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Research article

Treatment of frog farming effluent with *Eichhornia crassipes* (Mart.) Solms

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Abstract: Water, sediment, phytoplankton and Protozoa fauna from a constructed wetland used for the treatment of bullfrog farming effluent in southeastern Brazil, were sampled. The first sample was retrieved from the adult frog breeding area, fed on 16 kg day^{-1} feed and the second was retrieved from the tadpoles breeding, fed on 4 kg day^{-1} feed. Each period corresponded to 35 days (5 weeks each) of weekly sampling. The hydrological regime and decomposition processes had strong impact on the water quality in the wetland and on the composition of phytoplankton and Protozoa communities. Removal rate was lower than 42%, except thermo-tolerant coliforms, over 90%, due to flow in the wetland and the highest load in the inlet water. Plants and sediment had a strong effect on reducing nutrients in the wetland. Protozoa comprised three taxa, whilst phytoplankton comprised 36 taxa, with Bacillariophyceae featuring the highest relative abundance. Current study revealed relative removal of residue from the frog farm using the wetland in waste water treatment with floating plant (*Eichhornia crassipes*). The treatment system may be a tool in amphifarms due to management of frog framing.

Keywords: Wetland, Phytoplankton, Protozoa, Aquaculture, Abiotic parameters.

INTRODUCTION

One of the most important issues in the breeding of aquatic organisms is the control of effluents produced by feed management. In the case of frog culture, breeding management requires the daily cleaning of the area. In certain circumstances, it may have to be done more than once a day. Consequently, effluents from frog breeding produce high residue load with feed wastes, feces and other wastes which should be removed from the system due to negative impacts on the receiver water body.

Intensive frog farming techniques have been developed for the production of laboratory frogs used in medical and biological research, and for small-scale human consumption. Although indoor farming may be undertaken with certain frog species, the bullfrog (*Lithobates catesbeianus* Shaw) has the greatest potential rate due to large size. It is thus commonly used for farming. The single female may cover an area of approximately 3-5 square feet and include between 10,000 and 25,000 eggs, whilst the vegetarian larval frogs or tadpole spend most of their time grazing microscopic plants and bottom algae (Helfrich *et al.*, 2009).

Waste water may affect the health of the environmental and may cause changes in the water quality, directly affecting the development of the animals. In the case of aquaculture farm under analysis, the wastewater from frog culture flows directly into the pond located below. The treatment of wastewater from frog farming is mandatory. According to Borges & Sipaúba-Tavares (2017), constructed wetland in the effluent of frog farming improved the quality of wastewater by efficiently removing nutrients. In fact, many water treatment plants are introduced to clean polluted water.

Macrophytes plants act as biological filters that accumulate nutritional compounds and inorganic waste products (Velichkova & Sirakoy, 2013). Several free-floating macrophytes such as *Eichhornia crassipes* (Mart.) Solms, the effect of the leaves and stems, suppress algal growth and remove suspended solids by setting in the root zone. *Eichhornia crassipes* has rapid growth rate and high adaptability to extreme conditions (Kalubowila *et al.*, 2013).

Constructed wetlands are artificial ecosystems where macrophytes are widely used to treat different types of effluents. Plants and soil biota account for much of the advantages of the wetlands. Plants photosynthesize through their above ground organs, while their roots and rhizospheres drive the below ground productivity of the heterotrophic soil biota (Neori & Agam, 2016). Macrophytes are capable of decreasing all indicator of water quality in wastewater to a level would allow its use for irrigation (El-Din & Abdel-Aziz, 2018).

All aspects of water treatment play a significant role in intensive aquatic organism production since, control and monitoring of water quality is of vital importance to the success or failure of the production. Thus it is necessary to

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develop new research applications focused on avoiding or at least reducing the negative impacts of aquaculture effluents on the environment (Turcius & Papenbrock, 2014).

Due to the scarcity of studies in Brazil (Mercante *et al.*, 2014; Mello *et al.*, 2016; Borges & Sipaúba-Tavares, 2017) on effluents of indoor frog farming, current analysis is highly relevant for systems with aquatic organism production. The effluent of indoor frog culture was evaluated during two periods of bullfrog development: one occurred during the maintenance of adult animals and the other during the tadpole phase. Current study analyzes the effects of constructed wetlands on the treatment of wastewater from frog farming.

MATERIALS AND METHODS

Study area

Current study was carried out in the constructed wetland for indoor frog farming at the Aquaculture Center in southeast Brazil (21° 15′19″ S and 48° 19′21″ W) (Fig. 1). Monthly means of meteorological conditions during the study period (October 2016 - January 2017) corresponding to summer/rainy season were air temperature 24±0.3°C; water temperature 28±0.9°C and rainfall 178±80 mm. This sector is composed of two sheds constructed according to the amphifarm system with a total area of 144 m^2. One of the sheds has eight stalls measuring 12 m^2 each, totaling 96 m^2. It is used for housing animals during the fattening growth phase; the other shed has 16 stalls measuring 3 m^2 each, totaling 48 m^2. It is used for housing animals at a post-metamorphic phase. Stalls comprised shelters, feeding troughs and linear-arranged pools. An uninterrupted water flow was maintained in the pools to carry residues and excreta out of the stall. Cleaning was carried out daily and consisted of complete emptying and washing of ponds (Borges & Sipaúba-Tavares, 2017). The wetland is 30.55 m long and has a total area of 18.12 m^2. It is lined with a shallow and clayey bottom. The wetland presented four compartments with volumes of 0.84, 1.53, 1.40 and 1.27 m^3 and interconnected by channels with surface water flow. The first compartment was used only for solid residue sedimentation; the second and third compartments were filled with the floating macrophytes *Eichhornia crassipes*; the fourth compartment contained stones to retain the water flow (Fig. 1). The aquatic plant used in the constructed wetland came from a standard macrophyte culture pond. The plants were installed twice, once for the September sampling and once for the November one. They were completely removed between the two periods.

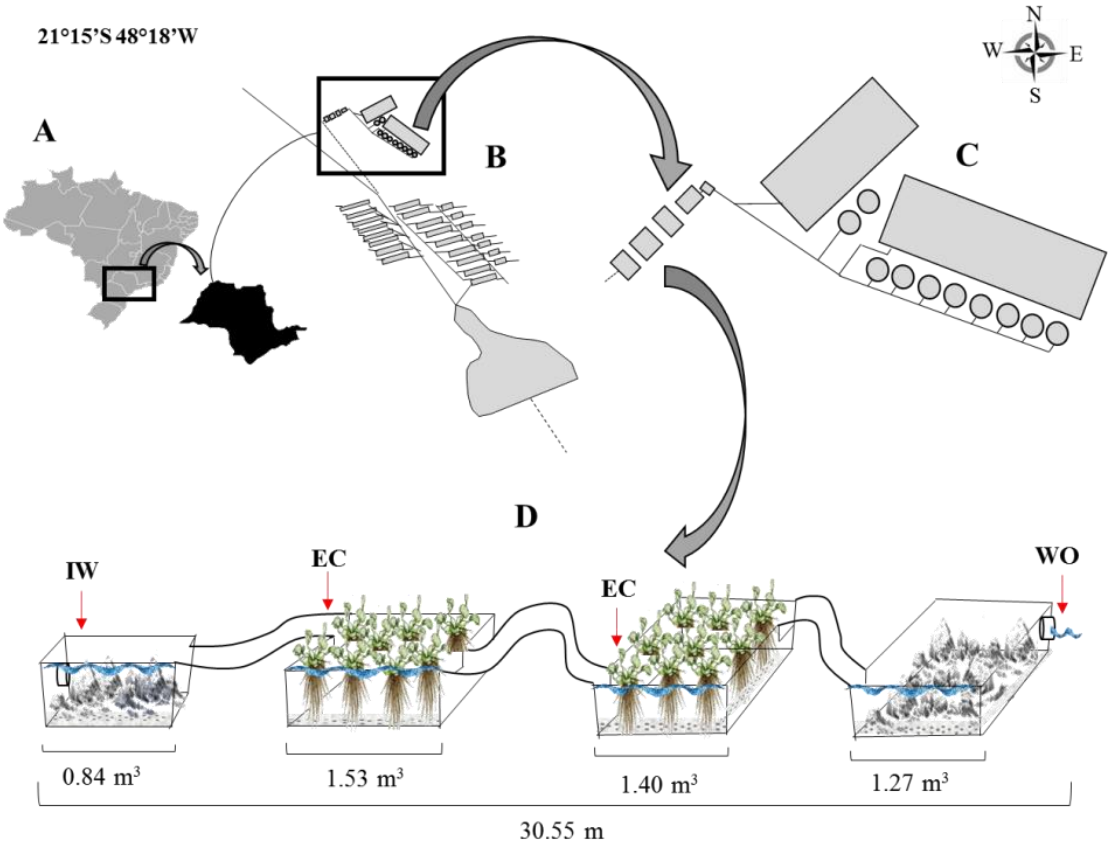

Figure 1. Diagram of constructed wetland: A = area inside the square indicate southeastern Brazil (the state of São Paulo). B = aquaculture farm of the Univ. of São Paulo State and constructed wetland. C = indoor bullfrog farming. D = constructed wetland showing different sites (IW = inlet water from indoor frog farming; EC = stand of *Eichhornia crassipes* (Mart.) Solms macrophyte into wetland; WO = water outlet that flows directly into the fishpond).

Period and sampling sites

Samples were retrieved in two periods; between October and November 2016 with regard to adult frog breeding with 2,700 animals, with diary feed of 16 kg day^{-1} and between December, 2016 and January, 2017 with regard to tadpoles breeding with 40,000 animals with diary feed of 4 kg day^{-1}. Each period corresponded to 35 days (5 weeks each) of weekly sampling. Samples were collected at three different sites, or rather, the inlet water (IW) of the wetland (water comes directly to the frog farm); site inside the *Eichhornia crassipes* stand; in the water outlet (WO) where water flows directly into a fishpond. Inlet (IW) and water outlet (WO) comprised only sediment samples.

Water and sediment

Water was sampled with a 1 L Van Dorn bottle and transported in refrigerated polyethylene bottles to laboratory. Water temperature (Temp), pH, dissolved oxygen (DO) and conductivity (Cond) were measured *in situ* with a multi-sensor Horiba U-10. Total phosphorus (TP) and total inorganic nitrogen (TIN) were quantified by spectrophotometry, following Koroleff (1976) and Golterman *et al*. (1978). Chlorophyll-*a* (Chloro-*a*) was extracted with alcohol 90% and quantified at 663 nm and 750 nm (Nusch, 1980). Total suspended solids (TSS), total dissolved solids (TDS) and 5-day biochemical oxygen demand (BOD_5) were determined according to Boyd & Tucker (1992), and alkalinity (Alkali) according to Mackereth *et al*. (1978). Chemical oxygen demand (COD) was determined according APHA (1995). Water samples for microbiological analysis using multiple-tube methods were collected in sterilized 500-mL flasks and taken to the laboratory in an isothermal container. The material used in the microbiological analysis (thermo-tolerant coliforms - TC) was sterilized prior to use (APHA 1995). Vertically mixed sediment samples were retrieved with a 4-cm diameter PVC core up to approximately 5 cm deep. Sediments were air dried, gently disaggregated and dried in a convection oven at 70°C until completely dry. Boron, Ca, Cu, Fe, K, Mg, Mn, N, P, S, Zn, pH and organic matter (OM) were determined following Raij *et al*. (2001). Analyses were performed immediately after sampling or samples were duly stored under refrigeration.

Hydraulic variables

The hydraulic loading rate (HLR) of the CW system was calculated for the two periods by:

$$q = \frac{Q}{A}$$

Where, q is the HLR (m h^{-1}); Q is the water inlet rate (m^3 h^{-1}) and A is the wetland land area (m^2) (Kadlec & Wallace 2009). The hydraulic retention time (HRT) for the two periods was measured by:

$$HRT = \frac{V}{Q}$$

Where, V is the maximum volume of the canal (m^3) and Q is the water inlet rate (m^3 h^{-1}). The removal rate (RR %) of mass loads by the CW system was measured by:

$$RR\ \% = \frac{C_i - C_0}{C_i} \times 100$$

Where, C_i is the inflow concentration (µg L^{-1}) and C_0 is the outflow concentration (mg L^{-1}), except for thermo-tolerant coliforms (MPN 10^4 100 mL^{-1}) and conductivity (µS cm^{-1}). Removal rate of main compounds related to water in the two frog farming periods was evaluated by the characteristics of their inlet and outlet data.

Biological data

The growth of *E. crassipes* biomass was determined twice a week during each sampling period, by measuring foliar height and width and rhizome length. Measurements were taken on the same 10 marked plants randomly chosen at the beginning of the experiment. The plants were collected using a 0.18 m^2 quadrant, dried at 60ºC until constant weight, and weighted. Plant nutrients composition was analysed according to Bataglia *et al*. (1983) at the beginning and end of each period. Phytoplankton and Protozoa samples were collected with polyethylene bottles and preserved in Lugol solution 1%. Phytoplankton and Protozoa abundance was estimated by counting cells in Utermöhl sedimentation chambers, following Lund *et al*. (1958), and counting was carried out under inverted microscope Axiovert 40 CFL (Carl Zeiss). Protozoa were grouped into two taxonomic categories: Testate Amoebae (Amoebozoa) and Ciliates (Ciliophora) (Adl *et al.*, 2005).

Data analysis

All data underwent one-way analysis of variance (ANOVA) with Statistica 10 package, to test differences between sites in the wetland of each period (Statsoft, 2011). Differences were considered significant at $p<0.05$. Results were expressed as means ± SD (Standard Deviation). Phytoplankton and Protozoa diversity was calculated with Shannon-Wiener (H') index (Pielou, 1975). Richness (S) was calculated as the total number of species present and evenness or equitability (E) was determined as H/H max, where H is the Shannon-Wiener index and H max = ln S. Species

dominance (D) and abundance (A) were analyzed for organisms between sites in the wetland. Species were considered dominant when density was higher than 50% of the total number of specimens in the sample; they were abundant when the number of specimens was higher than the mean density of all occurring species (Lobo & Leighton 1986).

RESULTS

HLR was higher during the adult period (0.85 m h^{-1}) than during the tadpole period (0.76 m h^{-1}). Consequently, HRT was faster during adult period (6.3 h) than during the tadpole period (7.14 h). Removal rate was lower than 42% except for thermo-tolerant coliforms (TC), or rather, 96% in adult period and 90% in tadpole period. During the tadpole period more compounds were removal such as BOD_5 (19%), conductivity (6%), TDS (16%), COD (36%) and TSS (16%). Besides TC, COD (28%) and TSS (42%) only were removed during the adult period.

Acidic pH was observed during the adult period and alkaline during tadpole period. Only TSS was reduced at the EC site in the two periods and DO only during the tadpole period (Table 1). Dissolved oxygen was below 3.7 mg L^{-1} in the two periods and conductivity was very high, or rather, above 114 µS cm^{-1} during adult period and 152 µS cm^{-1} during tadpole period. Low DO and high conductivity were due to the frog faeces and food waste. Total dissolved solids concentration was lower during the tadpole period ranging between 50 mg L^{-1} and 584 mg L^{-1}, with removal rate (16%) during the period, while in the adult period concentrations were higher and ranged between 105 mg L^{-1} and 412 mg L^{-1} without removal rate (Table 1). Alkalinity was high, or rather, above 105 mg L^{-1} in the two periods, and chlorophyll-*a* was below 4.7 mg L^{-1} during the adult period and 2.8 mg L^{-1} during the tadpole period. Highest concentrations during the two periods were observed in WO for COD and BOD_5 concentrations that were removed in the tadpole period, BOD_5 was similar ($p>0.05$) during the adult period. Although the number of TC was high in EC, the highest removal rate was obtained. In the case of nutrients, the wetland was not efficient since highest concentrations were observed in WO. Total inorganic nitrogen concentrations were higher than TP, and more than 1 mg L^{-1} of TIN concentrations were observed in the two periods (Table 1).

Table 1. Water variables and thermo-tolerant coliforms (means ± SD) in three sampling sites: IW = inlet water from indoor frog farming; EC = stand of *Eichhornia crassipes* (Mart.) Solms macrophyte in the wetland; WO = water outlet that flows directly into the fishpond, during two periods of frog farming (adult and tadpole).

VARIABLES	ADULT			TADPOLE		
	IW	EC	WO	IW	EC	WO
pH	6.9 ± 0.2^{a}	6.9 ± 0.1^{a}	6.9 ± 0.2^{a}	7.2 ± 0.1^{a}	7.1 ± 0.3^{a}	7 ± 0.4^{a}
Cond (µS cm^{-1})	170 ± 44^{ab}	167 ± 42^{b}	201 ± 30^{a}	185 ± 25^{a}	181 ± 20^{a}	174 ± 33^{b}
DO (mg L^{-1})	3.2 ± 0.4^{a}	1.8 ± 0.5^{b}	2.8 ± 0.6^{ab}	2.6 ± 0.6^{b}	1.6 ± 0.3^{c}	3.2 ± 0.64^{a}
TSS (mg L^{-1})	10.7 ± 5^{a}	4.1 ± 1^{c}	6.1 ± 3^{b}	9 ± 4^{a}	5.9 ± 3^{c}	7.6 ± 4^{b}
TDS (mg L^{-1})	168 ± 57^{c}	270 ± 122^{a}	212 ± 115^{b}	155 ± 79^{b}	245 ± 217^{a}	131 ± 58^{c}
Alkali (mg L^{-1})	116 ± 7^{b}	120 ± 9^{a}	120 ± 8^{a}	110 ± 5^{b}	110 ± 3^{b}	113 ± 4^{a}
TIN (mg L^{-1})	0.87 ± 0.2^{b}	0.88 ± 0.2^{b}	1.07 ± 0.1^{a}	0.75 ± 0.1^{b}	0.77 ± 0.2^{b}	0.83 ± 0.16^{a}
Total P (mg L^{-1})	0.27 ± 0.02^{c}	0.29 ± 0.08^{b}	0.33 ± 0.1^{a}	0.19 ± 0.07^{b}	0.25 ± 0.05^{a}	0.24 ± 0.06^{a}
Chloro-*a* (mg L^{-1})	2.9 ± 0.8^{b}	2.9 ± 1.2^{b}	3.8 ± 1.2^{a}	1.39 ± 0.7^{c}	1.86 ± 0.6^{b}	2.05 ± 0.9^{a}
COD (mg L^{-1})	25.4 ± 10^{a}	20.6 ± 9^{b}	18 ± 8^{c}	25.4 ± 6^{a}	19.7 ± 6^{b}	16.3 ± 5^{c}
BOD_5 (mg L^{-1})	51.3 ± 24^{a}	50.5 ± 37^{a}	50.1 ± 30^{a}	61.9 ± 20^{a}	51.4 ± 21^{b}	50.1 ± 10^{b}
TC (MPN 10^4 100 mL^{-1})	78 ± 35^{b}	2,020 ±3,045^{a}	3.0 ± 1.2^{c}	6.4 ± 3.1^{b}	11 ± 13^{a}	0.6 ± 0.4^{c}

Note: In each row, means followed by the same letter do not significantly differ ($p<0.05$).

Table 2. Means and SD concentrations of variables measured in the sediment during two periods of frog farming (adult and tadpole) in the inlet water (IW) and water outlet (WO) in the wetland.

Sediment (g L^{-1})	ADULT		TADPOLE	
	IW	WO	IW	WO
B	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.04 ± 0.01^{b}
Ca	2.09 ± 0.6^{a}	1.08 ± 0.7^{b}	3.67 ± 0.5^{a}	2.29 ± 0.05^{b}
Cu	0.16 ± 0.02^{b}	0.17 ± 0.03^{a}	0.22 ± 0.03^{a}	0.16 ± 0.1^{b}
Fe	2.6 ± 0.3^{b}	3 ± 0.3^{a}	2.65 ± 0.2^{b}	3.14 ± 0.3^{a}
K	0.1 ± 0.03^{a}	0.07 ± 0.02^{b}	0.09 ± 0.02^{a}	0.06 ± 0.02^{b}
Mg	0.16 ± 0.04^{a}	0.13 ± 0.02^{b}	0.20 ± 0.02^{a}	0.16 ± 0.01^{b}
Mn	0.02 ± 0.03^{b}	0.03 ± 0.01^{a}	0.01 ± 0.01^{b}	0.02 ± 0.01^{a}
N	1.04 ± 0.6^{a}	0.37 ± 0.04^{b}	0.68 ± 0.3^{a}	0.20 ± 0.09^{b}
P	1.62 ± 0.8^{a}	0.37 ± 0.1^{b}	1.66 ± 1^{a}	0.24 ± 0.1^{b}
S	4.1 ± 1.8^{a}	3.22 ± 0.7^{b}	2.33 ± 2^{b}	3.71 ± 1^{a}
Zn	0.04 ± 0.02^{a}	0.02 ± 0^{b}	0.03 ± 0.02^{a}	0.01 ± 0^{b}
pH	5.73 ± 0.2^{b}	6.33 ± 0.5^{a}	6.21 ± 0.4^{a}	6.21 ± 0.05^{a}
OM	48.47 ± 26^{a}	17.11 ± 2^{b}	26.11 ± 17^{a}	13.3 ± 0.1^{b}

Note: In each row, means followed by the same letter do not significantly differ ($p<0.05$).

A decrease of nutrients concentrations in the sediment was reported when compared to IW and WO, except B which was similar during the adult period. Iron and Mn were higher in WO during the two periods. However, Cu was higher in WO during the adult period and S was higher in WO during the tadpole period. The pH of the sediment was acidic and OM had a removal rate of 65% and 50% in the adult and tadpole periods, respectively (Table 2).

When plants nutrients at the beginning and at the end of the experiment of each frog farming period are compared, there is a great incorporation of Ca, K, Mn, N, P and S concentrations. However, such assimilation has not been observed for B, Fe, Mg, and Zn. Copper was absorbed by the plant mainly during the adult period. Nutrients in the aquatic plants biomass were much higher than those observed in the sediment, indicating incorporation of these nutrients in their growth, except B, Cu and Fe (Table 3). *Eichhornia crassipes* reached 109 cm and 119 cm (rhizome + aerial) during adult and tadpole periods, respectively in 35 days of growth (Fig. 2).

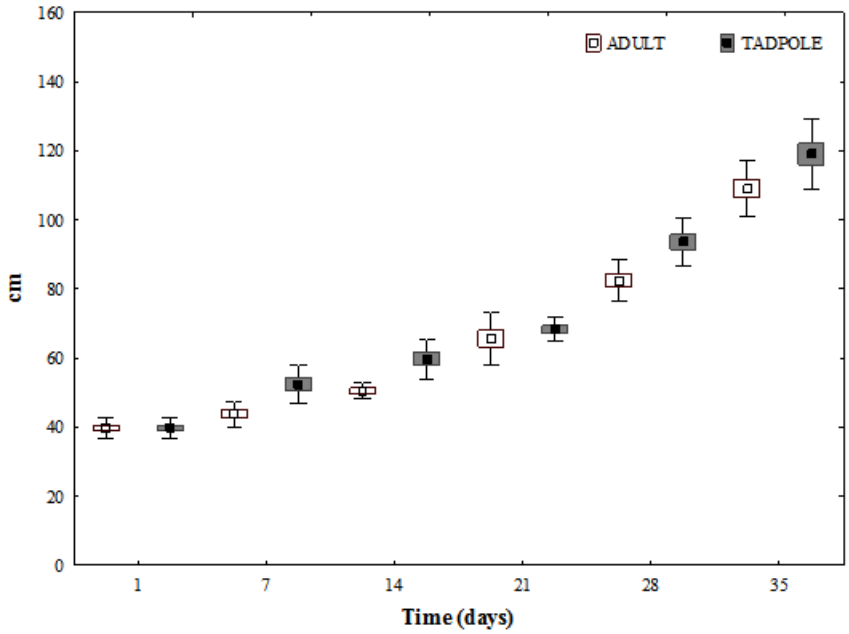

Figure 2. Total length (rhizome + aerial plant) growth of *Eichhornia crassipes* (Mart.) Solms during the two sampling periods of the frog farming (adult and tadpole). Measurements are means ± SD of 10 plants.

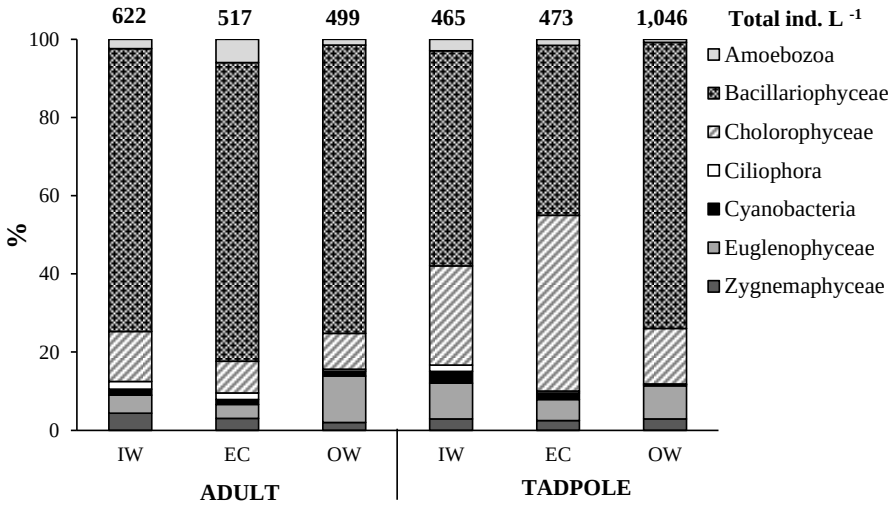

Figure 3. Relative abundance of phytoplankton and Protozoa at different sampling sites (IW = inlet water from indoor frog farming; EC = stand of *Eichhornia crassipes* (Mart.) Solms macrophyte in the wetland; WO = water outlet that flows directly into the fishpond) during two sampling periods of frog farming (adult and tadpole)

The phytoplankton community comprised 36 taxa, 10 Bacillariophyceae, 15 Chlorophyceae, 7 Cyanobacteria, 2 Euglenophyceae and 2 Zygnematophyceae. Protozoa consisted of 3 taxa with low contribution below 3% except in EC adult period which contributed with 5.9% due to *Arcella discoides*. The lattert was abundant and/or dominant in all sampling sites, except in IW during adult period (Fig. 3; Tables 3 & 4). The phytoplankton community had eight abundant/dominant species. However only *Navicula* sp. and *Pinnularia* sp. was abundant and/or dominant in all sampling sites. High number of Chlorophyceae species (15) were obtained, though Bacillariophyceae contributed with

more than 72% in the adult period and more than 43% in the tadpole period. Decrease in the contribution of Bacillariophyceae in the sampling sites IW (55%) and EC (43.5%) in the tadpole period was due to high contribution of Chlorophyceae in these sites, respectively with 25.9% and 44.9%, (Fig. 3). Diversity of phytoplankton species was high in IW during the adult period and in WO during the tadpole period. Cyanobacteria and Euglenophyceae were higher on the same site in both-periods, the first one in IW and the other one in WO. As a rule, diversity and evenness were high in IW. However, richness in EC and WO were similar (23) during adult period and higher in EC (29) during tadpole periods (Table 5). Protozoa richness was similar in all sampling sites, except in WO (2) in tadpole period; diversity was high in IW (0.69 and 0.55) and evenness was different. It was higher in IW (0.63) during the adult period and higher in WO (0.73) during the tadpole period (Table 5).

Table 3. Variables measured in *Eichhornia crassipes* (Mart.) Solms plants during two periods of frog farming (adult and tadpole) and the plant at the start of the experiment (IP).

Nutrient (g Kg^{-1})	IP	ADULT	TADPOLE
B	0.02	0.02	0.02
Ca	9.5	12.6	12.7
Cu	0.006	0.01	0.005
Fe	1.5	1.3	1.3
K	21.7	25.3	33.1
Mg	2.8	2.3	2.7
Mn	0.01	0.2	0.2
N	17.7	36.2	29.4
P	1.1	7.8	7.9
S	1.8	5.4	6.8
Zn	0.11	0.11	0.10

Table 4. Frequency of occurrence of phytoplankton and Protozoa at sites (IW = inlet water from indoor frog culture; EC = stand of *Eichhornia crassipes* (Mart.) Solms macrophyte into the wetland; WO = water outlet that flows directly into the fishpond) during two periods of frog farming (adult and tadpole).

Taxa	ADULT			TADPOLE		
	IW	EC	WO	IW	EC	WO
Bacillariophyceae						
Cyclotella sp.	+	+	+	A	A	A
Eunotia sp.	+	+	–	–	–	+
Fragilaria danica (Kützing) Lange-Bertalot	+	–	–	–	–	+
Gomphonema gracile Ehrenberg	+	+	+	+	+	+
Melosira sp.	+	–	+	–	–	–
Navicula sp.	A	A	D	A	A	A
Nitzschia amphibia Grunow	A	A	+	+	+	+
Pinnularia sp.	A	A	A	A	A	A
Surirella sp.	A	A	+	A	+	+
Synedra ulna (Nitzsch) Ehrenberg	+	+	–	–	–	–
Cholorophyceae						
Ankistrodesmus gracilis (Reinsch) Korshikov	+	+	+	+	+	+
Chlorella vulgaris (Beyerinck) Beijerinck	+	+	+	A	A	+
Coelastrum microporum Nägeli	–	–	–	–	+	–
Coelastrum reticulatum (Dangeard) Senn	–	–	–	+	+	+
Crucigenia quadrata Morren	+	+	+	–	+	+
Desmodesmus armatus (Chodat) Hegewald,	–	–	–	+	+	+
Dictyosphaerium pulchellum Wood	–	–	–	–	–	+
Kirchneriella lunaris Möbius	–	+	+	+	–	+
Pediastrum duplex Meyen	–	+	–	–	+	–
Scenedesmus acuminatus (Lagerheim) Chodat	–	–	–	+	–	–
Scenedesmus acutus Meyen	A	+	+	+	+	+
Scenedesmus bijugus (Turpin) Lagerheim	–	–	-	+	+	+
Scenedesmus quadricauda Brébisson & Godey	+	+	+	+	+	+
Sphaerocystis sp.	–	–	–	+	+	+
Tetraëdron trigonum (Nägeli) Hansgirg	–	–	–	+	+	–
Cyanobacteria						
Aphanocapsa sp.	+	+	+	+	–	–
Chroococcus limneticus Lemmerman	+	+	+	+	+	+
Lyngbia sp.	–	+	–	+	+	+
Microcystis aeruginosa (Kützing) Kützing	+	+	+	+	–	–

Oscillatoria sp.	–	–	–	+	+	–
Pseudoanabaena sp.	+	–	–	–	–	–
Sphaerocavum brasiliensis Azevedo & San't Anna	–	–	–	–	+	–
Euglenophyceae						
Euglena sp.	+	+	A	A	A	A
Phacus obolus Pochmann *:*	+	–	+	+	+	+
Zygnemaphyceae						
Closterium acutum Brébisson	+	+	+	+	+	+
Staurastrum sp.	+	–	+	–	+	–
Protozoa						
Amoebozoa						
Arcella discoides (Ehrenberg, 1830)	+	A	D	A	D	D
Difflugia sp.	+	+	+	+	+	–
Ciliophora						
Paramecium sp.	A	+	+	A	+	+

Note: + = present; – = absent; A = abundant; D = dominant.

Table 5. Quantitative analyses of phytoplankton and Protozoa communities: density, species richness, evenness, Shannon-Wiener diversity index (H') and total number of abundant and dominant species at sites (IW = inlet water from indoor frog farming; EC = stand of *Eichhornia crassipes* (Mart.) Solms macrophyte in the wetland; WO = water outlet that flows directly into the fishpond) during two periods of frog farming (adult and tadpole).

	ADULT			TADPOLE		
	IW	**EC**	**WO**	**IW**	**EC**	**WO**
Phytoplankton total (ind mL^{-1})	595	478	489	444	463	1036
Bacillariophyceae	450	395	368	256	205	765
Cholorophyceae	80	42	46	118	212	148
Cyanobacteria	9	7	6	14	8	3
Euglenophyceae	29	18	59	43	26	89
Zygnemaphyceae	27	16	10	13	12	30
Richness	19	23	23	27	29	26
Evenness	0.28	0.21	0.26	0.34	0.31	0.26
Diversity (H')	0.83	0.67	0.83	1.11	1.04	0.83
Total number of abundant species	5	4	2	6	5	4
Total number of dominant species	–	–	1	–	–	–
Protozoa total (ind mL^{-1})	27	39	10	21	10	10
Amoebozoa	15	31	7	14	7	8
Ciliophora	12	9	2	8	2	2
Richness	3	3	3	3	3	2
Evenness	0.63	0.48	0.51	0.59	0.51	0.73
Diversity (H')	0.69	0.53	0.56	0.65	0.56	0.51
Total number of abundant species	1	1	–	2	–	–
Total number of dominant species	–	–	1	–	1	1

Note: – = not found.

DISCUSSION

In current study the constructed wetland provided a relative removal rate using only floating plants, due to the high load from frog farming. In the effluent using two kinds of plants, floating and rooted, the removal efficiency was above 40% in the waste water of frog farming (Borges & Sipaúba-Tavares, 2017).

Gottschall *et al.* (2007) demonstrated that in constructed wetlands the absorption by plants might be considered the main form of nutrients removal due to macrophytes which would improve the removal of compounds by sedimentation. However, at greater water retention time in constructed wetland is one the most important factors in the removal efficiency of pollutants (Sindilariu *et al.*, 2009).

The low removal rate by constructed wetland in current research was effective for thermo-tolerant coliforms (>90%), BOD_5, conductivity, TDS, COD and TSS. However, the experimental period was during the rainy season with 178 ± 80 mm rainfall. According to Kuschk *et al.* (2003), precipitation affects detention time and consequently, influences the nutrients and solids removal efficiencies in the wetlands.

Variation in water volume and flow are factors that may affect the amount of pollutants discharged into the wetland and the capacity to remove compounds. Although the frog farming sector wastewater had higher concentrations of TIN, TP, solids and alkalinity, the hydraulic loading rate data indicated effluent with great flow due to the rainy season. Inlet water with high concentration of BOD_5, COD, and TSS was due to re-suspension caused by rain.

Water quality in the constructed wetland had great variability during each studied period so that most environmental parameters measured failed to show any significant difference between the adult and tadpole periods. The presence of thermo-tolerant coliforms in the effluent should have fostered detrital food where small size Protozoa have a successful favoured position in the circumstance. Only three Protozoa species were found in current study and were dominant or abundant at the different sites (IW, EC and WO). Protozoa are widespread in wetland rhizosphere where feeding on roots and microorganisms is enhanced. They are directly related to suspended solids produced by fast life cycle macrophytes such as *E. crassipes* (Neori & Argami, 2016). The continuous growth of the plants until the end of each sampled period and high wastewater from frog farming provided a large abundance of organisms mainly phytoplankton species.

High temperature, nutrients concentrations, solids and low dissolved oxygen concentrations were favorable to Protozoa, thermo-tolerant coliforms and Bacillariophyceae due to eutrophic conditions of constructed wetland. Protozoa in aquatic environments are directly related to suspended solids produced by fast life cycle macrophytes, such as *Eichhornia crassipes* (Mieczan, 2007).

Since the experimental period occurred during the rainy season, turbulence and water flow were more intense. It is a well-known fact that rainfall affects the physical and chemical parameters of water, also affecting the biomass of microorganisms. According Mello *et al.* (2016) the combination of aerobic and anaerobic bio-filters was essential for the removal of organic matter and toxic ammonia from the water of frog farm.

Phytoplankton in current study was characterized by Bacillariophyceae and Chlorophyceae. Dominance and differentiation between the groups and others microalgae groups are closely allied to the availability of their preferred physicochemical parameters. According to Zebek & Szymáriska (2017) the dominance of Bacillariophyceae is often correlated with high oxygen, ammonia concentration, alkaline pH. However, Travaini *et al*. (2016) observed that, in the constructed wetland the dominance of Bacillariophyceae was due to their high capacity to adapt low light intensity and their ability to persist on the sediment. Results were similar to those obtained in this research where *Navicula* sp. and *Pinnularia* sp. were abundant and/or dominant due to re-suspension of sediment caused by high HLR (0.76-0.85 m h^{-1}).

The high density of phytoplankton species in the constructed wetland was also due to conductivity, averaging 167-201 µS cm^{-1} at all sites. The above is beneficent to phytoplankton species. Rich nutrients such as K, Ca, N, P and Mg presently come from frog farming sector and maintain favorable conditions for phytoplankton production. According to Das *et al*. (2018) the densities of most of the phytoplankton species are associated with temperature, pH, TDS and conductivity.

Species natural to eutrophic environments in Protozoa and phytoplankton communities were frequent. However, low density of species linked to toxicity was detected. These included Cyanobacteria. During the adult period, the phytoplankton community decrease at the outlet of the wetland (WO). This fact has not reported during the tadpole period. In case of Protozoa, decrease in density was reported at the outlet (WO) of the wetland throughout the experimental period.

Although aquatic plants are important tools in the treatment of effluents in aquaculture, the single employment of *Eichhornia crassipes* in the frog farming wetland was not efficient for the removal organic; load, such as the join use of floating and rooted plants (Borges & Sipaúba-Tavares, 2017).

As may be perceived in current study, the aquatic plant *E. crassipes* has rapid growth (35 days). Changes in macrophytes had to be made so that the incorporation of nutrients in the metabolism could be efficient, confirming the assimilation of nutrients during two periods (adult and tadpole), with the exception of B, Fe, Mg and Zn. The roots of floating plants in the wetland may meet the sediment, with an exchange between the two compartments. Reactions in the sediment are complex and highly important particularly in the treatment of the effluent by the wetland. Current study revealed high concentrations of nutrients at the inlet of the wetland (IW) and a decrease of great number of nutrients at the outlet (WO) of the wetland, either on the sediment or in the water, such as in the absorption by the aquatic plant.

Current experiment revealed a relative removal of parameters analyzed through *E. crassipes* alone in the wetland. However, the system should be employed in frog farming effluents to minimize high pollutants loads. In fact, current study contributed towards an in-depth knowledge of waste-water from frog farming with regard to the removal rate of nutrients, the importance of sediment and performance of macrophyte *E. crassipes* in the wetland and the characterization of microorganisms, such as phytoplankton and Protozoa species.

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