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Zooplankton-based reactors for tertiary wastewater treatment: a pilot-scale case study

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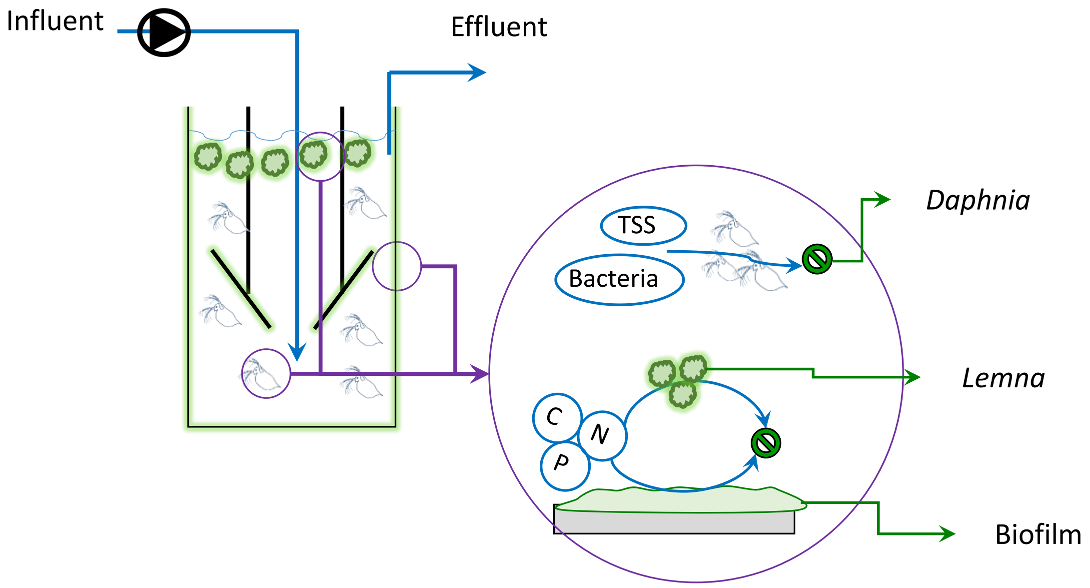
Graphical abstract

Zooplankton-based reactor at pilot-scale for treating secondary wastewater: Assessment as an environmental friendly technology for water reuse. The system is based on the coupling of zooplankton (*Daphnia*), *Lemna* and bacterial/microalgal biofilm.



Zooplankton-based reactors for tertiary wastewater treatment: a pilot-scale case study

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Abstract

Nature-based wastewater treatments are an economic and sustainable alternative to intensive technologies in rural areas, although their efficiency needs to be improved. This study explores technological co-operation between zooplankton (e.g., *Daphnia magna*) and bacterial and algal biofilms in a 1.5 m^3 zooplankton-based reactor for the on-site treatment of secondary urban wastewater. The efficiency of the reactor was evaluated over a 14-month period without any maintenance. The results suggest a low seasonality effect on nutrient polishing (organic matter and nitrogen) and the removal of solids (TSS and turbidity). The best performance, involving a decrease in organic carbon, nitrogen, *E. coli* loads, and solid content was achieved in winter when operating the reactor at 750 L d^{-1}. Under these conditions, the quality of the effluent water was suitable for its reuse for six different purposes in conformance with Spanish legislation. These results demonstrate that the zooplankton-based reactor presented here can be used as an eco-sustainable tertiary treatment to provide water suitable for reuse. On-site research revealed that the robustness of the reactor against temperature and oxygen fluctuations needs to be improved to ensure good performance throughout the year.

Keywords: *Daphnia*; biofilm; decentralized system; nutrient removal; soft treatment; wastewater bioremediation.

1. Introduction

A satisfactory standard of sanitation is still not available for approximately 2,500 million people globally (WHO and UN-Water, 2014), and approximately 40 % of the global population is affected by water shortages (Kummu et al., 2010; Rijsberman, 2006). Intensive wastewater treatments are effective for providing good sanitation in urban areas (e.g., activated sludge treatments), but their high installation and operational costs make them unaffordable for rural areas and developing countries. Low-cost, easy-to-use, nature-based treatment alternatives can play an important role in ameliorating this situation (Langergraber and Muellegger, 2005).

Nature-based depuration has been developed using media filters, lagoons/ponds, aerobic treatments, and wetlands (Hunter et al., 2019; Matamoros et al., 2012a; Stottmeister et al., 2003; Zhang et al., 2019; Zraunig et al., 2019). Although these systems have proved to be effective, their application is still challenging. Media filters and aerobic treatments require maintenance and consume electricity (Garfí et al., 2017). Lagoons and wetlands require large areas of land and present difficulties in meeting the discharge criteria all year around (Lutterbeck et al., 2018; Massoud et al., 2009; Young et al., 2017).

Natural ecosystem depuration is performed by a consortium of bacteria, fungi, algae, plants, microinvertebrates, and annelids among others (Blouin et al., 2013; Calbet and Landry, 2004; Read and Perez-Moreno, 2003). Some studies have also explored the use of animal organisms as filters, such as earthworms (vermifiltration) (Singh et al., 2019) and zooplankton (Serra et al., 2014).

Zooplankton (e.g., *Daphnia magna*) have been found to remove particles that do not settle in secondary clarifiers (Pau et al., 2013). This filtering of solids is associated with the removal of organic matter (Shiny et al., 2005) and pathogens, such as *E. coli* (Serra et al., 2014) and coliforms (Shiny et al., 2005). *D. magna* individuals are sensitive to common contaminants as organic matter (Pous et al., 2020), ammonia (Lyu et al., 2013), ammonium, nitrite (Serra et al., 2019a), and metals (Okamoto et al., 2015) when they are at raw wastewater levels, limiting their application to tertiary treatments (Maceda-Veiga et al., 2015; Matamoros et al., 2012b; Pous et al., 2020; Serra et al., 2014; Serra and Colomer, 2016).

The removal of suspended solids, *E. coli*, and emerging contaminants from secondary wastewater by *D. magna* has been evaluated previously (Matamoros et al., 2012b; Serra et al., 2014; Serra and Colomer, 2016), but no attention has been given to nutrient dynamics. The coupling of zooplankton with bacterial/microalgal biofilms at the laboratory scale has resulted in nutrient polishing and a higher effluent quality (Pous et al., 2020). The results reported by our group regarding the effects of temperature, hydrodynamics, and light on the filtration capacity of *D. magna* (Müller et al., 2018; Serra et al., 2018; Serra et al., 2019b) have been applied to design and operate a pilot-scale zooplankton-based reactor in this work to achieve nutrient elimination and to achieve high solid and pathogen removal rates. The long-term performance of this reactor has been evaluated for the on-site treatment of secondary wastewater with real conditions of varying wastewater supply and changing seasons. The objective of this work is to surpass preliminary laboratory scale, short time frame studies to assess whether zooplankton-based reactors can become a real alternative for the production of reusable water.

2. Materials and methods

2.1. Reactor set up and operation

A cylindrical 1,500 L zooplankton-based reactor was set up as shown in Figure 1 and installed at the wastewater treatment plant of Quart (NE Spain). It has a conical base and contains two flat rectangular plates to increase the internal surface area for bacterial and algal biofilm growth. This configuration also favours the settling of sludge particles. The inflow is located at the centre of the reactor and ensures minimum flow velocities inside the reactor. This design also avoids flow rates higher than 3.5 $mm \cdot s^{-1}$ so as not to affect the performance of *D. magna* (Serra et al., 2018). The outlet runs along the top of the entire cylindrical of the reactor to ensure a gentle water outflow. The positioning of the inlet and outlet allow water flow due to gravity. A passive aeration system was added on day 69 (Figure 1) to avoid anoxia: the inlet pipe was constrained to reduce the fluid pressure and promote air dissolution in the influent wastewater (i.e., the Venturi effect). The design has been registered as a utility model (Salvadó et al., 2019).

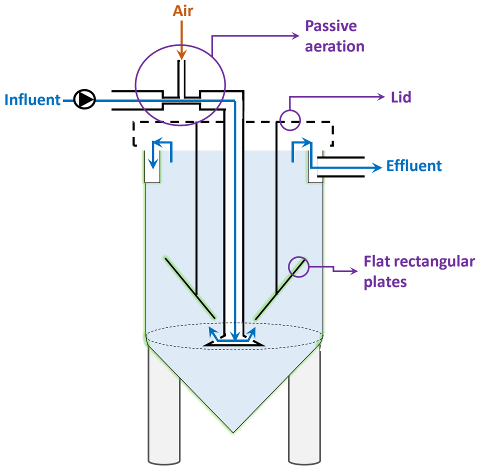

Figure 1. Scheme of the zooplankton-based reactor used in this study.

The reactor was connected to the secondary effluent of Quart's wastewater treatment plant, which had the following characteristics: pH 7.3 ± 0.3, 1387 ± 228 $\mu S\ cm^{-1}$, 68 ± 59 mg COD L^{-1}, 29.5 ± 14.6 mg $N\text{-}NH_4^+\ L^{-1}$, 0.5 ± 0.7 mg $N\text{-}NO_3^-\ L^{-1}$, 0.5 ± 1.0 mg $N\text{-}NO_2^-\ L^{-1}$ (total nitrogen = 30.4 ± 14.3 mg N-TN L^{-1}), 4.4 ± 7.3 mg $P\text{-}PO_4^{3-}\ L^{-1}$, 64 ± 170 mg TSS L^{-1}, and 105 ± 260 NTU.

The reactor was fed with secondary wastewater for 19 days to allow bacterial and algal biofilm growth. Approximately 1,000 *D. magna* individuals from a laboratory aquarium were then added, resulting in a *Daphnia* concentration of 1 individual L^{-1}. The reactor was operated for an experimental period of 412 d from April 2018 to June 2019, without the need for maintenance.

2.2. Zooplankton (*Daphnia*) collection, cultivation, and inoculation

D. magna were collected from Empuriabrava WWTP ponds (Serra et al., 2014), which receive inputs of secondary wastewaters, and were kept for 2 years in 50 L aquariums in the laboratory with a continuous air flow. *Daphnia* were fed twice a week with a mixture of *Spirulina* sp. and yeast, and 1/3 of the water was renewed every 15 d.

2.3. Evaluation of the wastewater flow rate effect

The reactor was designed to operate normally at 1,500 L d^{-1} as a nominal load. This implies a hydraulic retention time (HRT) of 1 d. The reactor was tested with four different flow rates (0, 750, 1,500, and 3,000 L d^{-1}) following the schedule described in Table 1.

Table 1. Wastewater flow rates tested during the experimental period.

Days of operation	**Calendar dates**	**Flow rate** (L d^{-1})
0 – 71	25 April – 5 July 2018	1,500
72 – 145	6 July – 17 Sept. 2018	3,000
146 – 161	18 Sept. – 3 Oct. 2018	0
162 – 216	4 Oct. – 27 Nov. 2018	1,500
217 – 222	28 Nov. – 3 Dec. 2018	750
223 – 232	4 Dec. – 13 Dec. 2018	1,500
233 – 239	14 Dec. – 20 Dec. 2018	3,000
240 – 264	21 Dec. 2018 – 14 Jan. 2019	0
265 – 412	15 Jan. – 11 June 2019	1,500

2.4. Chemical and microbiological analyses

Influent and effluent samples were taken twice a week to measure pH, conductivity, organic matter (COD), nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), phosphates ($P\text{-}PO_4^{3-}$), total suspended solids (TSS), and turbidity in accordance with the American Public Health Association (APHA) standards (APHA, 2005). The reactor was equipped with on-line sensors to monitor the internal dissolved oxygen (OD) (Oxymax COS61D, Endress-Hauser, Germany), turbidity (Turbimax CUS51D, Endress-Hauser), and temperature (Oxymax COS61D, Endress-Hauser).

Organic matter and phosphate removal were calculated by their differences in the influent and effluent. Ammonium removal was calculated as the difference between the influent and effluent ammonium content. Total nitrogen (N-TN) removal was calculated as the total nitrogen ($N\text{-}NH_4^+ + N\text{-}NO_2^- + N\text{-}NO_3^-$) difference between the influent and effluent. Organic matter removal, phosphate removal, ammonium removal, and total nitrogen removal rates were calculated depending on the different HRTs of the reactor.

Samples were taken periodically from the influent and effluent to analyse *E. coli*, coliforms, and *Enterococcus* content (Laboratori Cat-gairin, Girona).

3. Results and discussion

3.1. Survival of *Daphnia* inside the reactor over the entire year

While microalgae and bacteria are adaptable to different environments, zooplankton are more sensitive. The most significant parameters for *D. magna* survival and activity in wastewater are dissolved oxygen, water temperature (Müller et al., 2018), organic matter (Pous et al., 2020), ammonium, nitrite, nitrate, and phosphate (Maceda-Veiga et al., 2015; Serra et al., 2019a). Our first task was to evaluate whether the secondary wastewater characteristics inside the reactor were suitable for the *Daphnia* population. A qualitative summary of possible conditions for *D. magna* stress is shown in Table 2.

The full dataset of these parameters over the entire 412-day experimental period is shown in Table S1.

The maximum filtration capacity of *D. magna* is expected to be found at 20 °C (Burns, 1969; Müller et al., 2018) and significant activity is found in the 11 – 25 °C range (Müller et al., 2018). The filtration activity falls rapidly at temperatures higher than 25 °C, and the survival of *Daphnia* is compromised at 29 °C. When observing temperature data in the reactor (Table S1), it can be seen that temperatures > 25 °C were achieved during July (29.3 ± 1.6 °C), August (28.6 ± 1.1 °C), and September (26.8 ± 1.1 °C). The remainder of the year presented temperatures between 10.4 ± 1.8 °C and 23.5 ± 2.7 °C.

Daphnia, as aerobic organisms, require oxygen to survive. Although dissolved oxygen was detected inside the reactor at the beginning of the experiment, anoxic conditions were found in July. A passive aeration system based on the Venturi principle was installed to improve the performance on day 69 (Figure 1). After this modification, a concentration of 0.5 mg O_2 L^{-1} was maintained in the reactor for the remainder of the experimental period, except during March and April 2019.

Special care is needed in the presence of organic matter, nitrogen, and phosphorus in order to avoid the inhibition of the filtration capacity of *Daphnia* (Maceda-Veiga et al., 2015; Pous et al., 2020; Serra et al., 2019a). As can be seen in Table S1, only ammonium compromised *Daphnia* survival and activity (Serra et al., 2019a) given that concentrations higher than 40 mg N-NH_4^+ L^{-1} were achieved in five separate months (Table S1). The presence of ammonium also suggests a low oxygen concentration in the secondary effluent and inside the zooplankton-based reactor.

In summary, in-reactor conditions over the whole year (Table 2) suggest that *Daphnia* survival and activity were limited in 7 out of 15 months of operation due to high temperatures ≥ 29 °C (July 2018), dissolved oxygen concentrations ≤ 0.5 mgO_2 L^{-1} (June and July 2018 and March and April, 2019), and ammonium concentrations ≥ 40 mg N-NH_4^+ L^{-1} (June and July 2018 and January, February, and June 2019). All three of these stressors were present in June 2018, resulting in a synergistic effect (Buser et al., 2012; Serra et a., 2020). This impact was confirmed by the absence of *Daphnia* inside the reactor in June–July 2018 and June 2019, whereas concentrations of 295 and 899 individuals L^{-1} were found in November 2018 and April 2019. The population of *Daphnia* varied over the year without the need for further inoculation, as under stressed conditions, *Daphnia* produces resting eggs that can hatch when favourable environmental conditions return (Cuenca and Orsini, 2018).

Table 2. Possible stresses for *D. magna* activity and survival. Legend: Green shading indicates absence of *D. magna* stressing conditions and Orange suggests conditions of *D. magna* stress.

Stressors	**2018**									**2019**					
	Apr.	May	Jun.	Jul.	Ago.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.
Temperature (≥ 29 °C)	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Dissolved O_2 ($\leqslant$ 0.5 mg O_2 L^{-1})	🟩	🟩	🟧	🟧	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟧	🟩	🟩
COD (≥ 250 mg COD L^{-1})	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
N-NH_4^+ (≥ 40 mg N-NH_4^+ L^{-1})	🟩	🟩	🟧	🟧	🟩	🟩	🟩	🟩	🟩	🟧	🟧	🟩	🟩	🟩	🟧
N-NO_3^- (≥ 56 mg N-NO_3^- L^{-1})	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
N-NO_2^- (≥ 6 mg N-NO_2^- L^{-1})	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
P-PO_4^{3-} (≥ 50 mg P-PO_4^{3-} L^{-1})	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Possible *D. magna* stress	🟩	🟩	🟧	🟧	🟩	🟩	🟩	🟩	🟩	🟧	🟧	🟧	🟧	🟩	🟧

3.2. Filtering capacity of the zooplankton-based reactor

The zooplankton-based reactor relies on the capacity of *Daphnia* to reduce the amount of solids present in secondary effluents. The dynamics of the total suspended solids and turbidity over the entire experimental study are shown in Figure 2.

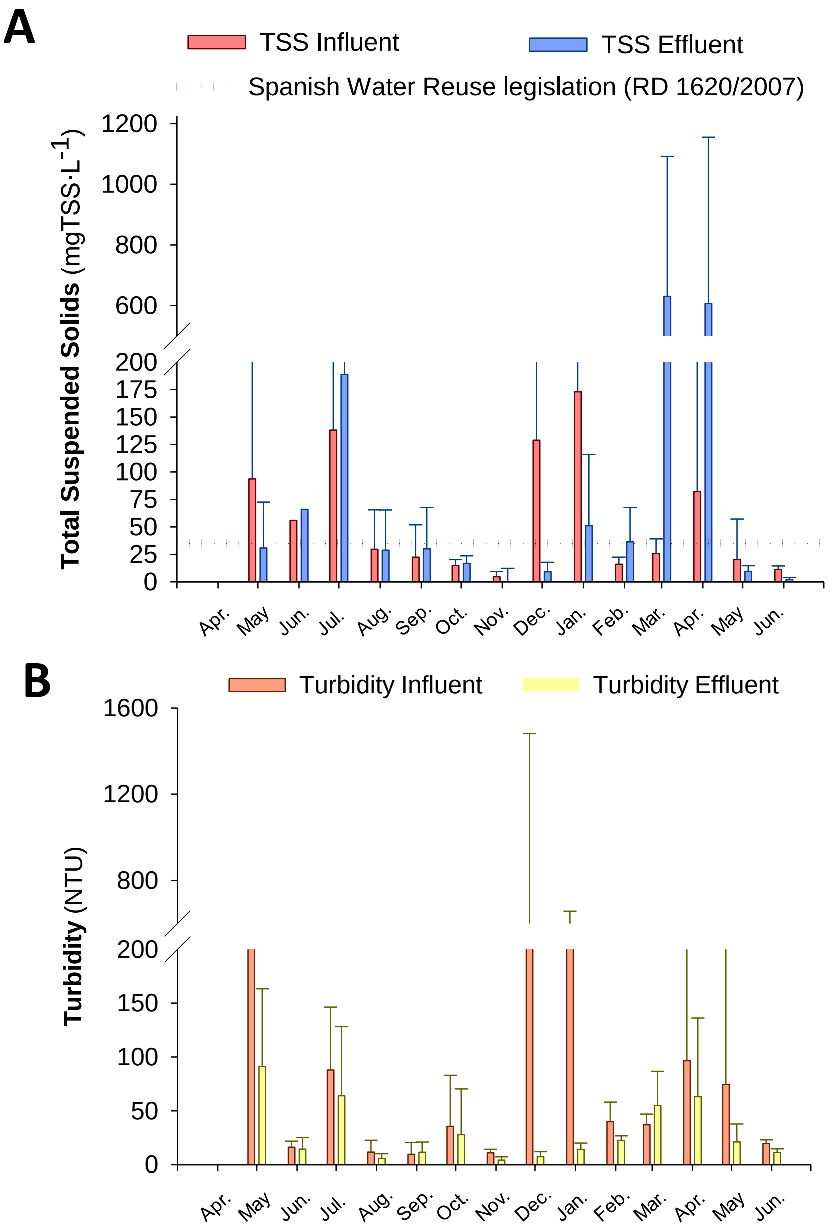

Figure 2. Monthly average data of: A) influent and effluent total suspended solid content (TSS), the dotted line represents the TSS standard for water reuse in the Spanish legislation (RD 1620/2007); and B) turbidity of the influent and the effluent. Error bars show standard deviation (n > 4).

Total suspended solids showed fluctuations over the year (Figure 2A), and the reactor performed better during autumn and winter. The removal of solids was especially noticeable in December 2018 and January 2019, where a higher population of

Daphnia was observed (between 209 and 295 individuals L^{-1}). TSS overflow was detected during February, March, and April 2019, while the evolution of turbidity over these months (Figure 2B) was relatively stable in the effluent, suggesting that the size of the solids leaving the reactor was larger than that detected by turbidity analyses. This may be explained by the uncontrolled growth of *Lemna* (duckweed) in the reactor, which was observed throughout the year.

The effect of the wastewater inflow rate on the effluent quality was evaluated over a year as seasonality is relevant for nature-based technology. Figure 3 shows the results obtained for TSS and turbidity. The system was resilient enough to keep effluent TSS within Spanish regulatory parameters in autumn and winter at all flow rates tested. On the other hand, the content of solids in the effluent was higher than that in the influent at the different flow rates tested in spring and summer (Figure 3), although only slight variations in turbidity were recorded. Turbidity values at all flow rates were acceptable throughout the year, and were especially low in winter.

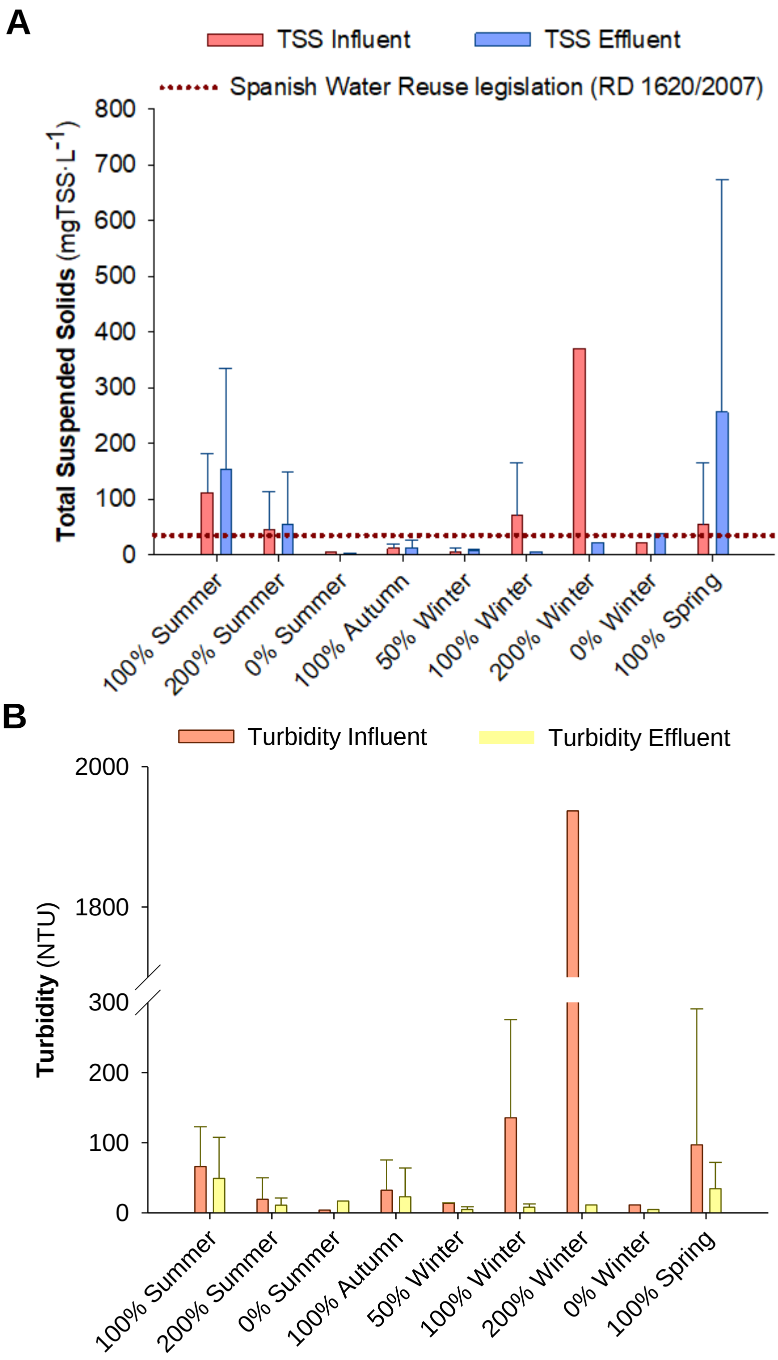

Figure 3. Average data (n > 4) of wastewater flow rate tests: A) total suspended solids (TSS) concentrations at the influent and effluent, the dotted line corresponds to the TSS standard for water reuse in the Spanish legislation; and B) turbidity of the influent and effluent. Error bars show the standard deviation.

The results indicate that the performance of removing solids is more dependent on seasonal changes than on wastewater flow rate. These dependencies could be seen with greater clarity in the case of the outcome obtained from TSS rather than from turbidity. A decrease in turbidity was observed in 12 out of the 14 months tested, whereas a reduction in TSS was observed for 6 months. Both turbidity and TSS refer to particles present in the water column, but turbidity does not include settled solids. In the zooplankton-based reactor, *D. magna* was responsible for the removal of small particles (< 30 µm), which contributed to the turbidity of the water (Pau et al., 2013). The slight variation in turbidity indicates that the filtration activity of *Daphnia* was relatively stable over the whole year, despite the potential stressors (Section 3.1.). Larger particles, such as floccular bacterial aggregates and *Lemna*, in the effluent of the reactor (measured by TSS), were observed over the entire experimental period. *Lemna* can contribute positively to the overall reactor ecosystem because it accumulates nitrogen and phosphorus (Ennabili et al., 2019) and fixes CO_2 derived from COD oxidation (Mohedano et al., 2019). However, the resulting increase in the effluent TSS concentrations gives values that exceed the regulatory limits. It is necessary to control the growth of *Lemna*, the excess of which can be recycled for use as animal feed or as a source of ethanol fuel (Cheng and Stomp, 2009).

3.3. Polishing the nutrient content of the zooplankton-based reactor

The quality of treated wastewater depends not only on the content of solids but also on the concentration of nutrients. By coupling the filtering capacity of *Daphnia* with the nutrient removal capacity of a microalgae/bacterial biofilm in the zooplankton-based reactor, organic matter (Figure 4) and nitrogen polishing (Figure 5) in addition to solids and pathogen removal were achieved, while significant phosphate removal was not detected (Figure S2).

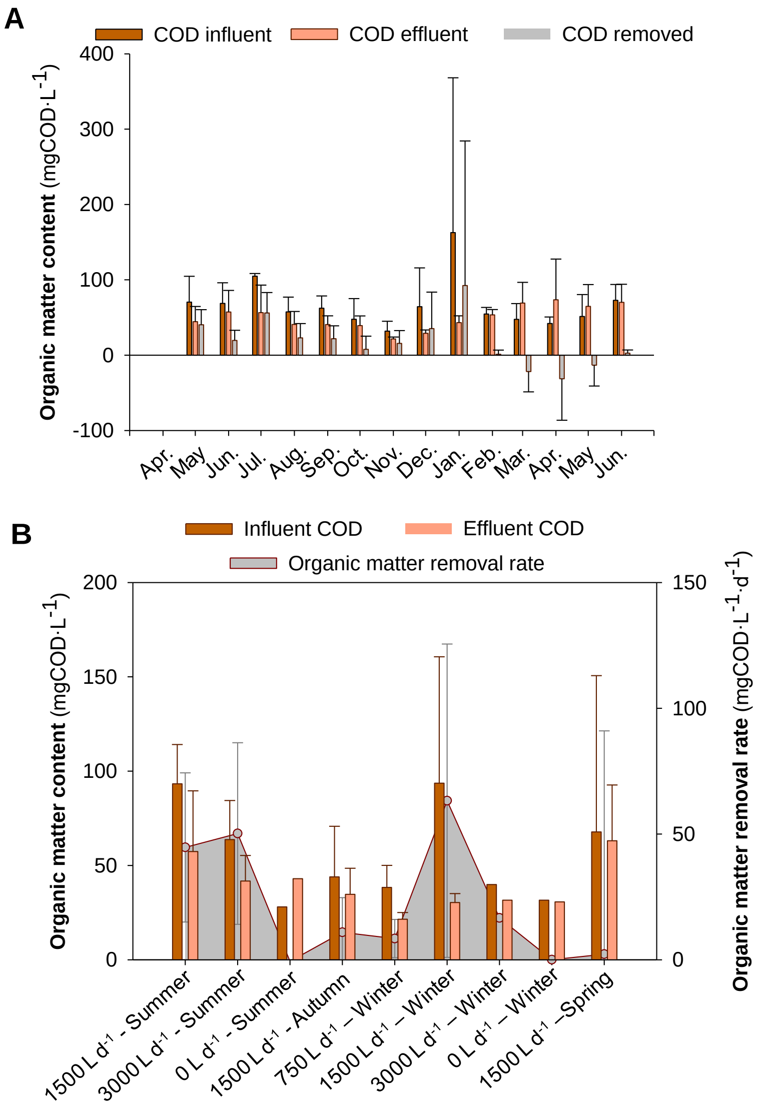

Figure 4. Organic matter removal performance. A) Monthly average data of organic matter (COD) content at the influent and effluent, and COD removed. B) Average data from wastewater flow rate tests on organic matter (COD) content at the influent and effluent, and removal. Error bars show the standard deviation (n > 4).

Similar organic matter removal (Figure 4A) was achieved between May and December 2018, with values fluctuating between 8 ± 17 mg COD L^{-1} (October) and 56 ± 27 mg COD L^{-1} (July). Effluent concentrations varied between 22 ± 3 and 57 ± 36 mg COD L^{-1} (November and July) during this period. A substantial increase in organic matter removal was observed in January 2019 (92 ± 192 mg COD L^{-1}), followed by a change in the reactor performance towards “negative” organic matter removal, indicating that biomass leaving the reactor was confirmed by TSS analyses (Section 3.2.). Seasonal trends were not observed for organic matter removal.

Organic matter removal rates were evaluated at different wastewater flow rates (Figure 4B). In the summer, similar organic matter removal rates (45 ± 29 and 50 ± 36 mg COD L^{-1} d^{-1}) were obtained at 1,500 and 3,000 L d^{-1}, respectively, mimicking previous findings at the lab scale (Pous et al., 2020). The change in flow rates in winter led to variations in the maximum organic matter removal rate being observed from those at 1,500 L d^{-1} (63 ± 62 mg COD L^{-1} d^{-1}), which decreased sharply to 9 ± 7 and 17 ± 0 mg COD L^{-1} d^{-1} at 750 and 3,000 L d^{-1}, respectively. Effluent concentrations in the three tests were similar (between 22 and 32 mg COD L^{-1}), suggesting that the difference in the removal rates was related to differences in the influent COD content (38, 94, and 40 mg COD L^{-1} at 750, 1,500, and 3,000 L d^{-1}). It can be assumed that similar rates would have been obtained at different flows if the influent COD remained constant. COD was not removed in the absence of flow, clearly showing that the reactor oxygen supply depended on the passive aeration system (Figure 1).

Ammonium removal (nitrification and $N\text{-}NH_4^+$ assimilation) and total nitrogen removal (denitrification and nitrogen assimilation) were considered as factors to explain the nitrogen dynamics inside the reactor. As can be seen in Figure 5A, the ammonium and total nitrogen removal trends are different. $N\text{-}NH_4^+$ removal ranged over the year from 0.4 ± 3.3 mg N L^{-1} in January (winter) to 5.1 ± 7.5 mg N L^{-1} in May (spring), and these variations were not considered to be temperature-dependent given that a value of 0.4 ± 3.3 mg N L^{-1} was also obtained in August. The N-TN removal performance shows a trend similar to that found for organic matter. From the beginning of the tests in May until December, nitrogen removal varied from 0.2 ± 0.6 mg N L^{-1} (August) to 3.5 ± 5.9

mg N L^{-1} (October). Average N-NH_4^+ removal in the first period was 2.5 ± 2.7 mg N L^{-1}, while the average N-TN removal was 2.1 ± 2.7 mg N L^{-1}, resulting in an average N-TN/N-NH_4^+ removal ratio of 0.8. This elevated ratio could be interpreted as: i) denitrification being limited by nitrification, or ii) nitrogen being removed by assimilation uptake. The most reasonable hypothesis seems to be the coexistence of both processes due to the presence of bacteria (nitrifiers and denitrifiers), and microalgae and *Lemna* (both of which are nitrogen uptakers) in the reactor.

A sudden change in nitrogen removal from positive to negative values was observed in January, indicating that nitrogen (in this case, nitrate) had been produced inside the reactor. The conjugation of organic matter and nitrogen escaping from the reactor in the month with the lowest temperature (January: 10.4 ± 1.8 °C in the reactor) suggests biomass death (due to low temperatures) or excessive biofilm growth.

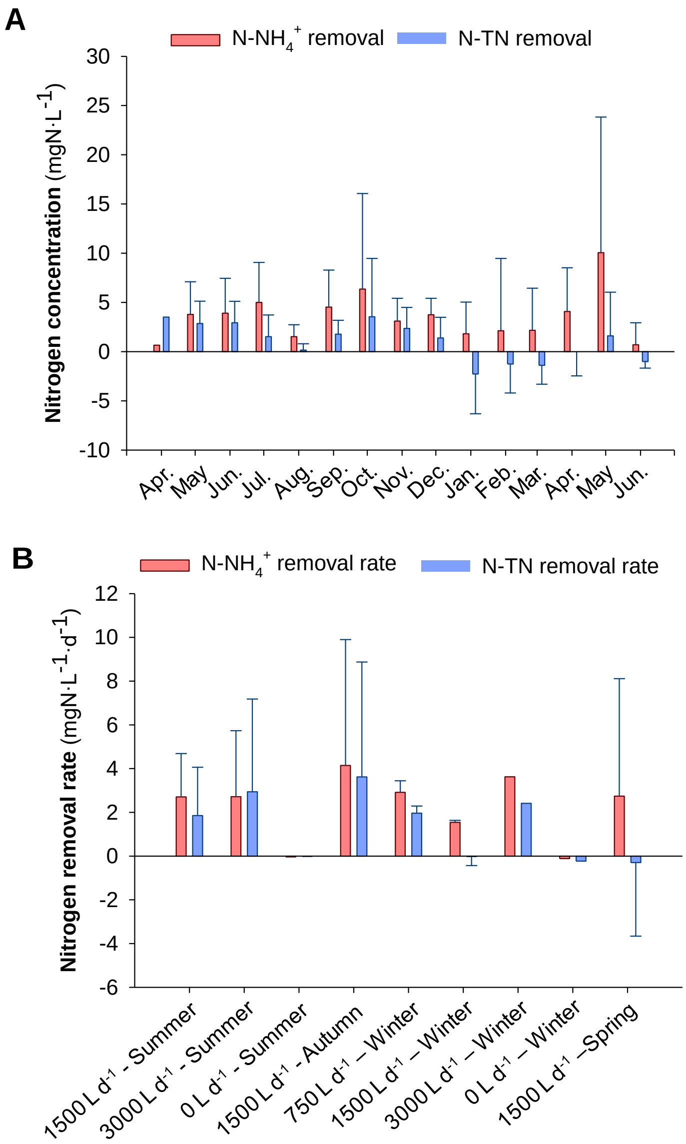

Figure 5. Nitrogen removal performance. A) Monthly average data of ammonium (N-NH_4^+) and total nitrogen (N-TN) removal. B) Average data from wastewater flow rate tests on ammonium (N-NH_4^+) and total nitrogen (N-TN) removal. Error bars show the standard deviation (n > 4).

With regard to the effect of the flow rate on the nitrogen dynamics, it can be observed that similar ammonium and total nitrogen removal rates were recorded in most of the conditions tested (Figure 5B). It can be hypothesised that either the denitrification performance was limited by nitrification or that the system was controlled by nitrogen assimilation, given that ammonium was the only nitrogen species determined in the influent. If nitrification processes are considered, the absence of significant differences in nitrogen removal rates at different flow rates (750, 1,500, and 3,000 L d^{-1}) could be interpreted as a malfunctioning of the passive aeration system. In principle, the design should provide more oxygen to the system when operated at higher flow rates. When the system was stopped (no flow), ammonium was not removed both in the summer (12-day test, with ammonium content increasing from 11.6 to 12.5 mgN-NH_4^+ L^{-1}) and the winter (28-day test, the ammonium concentration increased from 28.5 to 33.4 mgN-NH_4^+ L^{-1}). The lack of aeration does not affect nitrogen assimilation processes, but rather suggests that nitrification has a relevant role in nitrogen dynamics and that the oxygen needed for ammonium oxidation was mostly provided by passive aeration at the inlet rather than oxygen dissolution from the surface of the reactor. Higher ammonium and nitrogen removal rates could be achieved by improving the passive aeration system and by using *Lemna* or microalgae for nitrogen accumulation.

3.4. Suitability of the zooplankton-based reactor for water reuse

The aim of the zooplankton-based reactor was to produce an effluent suitable for reuse as defined by Spanish water reuse legislation (RD 1620/2007), which we used as a reference. This legislation sets different standards for *E. coli*, TSS, turbidity, and nitrogen depending on the end-use, and the reactor performance in attempting to reach these targets is presented in Table 3 (see Table S3 for the full dataset). The results achieved in May 2019 (1,500 L d^{-1}) indicate that the water quality standard was acceptable for the irrigation of crops not aimed at human consumption, forests, and for recreational use in private lakes. Acceptable reuse qualities were achieved since December 2018, although in June 2018, the microbiological standard values were not reached due to poor *E. coli* removal.

Higher flow rates were found to have a significant effect on effluent quality in winter. *E. coli* removal decreased because of a reduction in the concentration of *Daphnia* (295, 209, and 8 individuals L^{-1} at 750, 1,500, and 3,000 L d^{-1}, respectively). The best results were obtained when the system was operated at 750 L d^{-1}. (*E. coli* content of 400 CFU 100 mL^{-1}) and the effluent water could be reused for six different categories defined in the legislation, including agricultural irrigation of food products where water is not in direct contact with the edible product.

The results suggest that higher effluent quality can be attained by operating the reactor at the lowest flow rate. The zooplankton-based reactor requires a low initial input of capital, which is principally related to the reactor itself (< 1,000 €), *Daphnia* (< 5 € to inoculate the current reactor), and a small garden pump (< 150 €). The use of living organisms (i.e., zooplankton, *Lemna*, and bacteria/microalgae biofilm) and the development of a self-sustained ecosystem reduce the need for maintenance and technical assistance while requiring little operational expenditure. In fact, no maintenance was required during the entire experimental period. It is important to maintain appropriate temperature and dissolved oxygen levels inside the reactor. The oxygen provided by the passive aeration system was sufficient to avoid anoxic conditions, but can be optimised to achieve better effluent quality. Burying the reactor underground might be considered as a possible solution to the excessively high summer water temperatures in warmer countries. The adaptive capacity of *Daphnia* also needs to be considered, particularly with regard to temperature, as successive generations of the community may adapt to the specific conditions of the zooplankton-based reactor environment (Yampolsky et al., 2013). Members of the Cladocera order can be found in a wide range of different climates around the world (Forró et al., 2008) and the selection of appropriate species will be critical for the implementation of zooplankton-based reactors in hot countries.

Table 3. Comparison of legal requirements for different water reuse applications and qualities achieved in the reactor. Legend: Red indicates that the standards required were not reached. Green indicates that the standards required were reached; N/A = not applicable.

Uses		**Legal requirements for every use according to RD 1620/2007**					**Condition tested**				
		E. coli (CFU 100 mL^{-1})	**TSS** (mg TSS L^{-1})	**Turbidity** (NTU)	**Total N** (mg N L^{-1})	**N-NO$_3^-$** (mg N L^{-1})	**1,500 L d^{-1} Jun. 18**	**750 L d^{-1} Dec. 18**	**1,500 L d^{-1} Dec. 18**	**3,000 L d^{-1} Dec. 18**	**1,500 L d^{-1} May 19**
Urban	1.1. Residential	0	10	2	N/A	N/A	🟥	🟥	🟥	🟥	🟥
	1.2. Services	200	20	10	N/A	N/A	🟥	🟥	🟥	🟥	🟥
Agriculture	2.12 Direct contact edible parts	100	20	10	N/A	N/A	🟥	🟥	🟥	🟥	🟥
	2.2 No direct contact edible parts	1000	35	N/A	N/A	N/A	🟥	🟩	🟥	🟥	🟥
	2.3. Non-food uses	10000	35	N/A	N/A	N/A	🟥	🟩	🟩	🟥	🟩
Industrial	3.1.a Process water non-food processing	10000	35	15	N/A	N/A	🟥	🟩	🟩	🟥	🟥
	3.1.b Process water food processing	1000	35	N/A	N/A	N/A	🟥	🟩	🟥	🟥	🟥
	3.2. Refrigeration	0	5	1	N/A	N/A	🟥	🟥	🟥	🟥	🟥
Recreational	4.1. Golf	200	20	10	N/A	N/A	🟥	🟥	🟥	🟥	🟥
	4.2. Private lakes	10000	35	N/A	N/A	N/A	🟥	🟩	🟩	🟥	🟩
Environmental	5.1. Aquifers, indirect injection	1000	35	N/A	10	6	🟥	🟥	🟥	🟥	🟥
	5.2. Aquifers, direct injection	0	10	2	10	6	🟥	🟥	🟥	🟥	🟥
	5.3. Forest watering	N/A	35	N/A	N/A	N/A	🟥	🟩	🟩	🟩	🟩

4. Conclusions

A 1,500 L zooplankton-based reactor was designed and operated over a period of 14 months to treat secondary wastewater and to produce an effluent suitable for water reuse. This pilot-scale zooplankton-based reactor proved to be effective not only for the removal of solids and pathogens but also for nutrient polishing. Biologic organic matter and nitrogen removal were successfully promoted by the co-operation between zooplankton, *Lemna*, and bacterial/algal biofilm. The best performance, involving a decrease in organic carbon, nitrogen, *E. coli* loads, and solid content, was achieved in winter when the system was operated at 750 L d^{-1}, and the effluent water met Spanish standards for reuse in agricultural irrigation of food products where water is not in direct contact with the edible products among others.

Further optimisation of the reactor is needed to improve performance over the entire year, especially in summer. This may be achieved through better temperature control, zooplankton community acclimatisation, improved reactor aeration, and by optimising the role of *Lemna* in the reactor. The zooplankton-based reactor presented here is a low-cost eco-sustainable tertiary treatment that is able to provide good effluent qualities for water reuse in communities susceptible to water scarcity, which do not have access to centralised wastewater treatment systems.

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