	6.52×10^3
		2	N.D.	N.D.	1.85×10^3
		3	N.D.	N.D.	N.D.
		4	N.D.	N.D.	N.D.
		5	N.D.	N.D.	2.98×10^3
		6	N.D.	N.D.	N.D.
	18/01/2021	1	N.D.	N.D.	N.D.
		2	N.D.	N.D.	N.D.
		3	N.D.	N.D.	N.D.
		4	N.D.	N.D.	5.46×10^3
		5	N.D.	N.D.	5.57×10^3
		6	N.D.	N.D.	N.D.
Green roof	19/11/2020	R1	N.D.	N.D.	N.D.
		R2	N.D.	N.D.	N.D.
Football Field	03/12/2020	F1	N.D.	N.D.	N.D.
		F2	N.D.	N.D.	4.41×10^3
WWTP	14/12/2020	E1	N.D.	N.D.	N.D.
		E2	N.D.	N.D.	N.D.
		E3	N.D.	N.D.	N.D.
		E4	N.D.	N.D.	N.D.

Findings of high concentrations of NoV on grass samples are unexpected. A hypothesis for the presence of NoV in the grass samples could be the transfer of viruses to the grass surface through people's shoes. NoV has been known to display seasonality for the winter months (period during which samples were collected) and has been proven to persist in water and other surfaces during long periods of time (NoV genome was detected up to 3 months in surfaces and 1277 days in water) (Eftim *et al.*, 2014, Kauppinen *et al.*, 2017). Some studies have also assessed the transfer of viruses on different surfaces.

There are very few studies related to the presence of enteric viruses on grass. Sindhu et al. (2018) irrigated grass with sterile effluent seeded with known numbers of the target microorganisms, including bacteriophage MS2 that was used as an enteric virus surrogate, and assessed the survival of enteric microorganisms on grass surface. This study revealed that there was no significant seasonal variation observed in the inactivation of the bacteriophage MS2. Other studies point out temperature and relative humidity as the main influence on virus environmental survival on different surfaces (Escudero et al., 2012; Kotwal & Cannon, 2014; Lamhoujeb et al., 2009).

Previous research also determined that environmental contamination can lead to prolonged outbreaks, where environmental swabs tested positive for human NoV 14 days and even 9 weeks after outbreak initiation (Cheesbrough et al., 2000; Wu & Lin, 2005). However, in some studies infectivity experienced a prominent loss, showing that viral genetic material persists longer than infectious virus (Escudero et al., 2012; Fallahi & Mattison, 2011; Kotwal & Cannon, 2014).

Some studies assessed the role of hands and environmental surfaces in virus transmission (Barker et al., 2004; D'Souza et al., 2006; Kotwal & Cannon, 2014). D'Souza et al. (2016) studied the transfer of NoVGI from stainless steel to lettuce surfaces and found 8 out of 9 lettuce samples tested positive after transfer. Similarly, Barker et al. (2004) demonstrated that fingers contaminated with NoV were able to contaminate seven clean surfaces touched consequently. Door handles, faucets and telephone receivers touched with contaminated fingers also tested positive for virus. Two variables of transfer between surfaces, namely moisture and pressure applied, are important factors on the results obtained from these studies.

Cheesbrough et al. (2000) evaluated the presence of NoV on different surfaces during an outbreak. Results showed that 5 out of 8 samples (62%) tested positive from carpet where guests had vomited and 9 out of 12 samples (75%) tested positive from carpet with no definite record of direct contamination with vomit (Cheesbrough et al., 2000). Similarly, another study notes that viruses might have been carried to different locations through shoes (Kimura et al., 2011), which supports the hypothesis that NoV might have been transferred to the grass through people walking on it.

In terms of existent literature, there were no studies found on the concentration of enteric viruses on grass. In this study, the concentration of NoVGII ranged between 2.25×10^2 GU/g and 6.52×10^3 GU/g with a mean concentration of 3.43×10^3 GU/g. For NoVGI the mean concentration was found to be 5.55×10^3 GU/g. Comparing the

obtained results with concentrations of NoVGI, NoVGII and HAV after wastewater treatment stages, concentrations for both NoVGI and NoVGII in this study were above those reported after wastewater treatment. Eloy et al. (2019) reported a mean concentration for NoVGII after tertiary treatment of 6.45×10^2 GU/ml and 2.50×10^0 GU/ml for different WWTP. Other studies reported similar levels of NoVGI and NoVGII, around 10^5 copies/L in WWTP effluents (Da Silva et al., 2007; Laverick et al., 2004; Lodder & De Roda Husman, 2005; Pusch et al., 2005). For HAV, previous research shows that, in general, HAV is not detected after secondary treatment (Carducci et al., 2008; Grabow et al., 1983).

These studies highlight the fact that inactivated NoV may be detected due to the method used (qPCR), therefore results may overestimate the number of infectious virus present in the final effluent and thus underestimate the reduction of viable viruses and overestimate the infectious risk (Flannery et al., 2012; Gonzales-Gustavson et al., 2019; Kato et al., 2005; Lodder & De Roda Husman, 2005; Pusch et al., 2005).

A limitation of the method used to detect virus in this study (qPCR) is the inability to differentiate infective viruses from non-infective viruses. qPCR methods have become essential to detect the presence of genetic material from enteric virus in the environment, due to shorter detection times, high sensitivity and specificity and provide the possible detection and quantification method for nonculturable viruses or not easily culturable viruses, however this method is unable to differentiate between infectious and non-infectious viruses. Therefore, when viruses are detected by molecular techniques, it may not necessarily mean that there is a direct risk to public health, as the genetic material detected may not result in infectious viral particles. Some studies observed low global WWTP removals of enteric viruses, such as NoV (Francy et al., 2012; E. Haramoto et al., 2006; Katayama et al., 2008). Kato et al. (2005) states that after UV treatment NoV genes are damaged and virus toxicity is lost, nevertheless virus genetic material is still found after tertiary treatment.

Previous research has also reported NoVGII to be more prevalent than NoVGI in wastewater, with lower concentrations of both genogroups during the summer (Flannery et al., 2012; Gonzales-Gustavson et al., 2019; Eiji Haramoto et al., 2015; Katayama et al., 2008). Therefore, since green spaces are mainly irrigated during the summer months, the risk of contamination is lowered.

4.5. Overall assessment of bacteria and virus

According to all results obtained, there is also a significant fecal presence of human origin in the grass from “Parque Tejo”, possibly due to the use of the green spaces by pedestrians. The grass surrounding “Parque Aranha” (points 1 to 4) showed signs of lower dog fecal contamination (13%) and higher presence of enteric viruses (33%), which might reflect the use of this location as a pathway to access the playground. Other animals might have contributed to the presence of fecal contamination since

enterococci concentrations were higher than *E. coli*. In “Parque Aranha” points 5 and 6 tested positive for 58% of the samples for the presence of dog mtDNA and showed lower presence of enteric viruses (25%), which was expected as this area is not used as a pathway and is mainly used by dogs.

The grass from the WWTP, despite being previously irrigated with RW, tested negative for all viruses. However, samples from the WWTP, green roof and football field were much less representative than “Parque Aranha”, since in these locations it was only possible to do one campaign. Additionally, the grass from the WWTP was not being irrigated during this study. Nonetheless, the grass in this location has limited access, therefore the results obtained for FIB point to birds as a probable source of fecal contamination, as well as, contamination transferred through workers’ shoes (sample E3). For the green roof, due to the difficult accessibility, it is expected that birds, such as pigeons, are the source of fecal contamination.

5. Conclusions and Future Work

The present study assessed the microbiological quality of grass from different locations, irrigated with different water sources (groundwater, potable water and RW). Grass samples from all locations showed prominent occurrences of bacteria, with concentrations ranging between 6.73×10^2 GU/g and 6.14×10^5 GU/g for *E. coli* and for enterococci values ranged from 9.31×10^3 GU/g to 4.27×10^6 GU/g. One sample from the WWTP showed a higher concentration of *E. coli* compared to enterococci, suggesting the presence of fecal contamination from human origin. All other samples displayed higher concentration levels for enterococci compared to *E. coli*, suggesting the presence of fecal contamination from animal origin. Through the use of mtDNA markers, the presence of fecal contamination from dogs was determined in 28% of samples from "Parque Aranha", however this analysis was only performed for the presence of dog mtDNA, which limited the conclusions in regard to sources of pollution.

The high prevalence of FIB on grass compared to results obtained for RW quality suggests that irrigation with RW will not negatively affect the quality of the grass, in regard to contamination with *E. coli* and enterococci.

Enteric viruses were detected in various grass samples from "Parque Tejo" with NoVGII being the virus detected in the highest percentage of samples, followed by NoVGI. HAV was not detected in any samples. Grass samples from the WWTP were negative for all enteric viruses tested, despite being previously irrigated with RW. Results from "Parque Tejo" show the presence of NoVGII in all sampling dates and samples for the green roof at IST tested negative for all viruses, which is expected due to the difficult accessibility. However, samples were very limited, especially at the green roof, football field at "Parque Tejo" and WWTP, and at the same time samples were collected in a short period of time.

Viral contamination present on the grass from "Parque Tejo" was found to be higher than contamination levels of RW from previous studies. Although the presence of genetic material from viruses in the grass samples from "Parque Tejo" does not directly indicate that there is a real danger to public health, it indicates the presence of fecal contamination from human origin in the grass.

Overall, the high contamination values present on the grass from "Parque Tejo" from exogenous sources of fecal contamination, such as natural contamination from animals and use of the green spaces by people, suggest that irrigation with RW will not affect negatively the microbiological quality of the grass, since contamination levels for RW are lower to those found in this study. Nonetheless, the majority of previous studies note that NoV is still detectable after wastewater treatment (Francy et al., 2012; E. Haramoto et al., 2006; Katayama et al., 2008), which may pose a potential health risk.

Future evaluations of microbiological quality of grass should be based on extensive sampling, to be as most statistically relevant as possible, and for a period of time that encompasses the different seasons of the year, since results can vary seasonally and

during the sampling period none of the areas was being irrigated due to meteorological conditions. Having control grass samples irrigated with potable water and without access from people and animals would be ideal for comparison purposes. Future studies should also expand the use of mitochondrial markers for the presence of human, pigeon and cat mtDNA.

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7. Annexes

Annex A1 – Preparation of solutions

Phosphate-buffered saline (PBS):

Composition and preparation:

Table A 1 - Reagents and quantities used for the preparation of PBS.

Reagents	Quantity
NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4.7H_2O$	1.15 g
Distilled water	1000 ml

Dissolve the solids in the water. Adjust to pH 7.1 ± 0.1 at 25 °C. Sterilize by autoclaving.

Annex A2 – Grass samples results

Table A 2 - Concentrations of E. coli and enterococci for each sampling point for "Parque Aranha" by qPCR. N.D. – Not Detected.

Date	Sampling point	E. coli (GU/g)	Enterococci (GU/g)
26/10/2020	1	2.58×10^5	6.46×10^5
	2	2.98×10^5	3.65×10^6
	3	3.96×10^5	2.02×10^6
	4	9.38×10^4	4.66×10^5
	5	9.35×10^4	2.72×10^6
	6	6.14×10^5	4.02×10^6
10/12/2020	1	9.59×10^3	1.85×10^5
	2	1.07×10^4	1.61×10^5
	3	6.73×10^2	6.22×10^4
	4	6.15×10^3	6.78×10^4
	5	N.D.	2.65×10^5
	6	1.22×10^3	4.29×10^4
21/12/2020	1	8.93×10^2	1.81×10^4
	2	1.44×10^4	8.39×10^4
	3	8.39×10^3	3.05×10^5
	4	4.23×10^3	2.20×10^5
	5	7.45×10^4	3.69×10^6
	6	6.97×10^3	3.14×10^5
28/12/2020	1	2.72×10^3	1.28×10^5
	2	2.20×10^3	4.57×10^5
	3	6.81×10^4	6.87×10^5
	4	2.99×10^4	1.18×10^5
	5	4.58×10^4	3.72×10^5
	6	4.35×10^4	5.89×10^5
07/01/2021	1	1.58×10^3	4.05×10^5
	2	2.68×10^5	3.00×10^6
	3	3.70×10^4	4.27×10^6
	4	2.76×10^4	3.15×10^6
	5	2.70×10^4	3.09×10^5
	6	3.87×10^3	2.59×10^6
18/01/2021	1	1.42×10^3	1.25×10^5
	2	1.11×10^3	1.73×10^5
	3	3.51×10^4	4.39×10^5
	4	N.D.	8.14×10^5
	5	1.64×10^3	1.19×10^5
	6	2.45×10^4	5.83×10^5

Table A 3 - Concentrations of E. coli and enterococci for each sampling point for the Green Roof in IST, Football Field and WWTP by qPCR. N.D. – Not Detected.

Sampling Location	Date	Sampling point	E. coli (GU/g)	Enterococci (GU/g)
IST	19/11/2020	R1	7.29×10^3	1.06×10^5
		R2	7.92×10^3	1.24×10^5
Football Field	03/12/2020	F1	8.25×10^4	5.42×10^5
		F2	8.03×10^3	1.47×10^5
WWTP	14/12/2020	E1	N.D.	8.23×10^4
		E2	N.D.	2.27×10^4
		E3	1.40×10^5	4.03×10^4
		E4	N.D.	9.31×10^3