Ecology of duckweed ponds used for nutrient recovery from wastewater

C. C. Teles, R. A. Mohedano, G. Tonon, P. Belli Filho and R. H. R. Costa

ABSTRACT

The microorganism community that grows under duckweed shelter can play an important role on treatment processes. Therefore, the present study aimed to assess the zooplankton dynamic and microbial community in duckweed ponds (DPs) applied for domestic wastewater treatment under open field conditions. A pilot system comprised of two DPs in series (DP1 and DP2), with 10 m^2 each, received domestic wastewater through a flow rate of 200 L·day^{-1}. Thus, the system was monitored during 314 days through samples collected and analysed weekly. Also, the zooplankton organisms were identified and quantified. DNA sequencing was performed in order to identify the bacterial populations. The findings showed a high efficiency of nutrient removal with 93% and 91% of total phosphorus and total nitrogen, respectively. A high density of microcrustaceans was observed in DP1 reaching 4,700 org.100 mL^{-1} and rotifers (over than 32,000 org.100 mL^{-1}) in DP2, that could be related to the low suspended solids concentration (<30 mg·L^{-1}) and turbidity (<10 NTU). The bacterial community showed a strong heterogeneity between samples collected along the seasons. Through these findings, it is possible to realise that the understanding of ecology could help to enhance the operation and designs of DPs.

Key words | duckweed pond, microbial community, wastewater treatment, zooplankton

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INTRODUCTION

Currently, nutrient removal from wastewaters is one of the largest challenges for treatment plants. Most of wastewater treatment systems are designed based on organic load, thus nitrogen and phosphorus removals are often neglected (Metcalf & Eddy Inc. 2013). Moreover, nutrients removal through conventional systems involves energy expenditure and/or chemical additives causing additional costs to be a problem for poor municipalities and rural areas (Kneese et al. 2015). Thus, the effluent from treatment plants with low efficiency certainly carries residual nutrients, triggering the eutrophication process in receiving water bodies. Also, toxic algae blooms have been attributed to nutrients arising from unsatisfactory treated sewage released into water sources that could cause troubles for human health (Powley et al. 2016).

In the search for wastewater polishing alternatives, duckweed ponds (DPs) have arisen as an efficient and low-cost option, thus this group of aquatic plants has been successfully used in effluent treatment systems, mainly for agricultural and municipal wastewater (Alaerts et al. 1996;

Körner & Vermaat 1998; Mohedano et al. 2012a). DPs are a suitable and outstanding technology for tertiary wastewater treatment due to their great capacity on nutrients removal, mainly nitrogen and phosphorus compounds (Cheng et al. 2002a; Mohedano et al. 2012b). There are different ways for nutrient removal through biological means; however, in duckweed based systems, the plants uptake is the predominant mechanism. Complementarily, the nitrifying/denitrifying process could occur depending on some environmental conditions such as dissolved oxygen (DO) and ammonia concentration.

In addition to the high nutrient removal rate, the conditions created by duckweed mats provide a suitable microenvironment for microorganisms growth, thus improving nitrification and organic matter degradation (Körner et al. 2003; Dawidowicz & Ozimek 2013; Mohedano et al. 2014; Zhao et al. 2015). Besides the bacteria community, the zooplankton populations, such as microcrustaceans, rotifers and protozoans, are benefited due to the shelter provided by duckweed coverage. By contrast, the microalgae

community is strongly inhibited due to the shading under duckweed mat, changing the food chain commonly found in stabilization ponds.

While the numbers of studies concerning DPs have been increasing for the last four decades, a few researches focusing on the microorganisms associated with this ecosystem were developed. The understanding of the microbial community role and their behaviour facing seasonal changes, influent wastewater quality, applied loads, duckweed growth rate besides other parameters could help to improve ponds operation and their efficiency as well. Therefore, the present study aimed to assess the microbial community behaviour in DPs applied for domestic wastewater treatment under real (open field) conditions.

METHODS

Pilot system description and operation

This study was developed through a pilot-scale system located at the Federal University of Santa Catarina (27 °35′46.74″ S; 48 °30′58.64″ W, under a sub-temperate climate) at the municipality of Florianópolis in southern Brazil. Two duckweed ponds (DP1 and DP2) were made of fiberglass with dimension of 4.2 (length) × 2.4 (width) × 1 m (depth) and 0.40 m of water column. Considering the wall ponds slope (30 °), the total useful volume was about 3,400 L for each one. The ponds run in series interconnected with PVC pipes (50 mm) and were designed from ammonia loading rate of 15 $kgNH_3 \cdot ha^{-1} \cdot d^{-1}$ (about 60 $kgBOD \cdot ha^{-1} \cdot d^{-1}$).

Real domestic wastewater from residential condominium was applied through peristaltic pump providing a continuous flow rate of 200 $L \cdot d^{-1}$ and resulting in hydraulic retention time (HRT) of 17 days in each pond, or 34 days considering both ponds together. However, before being applied in DPs, the sewage was stored in equalization tanks simulating a pretreatment (25 days of HRT). The duckweed population from *Landoltia punctata* species (a native species) was adapted in this system along 5 years.

Duckweed growth

During the experimental period the duckweed biomass was collected twice a week, intending to preserve the coverage density between 400 and 600 $g \cdot m^{-2} \cdot d^{-1}$ of fresh biomass.

The maintenance of a suitable biomass density is a key factor for the success of the treatment, because both high and low densities could detract the water quality (Driever *et al.* 2005). According to the method used by Mohedano *et al.* (2012a), the biomass density was sampled trough a floating plastic square with internal area of 0.25 m^2, which was released randomly on the pond surface and the trapped biomass was collected, dried and weighed, providing biomass weight per area ($g \cdot m^{-2}$ dw).

The assessment of duckweed biomass productivity was made by determining the relative growth rate (RGR in $g \cdot m^{-2} \cdot day^{-1}$). Thus, all the biomass harvested during the treatment period was oven dried for 24 h under 55 °C and it was weighed right after. The RGR was obtained by calculating the weigh variation in a known time in determined area that could be expressed by the Equation (1). Also the biomass nitrogen content was determined through samples collected once a moth according to AOAC Method n° 991.20 (AOAC, 2005).

$$RGR = \frac{(HB/D)}{A} \tag{1}$$

where:

RGR = Relative growth rate
HB = Harvested biomass (dry weigh in grams)
D = Number of days between harvest
A = Pond area (m^2)

Monitoring

The experiment was run during 314 days where samples were collected once a week in each pond and forwarded to the laboratory for analysis. The parameters selected for performance evaluation were pH, temperature, oxidation-reduction potential (ORP) and DO, by electronic probes (*in situ*) and chemical oxygen demand (COD), biochemical oxygen demand (BOD), dissolved organic carbon (DOC), total phosphorus (TP), $N-NH_3$, total nitrogen (TN), according to *Standard Methods* (APHA 2005) and PO_4-P, NO_3-N, NO_2-N were determined by liquid chromatography using Dionex® chromatographer.

The zooplankton community assessment in DPs was made through qualitative and quantitative analyses of samples (200 mL) collected in surface (rhizosphere) and in the middle of the water column (25 cm). To concentrate the organisms, the samples were filtered through sieve for plankton separation with porosity of 30 μm. After

concentration, each sample was preserved in 4% formaldehyde solution. A portion of 1 mL was transferred to a Sedgwick-Rafter chamber to count all organisms present in a microscope (×100 magnification). Total number of each genus was extrapolated to obtain the concentration in terms of organisms per litter. This described analysis was carried out fortnightly.

Samples of duckweed rhizosphere and sludge settled in the bottom were collected for bacterial community identification, in both ponds. The monitoring comprised three seasons through collections made in March 2015 (summer), June 2015 (winter) and September 2015 (spring). For samples collected in the autumn, the amount of genetic material was not enough to carry out the DNA sequencing with assurance. Thus, DNA sequencing was performed using MiSeq® Illumina technology for sequencing by synthesis (SBS). It consisted of the rRNA 16S V3/V4 region amplification using the 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) primers, with Illumina adapters, necessary for sequencing. The amplification was performed in 35 cycles at 50 °C of annealing temperature, where, each sample was amplified in triplicate. The sequencing was performed in MiSeq® Illumina, using V2 kit, with a single-end 300 nt run. The system ensured the 100,000 sequences reading with sampling taxonomic identification and quantification of the number of the sequences obtained from each taxon. OTU Picking was performed using BLASTN 2.2.28 against GreenGenes 13.8 database. To attribute taxonomy, only the sequences with hits of 99% of identity in an alignment covered over 99% were considered.

RESULTS AND DISCUSSION

DPs performance

Organic matter

During the whole experimental period, the DPs pilot system performed a satisfactory efficiency with a high nutrient removal rates and fitting the final effluent in more restrictive standards legislation (<4 mgTP·L^{-1} in State law 14.675/14/ CONSEMA and <20 mg NH$_3$-N in CONAMA 430/11). The summary of the mean values could be seen in Table 1. The pH values remained near to neutrality (6.5 and 7.5), featuring a stable biological process. Due to their influence in NH$_3$/NH$_4^+$ equilibrium, a neutral pH is an important factor for nitrogen absorption and also for duckweed health (Caicedo *et al.* 2000). DO concentration in DPs strongly depends on the organic load applied, thus DO concentrations below 1.0 mg·L^{-1} were detected during some periods (during spring) with high loads. However, DO mean values between 1.2 and 2.8 mg·L^{-1} were found during summer, autumn and winter, showing an aerobic environment. Moreover, the strong presence of microcurstaceans and others organisms mentioned bellow corroborate to define the DPs evaluated as a constant aerobic environment, considering the surface layer (10 cm).

According to Mohedano *et al.* (2014), there is not a consensus among authors about DO increasing in DPs. In some cases, the biomass coverage in water surface may obstruct the diffusion of atmospheric oxygen through the water column causing DO depletion. Also, it is important to

Table 1 | Mean values of variables concentrations and duckweed pond (DP1 and DP2) efficiencies

Variables (mg·L^{-1})	n[a]	Influent	DP1 Effl.	(%)[b]	DP2 Effl.	(%)[c]	Final efficiency (%)
COD	41	99.7 ± 52.6	37.4 ± 11.6	62.4	32.9 ± 13.7	12.2	**67.0**
BOD	41	76.1 ± 61.7	26.7 ± 22.6	64.9	10.5 ± 8.5	60.6	**86.2**
TN	41	59.7 ± 19.1	20.6 ± 10.9	65.4	5.3 ± 5.0	74.2	**91.1**
NH$_3$-N	41	49.4 ± 16.2	15.5 ± 6.3	68.5	2.2 ± 2.8	86.2	**95.6**
NO$_2$-N	41	0.07 ± 0.3	0.1 ± 0.2	–	0.1 ± 0.1	–	–
NO$_3$-N	41	0.02 ± 0.1	0.3 ± 0.5	–	0.4 ± 0.7	–	–
TP	41	6.7 ± 1.9	2.1 ± 1.2	68.9	0.5 ± 0.7	76.6	**92.7**
PO$_4$-P	41	4.1 ± 1.9	1.4 ± 0.8	68.8	0.2 ± 0.5	80.0	**95.1**
DO	30	0.0 ± 0.0	1.8 ± 1.5	–	2.0 ± 1.2	–	–
Turbidity (NTU)	41	45.9 ± 37.4	3.9 ± 2.3	91.4	2.3 ± 1.5	42.1	**95.0**

[a]Number of campaigns.
[b]DP1 efficiency.
[c]DP2 efficiency.

highlight that the microalgae concentration is often low in DPs due to the barrier for sunlight penetration provided by duckweed coverage. Thus, the daily DO and pH variation due to algal photosynthesis is commonly avoided in DPs.

Therefore, the DO supply could promote a suitable COD and BOD removal reaching 67.0 and 86.2% respectively. Regarding the stabilization stage (pretreatment) the organic matter in DP1 influent presented low concentrations values (about 100 mgCOD L^{-1} in average). The surface loading rates applied along the seasons are shown in Table 2. A low carbon concentration is expected for polish treatment stage and considering duckweed as autotrophic organism this fact does not detract nutrient uptake efficiency. By contrast, heterotrophic organism populations could decrease in low carbon environment. In similar conditions Sims *et al.* (2013) studied two DPs in series cites 83% of COD removal from average concentration of 111 mg·L^{-1}. Considering higher inlet concentration, Ran *et al.* (2004) using *Lemna gibba* found a COD removal efficiency of 67.5% from 98.2 mg·L^{-1}. The COD measured in this study and in the others mentioned comprises the total COD, in other words, particulate and soluble organic matter.

Nutrient removal

Regarding phosphorus removal (TP and PO$_4$-P) the average efficiency of the whole system reached over 93% of TP and 95% of PO$_4$-P (from 6.7 to 0.5 mgTP·L^{-1} and from 4.1 to 0.2 mg PO$_4$·L^{-1}), this efficiency could be considered one of highest reported to similar conditions. TP is the main parameter to be observed in effluents considering their strong role to avoid eutrophication process in receiving water bodies. The highest removal efficiency was observed during summer and fall being in agreement with Zhao *et al.* (2015). Throughout the monitoring period, the TP concentration remained below of the State legislation standard (4 mg TP L^{-1} in State low 14.675/14) for effluent discharges into water bodies (Figure 1). Comparing with

Table 2 | Mean surface loadings rates applied in pilot system during each season

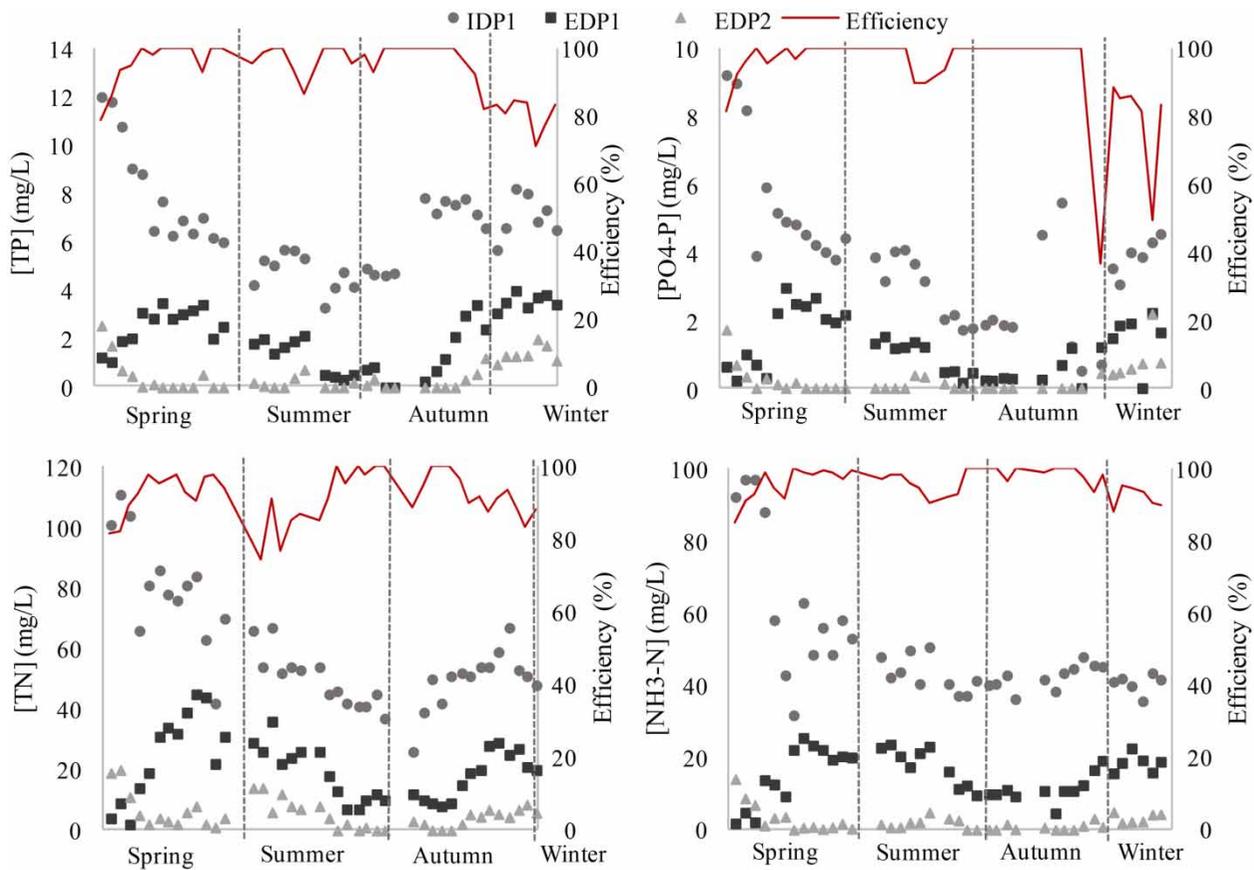| Parameters	SLR spring	SLR summer	SLR autumn	SLR winter
COD	22.2	17.7	22.1	44.7
TN	19.8	12.7	10.8	14.3
NH$_3$-N	15.8	10.6	10.7	10.1
TP	2.0	1.2	1.6	1.8

SLR: Surface loading rate (kg·ha^{-1}·day^{-1}).

similar studies Priya *et al.* (2012) obtained a removal of 79% using *Lemna minor*, also El-Shafai *et al.* (2007) using a pilot DPs as post-treatment of a UASB reactor reported 78% of phosphorus removal. Iqbal (1999) states that the higher the duckweeds growth rate, the higher the phosphorus removal through the PO$_4$ absorption mechanisms, thus highlighting the importance of harvesting frequency to maximize the efficiency.

Due to the ammonification occurred in the stabilization tank (pretreatment) the mean of ammonia was close to the TN concentration in influent being 49.4 and 59.7 mg·L^{-1}, respectively. From these concentrations, the system performs a high removal efficiency reaching 91% of TN and 96% of NH$_3$-N resulting in output concentration of 5.3 and 2.2 mg·L^{-1} (Figure 1). For both NH$_3$ and TN, the DP2 performs the highest efficiency (86 and 74%, respectively – Table 1). Indeed, a high rate of nitrogen removal by DP system is expected and often reported in literature being the greatest advantage of this technology (Cheng *et al.* 2002b; Zimmo *et al.* 2004; Caicedo *et al.* 2000; Mohedano *et al.* 2012a, Zhao *et al.* 2014). For instance, Xu & Shen (2011) using *S. oligorrhiza* to treat pig manure, from initial concentration of 52.1 mg NH$_4$·L^{-1} (similar to present work), obtained removals of 85.3% and 100% after 1 and 2 weeks, respectively. Is important to highlight that ions adsorption could occur in plant roots but this mechanism is stronger in constructed wetlands with filter sediment, such as sand or argil.

Differently from what occurred in biological reactors, the nitrification in DPs could not be verified just by the presence of nitrate, because these plants are able to uptake nitrate way faster than the nitrifying bacteria can produce it (Mohedano *et al.* 2012a). Therefore, in the present study NO$_3^-$ concentrations were always low, ranging from 0.01 to 0.30 mg·L^{-1}, whereas an important nitrification may have occurred but it will be discussed later against nitrifying bacteria community assessment. In previous studies, as mentioned by Caicedo *et al.* (2000) and Mohedano *et al.* (2012a), the nitrogen uptake by plants could be the main route for nitrogen removal in the conditions presented. However, the calculation of nitrogen removed by duckweed absorption brings out a different result, as it will be explained in the following.

Biomass yield and content are important parameters to estimate the nutrients removed by plant absorption. Thus, to the end of the experimental period, about 10.6 kg of dry biomass were obtained from DP1 and 7.2 kg from DP2. The average nitrogen content in biomass was 5.4% ($\pm$1.4), therefore it was possible to estimate the amount of nitrogen

Figure 1 | Nutrient concentration (TP, PO$_4$-P, TN and NH$_3$-N) and removal efficiency across the seasons (IDP1 = Influent of duckweed pond 1; EDP1 = effluent of duckweed pond 1 and EDP2 = final effluent).

removed by plant uptake which were 585 g and 390 g in DP1 and DP2, respectively. These data mean that about 27% of TN removed (3.5 kg) was due to plant absorption. This percentage complies with Mohedano *et al.* (2012b) who conclude that the route of nitrogen mass removed depends on the ammonia load applied (higher loads favor nitrification/denitrification). Zimmo *et al.* (2004) also mention denitrification as the main route. During the spring plant absorption could be stronger than in others seasons due to the higher growth rate as demonstrated in Table 3.

Besides climate conditions, RGR depends on a lot of other points such as biological species (strains) and effluent composition, therefore, a wide RGR range is observed in

scientific literature. Iatrou *et al.* (2013) investigated the duckweed's *Lemna minor* growth using human urine and treated domestic wastewater under different conditions obtaining maximum values of 7 g·m^{-2}·day^{-1} to RGR. In contrast, Mohedano *et al.* (2012b) studying a full-scale DP for swine waste treatment found higher growth rates reaching 18 g·m^{-2}·day^{-1}. Considering the subtropical climate in Southern Brazil with milder winter the RGR variation is more related to influent characteristics, duckweed density, and frequency of harvesting than temperature. Otherwise, Zhao *et al.* (2015) in an experimental field in China highlighted that the seasonal variation in growth rates ranged from 3.4 in the coldest month to 8.9 g·m^{-2}·day^{-1}.

Zooplankton assessment

The zooplankton community's characterization was made through quantitative and qualitative means intending to correlate the organism's relative frequency with physical-chemical parameters (treatment efficiency) as well as the
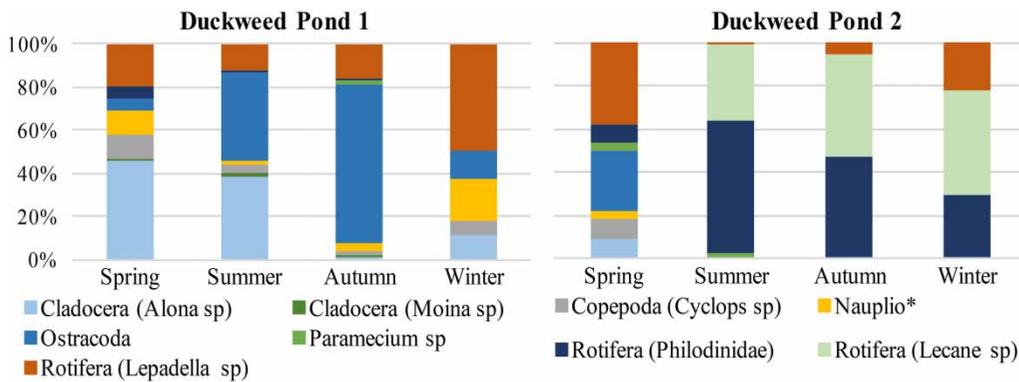
Table 3 | RGR of duckweed in different seasons

		Spring (*n* = 23)	Summer (*n* = 18)	Autumn (*n* = 11)	Winter (*n* = 5)
RGR (g·m^{-2}·d^{-1})	DP1	5.91	4.99	3.83	4.52
RGR (g·m^{-2}·d^{-1})	DP2	4.88	2.26	2.49	2.06

Figure 2 │ Relative frequency (%) of organisms from zooplankton found in the rhizosphere.

seasonal variations. The most frequent organisms found during the experimental period were microcrustaceans being represented by the Class Ostracoda, Copepoda and Branchiopoda (Cladocera), but also the presence of rotifers was abundant. When in natural environment the microalgae are the main source of food for these organisms; however, in DPs (where algae are often rare) the microcrustaceans probably consume a lot of particulate organic matter. Therefore, zooplankton populations act as a powerful filter removing particles (turbidity and suspended solids) and making the carbon source easily available for heterotrophic bacteria degradation (ANSA *et al.* 2012; Dawidowicz & Ozimek 2013).

The relative frequency of zooplankton found in surface (rhizosphere) for both ponds is shown in Figure 2. In DP1, the cladoceran *Alona* sp. appeared in high density during spring and summer reaching the average 2,144 org.100 mL^{-1} (45%) and 1,065 org.100 mL^{-1} (42%). Along the summer and early autumn, this genera was replaced by ostracodes (1,630 org.100 mL^{-1}) representing 75% of zooplankton community. In spite of these changes regarding the dominance, the total amount of organisms did not present a wide ranges in DP1 remaining between 4,700 and 3,000 org.100 mL^{-1} in average (Figure 3). Also, the turbidity and suspended solids were always low along the whole period (<30 mg TSS·L^{-1} and <10 NTU) and no relation with these parameters could be attributed to the species' relative frequency. However, the high amount of microcrustaceans and rotifers is probably related to food available, in other words, total suspended solids (TSS) consumption (Dole-Oliver *et al.* 2000).

In DP2, it was possible to highlight the increase of the free swimmers rotifers such as *Lecane* sp. and *Lapadella* sp. and also fixed rotifers belonging to the Philodinidae family, mainly after spring (Figure 2). In the same period it
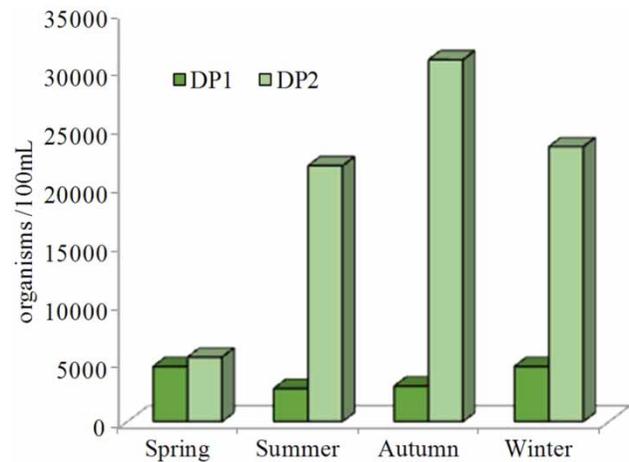

Figure 3 │ Concentration of organisms from zooplankton found in the rhizosphere.

was observed bloom of cyanobacteria (*Oscilatoria* sp.) in DP2, so both events may be correlated considering the food supply. Most likely, the zooplankton community composition, presented in Figure 2, was due to specific conditions found along the evaluated period. Considering the interaction with environmental conditions and wastewater composition, the biological community dynamic in DPs is complex and these results are not reproducible.

Moreover, the total number of organisms increased around ten times in DP2 comparing with DP1 (Figure 3). This fact was opposite to the one related by Ansa *et al.* (2012) who found a decrease of micro invertebrate through a series of four ponds (DPs, algal ponds and hybrid). Besides the food and shelter provided by filamentous cyanobacteria, the stress caused by high ammonia concentration (close to 20 mg·L^{-1}) inside DP1 probably was a restrictive factor. Unfortunately, studies with focus on duckweed ecology are rare to enrich this discussion.
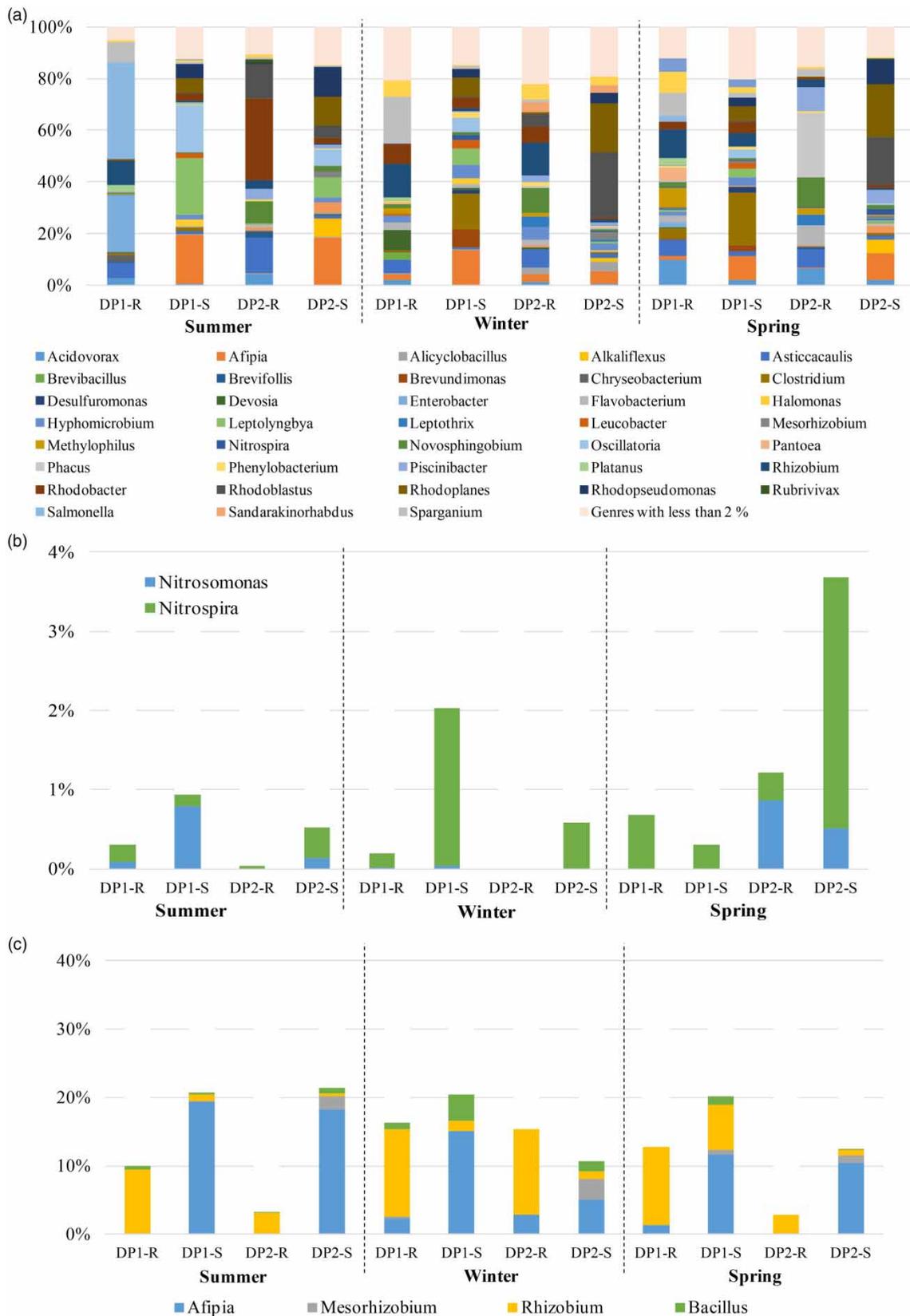
Figure 4 | Relative abundance of bacteria at genus level in DPs. (a) All genera over 2% of abundance; (b) nitrifying bacteria; (c) nitrogen fixing bacteria. DP-R = samples from rhizosphere (surface); DP-S: samples from sludge (bottom).

Samples collected in water column (25 cm) revealed a very different composition when compared with the surface, with predominance of ciliates protozoan such as *Paremeceum* sp. (883 org.100 mL^{-1}) and rotifers Philodinidae (691 org.100 mL^{-1}) in DP1 and DP2, respectively. This fact pointed to the importance of duckweed rhizosphere as microenvironment supporting larger diversity and biomass of zooplankton. Also, defined layers of communities could be observed where DO, competition and predation play an important role.

DNA sequencing and microbial assessment

The DNA sequencing, which was assessed against Green-Genes database, demonstrated a strong heterogeneity among samples collected in the seasons. In Figure 4(a), 40 genera of bacteria are shown which have occurred in frequencies over than 2%, including functional groups as nitrifying (*Nitrospira*), denitrifying (*Rhodopseudomonas*), nitrogen fixing (*Rhizobium*) and pathogenic (*Salmonella*). Nitrifying bacteria was found in both samples, sludge and rhizosphere being more abundant in the spring, mainly for *Nitrospira* reaching 4.8% of abundance (Figure 3(b)). However, due to the complexity of processes that occur simultaneously in open field pilot system (with variance of OD, NH$_3$ concentration as well as temperature) it is not possible to conclude that the higher *Nitrospira* abundance, the higher ammonia removal efficiency. On the other hand, the nitrogen mass balance remarked before (DPs performance) pointed that nitrification/denitrification process surpass plant uptake along the seasons showing the importance of *Nitrospira* for nitrogen removal. Zhao et al. (2015) found 1.5% of nitrifying bacteria in DPs with good efficiency for nitrogen removal; however, this percentage was enhanced by using artificial carrier for biofilm attachment (to 3%). Considering the frequent harvesting of duckweed biomass and the slow growth of nitrifying bacteria, it is possible to conclude that these organisms are often removed together and the population could not be replaced without enough growth velocity. Nevertheless, nitrification may have occurred and evidenced by alkalinity consumption (from 320 to 60 mgCaCO$_3\cdot$L^{-1}) being related by many authors (Zimmo et al. 2003; Caicedo et al. 2000; Zhao et al. 2014). Therefore, the nitrogen uptake by duckweeds is supported as the main route of N removal.

Moreover, nitrogen fixing bacteria populations were observed with high frequency reaching 11.6% in DP1 during spring (Figure 4(c)). Also, Zhao et al. (2014) showed a high amount of nitrogen fixing bacteria in DPs compared with water hyacinth ponds representing an important nitrogen contribution. In DP1, it was observed also a very high concentration of pathogenic bacteria such as *Clostridium* sp. and *Salmonella* sp. whereas *Escherichia coli* was low; this fact could indicate that *E. coli* is not a good indicator for pathogenic contamination, mainly after long HRT.

CONCLUSION

The DPs system performed a high wastewater treatment efficiency highlighting the unexpected high phosphorus removal (93%). The low carbon/nutrient ratio (expected for tertiary treatment) did not detract the nutrient removal efficiency, considering duckweed uptake (autotrophic metabolism) as the main removal route. The community of microcrustaceans such as cladocerans (*Alona* sp.) and ostracodes were present in high concentration in DP1, resulting in a low turbidity and TSS across the seasons. A high diversity of bacteria was noted including pathogenic, nitrifying, denitrifying, and nitrogen fixing; however, a strong dynamic of populations could be highlighted along the period due to the wide variation among the samples. It is recommended that a deeper statistical analysis to assess the relation between microorganisms and the treatment efficiency.

ACKNOWLEDGEMENTS

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