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Wastewater Discharge through a Stream into a Mediterranean Ramsar Wetland: Evaluation and Proposal of a Nature-Based Treatment System

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Abstract: Impacts on wetlands are becoming more pressing every day. Among them, habitat loss, overexploitation of aquifers and changes in land use are considered the most important. However, the impacts linked to wastewater discharges are increasing worldwide. In this context, this study analyses the impacts of input of wastewater to a Mediterranean Ramsar temporary wetland (Fuente de Piedra, south of Spain). To this end, systematic sampling was carried out in the Charcón stream which receives water from a wastewater treatment plant (WWTP) and discharges it into the wetland. The results showed a slight decrease in the nutrient concentrations, particularly for nitrogen compounds. Heterotrophic and fecal bacteria concentration, as well as phytoplankton and zooplankton abundance and biomass, all significantly decreased from the treatment plant to the wetland. When comparing the effect of this discharge with other similar occurring to the same wetland, it was evident that the Charcón stream was responsible for a greater impact. At this point, it is relevant to note that the main difference among both treated wastewater discharges lies in the different water retention time once the wastewater was released from the WWTP. In fact, we recommend an increase in the water retention time by building seminatural ponds, together with the use of biofilters, which will notably contribute to improve the processes of assimilation of nutrients and to decrease the impact generated in the wetland by this spill.

Keywords: cultural eutrophication; management; seminatural ponds; temporary wetlands; wastewater; water quality

1. Introduction

Inland freshwater ecosystems are hotspots of biodiversity as they show higher relative species richness than marine or terrestrial ecosystems [1]. They provide important ecosystem services, with a global estimated annual economic value of USD 44,000 per

hectare [2]. In spite of their importance, almost 50% of inland water environments have been lost during the twentieth century, which makes them one of the most threatened ecosystems [1]. The global Living Planet Index (LPI) shows that 60% of species have shown population declines between 1970 and 2014, with freshwater species showing an 83% decline [3]. These authors and others indicate that the biggest drivers of biodiversity decline are habitat loss, habitat degradation, and overexploitation [4,5]. In this sense, wetlands biodiversity is mostly affected by changes in land use and eutrophication processes [6–9]. One of the main problems of the discharge of wastewater to aquatic ecosystems is the increase in eutrophication [10], a current problem suffered by wetlands around the world that cannot be resolved despite the existence of legislation for wetland protection [11]. Cultural eutrophication can be caused, among others, by (i) changes in the land use of the catchment area, where farming and the use of fertilizers increase the runoff of nutrient rich water into the wetlands [8,9], and (ii) the direct spill of urban wastewater into the wetland [12,13]. Attending to the second one, within the EU urban wastewater is supposed to be treated according to the urban wastewater Council Directive [14]. This requires that wastewater delivered into sensitive freshwater and estuaries should not be higher than $1 \text{ mg}\cdot\text{L}^{-1}$ of total phosphorus (TP) and $10 \text{ mg}\cdot\text{L}^{-1}$ of total nitrogen (TN). Wastewater treatment is a critical issue in Spain and supervision and improvement of wastewater treatment as well as reuse are urgently needed [15]. This is especially true for the effluents of the wastewater treatment plant (WWTP) that spill into sensitive inland waters, such as the Ramsar wetlands. When this occurs, the administration tries to find solutions to improve the quality of the water discharged, as is the case of building artificial wetlands which can decrease concentration of TP, TN and fecal bacteria [12]. Nevertheless, sometimes the wastewater is directly discharged in those sensitive wetlands. This is the case of Fuente de Piedra (south of Spain), a Ramsar wetland that receives the wastewater of two nearby villages, Fuente de Piedra and Humilladero. From the first one, the wastewater runs through a set of seminatural ponds constructed during a Life project (LIFE03NAT/E/000055) which improved considerably the water quality of the wastewater before reaching the wetland [12]. However, the wastewater from Humilladero village reaches the wetland directly through a temporary stream, named Charcón. Thus, the present study is focused on the wastewater selfdeposition processes that occurs during the transit through the Charcón stream connecting the Humilladero WWTP to the Ramsar wetland. These processes have been evaluated through the study of a series of biotic and abiotic variables along the stream, which has allowed us to compare the results with those previously obtained of the discharge from Fuente de Piedra village to the same wetland [12], with the aim to propose nature-based facilities in order to achieve Sustainable Urban Water Management [16].

2. Materials and Methods

2.1. Study Area and Sampling Sites

Fuente de Piedra is a Mediterranean temporary endorheic ecosystem [17] located in Andalusia (southern Spain). It was included in the list of Ramsar sites in 1983, declared as a Nature Reserve in 1984, and achieved Special Areas of Conservation (SAC) status in 2013 [18,19]. This ecosystem receives the discharge of three wastewater treatment plants (WWTP); two from the Fuente de Piedra village [12] and the other one from the Humilladero village through the Charcón stream, this last being the objective of the present study (Figure 1). The Humilladero WWTP is composed of two aerobic basins and treat the wastewater of a population of approximately 3300 people. Data for biochemical oxygen demand, chemical oxygen demand and suspended solids are available by Andalusian Administration (Table 1).

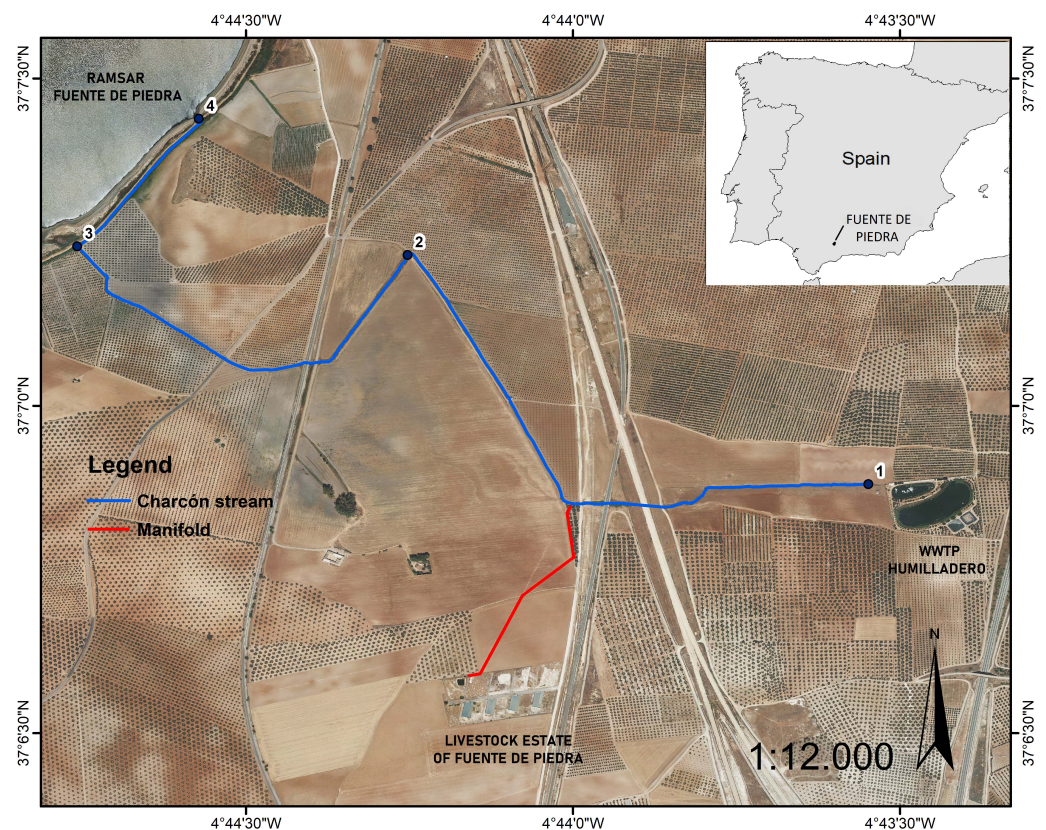


Figure 1. Location of the sampling points (1 to 4) into the Charcón stream, from the Humilladero WWTP (wastewater treatment plant) to Fuente de Piedra wetland. In this figure is also represented a manifold that discharges into the Charcón stream from a livestock estate.

Table 1. Characteristics of input and output wastewater from the wastewater treatment plant of Humilladero. Efficiency (EFF) (data supplied by Junta de Andalucía). BOD5: biochemical oxygen demand for 5 days ($\text{mg}\cdot\text{L}^{-1}$); COD: chemical oxygen demand ($\text{mg}\cdot\text{L}^{-1}$); SS: suspended solid ($\text{mg}\cdot\text{L}^{-1}$). Mean values of the year of study (2018) and values obtained from the day closest to the sample in which data is available.

Date	BOD5			COD			SS		
	Input	Output	EFF	Input	Output	EFF	Input	Output	EFF
Mean 2018	87.4	12.6	71%	252.3	56.2	69%	109.9	59.9	38%
11/06/2018	90	6.4	93%	184	60	67%	92	78	15%

Charcón is a temporary stream that comes from the nearby Sierra de Humilladero and ends at the Fuente de Piedra wetland. Next to this stream is located the WWTP of Humilladero. From this treatment plant to the Fuente de Piedra wetland, the stream flows along 2905 m with an unevenness of 11 m. In this travel, four sampling stations were taken; the first one (point 1) next to the treatment plant and the last (point 4) at the entrance to the Fuente de Piedra wetland (Figure 1). The second sampling station (point 2) is located in an intermediate section of the stream, and the third (point 3) in the entrance to the nature reserve. In order to know the time required for the effluent to travel from the WWTP to the Ramsar wetland, the stream was divided in two homogeneous sections, the first with a length of 1072 m and an average slope of $0.65 \text{ cm}\cdot\text{m}^{-1}$, and the second with a length of 1833 m and an average slope of $0.22 \text{ cm}\cdot\text{m}^{-1}$. In both sections the water velocity and traveling time of the wastewater was measured by the dilution method using tracers [20]. The samplings were carried out in June 2018, working upstream to avoid contamination, that is, from the Fuente de Piedra wetland (point 4) to the WWTP (point 1).

2.2. Abiotic Variables

At each sampling point, conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$) and pH were measured with a Hanna Multiparameter sensor HI 9829 (Hanna Instruments, Woonsocket, RI, USA). Three water samples for nutrients concentrations were taken into sterile polyethylene vials and immediately frozen ($-20\text{ }^{\circ}\text{C}$). In the laboratory, dissolved inorganic phosphorus (DIP) was determined by using the molybdenum blue method [21] and TP was measured after the digestion with potassium persulfate of unfiltered and filtered water (Whatman GF/F), respectively, [22]. Ammonium (NH_4^+) was measured by phenate method [23], nitrates (NO_3^-) was analyzed using the ultraviolet spectrophotometric screening method [22] and nitrite (NO_2^-) was determined using the sulfanilamide method [23]. Lastly, TN was analyzed by ultraviolet method of digested unfiltered and filtered water, respectively, [22].

2.3. Biotic Variables

For biotic variables three samples were also taken in the same sampling point for the evaluation of the biotic variables. Total chlorophyll-a concentration (Chl-a) and phytoplankton composition were estimated with a submersible FluoroProbe (bbeMoldaenke GmbH, Schwentinental, Germany), which discriminates between four main phytoplankton groups (i.e., diatoms and dinoflagellates, blue green algae, green algae, and cryptophytes) [24,25]. Abundance of phytoplankton $< 20\text{ }\mu\text{m}$ equivalent spherical diameter (ESD), was measured by passing the sample through a BD Acurri C6 flow cytometer, counting at least 10,000 events. Abundance and size of phytoplankton cells (5 and $100\text{ }\mu\text{m}$ ESD) and zooplankton (250 and $1000\text{ }\mu\text{m}$ ESD) were analysed with a FlowCAM (Fluid Imaging Technologies, Inc. Scarborough, ME, USA) using the auto image mode. For phytoplankton, 30 mL of the sample preserved with formol (4% f.c.) were passed through a $100\text{ }\mu\text{m}$ flow cell and 1 mL was analysed with a 100-fold magnification ($10\times$ objective). For zooplankton, 50 mL of the samples preserved with formaldehyde (4% f.c.) were passed through a $1000\text{ }\mu\text{m}$ flow cell and analysed with a 20-fold magnification ($2\times$ objective).

The enumeration of heterotrophic cultivable microorganisms was carried out by adding 0.1 mL of serial dilution of the samples in Trypticase soy agar (Oxoid Ltd., Wade Road, Basingstoke, UK) plates. The plates were cultured at $22\text{ }^{\circ}\text{C}$ for 48 h (ISO 6222:1999). Coliforms and fecal streptococci concentrations were determined by water filtration through sterile nitrocellulose filters (47 mm diameter, $0.45\text{ }\mu\text{m}$ pore size; Millipore Corp., Bedford, MA, USA). Membranes were incubated in Chromocult coliform agar (Merck, Darmstadt, Germany) at $37\text{ }^{\circ}\text{C}$, 24 h for the determination of total coliforms and *Escherichia coli* (ISO 9308-1:2000), or in *m-Enterococcus* agar (Merck) at $37\text{ }^{\circ}\text{C}$, 48 h for the determination of fecal streptococci (ISO 7899-2:2001). After incubation, colonies were counted on each medium, and concentrations of the different groups of microorganisms were determined.

For the identification of the fecal streptococci species, isolates were identified to species level by the amplification and sequentiation of a fragment of 16S rDNA. The Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for the extraction of total genomic DNA from bacteria. Afterward, this fragment was amplified using the universal primers SD-Bact-0008-a-S20 (5' AGA GTT TGA TCC TGG CTC AG 3') and SD-Bact-1492-a-A-19 (5' GGT TAC CTT GTT ACG ACT T 3') [26]. Polymerase chain reactions were carried out in a $50\text{ }\mu\text{L}$ reaction mixture that included 5 pmol of each primer, 0.2 mM dNTPs mix, 10x DreamTaq Buffer, 2 mM MgCl_2 , 1.25 U DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μL of colony DNA ($100\text{ ng}/\mu\text{L}$). The PCR profile was as follows: 2 min at $95\text{ }^{\circ}\text{C}$ and 35 cycles of 30 s at $95\text{ }^{\circ}\text{C}$, 40 s at $52\text{ }^{\circ}\text{C}$ and 1.3 min at $72\text{ }^{\circ}\text{C}$ and a final step 5 min at $72\text{ }^{\circ}\text{C}$. Polymerase chain reaction products were electrophoresed on a 1% agarose gel and visualized via ultraviolet transillumination. PCR products were sequenced by Macrogen Spain (Madrid, Spain). The resulting sequences were compared with those in the GenBank database (www.ncbi.nlm.nih.gov/genbank/ accessed on 16 March 2021) by using the BLAST program (www.ncbi.nlm.nih.gov/blast accessed on 16 March 2021). Finally, water acute toxicity test was carried out by modification of method described by Johnson [27]

using the bioluminescent bacteria *Vibrio fischeri* and the Microtox[®] M500 test (Microbics Corporation, Carlsbad, CA, USA). Microtox test is widely used for the toxicity assessment of environmental samples and is based on the measurement of *V. fischeri* bioluminescence inhibition after sample exposure at various contact times [28]. Cuvettes with 1 mL of each water sample (without dilute, or diluted at 1/50 and 1/100 in 2% saline solution) were maintained at 15 °C. *V. fischeri* growth for 24 h in TSA with 2% of NaCl (TSAs), was suspended in 2% saline solution at 0.6 of optical density (600 nm) and maintained at 5 °C prior use. Then, the cuvettes with the water samples and controls were inoculated with 20 µL of the *V. fischeri* suspension. The samples were incubated at 15 °C for 15 and 45 min. After incubation, bioluminescence of *V. fischeri* in each cuvette was measured by Microtox luminometer. Bioluminescence in each sample was relativized with bioluminescence in control cuvettes, obtaining a percent decrease of bioluminescence. The inhibition of the luminescence was assumed to be correlated with the toxicity of the samples. The percent reduction in bioluminescence of *V. fischeri* produced by the samples were recorded as median effective concentration (EC50) values.

2.4. Statistical Analysis

Residual normality (Shapiro–Wilk test) and homogeneity of variances (Levene test) were checked before performing the statistical analysis of the data. Differences in pH, TN, Chl-a, phytoplankton composition and phytoplankton 1–20 µm abundance between sampling stations were tested by using one-way ANOVA test, followed by Bonferroni's post hoc test. Since all other variables did not satisfy homoscedasticity assumptions (Levene test, $p < 0.05$) or normality distribution (Shapiro–Wilk test, $p < 0.05$), Kruskal–Wallis and post hoc Dunn's test with Bonferroni adjustment were carried out to test the differences between sampling stations. To perform this statistical analysis, the Statistica 7.1 software (StatSoft Inc., Tulsa, OK, USA) was used.

3. Results

3.1. Abiotic Variables

Results obtained shown significant conductivity differences (Table 2) from point 1 to point 4 (Kruskal–Wallis test, $p < 0.001$) with a significant increase among point 1 and points 3 and 4, and among point 2 and point 4 (Dunn–Bonferroni post hoc, $p < 0.05$). The pH in contrast decreased significantly (Table 2) from the wastewater treatment plant spill (point 1) to the Ramsar inflow at point 4 (one-way ANOVA, $p < 0.001$) with a significant decrease in each sampling point (Bonferroni post hoc, $p < 0.001$).

Table 2. Conductivity (Cond.) and pH measured at the four sampling points in the Charcón stream. Kruskal–Wallis test Median (25, 75 percentiles) and one way ANOVA (mean ± standard deviation) Different letters show significant differences between sampling points (mean ± standard deviation).

Abiotic Variables	Point 1	Point 2	Point 3	Point 4
Cond. ($\mu\text{S}\cdot\text{cm}^{-1}$)	2549 (2545; 2569) ^a	2585 (2579; 2585) ^{ab}	2657 (2657; 2656) ^{bc}	2668 (2686; 2688) ^c
pH	8.12 ± 0.03 ^a	7.72 ± 0.02 ^b	7.63 ± 0.03 ^c	7.36 ± 0.01 ^d

The time required for the effluent to travel the total length of the stream from the WWTP to the Ramsar wetland was approximately 9.5 h. In this time, TP and TN decreased from the spilling point (point 1) to the Ramsar inflow point (point 4, Table 3) by 12% and 30% respectively. However, these decreases were not significant (Kruskal–Wallis test and one-way ANOVA, respectively, $p > 0.05$). Similarly, nitrogen dissolved compounds decreased in general, while the phosphorus compounds increased (Table 3). These changes in dissolved nutrients were significant for DIP (Kruskal–Wallis test, $p < 0.05$), among point 1 and point 4 (Dunn–Bonferroni post hoc, $p < 0.05$). Regarding to the nitrogen forms, there were not significant differences for NO_3^- and a significant decrease was found for NO_2^- and NH_4^+ (Kruskal–Wallis test, $p < 0.05$). Concerning NO_3^- concentration, it significantly changed among points 3 and 4 (Dunn–Bonferroni post hoc, $p < 0.05$). In addition, NO_2^-

showed significant differences among the sampling points 2 in comparison to points 4 (Dunn–Bonferroni post hoc, $p < 0.005$), while NH_4^+ showed a significant decrease among point 1 and point 4 (Dunn–Bonferroni post hoc, $p < 0.05$).

Table 3. Nutrient concentrations measured in the sampling points in the Charcón stream. Kruskal–Wallis test Median (25, 75 percentiles) and one way ANOVA (mean \pm standard deviation). Different letters show significant differences between sampling points.

Nutrient Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Point 1	Point 2
Dissolved Inorganic Phosphorus	0.70 (0.68; 0.70) ^a	0.75 (0.68; 0.76) ^{ab}
Total Phosphorus	1.45 (1.42; 1.66) ^a	1.44 (1.41; 1.47) ^{ab}
Nitrates	0.56 (0.56; 0.57) ^{ab}	0.47 (0.45; 0.50) ^{ab}
Nitrites	0.51 (0.48; 0.59) ^{ab}	0.55 (0.54; 0.55) ^a
Ammonium	10.92 (9.97; 13.6) ^a	9.27 (9.18; 9.92) ^{ab}
Total Nitrogen	15.48 \pm 1.56 ^a	13.92 \pm 2.63 ^a
Nutrient Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Point 3	Point 4
Dissolved Inorganic Phosphorus	0.86 (0.82; 0.87) ^{ab}	1.04 (0.97; 1.04) ^b
Total Phosphorus	1.48 (1.47; 1.54) ^a	1.34 (1.30; 1.36) ^a
Nitrates	1.14 (1.12; 1.17) ^a	0.10 (0.09; 0.13) ^b
Nitrites	0.16 (0.16; 0.16) ^{ab}	0.02 (0.02; 0.02) ^b
Ammonium	8.55 (8.50; 8.64) ^{ab}	7.68 (6.61; 8.22) ^b
Total Nitrogen	10.53 \pm 2.86 ^a	10.16 \pm 2.73 ^a

3.2. Biotic Variables

Chl-a concentration (Figure 2a) decreased significantly from point 1 to point 4 (one-way ANOVA, $p < 0.05$); although, this decrease was not uniform since a slight increase was observed in point 2. The differences of the successive sampling stations were significant as well as among all the sampling points (Dunn–Bonferroni post hoc, $p < 0.001$). Regarding the phytoplankton groups (Figure 2a), significant changes were also observed (one-way ANOVA, $p < 0.05$). Green algae showed similar Chl-a concentration at point 1 and point 2 and decreased significantly at point 3, reaching the lowest concentration at point 4 (Dunn–Bonferroni post hoc, $p < 0.001$). The Chl-a concentration of blue green algae increased significantly from point 1 to point 2 and decreased significantly to point 3 and point 4 (Bonferroni post hoc, $p < 0.001$). Similarly, diatoms and dinoflagellates Chl-a concentration increased significantly from point 1 to point 2 and decreased significantly from point 3 to point 4 (Bonferroni post hoc, $p < 0.05$). The Chl-a concentration of cryptomonads showed the lowest concentrations of the four groups and was similar at the first three sampling points, decreasing significantly at sampling point 4 (Bonferroni post hoc, $p < 0.05$). Phytoplankton abundance for 1–20 μm ESD showed significant differences (one-way ANOVA, $p < 0.05$), with similar abundances at sampling points 1 and 2, and a significant decrease from point 2 to the half at sampling point 3 and from point 3 to the lowest abundance at sampling point 4 (Bonferroni post hoc, $p < 0.001$). Concerning biovolume of phytoplankton 5–100 μm ESD, significant differences among the sampling points were detected (Kruskal–Wallis test, $p < 0.05$), but it was not possible to determine which sampling stations were significant different (Dunn–Bonferroni post hoc, $p > 0.05$) (Figure 2b). Finally, although a decrease in zooplankton biovolume, can be observed from point 1 to point 2 (Figure 2b) and there were detected significant differences among the sampling points (Kruskal–Wallis test, $p < 0.05$), the post hoc analysis was not able to determine these differences (Dunn–Bonferroni post hoc, $p > 0.05$).

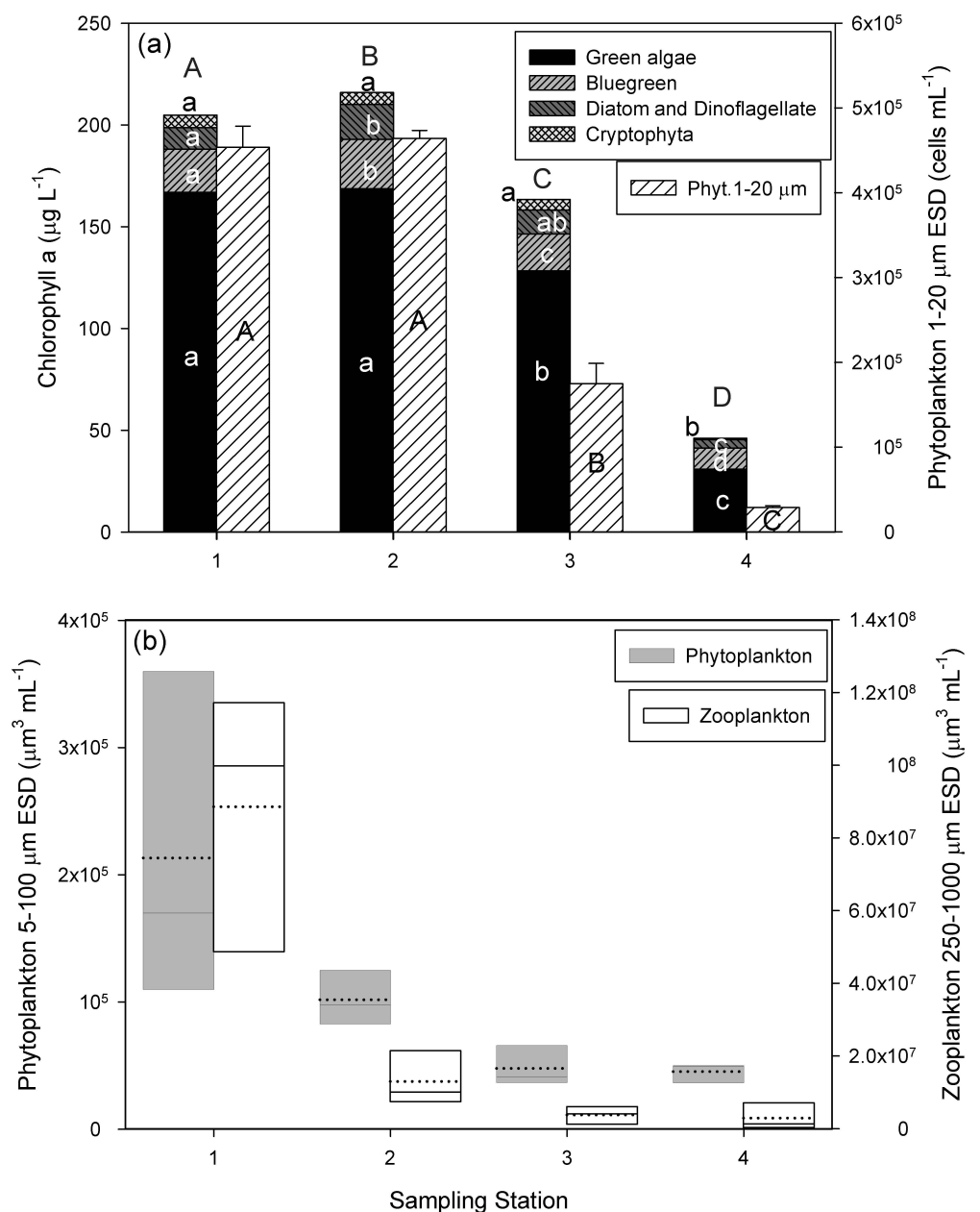


Figure 2. (a) Chl-a concentration (stacked mean) of the four phytoplankton groups, and abundance of phytoplankton < 20 μm in the four sampling stations at the Charcón stream. Capital letters indicates significant differences between total Chl-a concentration and lowercase letter indicates significant differences of the respective phytoplanktonic groups among the sampling points. (b) Biovolume of phytoplankton (5–100 μm ESD) and zooplankton (250–1000 μm) in the four sampling stations at the Charcón stream. The horizontal continuous line indicates the median and dotted line the mean.

Significant differences were tested in microorganism (Figure 3) by Kruskal–Wallis test ($p < 0.05$). A progressive and significant decrease in the concentration of heterotrophic bacteria was seen between points 1 and 4 (Dunn–Bonferroni post hoc, $p < 0.05$). In the case of total coliforms, no significant differences were observed among any point (Kruskal–Wallis test, $p > 0.05$), while *E. coli* showed a significant decrease in their titer from point 2 to point 4 (Dunn–Bonferroni post hoc, $p < 0.05$). In the case of fecal streptococci, concentration values showed a significant peak at point 2 (Dunn–Bonferroni post hoc, $p < 0.05$). At point 4

fecal streptococci concentrations were between the values reached at point 1 and point 3. Finally, the *E. coli*/fecal streptococci ratio showed a progressive decrease in its values from point 1 to 4 that was significant among point 3 and point 4 (Dunn–Bonferroni post hoc, $p < 0.05$).

A total of 24 strains were isolated from m-*Enterococcus* agar for their identification. The 25% were identified as *Enterococcus hirae* and the 16.7% as *E. faecium*. The rest were identified as *E. mundtii*, *E. haemoperoxidus*, *E. plantarum* and *Bacterium* BEL B14 (each of them at 8.3%). Furthermore, a strain of *E. casseliflavus*, *E. durans* and 4 strains of *Enterococcus* sp. were isolated. The water acute toxicity assessment showed no decrease in *V. fischeri* bioluminescence, regardless of the dilution used or the incubation time; thus, indicating absence of toxicity. Furthermore, a significant activation of bioluminescence compared to control was detected in the samples at dilution 1:50 (data not shown).

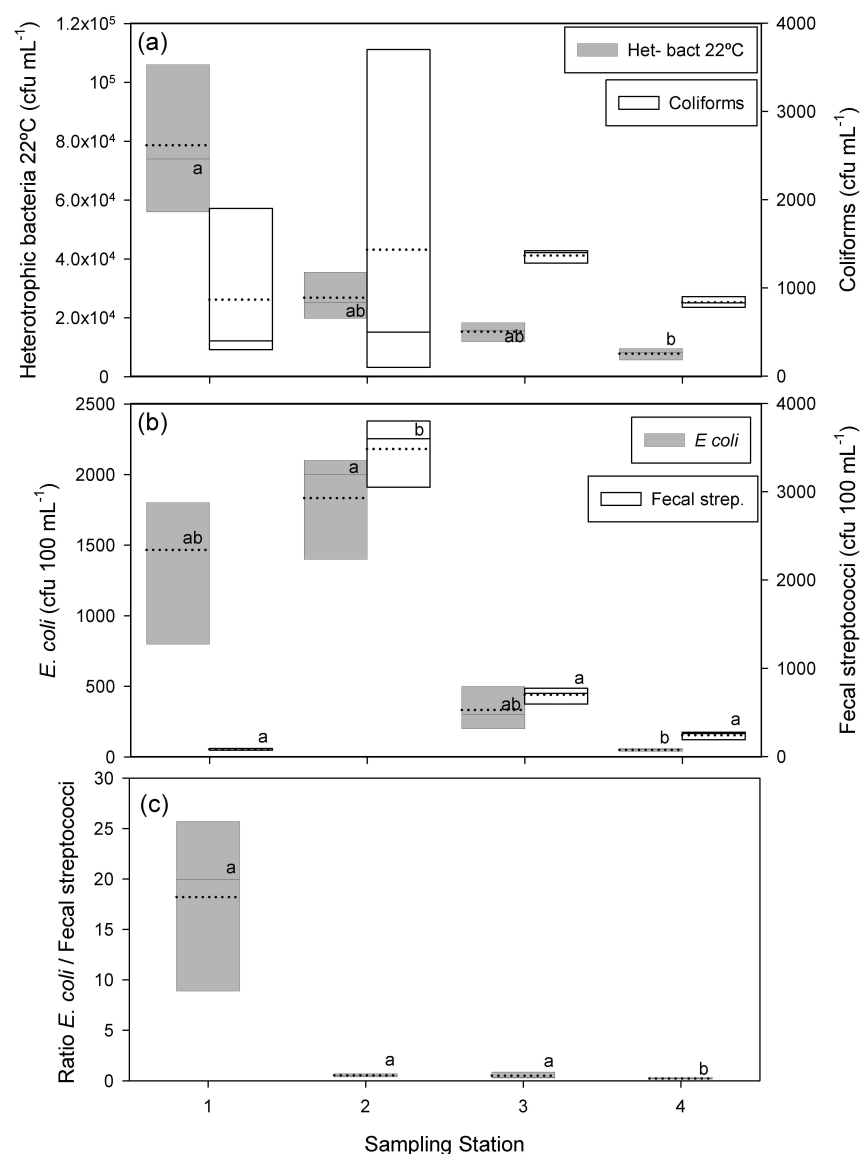


Figure 3. Bacterial concentration measured in all the sampling points in the Charcón stream. (a) Heterotrophic bacteria growth at 22 °C and total coliforms (cfu mL⁻¹). (b) *E. coli* and fecal streptococci (cfu 100 mL⁻¹). (c) Ratio *E. coli*/fecal streptococci. The horizontal continuous line indicates the median and dotted line the mean. Different letters show significant differences between sampling points into each bacterial group.

4. Discussion

Impacts on wetlands due to wastewater discharges are increasing worldwide and becoming a real problem that has been not resolved yet, despite legislation for the protection of wetlands [11]. In this study, we analyze the effect of a direct discharge of wastewater from a village located in the watershed of the Fuente de Piedra Ramsar site through a stream named Charcón. The results show that in general, along the Charcón stream, the measured environmental variables show few significant changes downstream of the discharge. In fact, the main variables related to the cultural eutrophication of aquatic ecosystems (TN and TP) show similar values at the outlet of the wastewater treatment plant and at the in-put to the wetland. Only significant changes in NO_3^- due to their decrease at input to the wetland as well as a significant increase in DIP and significant decreases in nitrite and ammonia concentrations were observed. Attending to the biological variables, a decrease in Chl-a concentration, abundance and biovolume of the phytoplankton and zooplankton community and in the abundance of indicator microorganisms is observed. Comparing these results with those obtained in another of the discharges that pours into the same wetland [12], it is observed that the differences are important. The water coming out the WWTP, travels the 2905 m transect from the spill to the Ramsar wetland in less than 10 h. This is a very short time for self-depuration process through the stream in comparison to those observed with the use of constructed wetlands [12] and it is unsurprising that few variables showed downstream changes in our studies. In this sense, Søballe and Kimmel [29] separates the ecological structure and function of natural rivers, river impoundments, and natural lakes, where natural lakes and river are in opposite extremes. According to these authors, algal abundance per unit of P is lower in rivers than in impoundments and lakes as long as residence time are different. The authors conclude that residence time is a useful system-level index and it has similar ecological implication for rivers, lakes and reservoirs. This explains why, due to longer residence time, effective P and N reduction by biological processes were observed in the adjacent pond system coupled to the wastewater plant of Fuente de Piedra village [12] in comparison to the low nutrient reduction observed in this study in the Charcón stream, with a lower residence time.

Indicator microorganisms are used to assess the effectiveness of water and wastewater treatment processes [30]. These microorganisms are decisive to determine the degree of fecal pollution and the load of organic matter of the water. According to data from the Andalusian administration (Table 2), the treated wastewater discharged into the Charcón stream has a load of suspended solids above the reference levels of the European Directive 91/271/EC [14] (EC 1991). BOD5 and COD values are also high, although within the limits of such legislation. Thus, it is reasonable that indicators microorganisms of fecal contamination can still be isolated in the outflow water (point 1). High heterotrophic and fecal concentration bacteria (coliforms and fecal enterococci) are observed at the spill to the Charcón stream. Strong positive correlations between Chl-a and bacterial production rates suggest that bacteria are mainly controlled by organic substrates released during phytoplankton photosynthesis [31]. Total coliforms decrease no significantly along the Charcón stream, while mesophilic heterotrophs and *E. coli* decrease from points 1 and 2 to point 4. On the other hand, an increase in the bacterial concentration occurs at sampling point 2, being 42 times higher in the case of fecal streptococci. This significant increase in fecal bacteria at sampling point 2 could be due to livestock estate spill released into the Charcón stream between sampling point 1 and sampling point 2 (Figure 4). After this spill, and following our previous interpretation, from point 2 to point 4 the concentration of fecal bacteria decreases progressively. At the Ramsar inflow, the water shows a concentration of fecal microorganisms suitable for bathing, according to Spanish regulations [32]. These results show that the Charcón stream, unlike what happens with the concentration of nutrients, acts as an efficient natural purification system in relation to microorganisms.

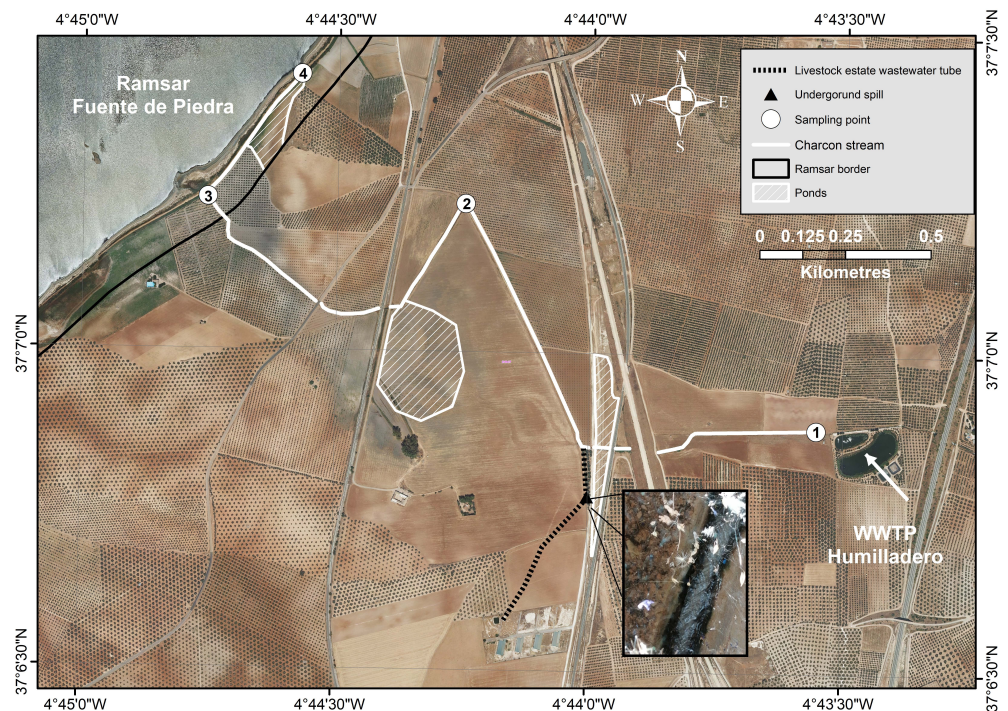


Figure 4. WWTP, Charcón stream, livestock estate spill (inlet) and proposed seminatural ponds system along the Charcón stream.

Wastewater can contain indefinite toxic substances, therefore toxicity tests have to be performed for an integrated evaluation of the environmental impact of contaminants. The toxicity analyses have not shown a decrease in bioluminescence in the different treatments. However, an activation of bioluminescence with respect to controls has been observed, possibly due to the existence of nutrients in the water [33]. It can be concluded that the waters do not have environmental toxic substances in enough concentration as to generate an acute toxic action. Cultural eutrophication is greatly affecting the structure and function of Mediterranean wetlands [34–36]. These ecosystems have been considered as “hotspot” sites for biogeochemical processes [37] due to their intrinsic characteristics. In the case of Fuente de Piedra, an endorheic wetland, it is needed to reduce the entrance of nutrients. This has been efficiently achieved in the other discharge that this wetland receives thanks to seminatural ponds, which increase the residence time of the wastewater [12]. Therefore, in a similar way, it is proposed a system with three seminatural ponds along the Charcón stream, with an area of 21,048 m², 63,549 m² and 15,250 m², respectively, (Figure 4). If the mean depth of the ponds were 1.5 m, 1.0 m and 0.5 m, respectively, the first pond would have 31,572 m³, the second pond 63,549 m³ and the third pond 7625 m³. With a mean flow of 15 m³·h^{−1}, the residence time in the first, second and third pond would be 88, 177 and 21 days, respectively. These values are higher than those calculated for the seminatural ponds system in the other spill of the adjacent Fuente de Piedra WWTP (65–85 days for the whole pond system) [12]. The site for the first proposed pond is located in an uncultivated area adjacent to a new road (Figure 4). The second proposed pond would be located near to the sampling point 2, where highest bacteria concentration was observed, and downstream of the possible agricultural discharge. In this place is located a temporary wetland disappeared due to the action of a drainage; therefore, in order to recover or restore this wetland, the installation of this pond would be more feasible than that of any other (Figure 4). Finally, the third pond would be located in the Ramsar area, immediately above the wetland. This pond would further enhance water remediation and might facilitate further benefits the Ramsar wetland. In this pond it would be suitable to use helophytic vegetation that plays an excellent role in Mediterranean wastewater

purification [38]. The construction of two new ponds and the recovery of a temporary pond will contribute to increasing biodiversity in the study area. In addition, this action will reduce the tourist pressure suffered by the Interpretation Center of the Nature Reserve since visitors, mostly ornithologists, will have more wetlands for bird watching. Furthermore, the presence of a greater number of ponds around the Ramsar wetland will lead to an increase in the environmental heterogeneity, in terms of hydroperiod and salinity, a fundamental aspect since the stability and recovery of some ecosystems depends to some extent on environmental heterogeneity [39]. In addition, this increase in the heterogeneity of the environment will mean an enrichment in the number of species present and also the conservation of numerous species that follow a metapopulation-metacommunity dynamic, as the plankton community [40,41].

Finally, and attending to the restoration of eutrophicated systems, other methods could also be used. Between them, one of the most promising methods is the application of magnetic particles (MPs) for removing P from aquatic ecosystems. MPs have shown a high P removal efficiency in synthetic, natural and wastewaters, see among others, [42–44]. P loaded MPs can be recovered and washed, allowing MPs reuse [42] which reduces the cost of this method. Álvarez-Manzaneda et al. [44] have estimated a final cost of EUR 6601 for the application of MPs (reused four times) in the seminatural pond located in the wastewater spill from Fuente de Piedra village. This cost would be similar to Phoslock[®], that has the disadvantage of not being able to recover the P from the system which may be used to make fertilizers, facing the problem of P reserves exhaustion. For all this, the combination of seminatural ponds and application of MPs along the Charcón stream could provide a solution for the release of wastewater by the WWTP in Andalusia.

5. Conclusions

The short residence time of the wastewater in the Charcón stream (<10 h) does not allow an efficient self-depuration process. Therefore, little reduction of TP and TN occurs during the time that the wastewater reaches the Ramsar site. Although no toxic effect has been observed in the water and the concentration of bacteria decreased along the stream, a better purification of the discharge from the wastewater treatment plant is necessary to avoid cultural eutrophication. A combination of bioremediation by increasing the residence time through building seminatural ponds, introducing biofilters with more vegetation such as *Phragmites australis* [45], *Typha latifolia* [46], *Lemna minor* and *Azolla filiculoides* [47], as well as techniques to absorb phosphate by MPs, should be urgently applied to the wastewater spill of the Charcón stream. For this, as a first step, a set of semi-natural ponds system is proposed to be built along the Charcón stream.

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