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Assessment of zooplankton-based eco-sustainable wastewater treatment at laboratory scale

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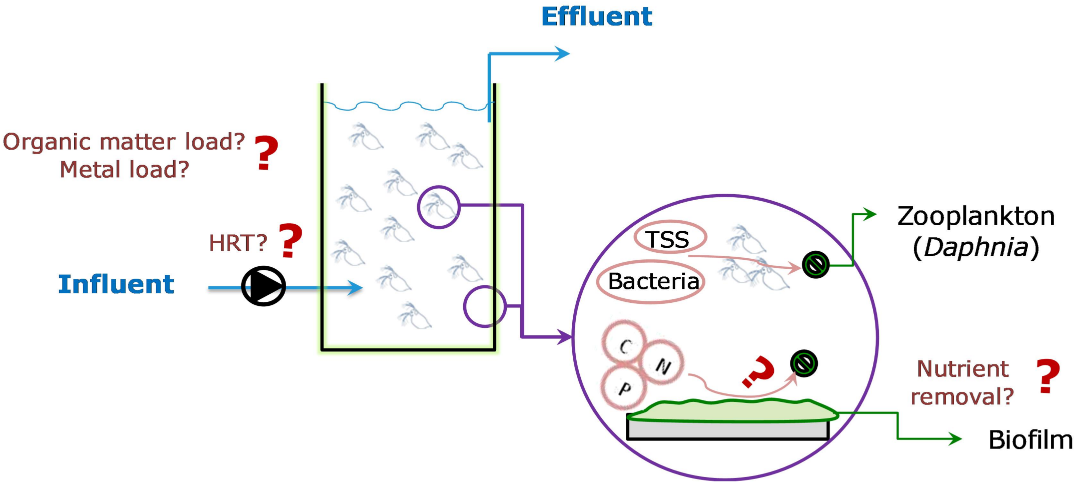
Graphical abstract

Zooplankton-based reactor: i) Evaluation of nutrient removal at different HRTs; ii) Organic matter and metal inhibition tests.



Water impact statement

- Significant nutrient removal (C and N) when operated at HRTs longer than 1.1 days.
- Organic matter concentrations > 250 mg COD L^{-1} inhibit zooplankton.
- Copper overload (380 µg Cu L^{-1}) inhibit nutrient removal.
- Zooplankton-based reactor as alternative for tertiary natural-based treatments.

Assessment of zooplankton-based eco-sustainable wastewater treatment at laboratory scale

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Abstract

The combination of the filtration capacity of zooplankton (e.g. *Daphnia*) with the nutrient removal capacity of bacterial/algal biofilm in a zooplankton-containing reactor could provide a natural-based alternative for wastewater treatment.

A laboratory-scale zooplankton-based reactor was tested at different HRTs resulting in a significant reduction in nutrient concentrations in wastewater when the system was operated at HRTs longer than 1.1 days (preferably of between 2-4 days). However, the presence of high concentrations of organic matter (>250 mg COD L^{-1}) in the wastewater inhibited zooplankton activity, limiting its use to tertiary treatment. Therefore, in combination with other natural treatments that can perform primary and secondary treatments, zooplankton may provide a solution for wastewater clarification and nutrient polishing. The effect of a common metal such as copper on the filtration capacity of Daphnia was also evaluated. *Daphnia*, as well as the whole zooplankton-based reactor, adapted to copper concentrations of up to 70 µg Cu L^{-1} but an overload of 380 µg Cu L^{-1} for two-weeks severely affected the biological system.

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

1. INTRODUCTION

Whereas the implementation of wastewater treatments is widespread in urban areas, rural communities still encounter difficulties in finding appropriate technologies, given the high installation and operational costs that are typically involved in centralized wastewater treatment solutions. In consequence, about 2500 million people still do not have access to improved sanitation (WHO and UN-Water, 2014). Hence, the development of mild, affordable and easy-to-operate systems that are sustainable for small communities is an important issue in the field of wastewater treatment (Langergraber and Muellegger, 2005). Different strategies, such as media filters, lagoons/ponds, aerobic treatments and wetlands, have been proposed (Massoud et al., 2009; Singh et al., 2015). However, media filters and aerobic treatments require regular maintenance and consume electricity and, unlike ponds and wetlands, have a significant environmental impact (Garfí et al., 2017). Innovative, low-maintenance and low-energy input solutions to treat wastewater should preferably be based on natural depuration systems. Although the implementation of lagoons and ponds (Young et al., 2017) and constructed wetlands (Lutterbeck et al., 2018) has grown over time, they still have the disadvantage of requiring large areas of land, sludge accumulation and problems in meeting the criteria for discharge over the whole year (Massoud et al., 2009) and so it is necessary to improve the efficiency of natural-based treatments. In order to achieve this objective, the role of the different living organisms involved in decontamination processes in natural ecosystems should be investigated, as well as the factors affecting these processes. It is known that zooplankton, specifically daphnids, is of fundamental importance in improving the efficiency of water treatment in ponds (Kampf et al. 2007).

Daphnia is a planktonic crustacean belonging to the Cladocera order that is widely used for water ecotoxicology tests (Häder and Erzinger, 2017; Martins et al., 2007;

Harmon and Wiley, 2010). However, this zooplankton can also be exploited for wastewater treatment purposes given that it is a filter feeder organism whose diet is based on ingesting algae and other organic detritus, including protists and bacteria (Ebert, 2005; Gliwicz, 2008). *Daphnia* have been found to be able to remove small particles that are not sufficiently large as to settle on the bed of the secondary systems (diameter < 30 μm) (Pau et al., 2013) and has also been reported to reduce bacterial loads such as *E. coli* (Burnet et al. 2017; Serra et al., 2014) and coliforms (Shiny et al., 2005). These findings suggest that *Daphnia* (i.e. zooplankton) could be used as a clarifier (reducing the suspended solid content) and disinfectant (reducing the bacterial load), and optimization of these processes would make *Daphnia* suitable for tertiary wastewater treatment (Maceda-Veiga et al., 2015; Matamoros et al., 2012; Müller et al., 2018; Serra et al., 2014; Serra and Colomer, 2016). It has also been pointed out that *Daphnia* could contribute to reducing BOD content through the consumption of particulate organic matter (Shiny et al., 2005). However, daphnids are sensitive to other wastewater contaminants such as ammonia (Lyu et al., 2013), ammonium and nitrite (Serra et al., 2019a), organic matter and metals (Okamoto et al., 2015). In consequence, a reactor aimed at treating wastewater using zooplankton would be vulnerable to organic matter and nutrient overloads restricting their implementation to a limited section of the wastewater treatment line. As no evidence has been described so far about the capacity of *Daphnia* to remove nitrogen and phosphorus, the coupling of the zooplankton population with a microalgal/bacterial community that is able to remove the nutrient content may overcome this problem. Despite zooplankton being a microalgal/bacterial predator, filter-feeder organisms have little access to microorganisms attached to the surface as biofilm (Langis et al., 1988) and so, this community can only survive as biofilm inside the reactor. This study aims to evaluate the feasibility of combining the filtration capacity of zooplankton with bacterial and algal biofilms in a reactor for wastewater treatment by investigating: i) the characteristics of wastewater in which the filtration capacity of *Daphnia* is not inhibited by the organic matter content nor the presence of a common wastewater metal (copper); ii) the removal of nutrients in secondary wastewater at different hydraulic retention times (HRTs), and iii) the effect of an overload of a metal (copper) on a zooplankton-based reactor treating secondary wastewater.

2. MATERIALS AND METHODS

2.1. Zooplankton (*Daphnia*) collection and cultivation

Daphnia were collected from Empuriabrava WWTP ponds, receiving inputs of secondary wastewaters, (Serra et al., 2014) and they were kept in 50 L containers in the laboratory with a continuous air flow to prevent anoxia. The *Daphnia* were fed twice a week with a mixture of *Spirulina* sp. and yeast and 1/3 of the water was renewed every 15 days. When a population of zooplankton developed, *Daphnia* individuals with a mean size of around 1.8 mm were collected from the laboratory container with a 1 mm mesh size plankton net. No specific procedure was followed to detect the possibility of an adaptation of *Daphnia* to contaminants (maternal effect), increasing their resistance to toxics (Marshall, 2008).

2.2. Experimental set-up for zooplankton inhibition experiments (organic matter and copper)

Inhibition tests were performed in triplicate in 1L beakers containing 20 *Daphnia* per litre, which is the average *Daphnia* population observed in a previous study carried out at a pilot plant installed at Empuriabrava WWTP (Serra et al., 2014). Inhibition tests had duration of 24 h with a photoperiodic illumination of 16 h of light and 8 h of darkness (Serra et al., 2019b). Tests were performed at a controlled temperature of 20 ± 1 °C.

In order to evaluate the effect of organic matter on zooplankton, synthetic domestic wastewater with different COD concentrations, ranging from 0 (control) to 250 mg COD L^{-1}, was prepared by adding different quantities of CH_3COONa:CH_3CH_2OH: Dehydrated meat extract (DME):milk in a proportion 1.3:1.0:3.7:2.7 to mineral water (Puig et al., 2007). The effect of nutrients such as nitrogen and phosphorus species on the filtration capacity of *Daphnia* has been studied before (Serra et al., 2019a) and was not evaluated here.

Different metals such as copper (10-80 μg Cu L^{-1}) are often present in real wastewater (Cantinho et al., 2016; Hargreaves et al., 2018). Copper is particularly toxic for *Daphnia* with an EC50 of 13 μg Cu L^{-1} (Okamoto et al., 2015). Taking into account these values as well as the standards for copper in the WWTP's receiving bodies (0.18-28 μg Cu L^{-1} (USEPA, 2007)), in the present study we evaluate the effect of Cu(II) on zooplankton filtration capacity at different concentrations ranging from 0 (control) to 25 μg Cu L^{-1} in secondary wastewater containing 20 *Daphnia* L^{-1}.

2.3. Experimental set-up for nutrient removal tests

Biological nutrient removal was evaluated in a 2 L reactor inoculated with 100 *Daphnia*. The reactor was fed with synthetic wastewater that simulated secondary wastewater (133 ± 64 mg COD L^{-1}, 14 ± 5 mg N-NH_4^+ L^{-1} and 7 ± 1 mg P-PO_4^{3-} L^{-1} and *Spirulina* sp.). This composition was selected taking into account the results of the inhibition tests for COD, and based on Serra et al., (2019a) with regards to N and P. In order to evaluate the nutrient removal capacity of the system, the reactor was operated at a hydraulic retention time (HRT) of 3.7 days for 25 days to permit the growth of a bacterial and algal biofilm. During this period the *Daphnia* population was stable at around 100 individuals and size monitoring was avoided so as not to harm the population. After the acclimatization period, the influent water flow rate was sequentially increased to test the reactor performance at different HRTs: 2.1, 1.0 and 0.7 days. The system was operated at each HRT for 14 days.

2.4. Experimental set-up to evaluate the effect of a toxic metal overload (Cu(II)) on nutrient removal

After the tests to evaluate the HRT effect on nutrient removal were performed, the system was operated at the initial conditions (1.1 days HRT) for 14 days with real secondary wastewater, which contained 24 µg Cu L^{-1}, 70 ± 35 mg COD L^{-1}, 16 ± 10 mg $N\text{-}NH_4^+$ L^{-1}, and 6 ± 8 mg $P\text{-}PO_4^{3-}$ L^{-1}.

The effect of an overload of Cu(II) on the removal of nutrients in the zooplankton-based reactor was tested by sequentially increasing Cu(II) concentrations (24, 70, 380 µg Cu L^{-1}) and then decreasing them (70 and 24 µg Cu L^{-1}). The effect of each Cu(II) concentration on the performance of the system was tested over a period of two weeks.

2.5. Analyses

During the inhibition tests, water samples were taken from the reactor to determine organic matter (COD) concentrations, analysed in accordance with the American Public Health Association (APHA) standards (APHA, 2005), and turbidity (Turbidimeter TN-100, Eutech Instruments).

During nutrient removal tests, standard wastewater parameters (pH, conductivity, organic matter (COD), nitrogen (nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), ammonium (N-

NH_4^+)) and phosphates (P-PO_4^{3-})) were monitored twice a week also following the APHA guidelines (APHA, 2005). During the tests performed under copper overload conditions, Cu(II) content was determined by ICP-MS (Agilent 7500c, Santa Clara CA, USA).

3. RESULTS AND DISCUSSION

3.1. Zooplankton inhibition tests

3.1.1. Effect of COD

The effect of COD on *Daphnia*, was tested in triplicate by varying the COD concentration in the vessels from 0 (control) to 250 mg COD L^{-1}, which is the expected value of a low strength urban wastewater (Metcalf and Eddy, 1991). The variations over time of organic matter content, as well as the filtration efficiency in terms of turbidity, are shown in Figure 1. All *Daphnia* individuals died in two of the three replicates when they were exposed to the highest COD concentration (250 mg COD L^{-1}). However, at COD concentrations <160 mg COD L^{-1}, *Daphnia* were not inhibited and a slight reduction in the COD content was found in the different tests (at 50 mg COD L^{-1} no major differences were observed, but at these values the method to determine COD was not sufficiently precise to determine small variations). According to Metcalf and Eddy (1991), a weak load influent urban wastewater contains around 250 mg COD L^{-1} and in the primary treatment only around 30% of the BOD is treated (normally BOD:COD influent ratio is around 0.5). Thus, *Daphnia* cannot directly treat influent and primary wastewater. However, theoretically it is worth noticing that the inhibition of *Daphnia* in the presence of COD could be related to organic matter toxicity itself, oxygen depletion due to heterotrophic growth, or to a combination of both. For example, *Daphnia* has been found to be more or less sensitive to ammonia depending on whether exposure takes place under hypoxia or normoxia (Lyu et al., 2013). In COD removal experiments, it has been observed that oxygen available at the beginning of the test in the vessels (saturation of 9.1 mg O_2 L^{-1} at 20ºC) was not able to remove all the COD during the full duration of the test (59 ± 9, 64 ± 13 and 101 ± 11 mg COD L^{-1} removed in 130, 160 and 250 mg COD L^{-1} tests, respectively). Vessels were not mechanically aerated but were rather open to the air and so additional oxygen will have been supplied through diffusion from the upper layer. Taking into account that microorganisms can grow on the upper layer of the vessel, an oxygen barrier may have been created that harmed the survival of the daphnids. Moreover, an excessive growth of heterotrophic bacteria could also be the source of toxins that affected *Daphnia* (Rodriguez da Silva, et al, 2004). Thus, there are different reasons why daphnids could be inhibited by the presence of COD concentrations higher than 160 mg COD L^{-1} (organic matter itself, hypoxia, or toxins generated from eutrophication), which indicates that a reactor containing daphnids should be externally aerated when the wastewater contains > 160 mg COD L^{-1}. However, in order to decide which aeration strategy should be followed, we need to consider that background turbulences higher than 1 cm^2 s^{-3} have a negative impact on mobility and the filtration capacity of *Daphnia* individuals (Serra et al., 2019c). Thus, if applied, aeration should be as gentle as possible in order to reduce the generation of turbulence.

Daphnia is known to remove particulate matter (Evers and Kooijman, 1988), and can also reduce the particulate BOD content (Shiny et al., 2005). However, it should be taken into account that synthetic COD was used for this specific inhibition study, which reduces the amount of particulate organic matter. In fact, an elapsed lag time can be observed between the beginning of the inhibition tests and the observation of COD removal, suggesting that organic matter was mostly removed by a heterotrophic microorganism that grew during the test. Nevertheless, it cannot be discarded that *Daphnia* activity may contribute to COD removal when real wastewater is used, since it is expected that more particulate organic matter will be available.

The results of the inhibition tests suggest that COD values higher than 160 mg COD L^{-1} in influent wastewater affect *Daphnia* and that *Daphnia* can complete the wastewater treatment after a secondary treatment, where COD discharge limits are usually below 100 mg COD L^{-1}.

3.1.2. Effect of copper (Cu(II))

The presence of different metals has been detected in some wastewater influents (Hargreaves et al., 2018). Among these metals, copper may represent a significant threat for the use of *Daphnia* in wastewater treatment, since: i) it is commonly found in urban wastewaters (Hargreaves et al., 2018), ii) it tends to be is adsorbed on sludge (Cantinho et al., 2016), which is the *Daphnia* substrate in wastewater, and iii) it is one of the most toxic metals for pure *Daphnia* cultures (EC50 of 13 µg Cu L^{-1}) (Okamoto et al., 2015). In urban wastewater influents, copper can generally be found at levels between 10 and 80 µg Cu L^{-1} in the influent, of which around 30 to 80 % can be removed in primary and secondary treatments (Cantinho et al., 2016; Hargreaves et al., 2018). Standards for copper in freshwater (WWTP receiving body) range between 0.18 and 28 µg Cu L^{-1} (US-EPA, 2007). Taking into account these values, the effect of copper on the *Daphnia* filtration capacity was studied from 0 (control) to 25 µg Cu L^{-1} over a 24 h period. The evolution of copper content and its effect on filtration efficiency (turbidity variation) are shown in Figure 2. At 2 hours a slight decrease in copper concentration was observed before stabilizing. Given that the short time period would not seem to not allow for the possible intervention of bacteria growth, this removal was considered to be linked to the adsorption of Cu(II) ions on particles that were ingested by *Daphnia* (Zhao et al., 2009; Cantinho et al., 2016). In terms of turbidity, no major differences were detected at the end of the experiment when copper was added and mortality was not observed in these 24 h of exposure in any of the copper concentrations tested. These results differ from those of Okamoto et al. (2015), who reported an EC50 of 13 µg Cu L^{-1}. However, it should be taken into account that the *Daphnia* used in this study were collected in the polishing ponds of Empuriabrava WWTP (Serra et al., 2014), while Okamoto et al. (2015) used a single genetic stock of *Daphnia magna*.

In order to find out if this effect continues over time we extended the period of exposure to 168 hours (Figure 3). Similar turbidity was found in all tests and no mortality was observed, indicating that the presence of Cu(II) at concentrations equal to or lower than 25 µg Cu L^{-1}did not affect the filtration capacity of *Daphnia*. Different studies have shown that daphnids exposed to a range of copper exposure levels acclimated well and their offspring became more resistant to copper than pure *Daphnia* (Bossuyt and Janssen, 2004; Zhao et al., 2009). In the present study the *Daphnia* population were highly resistant to copper, presumably due to having been collected in a polishing pond that was exposed to this metal.

3.2. Nutrient removal in a zooplankton-based reactor - Effect of HRT

The zooplankton-based reactor (section 2.3) was operated with synthetic secondary wastewater under continuous flow mode at an HRT of 3.7 days until reaching steady-state conditions (11 days). During this period, the zooplankton population evolved and a bacterial/algal biofilm grew on the reactor surface. The capacity of the resulting system, consisting of daphnids and bacterial/algal biofilm, to remove organic matter and nitrogen and phosphorus species was then evaluated at different HRTs (2.1, 1.0 and 0.7

days), operating the system at each HRT for 14 days. The results obtained under continuous flow mode are presented in Figure 4.

The organic matter (COD) removed was mostly constant over the different HRTs tested (Figure 4A). The highest COD removal percentage, 89 ± 2 % (39 ± 13 mg COD L^{-1} d^{-1}), was obtained at an HRT of 3.7 days. When the HRT was decreased to 0.7 days, COD removal decreased slightly (74 ± 3 %). Nevertheless, this meant that from 3.7 to 0.7 days HRT, the COD removal rate increased from 39 ± 13 mg COD L^{-1} d^{-1} to 261 ± 98 mg COD L^{-1} d^{-1}. The increase in the organic matter removal rate at lower HRTs indicates that the microorganisms responsible for this removal, whether microalgae or bacteria, benefited at higher water fluxes. In contrast to a system with suspended biomass, where the decrease in the HRT would have led to lower performances due to biomass wash-out. However, in the system proposed here, biomass had difficulty in growing in the bulk liquid because of the presence of a predator (zooplankton). Nevertheless, biomass was able to find protection against zooplankton on the reactor surface and could grow as biofilm (Langis et al., 1988), which can have a positive effect at low HRTs (Liu et al., 2002).

In the case of phosphate (Figure 4B), the removal performance was low at around 12 % with an HRT of 3.7 days, but no P removal was observed at lower HRTs.

The nitrogen removal performance (Figure 4C) was more affected by changes in the HRT. A total nitrogen removal percentage of 45 ± 13 % (8.8 ± 3.8 mg N L^{-1}) was observed at an HRT of 3.7 days, giving a total nitrogen removal rate of 2.3 ± 1.0 mg N L^{-1} d^{-1}. However, in percentage terms, nitrogen removal was lower at higher water-flow rates (7 ± 4 % at 0.7 days HRT) although the total nitrogen removal rates at 0.7 and 3.7 days were similar (1.9 ± 0.5 and 2.3 ± 1.0 mg N L^{-1} d^{-1}, respectively), suggesting that the nitrogen removal activity was independent of the HRT. These results are different with respect to the behaviour of organic matter removal, where an increase in the removal rate at lower HRTs has been observed.

For a deep understanding of the nitrogen removal in the reactor, which is based on two processes, nitrification and denitrification, the variation of the different nitrogen species over time was studied (Figure 4D). Nitrogen speciation revealed two different general trends. On the one hand, it was found that ammonium removal decreased from 12.6 ± 3.9 to 1.4 ± 0.3 mg N-NH_4^+ L^{-1} when the HRT was lowered from 3.7 to 0.7 days. However, it was observed that the modification of the HRT did not affect the rate of removal of ammonium (3.3 ± 1.0 and 2.4 ± 0.5 mg N-NH_4^+ L^{-1} d^{-1} at 3.7 and 0.7 days, respectively). In consequence, the ammonium content in the effluent increased at lower HRTs. Hence, similar rates at different HRTs indicate that the ammonium removal activity inside the reactor was constant over the whole period. In the case of organic matter oxidation, the decrease in the HRT benefited the process although no difference was observed for the removal of ammonium. In principle, the increase in the water flux should have provided a better substrate (organic matter and ammonium) and electron acceptor (oxygen) mass transfer. However, only heterotrophic microorganisms were found to benefit. One explanation could be the existence of competition for the electron acceptor (oxygen). Heterotrophic microorganisms have a faster growth rate (2 - 6 d^{-1} at 20 ºC) than autotrophic organisms (1 d^{-1} at 20 ºC) (Gujer et al., 1999, Henze et al., 1987) and, hence, have a competitive advantage when a change in environmental conditions takes place.

On the other hand, with regards to the denitrification process, an accumulation of both nitrates and nitrites was observed at high HRTs. For example, at an HRT of 3.7 days, 12.6 ± 3.9 mg N-NH_4^+ L^{-1} was removed, but only 70 % of this was denitrified, hence, the concentration of oxidized forms of ammonium (NO_3^- + NO_2^- = NO_x^-) was of

3.8 ± 3.3 mg $N\text{-}NO_x^-$ L^{-1} at the effluent of the reactor. At the lowest HRT (0.7 days), 1.4 ± 0.3 mg $N\text{-}NH_4^+$ L^{-1} was removed and, in this case 79 % was denitrified (0.3 ± 0.1 mg $N\text{-}NO_x^-$ L^{-1} at the effluent). At higher HRTs, despite nitrate and nitrite being available for denitrification, this process was impeded by the lack of an electron donor (organic matter). On the other hand, at lower HRTs, the denitrification process was limited by substrate availability (nitrates and nitrites) due to poor nitrification performance.

In conclusion, the zooplankton-based reactor presented a significant removal of COD (89 %) and nitrogen (45 %) at an HRT of 3.7 days, with low phosphate removal (12 %). Decreasing the HRT to 1.1 days resulted in a slight decrease in COD removal (74 %) and nitrogen removal decreased to 20 %. At even lower HRTs, the levels of nitrogen removal progressively decreased. Therefore, it is seen that a reactor containing bacterial/algal biofilm and zooplankton operating at HRTs of between 2-4 days is able to remove the organic matter and nitrogen content of secondary wastewaters.

3.3. Nutrient removal in a zooplankton-based reactor - effect of a Cu(II) overload

In order to further evaluate the possible effects of copper in a zooplankton-based reactor, an overload of copper was induced by adding increasing concentrations of this metal in a system operated with an HRT of 1.1 days. The reactor was initially fed with real secondary wastewater which contained 24 μg Cu L^{-1} before being increased to 70 μg Cu L^{-1}, and then finally 380 μg Cu L^{-1}. After that, the concentration was again reduced to 70 μg Cu L^{-1} and then to the starting concentration. Each concentration was tested over two weeks. Results in terms of copper, organic matter, nitrogen and phosphate dynamics during the different tests are presented in Figure 5.

The dynamics of copper inside the zooplankton-based reactor followed the results observed in inhibition tests (Figures 2 and 3), where copper removal was observed in the reactor. When the reactor was operated under continuous-flow mode, a copper removal average ranging from 65 to 71 % was achieved when the copper concentration in the influent was increased to 380 µg Cu L^{-1} although it should be noted that the standard deviations were relatively high (Fig. 5 A). At this maximum concentration, most of the daphnids died during the test and the microalgal/bacterial biofilm also disappeared. However, when 70 µg Cu L^{-1} was tested for a second time for two weeks, the average of copper removal percentage decreased to 26% although this value has a high standard deviation. These results can be explained by the fact that a fraction of Cu(II) was being removed from the liquid phase by either uptake by the zooplankton and accumulation inside the biofilm. The removal of Cu by zooplankton have been reported to occur via the ingestion of small organic particles where copper was attached (Cantinho et al., 2016). Copper would then be accumulated by *Daphnia*, which will have a multi-generation effect through the maternal transfer of metals to the offspring improving the metal tolerance in the next generation (Zhao et al., 2009; Araujo et al., 2019; Pérez and Hoang, 2018).

The copper overload not only affected zooplankton survival but also the overall performance in removing nutrients. In terms of organic matter, COD removals of 50 and 58 % were observed at the first tests with 24 and 70 µg Cu L^{-1}, respectively. When the Cu increased to 380 µg Cu L^{-1}, the COD removal decreased to 35 %, and did not recover during the second test at 70 µg Cu L^{-1} (42 % COD removal). However, when the system was set at the initial conditions of 24 µg Cu L^{-1}, a massive increase in the effluent COD was observed, which might be indicative of considerable biomass degradation as a result of both zooplankton and bacterial/microalgal biofilm death.

With respect to nitrogen removal performance, significant variations were observed. At 24 µg Cu L^{-1}, the nitrogen removal was 6.6 ± 16.9 mg N L^{-1}. As the Cu concentration was increased to 70 µg Cu L^{-1}, the nitrogen removal remained stable at 6.2 ± 6.7 mg N L^{-1}. However, as the copper content was further increased to 380 µg Cu L^{-1}, the nitrogen removal depleted to 2.0 ± 7.2 mg N L^{-1}, suggesting that the nitrifying/denitrifying and/or nitrogen assimilator community was severely inhibited at high Cu concentrations (380 µg Cu L^{-1}). Confirmation of this was obtained on the nitrogen removal decreasing to zero in the following experiment when the copper content was set at 70 µg Cu L^{-1}. Copper inhibition of the nitrogen cycle has been reported in different processes, such as nitrification (Premi and Cornfield, 1969), denitrification (Sakadevan et al., 1999) and Anammox (Zhang et al., 2019). Hence, it is logical to conclude that the presence of copper had a severe effect on the nitrogen removal community in the reactor. When the copper concentration was returned to the starting conditions (24 µg Cu L^{-1}), the nitrogen removal suddenly increased to 91 %, which can be explained by the massive increase in organic matter available due to the dead biomass.

Finally, it is difficult to discern the effect of Cu(II) overload on phosphorus removal given that the error in the phosphorus measurements are too large to evaluate the variations.

4. CONCLUSIONS

Wastewater treatment based on the combination of the filtration capacity of zooplankton (e.g. *Daphnia*) with the nutrient removal capacity of bacterial/algal biofilm in a zooplankton-containing reactor could provide good efficiency as a tertiary system. This type of natural-based system is an interesting alternative for isolated communities given its low operation and installation costs and the little maintenance required. In order to use zooplankton for this function, a previous treatment should be made as high concentrations of organic matter (> 250 mg COD L^{-1}) can inhibit their feeding activity due to either the organic matter itself or hypoxia generated from heterotrophic growth. Therefore, this type of system can suitably be applied downstream of a primary/secondary treatment.

The results presented here indicate that this system allows a significant reduction in nutrient concentrations in wastewaters when it is operated at HRTs longer than 1.1 days (preferably of between two to four days).Another important point evaluated here was the effect of a common metal such as copper on the overall system. *Daphnia*, as well as the whole zooplankton-based reactor, adapted to Cu concentrations of up to 70 µg Cu L^{-1}. However, a Cu(II) overload (380 µg Cu L^{-1}) for two weeks severely affected the whole system (zooplankton and microalgal/bacterial biofilm).

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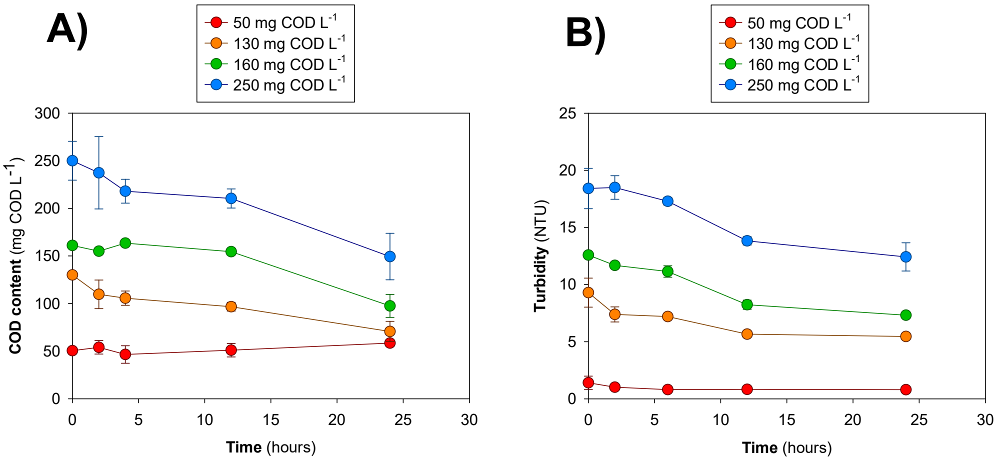

Figure 1. COD inhibition tests over *Daphnia* in vessels. A) Evolution of COD concentration over time. B) Evolution of turbidity over time.

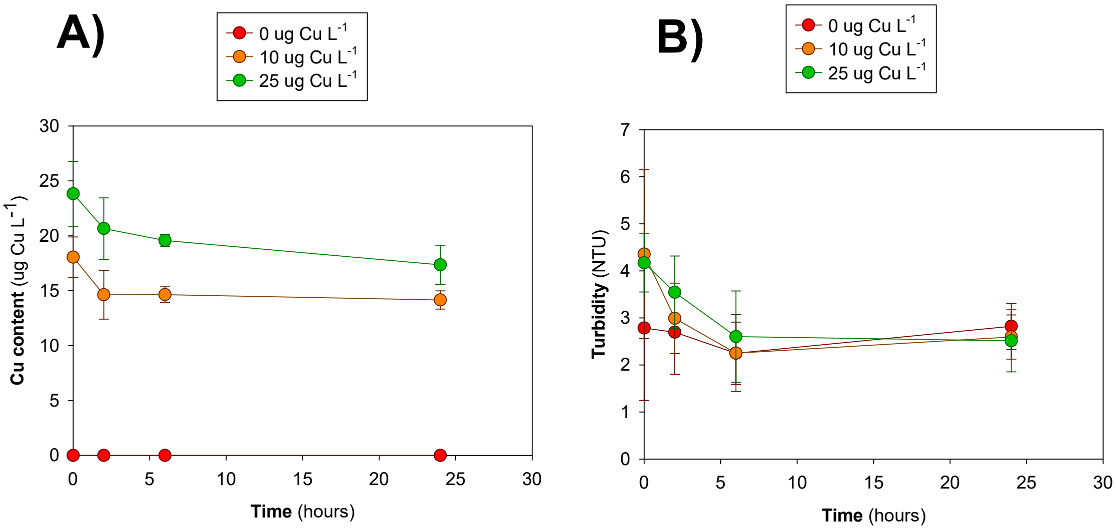

Figure 2. Cu(II) inhibition tests over *Daphnia* in vessels. A) Evolution of Cu concentration over time. B) Evolution of turbidity over time.

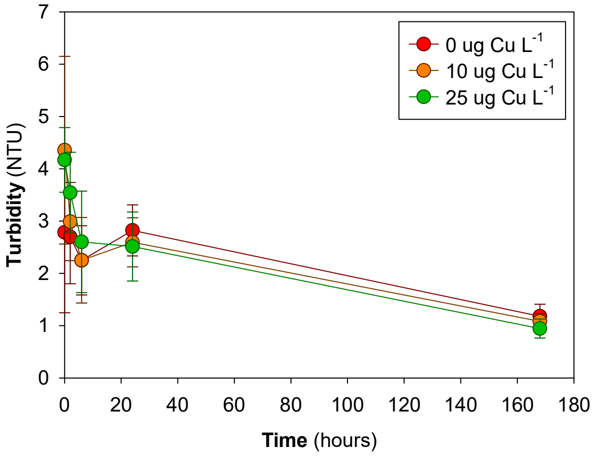

Figure 3. Cu(II) inhibition tests over *Daphnia* in vessels. Evolution of turbidity at long-term *Daphnia* exposure to copper.

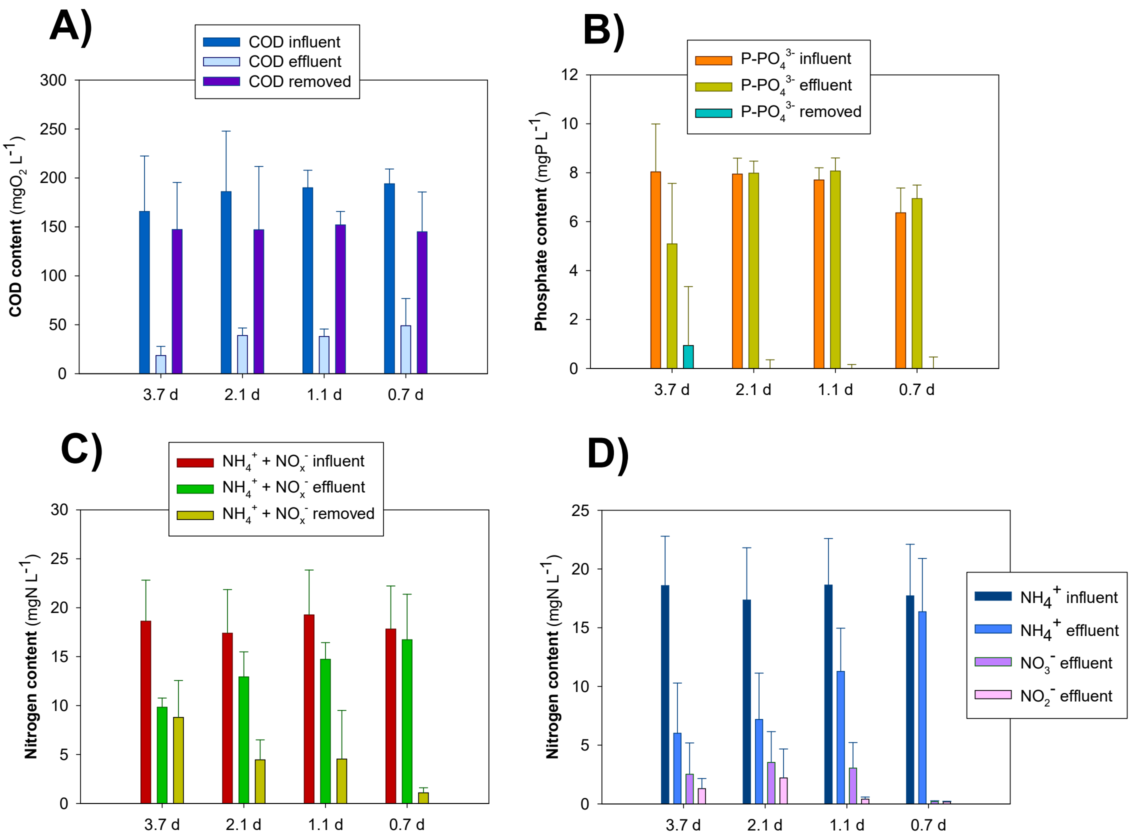

Figure 4. Zooplankton-based reactor performance - Effect of different HRTs. Dynamics of parameters evaluated: A) Organic matter (COD) removal. B) Phosphorus (PO_4^{3-}) removal. C) Nitrogen (NH_4^+ + NO_x^- (NO_2^- + NO_3^-)) removal. D) Nitrogen (NH_4^+ + NO_x^- (NO_2^- + NO_3^-)) speciation.

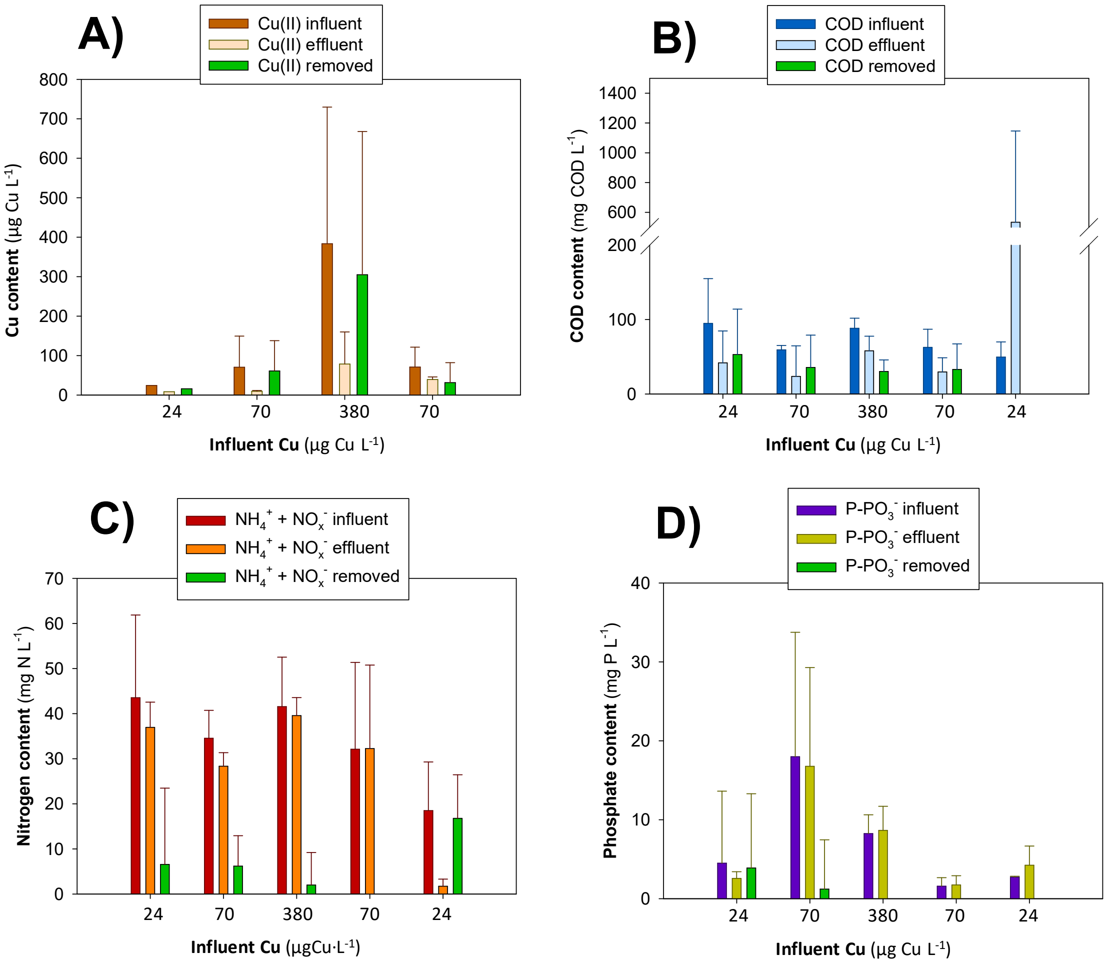

Figure 5. Zooplankton-based reactor performance - Effect of a Cu(II) overload. Dynamics of parameters evaluated: A) Cu(II) removal. B) Organic matter (COD) removal. B) Nitrogen (NH_4^+ + NO_x^- (NO_2^- + NO_3^-)) removal. C) Phosphorus (PO_4^{3-}) removal.