Microbiological quality of grass irrigated with different water sources

Magda Ferreira Marques
magda.f.m95@gmail.com
Instituto Superior Técnico, Universidade de Lisboa, Portugal

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.

1. Introduction

Water is a fundamental resource that affects populations living conditions and public health. Globally, the need for freshwater is increasing, often exceeding availability. Factors such as population growth and climate change increase the pressure on the world´s freshwater sources (Raso, 2013). 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). 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 (EEA, 2017). Therefore, it becomes imperative to consider alternative water sources such as the use of Reclaimed Water (RW), especially for these sectors. 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). RW represents one of the most readily available sources of water to meet the increasing demands of water for non-potable uses. 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 (Amec Foster Wheeler et al., 2016; Maurer & Davies, 1993). However, it is important to consider the potential risks of water reuse for the environment and public health. 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. 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 water used for irrigation and 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). In May 2017, Lisbon became a member of the Urban Water Agenda 2030 network of cities. 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 from a WWTP.

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.

2. Methods

2.1. Sampling Locations

Samples were collected from different green spaces in Lisbon, irrigated with different water sources and different accessibilities: "Parque Tejo" (children´s playground and football field), WWTP and green roof located in the Instituto Superior Técnico (IST) campus. "Parque Tejo" is a metropolitan park open to the practice of various sports, leisure and educational activities. The park is being irrigated with water from two wells. The WWTP[1] has an internal reuse policy, for non-potable purposes, such irrigation of green spaces. The wastewater that is reused inside the WWTP is subjected to complementary treatment, through ultraviolet (UV) irradiation and addition of sodium hypochlorite. The green roof at IST is not easily assessible and is irrigated with potable water.

2.2. Grass samples

2.2.1 Sampling

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

2.2.2 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

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

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 solution was kept at (-30±3) ºC until further processing.

2.2.3 Enumeration of Fecal Indicator Bacteria

A portion of the concentrated samples was filtered under vacuum through sterile membranes, and the membranes were placed on the respective plates. *E. coli* was detected on Tryptone Bile X-glucuronide (TBX) agar and enterococci on Slanetz and Bartley agar and incubated at 37 ºC. *E. coli* samples were incubated overnight and enterococci for 48 hours. After the incubation period, colonies were quantified. To verify enterococci colonies, the membranes were transferred to Esculin medium and incubated at 44 ºC for 2 hours.

2.2.4 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.

2.2.5 Microbial Detection and Quantification by Real-Time Polymerase Chain Reaction

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.

2.2.6 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. 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.

2.3. Soil Samples

2.3.1 Sampling

Two soil samples were collected from "Parque Aranha" using a 100 ml sterile container. Both samples were collected in December.

2.3.2 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.

2.3.3 Nucleic Acid Extraction and qPCR

The extraction of nucleic acid was performed using Instagene accordingly to manufacturer's instructions. For detection and quantification of *E. coli* and enterococci the method described in 2.2.5. for bacteria was performed.

3. Results and Discussion

3.1. *E. coli* and Enterococci

3.1.1. Grass Samples

Results of the analysis for the presence of bacteria (*E. coli* and enterococci) in the grass samples are displayed in Figure 1.

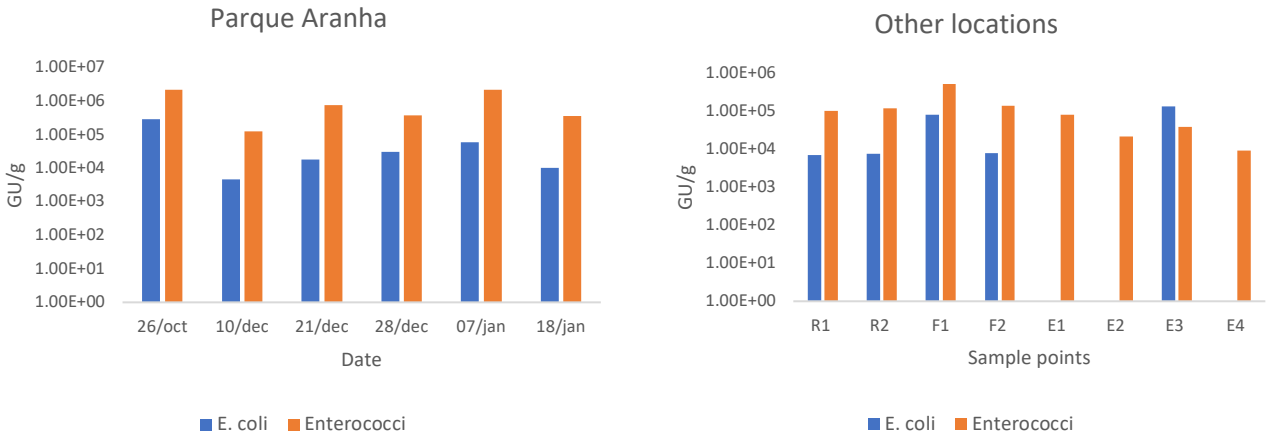

Figure 1 - Mean concentrations of E. coli and enterococci for the 6 campaigns from "Parque Aranha" and FIB results for the Green Roof (R1 and R2), Football Field (F1 and F2) and WWTP (E1, E2, E3 and E4).

All locations showed prominent occurrences of enterococci and *E. coli*. 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

$\times 10^4$ GU/g and for enterococci 1.15×10^5, 3.44×10^5 and 3.86×10^4 GU/g, respectively. For "Parque Aranha" mean concentrations for E. coli and enterococci were, respectively, 6.97×10^4 and 1.04×10^6 GU/g. A higher number of enterococci compared to *E. coli* suggests a fecal contamination from animal origin (Geldreich & Kenner, 1969; Scott et al., 2002). Sample E3 was the only sample that displayed higher concentration of *E. coli* compared to enterococci values, suggesting a fecal contamination from human source. This sample was collected from a pathway area, where the grass was visibly stepped on. Mean concentration values for *E. coli* and enterococci in "Parque Aranha" were higher than the concentrations obtained for the other locations, however samples were much more representative in "Parque Aranha". 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 fecal indicator bacteria (FIB) in irrigation waters does not influence FIB concentrations on soil and plants (Holvoet et al., 2014; Lopez-Galvez et al., 2016). Other studies 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 (Forslund et al., 2013; Ishii et al., 2006; Vergine et al., 2015).

Due to high concentration values for bacteria, in all locations, the method for quantification of bacteria had to be changed. Initially, the method described in 2.2.3. was performed, however 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 2.2.5., was performed for the rest of the samples.

3.1.2. 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).

3.2. Microbial Source Tracking

Results, for dog DNA presence, show that 28% of total samples contained fecal contamination from dogs. Considering the two green areas in "Parque Aranha" separately, points 5 and 6 tested positive for 58% of the samples and points 1 to 4 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.

3.3. Environmental variables

A correlation analysis was performed to assess the effect of precipitation, temperature and solar radiation on contamination present on the grass (Table 1 and Table 2). 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 this meteorological station during October. For solar radiation it was not possible to obtain data for the month of October. Since samples were collected early in the morning, data from the sampling day was not considered for the correlation analysis.

Table 1 - Correlation analysis between environmental variables and E. coli concentrations. 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 until a campaign.

E. coli						
	Point 1	Point 2	Point 3	Point 4	Point 5	Point 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 2 - Correlation analysis between environmental variables and enterococci concentrations.

Enterococci						
	Point 1	Point 2	Point 3	Point 4	Point 5	Point 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).

In general, maximum and mean radiation from the day prior to sampling also did not show significant correlation. 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*. According to previous research *E. coli* is more susceptible to solar radiation decay than enterococci (McCambridge & McMeekin, 1981). 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.

3.4. Enteric viruses

In terms of enteric viruses, all tested samples for HAV were found to be negative. Samples from the WWTP and IST tested negative for all viruses, however, only one campaign was carried out for each of these locations. NoVGII occurred the most often, 26% of all samples (12/46), which represents approximately 32% of positive samples from "Parque Tejo" (12/38), including the football field. Samples that tested positive for NoVGI also tested positive for NoVGII, with samples containing only one 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". 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. The presence of NoV suggests the presence of fecal contamination from human origin.

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 (Eftim *et al.*, 2014). Some studies have also assessed the transfer of viruses on different surfaces. Previous studies note that viruses might be carried to different locations through shoes (Cheesbrough et al., 2000; Kimura et al., 2011), which supports the hypothesis that NoV might have been transferred to the grass through people walking on it.

In this study, the mean concentration for NoVGII was 3.43×10^3 GU/g and 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 to those reported after wastewater treatment. Studies report levels of NoVGI and NoVGII, around 10^5 copies/L in WWTP effluents (Da Silva et al., 2007; Laverick et al., 2004). For HAV, previous research shows that, in general, HAV is not detected after secondary treatment (Carducci et al., 2008; Grabow et al., 1983).

3.5. 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.

4. Conclusions

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; Haramoto et al., 2006; Katayama et al., 2008), which may pose a potential health risk.

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