Dear Editor.

Thank you for considering the manuscript interesting.
Please find below the responses to your comments.
We introduced some changes and revised the manuscript again.
If you consider the manuscript acceptable for publishing in the Journal Water, we would be glad to do so.
However, in spite to publish in other journals linked the online congress but not considered in SCI index we prefer submitting the manuscript independently to the journal Water.

Sincerely

Jesús de los Ríos Mérida.

016-10-16 11:16:50 Editor
→ Abstractaccepted
Editor decision: Approve
Editor comments:

Dear authors,

Could you please add 1-2 sentences in the abstract stating natural wetland area and also the age. In addition, for how many years the natural wetland has been receiving wastewater?

Response: The natural RAMSAR wetland " Laguna de Fuente de Piedra" is an endorreic wetland, has a surface area of 13.5 km^2 and an endorheic basin of 150 km^2 [15]. Since 1996 the treatment plant of the Fuente de Piedra village (Andalusia, Spain) discharges its wastewater into the natural RAMSAR wetland, passing previously through the Laguneto wetland. In order to mitigate wastewater impact in 2005, the Laguneto was restored and a system of canals, water dams and several semi-artifical wetlands were constructed [16] and wastewater can be spilled alternatively through them or directly to the RAMSAR wetland.

Thank you very much.

2016-11-10 23:53:11 Jesús De los Ríos Mérida
→ Manuscript pending approval
New file(s) uploaded (manuscript [pdf], manuscript [doc,docx,zip])
2016-11-11 00:11:32 Jesús De los Ríos Mérida
Authorinformationupdated
2016-11-11 11:46:42 Editor
→ Pendingauthorrevision
Editor decision: Revision
Editor comments:

Thisis a very interesting manuscript. However some minor revisions are necessary. Please see below:

Formatting: please correct page numbers. Your total is 11 pages not 5.

Done

Lines 70-74 please reword. Either add “Constructed” wetlands at the beginning of this paragraph (line 70) as both references refer to constructed wetlands. Or reword to explain that “natural wetlands have long been recognized for their ability to purify wastewater and that in early 1980s constructed wetland technology was developed…”

Response: Rewritten as: Natural wetlands have long been recognized as “natural purifiers of water” systems, providing an effective treatment for many kinds of water pollution leading in the 1980s to the development of constructed wetland technology [12].

Line 80: are these small wetlands constructed wetlands or natural ones?

Response: we stated semi-artificial see Line: 150

Line 89: Results section

You should start with Figure 1 not 2. It is confusing that you refer to Figure 1 on page 8 for the first time.

Response: Figure 1 is mentioned in the Methodology.

According to the ecws-1_ECWSWordTemplate.doc. Methodology is part 4 of the article.

However, looking in the published paper of the journal “Water“ methodology is part 2, located after Introduction and before Results. This is How we think the paper might be published.

Therfore we changed now the methodology part to part 2. Thus figure numbers remain, but the order of citation has been changed.

Also please provide more explanatory captions under Figure 2 (which should be Figure 1 after the revision), such is “Temperature, pH and Conductivity in Ramsar wetland longitudinal profile” (or some thing like that to explain what points A to D are in the Figure captions). Same for Figure 3a (nutrients profile).

Response: After changing the order of methodology, now figure 1 appears before results, and the sampling points A, B, C and D of figure 2, 3, 4 and 5 are familiar to the reader.
Additionaly the x-label “Sampling station” have been included in the figure.
Furthre more the caption of figure 1 has been improved to clarify the water flow direction and sampling sites.
Additionally, according to the recomendation of the editor, the caption of figure 2, 3, 4 and 5 have been changed.

Type of the Paper (Article, Review, Communication, etc.)

Wastewater assimilation by semi-natural wetlands next to the RAMSAR area of Fuente de Piedra (southern Spain)

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Abstract: Urban wastewater treatment is one of the most important challenges in villages of southern Spain. This is especially outstanding in arid and semiarid regions in which wastewater are discharged to temporary streams or wetlands. The natural RAMSAR wetland " Laguna de Fuente de Piedra" is an endorreic wetland, has a surface area of 13.5 km^2 and an endorheic basin of 150 km^2 [15]. Since 1996 the treatment plant of the Fuente de Piedra village (Andalusia, Spain) discharges its wastewater into the natural RAMSAR wetland, passing previously through the Laguneto wetland. In order to mitigate wastewater impact in 2005, the Laguneto was restored and a system of canals, water dams and several semi-artifical wetlands were constructed [16] and wastewater can be spilled alternatively through them or directly to the RAMSAR wetland. In spring 2016, a very dry year, water affluent to Fuente de Piedra was limited to wastewater plant effluents without dilution. In order to study the natural assimilation capacity of the wetland system, four key points were sampled. Physico-chemical and biological indicators were analyzed (temperature, pH, conductivity, total phosphorus, total nitrogen, bacteria, phytoplankton and zooplankton). The results show very high chlorophyll *a* concentration (>500 µg l^{-1}) at the water inlet, which decreased to concentration lower than <20 µg l^{-1} before discharging into the RAMSAR wetland. Total nitrogen and phosphorus concentration was (14 mg l^{-1} and 5 mg l^{-1} respectively) at the wastewater inlet point and decreased in the last wetland (7 mg l^{-1} and 2 mg l^{-1} respectively). Fecal streptococci were highest at the inlet point (1033 ± 351 ufc/100 ml) and decreased to 1 ± 1 ufc/100 ml before entering in the RAMSAR wetland. In contrast, zooplankton, dominated by cladocerans (*Daphnia* sp.) was lowest in the inlet wetland and highest in the last wetland. In conclusion, during the wetlands circuit (i) phytoplankton reduced the total phosphorus and nitrogen concentration, (ii) then phytoplankton is controlled by zooplankton decreasing drastically the input of nutrient and biomass into the RAMSAR wetland. Fecal bacteria decreased three orders of magnitude. Thus, the negative impact from wastewater treatment plant is reduced. The waterbirds, one of the major tourists attractive of this wetland, benefits from food and water supply in dry years, guaranteeing the possibility of bird watching during high season.

Key words: Wastewater; Seminatural wetlands; Natural assimilation capacity; Phytoplankton; Zooplankton; Fecal bacteria; RAMSAR.

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1. Introduction

Cultural eutrophication is an increase in the biological production caused by human activity that incites changes in the community ecosystem succession, as a consequence of the increment of nutrient input into a water body. The cultural eutrophication of freshwater ecosystems worldwide has been recognized as a serious environmental issue for more than half a century [4,10,14,21], and it remains a major water quality problem; constituting a key problem in limnology. In Europe, general concerns about water quality led to the Water Framework Directive [7], which aims for 'good status' for all ground and surface waters (rivers, lakes, transitional waters, and coastal waters) in the EU. Apart of eutrophication, pollutants, and waterborne pathogens associated with suspended sediments are also of particular concern to public health [17,22]. This is especially interesting in the context of wastewater treatment and spilling afterwards of the treated water into natural water bodies. Andalusian water treatment is a challenging task, as during high tourism season habitant might duplicate resident population and water treatment capacity can be exceeded. Thus besides the widely known adverse effects of nutrients enrichment in the ecosystem, it represents also a serious threat to public health [9]. In order to protect the Andalusian wetlands, the Andalusian Wetland Plan was approved in 2002, with the aim to make a complete inventory of Andalusian wetlands and protect them. At this time Fuente de Piedra (Andalusia, Spain) was already protected and recognized as a RAMSAR wetland. The Plan of Arrangement of Natural Resources [5,6] has the objective to protect the natural resources of this area by arrangement and regulation of uses that promotes: (i) the activities compatible with the conservation of such resources, and, (ii) limit the activities that a deterioration of the same ones supposes. Also it is promoted to maintain or improve the quantity and quality of the water resources.

Natural wetlands have long been recognized as “natural purifiers of water” systems, providing an effective treatment for many kinds of water pollution leading in the 1980s to the development of constructed wetland technology [12]. In fact, efficient reduction of large amounts of pollutants (e.g. municipal and certain industrial effluents, mining, agricultural and urban runoff) including organic matter, suspended solids, excess of nutrients, pathogens, metals and other micropollutants have been reported [11]. This pollutants removal is accomplished by the interdependent action of several physical, chemical and biological processes. However, wetlands are generally a sink of dissolved nutrients, as biological activity (photosynthesis) incorporate dissolved nutrients in particulates (phytoplankton) or macrophytes. In our case study, the wastewater treatment plant of the Fuente de Piedra village, located adyacent to the Fuente de Piedra RAMSAR wetland releases the treated water into the RAMSAR wetland. This can occur directly, or passing the water previously through several small semi-artifical wetlands. During the voyage through these small semi-artifical wetlands, the spilled and treated wastewater is usually diluted and contaminants nutrient charge might disminish by biological activity. However, until now, no study of the effect of the small wetlands on the water quality has been carried out. After a dry year, without rain during 2016, the RAMSAR wetland was dry and no water affluent could dilute the spilled wastewater. This condition were optimal to study the effect of biologic processes on the water quality, and four sampling sation were sampled in april 2016 in order to determine the purifying effect of these wetlands in contrast to spilling the wastewater directly in te RAMSAR wetland.

2. Materials and Methods

In order to follow the assimilation capacity of the lagoon system, four sampling points were sampled on 27-29 of april 2016, covering from the entrance of wastewater into the semi-natural wetlands system to the release of the water to the RAMSAR ecosystem "Laguna Fuente de Piedra" (Figure 1). The chosen sampling points correspond to the entrance of wastewater into the first wetland called "Laguneto" (A), the second to the exit of the "Laguneto" (B), allowing the measurement of the assimilation capacity of the first wetland. The third (C) and fourth (D) sampling point were located at the "Los Juncares". The last point is the way by discharge to "Laguna de Fuente de Piedra".

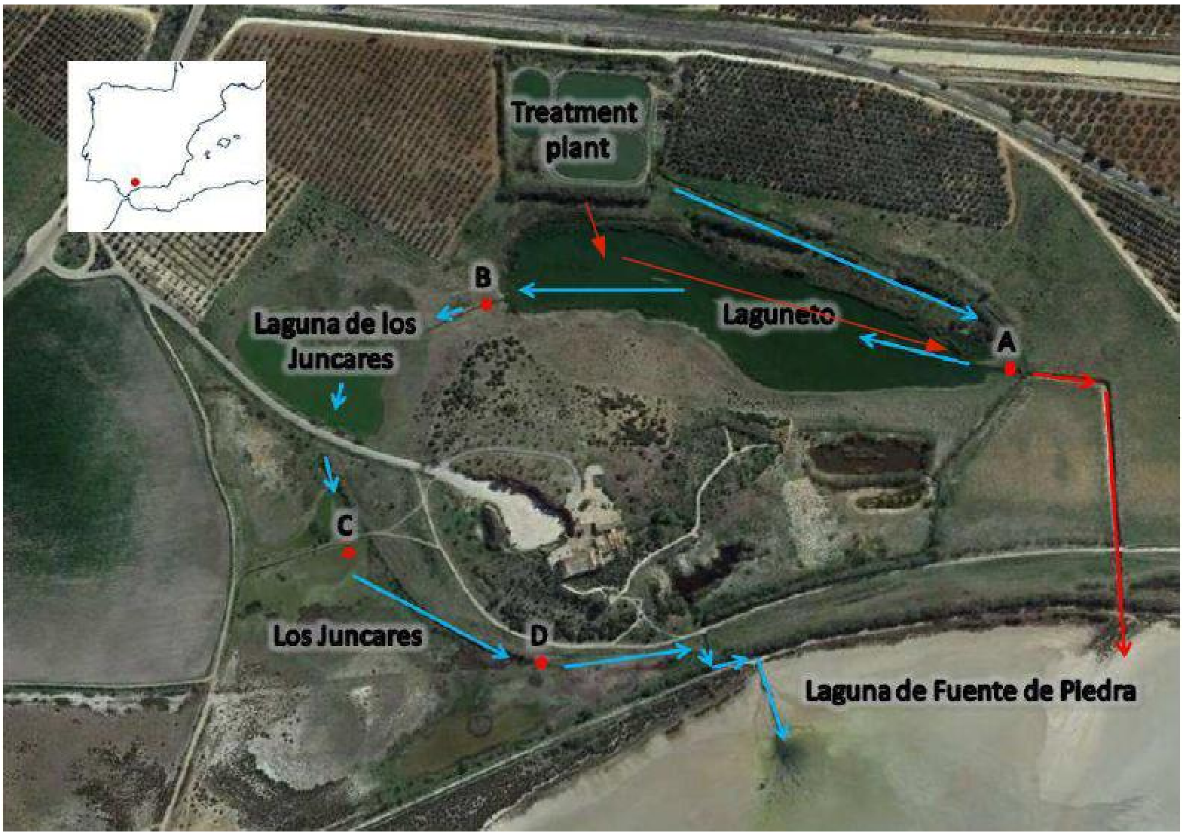


Figure 1. Map, location and water flow through the wetland system (blue arrows), direct to the RAMSAR wetland previous the restoration (red fine arrows), nowadays it is not possible pass through the Laguneto, only by red gross arrows.

Physical data

At each sampling point physical parameters (temperature, conductivity, pH) were measured with a Hanna HI 9829 Multiparameter sensor.

Total Nutrients

Water samples for total nutrient analyses were taken in sterile polyethylene vials and immediately frozen (-20 ºC). Total nitrogen and total phosphorus were analyzed later in the laboratory with a kit analysis Nanocolor 985 083 and Nanocolor 985 076, respectively.

Total Chlorophyll *a* and phytoplankton groups

Total chlorophyll concentration and phytoplankton composition were measured with a submersible fluorospectrometer. The fluorospectrometer discriminated between the main phytoplanktonic groups (i.e. diatoms and dinoflagellates, dinoflagellates, blue-green algae, green algae and cryptophytes) based on the relative fluorescence intensity of chlorophyll a (Chl *a*) at 680 nm, following sequential light excitation by 5 light-emitting diodes (LEDs) emitting at 450, 525, 570, 590 and 610 nm [3,13].

Phytoplankton 15-100 micrometer

For abundance and size estimation of phytoplankton with cell sizes between 15 and 100 µm ESD, 30 ml of each sample was filtered through a 100 µm mesh and passed through the FlowCAM equipped with a 100 µm flow cell and a 100-fold magnification (10x objective). The analysis was performed in autoimage mode, where individual picture of each particle in the vision field is taken. Afterwards phytoplankton abundance and size was estimated by manual reprocessing of the original data fields [20].

Zooplankton biovolumen 250-1000 micrometer.

For abundance and size estimation of zooplankton between 250 and 1000 µm ESD, 2 litres of samples were concentrated in 50 ml by passing the sample through a 45 µm mesh. Then for sample point A and B, 20 ml of the concentrate sample was passed through the flow CAM using a 1000 µm flow cell and 2x amplification. Due to high zooplankton concentration at sample point C and D 50 ml of unconcentrated samples was passed through the FlowCAM. The analysis was performed in autoimage mode, where individual picture of each particle in the vision field is taken. Afterwards zooplankton abundance and size was estimated by manual reprocessing of the original data fields.

Heterotrophic and fecal bacteria

The bacteriological analysis was carried out using the filtration technique with nitrocellulose membranes of 0.45 µm pore size. Membranes were incubated in nutrient agar and incubated at 22 and 37 °C for the determination of heterotrophic bacteria, mFC medium for 24 hours and in m*Enterococcus* medium for 48 hours. Fecal coliforms and fecal streptococci were analyzed according to the APHA 9222-D and APHA 9230-C regulations, respectively.

3. Results

3.1. Abiotic conditions

At the four sampling points the temperature was between 19.5 and 22 °C. Lowest temperature was observed at point A where the wastewater enters into the first small wetland called “Laguneto” and warms up according it passes throuht the wetland system (Figure 2a). pH was lowest at the entrance (point A) and reached its highest value at the exit of “Laguneto” (point B). Then it decreased as it flows towards the RAMSAR wetland “Laguna de Fuente de Piedra” (Figure 2b). Conductivity was between 2500 and 4500 µS cm^{-1}. Conductivity decreased from the entrance (point A) to the exit of “Laguneto” wetland (point B) and increased as it approaches to the RAMSAR wetland (Figure 2c).

Total phosphorus was high (5 mg l^{-1}) at the entrance to the semi-natural wetlands system (point A), then it decreased to values arround 2 mg l^{-1} at point B and C, and finally is relased with 3 mg l^{-1} to the RAMSAR ecosystem (point C, Figure 3a). On the other hand, total nitrogen was highest at the point A with a value of 14.7 mg l^{-1}, and decrease at the exit of “Laguneto” (point C), increasing afterwards to 11.3 mg l^{-1} and maintenance this value along the circuit towars the RAMSAR wetland (Figure 3b).

3.2. Phyto and zooplankton response

Tota chlorophyll *a* (Chl *a*) concentration was very high (arround 500 µg l^{-1}) at the entrance (point A) and exit (point B) of "Laguneto", the first wetland reciving the wastewater. Then it drops to values arround 100 µg l^{-1} at point C and is relased to the RAMSAR wetland with Chl *a* concentration <20 µg l^{-1} (point D, Figure 4). Except for sampling point D, the phytoplankton composition is dominated by green algae, which decreases considerably from point B to point C. Finally at point D bluegreen algae predominate the phytoplankton community (Figure 4).

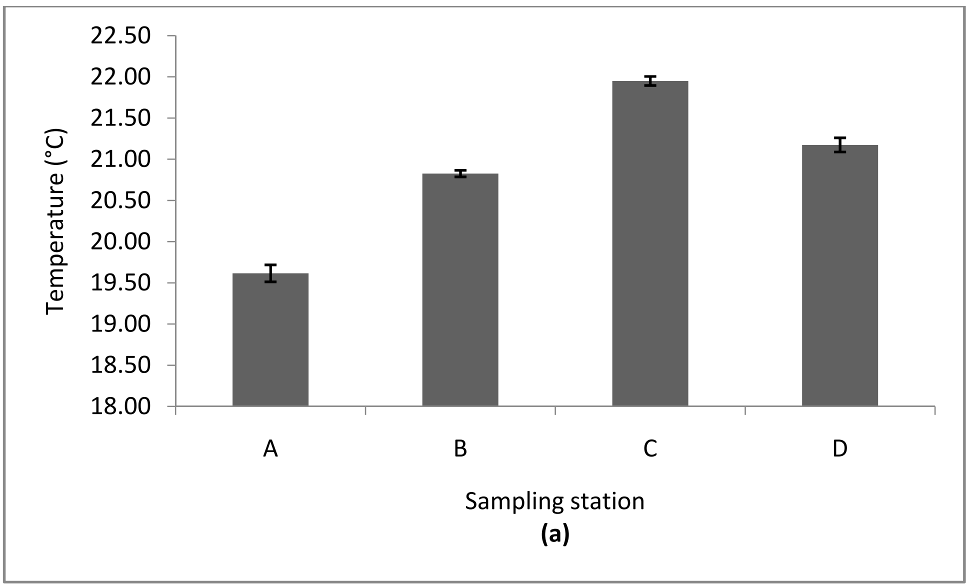
Also phytoplankton biovolumen of cells between 5-100 µm Equivalent Spherical Diameter (ESD), reached highest values at the entrance to the wetland system (point A) (>$5x10^{10}$ µm^3 ml^{-1}) decreasing to concentration arround ($1.5x10^{10}$ µm^3 ml^{-1}) at the exit of the first wetland (point B, Figure 5a). Then phytoplankton biovolume decreased to $4.3x10^9$ µm^3 ml^{-1} at point C and is released with the same value to the RAMSAR wetland (point D, Figure 5a).

Zooplankton biovolume shows an opposite distribution as phytoplankton biovolumen, being lowest ($3.5x10^7$ µm^3 ml^{-1}) at the entrance to the wetland system (point A), increasing slightly ($1.7x10^8$ µm^3 ml^{-1}) at the exit of "Laguneto" wetland (point B). Then zooplankton biovolume increased 15 times to values of $2.6x10^9$ µm^3 ml^{-1} and decreased slightly ($1.6x10^9$ µm^3 ml^{-1}) to point D, before releasing to the RAMSAR wetland. The increase of zooplankton biovolume was due to proliferation of *Daphnia* sp. which dominated the zooplankton community.

3.3. Heterotrophic and fecal bacteria

The total of heterotrophic bacteria, both growth at 22 °C and 37 °C, decreased three orders of magnitude from point A ($1.29x10^5$ and $2.10x10^5$ cfu ml^{-1}, respectively) (Table 1). Abundance of fecal coliforms was highest (655 ± 18 cfu/100 ml) at the exit of Laguneto wetland (point B) being 1 orden of magnitude less abundant at the entrance of wastewater (point A) and the water released to the RAMSAR wetland (point D, Table 1). Fecal streptococci, in contrast, showed highest abundances at the entrance (point A) of the wastewater (1033 ± 351 cfu/100 ml), decreasing three times towards the exit of "Laguneto" wetland (point B). Finally fecal streptococci concentration released to the RAMSAR wetland (point D) was about 1 cfu/100 ml (Table 1).

3.4. Figures and Tables



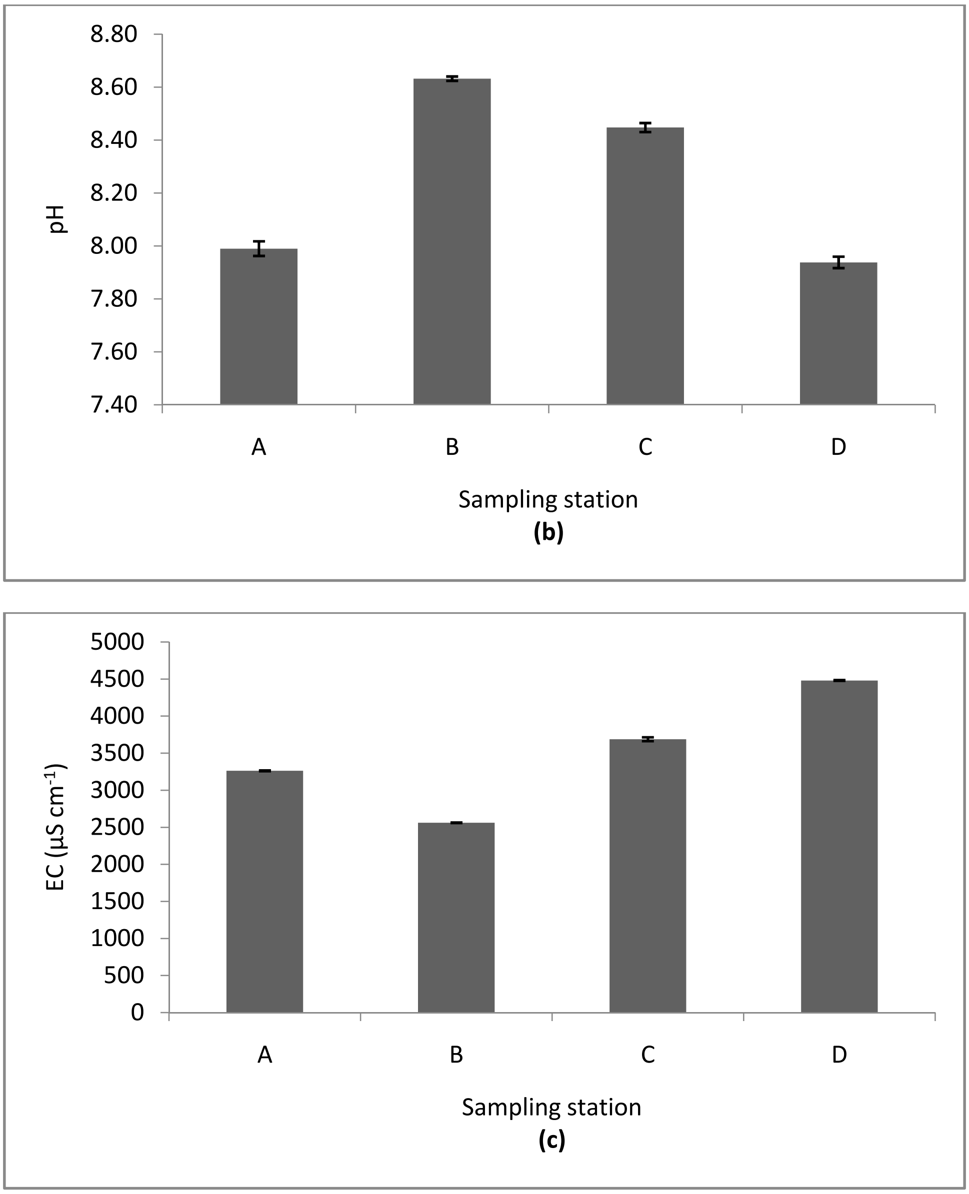


Figure 2. Longitudinal profile through the semi-artificial wetland system: (**a**) Temperature; (**b**) pH; (**c**) Electric conductivity.

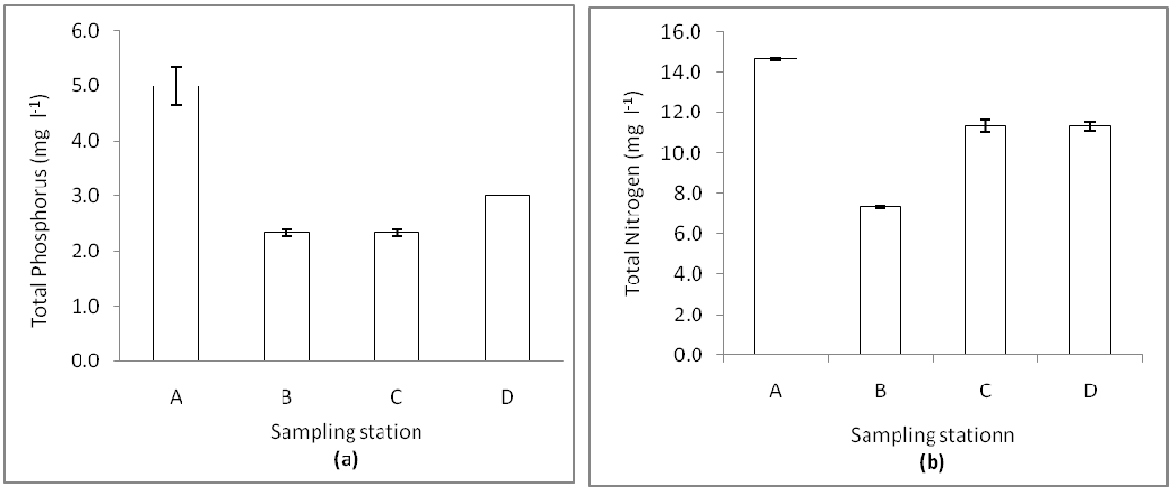


Figure 3. Longitudinal profile of nutrients: (**a**) Total Phosphorus; (**b**) Total Nitrogen.

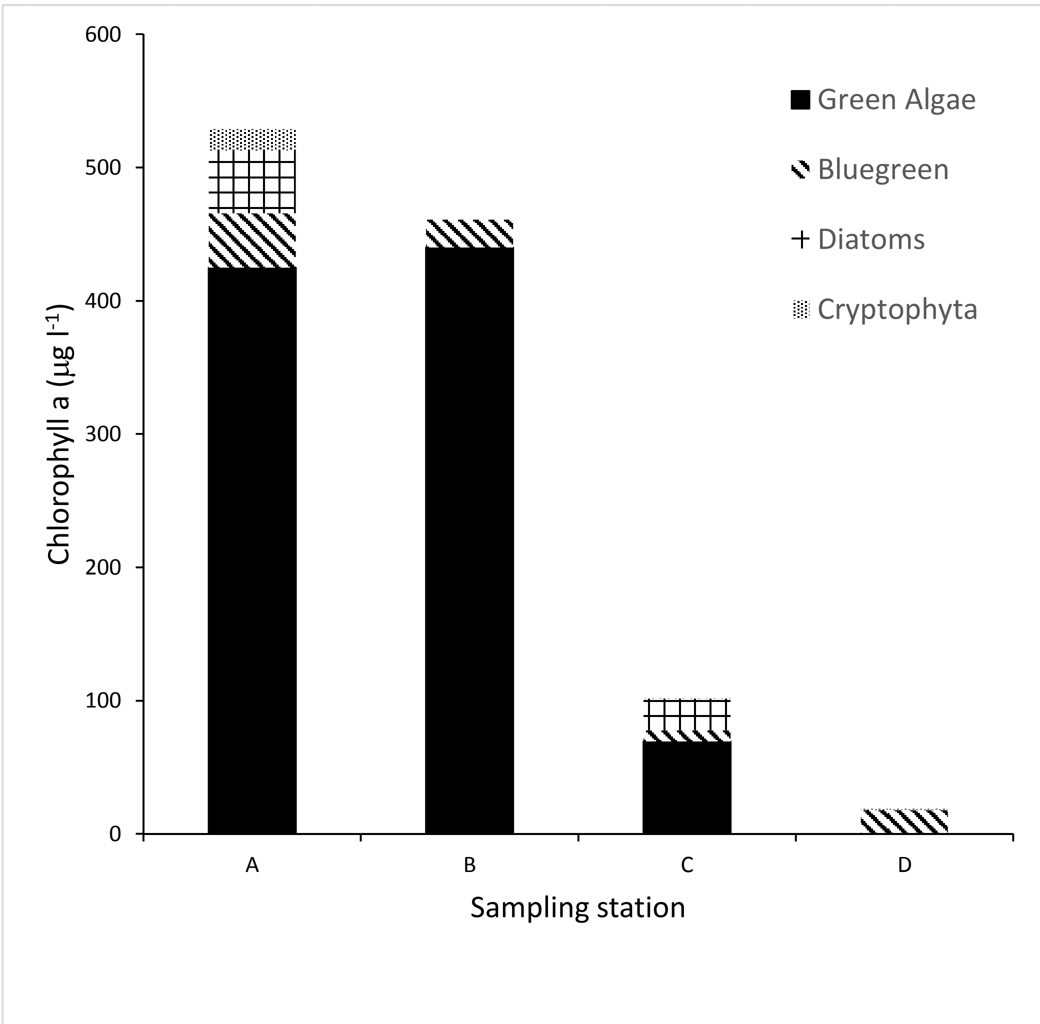


Figure 4. Longitudinal profile of total chlorophyll *a* and relative contribution of groups identificable by fluorescence fingerprints.

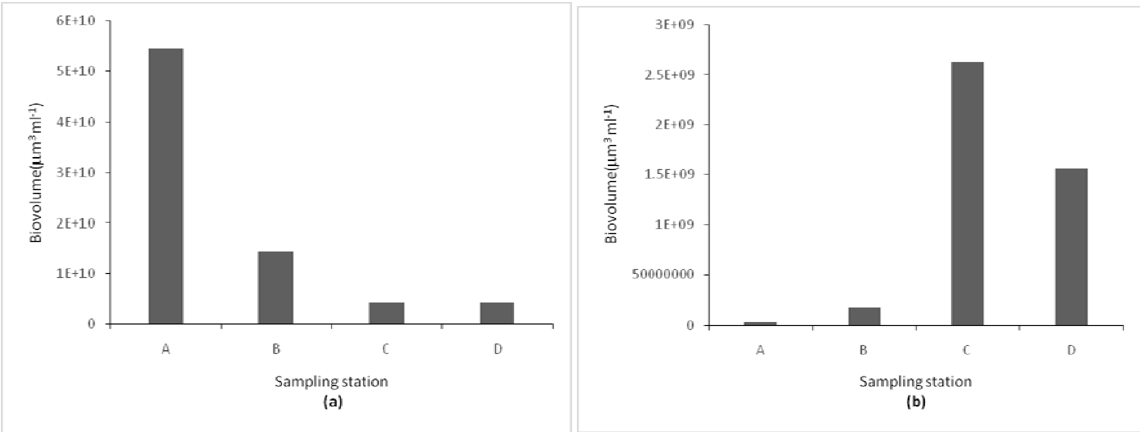


Figure 5. Longitudinal profile of: (**a**) Phytoplankton biovolumen 5-100 μm ESD; (**b**) Zooplankton biovolumen 250-1000 μm ESD.

Table 1. Quantifying colonial-forming units.

Bacteria	A	B	D
Heterotrophic bacteria at 22 °C (cfu ml^{-1})	(1.29 ± 0.60) x 10^5	(2,.10 ± 1.39) x 10^4	388 ± 151
Heterotrophic bacteria at 37 °C (cfu ml^{-1})	(2.39 ± 2.23) x 10^5	(3.18 ± 1.17) x 10^4	247 ± 135
Fecal coliforms (cfu/100 ml)	65 ± 40	655 ± 18	17 ± 21
Fecal streptococci (cfu/100 ml)	1033 ± 351	388 ± 68	1 ± 1

4. Discussion

As consequence of the dry year 2015-16, increasing temperature and conductivity from the spilling point to the releasing point into RAMSAR wetland, indicate that the system acts even in spring 2016 as a concentration basin. Thus the chemical and biological patterns described in the result section are not due to dilution, but only to biological processes acting on the pure spilled wastewater from the treatment plan.

The increasing pH from point A to point B can be explained by elevate phytoplankton biomass and associated primary production, which retire CO_2 from the water column which increases the pH. From point B to C and D, phytoplankton diminished and the primary production-respiration balance decreases, releasing again CO_2 to the water and dimishing the pH.

Through the wetland system total nitrogen and total phosphorus in the water column decreases by 3.4 mg l^{-1} and 2 mg l^{-1}, indicating net removal of nitrogen and phosphorus. According to our results, the wetland system operates in two phases. In the first wetland phytoplankton bloom remove nutrients. Afterwards, during its voyage through the following basins, a zooplankton bloom, dominated by *Daphnia* sp., controls phytoplankton proliferation. With high cleanse rate the dense *Daphnia* population channel the phytoplankton biomass to higher trophic levels (invertebrates and abundant avifauna) and turns the water transparent before it is released to the RAMSAR wetland.

As the spill comes from a wastewater treatment plant, high heterotrophic and fecal bacteria (fecal coliforms and fecal streptococci) were observed at the entrance of the wastewater. Despite the amounts of total heterotrophic bacteria decreased from point A to D, the presence of the elevate fecal bacteria concentration could indicate a incomplete functioning of the treatment plant. According to

[18] bath water requires concentration of fecal bacteria lower than 330 cfu/100 ml. Thus the sampling points A and B are not suitable for swimming or any other water activities.

Fortunately, the "Laguneto" wetland is not accessible to visitors, as it is included in a protected area acting as observation site of avifauna. While fecal sterptococii decreases continuously from the spilling point to the releasing point into the RAMSAR wetland, fecal coliforms increased by factor 10 from point A to B. Part of this pollution could be produced by the birds faeces [1,8]. This wetland has an elevate density of avifauna, among others *Phoenicopterus roseus*, and some gull species (*Larus ridibundus*, *Larus fuscus* and *Larus michahellis*). However, both fecal bacteria decreased to values lower than 200 cfu/100 ml at sampling station D, which is an excellent value for bath water, and shows with concentration of 17 ± 21 and 1 ± 1 cfu/100 ml concentration close to values permitted for drink water (0 cfu/100 ml) [19].

If the wastewater treatment spill would be introduced directly into the RAMSAR wetland (red arrow Figure 1) fecal bacteria would be 60 times times higher than if the wastewater is spilled through the wetland system (blue arrows Figure 1).

Thus, the use of artificial wetlands for the treatment of wastewater with lower costs of installation, operation, and maintenance make them an appropriate alternative to traditional treatment plants [23] or could be used, as in our case, to minimize bad function or temporally overload of treatment plant capacity.

Additionally, in our case, the spilled wastewater garantize small wetlands during dry summers, being an attraction point for birds. In fact, the small wetland system adyacent to the RAMSAR wetland Laguna Fuente de Piedra, include fix bird-watching points with guided observation during high season (summer). The guarantee of wetland and diverse avifauna throughout the year is a keyfactor for local tourism.

5. Conclusions

The wetland system fulfill two functions, (i) improves the water quality of spilled water of the treatment plant, and (ii) provide water during dry years guaranteeing the presence of avifauna, important for local tourism. The obtained results allow us to recommend that this semi-natural or artificial laggons should be extrapolable to other aquatic ecosystems (wetlands) that receive contributions of residual waters. However, it must be said, that a better functioning of the treatment plant would be desirable and improve the conservation of the RAMSAR and adyacent wetland system.

Author Contributions: JMR, AR, MM, MRM and FG sampling design and sampling. MM FlowCAM analysis, JMR, total nutrient analysis, SAA and ST-P heterotrophic bacteria analysis, FG multiparametric data analysis. JRM, AR, MM, SAA, ST-P, MRM and FG interpretation of data and wrote the paper."

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. The acquisition of the FlowCAM by the University of Málaga was co-financed by the 2008–2011 FEDER programme for Scientific-Technique Infrastructure (UNMA08-1E005).

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