# Sewage pollution: genotoxicity assessment and phytoremediation of nutrients excess with *Hydrocotyle ranunculoides*

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## Abstract

The discharge of sewage effluents into low-order streams has negative effects on water quality. Macrophytes can be efficient in the treatment of this wastewater due to the removal of the main pollutants. The genotoxicity of sewage-polluted water discharging into La Choza stream was evaluated by testing with *Allium cepa*. Also, a phytoremediation assay with continuous recirculation of the residual water was conducted for 12 days. Three treatments were carried out. One treatment (Hr) was performed with a macrophyte (*Hydrocotyle ranunculoides*); and two treatments were conducted without macrophytes: with lighting (Ai) and without lighting (Ao). The wastewater was toxic according to all the evaluated indexes (mitotic index, frequency of chromosomal aberrations and micronucleus). High concentrations of ammonium, dissolved inorganic nitrogen (DIN), total (TP) and soluble reactive phosphorous (SRP) and indicators of faecal contamination were determined in wastewater. The ammonium, DIN, SRP and TP loads at the end of the assay were significantly lower in the treatments with light (Hr and Ai). So, the nutrient removal was due to their absorption and adsorption by the periphyton and *H. ranunculoides*. Our results lead us to recommend the maintenance and planting of macrophytes in lowland streams subject to sewage pollution.

**Keywords**: Domestic wastewater; Genotoxicity; Nutrients; Macrophytes.

## Introduction

Domestic wastewater is a complex mixture of organic, inorganic, dissolved and suspended matter. The main components are organic matter, microorganisms, suspended solids, inorganic compounds (chlorides, sulfates, nitrogen, phosphorous, carbonates and bicarbonates) and toxicants. The organic matter in sewage reaching wastewater treatment plants (WTP) consists mainly (90%) of proteins and carbohydrates (Henry 1999). Unfortunately, in many cases the treatment is insufficient and dumping has negative consequences on the water quality of the receiving water body, particularly when it is a shallow lake or a low-order stream. If the levels of organic matter, nutrients and other pollutants in the treated effluent are high, the self-purification process in the receiving water bodies and the biological community structure and function may be affected significantly (Bunzel et al. 2013; Taylor et al. 2014).

An increase in the sanitation services is seen in many developing countries (WHO/UNICEF JMP 2016). In Argentina, 48.8% of the population has access to improved sewage services (INDEC 2010 a). According to the Agenda for Sustainable Development (UNDP 2015), countries must achieve universal and equitable access to water and sanitation by 2030. The increase in access to improved sanitation implies a reduction in water-borne diseases, but it also means an increase in the volume of sewage effluent that has to be disposed of.

Lowland streams in the Pampean region have a low discharge (Arreghini et al. 2007; Leggieri and Ferreiro 2015), and therefore the effluent from treatment plants of sewage liquids discharged into these streams may account for much of its flow, particularly in periods when the discharge is lowest. These streams are naturally rich in nutrients, particularly phosphorus, and eutrophication of the water may cause ecological changes, such as an increase in the total density of diatoms and a decline in biodiversity (Licursi et al. 2016). La Choza stream (Reconquista river basin, Buenos Aires, Argentina) is a typical lowland stream with a high concentration of nutrients, due to the inadequate management of effluent received (poultry industry, among others) (Basílico et al. 2013) and to nonpoint pollution from agriculture and intensive livestock activities in the watershed (Vilches et al. 2013). Whereas concentrations of dissolved inorganic nitrogen are relatively low (0.65 ± 0.43 mg/l; Cochero et al. 2013) in the upper basin, the discharge of industrial effluents with little treatment leads to a situation of chronic pollution in the middle and lower basins, with very high concentrations of ammonium, total phosphorus and organic matter (Basílico et al. 2013; 2015).

Floating macrophytes have been shown to be efficient in the treatment of wastewater by removing nutrients, organic and toxicants (Martelo and Lara Borrero 2012). *Hydrocotyle ranunculoides* is a native species distributed in South America and frequently found in Pampean streams, as La Choza stream (Basílico et al. 2015). This macrophyte grows rooted in the banks, but also as a floating plant covering the width of the water courses in some sections. *H. ranunculoides* has been used successfully in the phytoremediation of effluent from cattle feedlots (Rizzo et al. 2012), but there are few studies on its ability for treating other types of wastewater (Basílico et al. 2016 a) or for the *in situ* remediation of polluted water bodies (Basílico et al. 2016 b).

This paper aims to assess the toxicity of mixed wastewater mainly composed of effluent from a liquid sewage treatment plant and to characterize the use of *H. ranunculoides* for its phytoremediation.

## Materials and methods

### Study area

La Choza stream (Buenos Aires, Argentina, **Fig. 1**) receives industrial and sewage effluents, for example from the sewage treatment plant (STP) in General Rodriguez (6720 households with sewer, INDEC (2010 b)) and poultry industry (Basílico et al. 2013). Basílico (2015) found some evidence of detrimental effects of sewage discharge over La Choza stream water quality after sampling (n=1) surface water at a site upstream (U) and downstream (D) of the mouth of a semi-natural channeled tributary receiving STP effluent. Nutrients concentrations were several times higher downstream than upstream, with D/U ratios of 40.8 for ammonium nitrogen, 25.5 for dissolved inorganic nitrogen (DIN), 3.49 for soluble reactive phosphorus and 2.55 for total phosphorus. On the other hand, the indicators of faecal contamination increased downstream, with D/U ratios of 16.8 for faecal coliforms, 25.0 for *Escherichia coli* and 20.0 for enterococci (ratios calculated from Basílico (2015)).

By comparing the results of Basílico (2015) with those found by other authors in similar watercourses in the region (Arreghini et al. 2007; Feijoó and Lombardo 2007; Rigacci et al. 2013; Vilches et al. 2011), it was observed that the physicochemical characteristics of site upstream the tributary discharge are similar to those of other sites upstream in the same catchment area as La Choza stream and other basins, such as El Durazno stream, which is considered as a reference for water quality in the Reconquista river basin (Arreghini et al. 2007). In these less contaminated sites, typical ammonium values are lower than 0.6 mg/L, whereas those of the SRP are below 0.4 mg/L.

On the other hand, the water quality downstream the tributary discharge was comparable to that found in contaminated sections of the same basin, downstream from polluted tributary discharge (Arreghini et al. 2007; Basílico et al. 2015; Rigacci et al. 2013). Characteristic values of ammonium in the contaminated sites of the Reconquista river basin are in the range of 2.78 mg/L to 11.067 mg/L and those of SRP are greater or close to 1 mg/L. Levels found by Basílico (2015) are even similar to those found in sections of the lower Reconquista basin which have been severely contaminated for decades (Arreghini et al. 2007).

Although the information corresponds to a single sampling date, given the very sharp increase in the concentrations of ammonium nitrogen, DIN, SRP, TP and indicators of faecal contamination in a stretch of 200 m that receives a single discharge of water from sewage contaminated tributary, there is evidence to suspect that this is one of the main sources of pollution in La Choza stream.

### Genotoxicity test in *Allium cepa* meristematic root cells

Water samples were taken in the last section of the sewage-polluted tributary, 25 m upstream of its discharge into La Choza stream (site T, **Fig. 1**). A mutagenicity test of the wastewater was performed using seeds of *A. cepa* following the methodology described by Matsumoto et al. (2006). A homogeneous lot of seeds (Valcatorce variety) grown under organic conditions was used. The seeds of this species are preferred because of their genetic and physiological homogeneity and their availability throughout the year. A total of 100 seeds were placed in Petri dishes containing a filter paper with 5 ml of the sample or control. Distilled water was used as a negative control and methyl methanesulphonate (MMS) (2 x $10^4$ M concentration) as a positive control. The plates were kept at 22-24 °C for 4 days. The seeds with their roots were fixed for 24 h in acetic Carnoy, then washed and preserved in 70% ethanol for later observation (Fiskesjö 1985). Chromosomes of the meristematic root cells were stained with orcein in 2% acetic acid. The mitotic index (MI) was calculated by counting all stages of mitotic cells with respect to the total number of cells. For the chromosome aberration (CA) analyses, several aberrations such as bridges, fragments, delayed chromosomes and others in the anaphase and telophase were analyzed. The micronuclei (MN) induction was recorded by observing the interphase cells. The analyses were performed by scoring 5000 cells per treatment, i.e. 1000 cells per slide and a total of 5 slides. Cytotoxicity was assessed based on the MI values, and genotoxicity was evaluated based on the CA and MN frequencies, as frequency = (A/B) x 100; where A is equivalent to the total number of cells with a parameter to be analyzed (CA or MN), and B corresponds to the total analyzed cells.

### Bioassay with continuous recirculation of polluted water

Sixty liters of mixed wastewater were collected from site T (**Fig. 1**). A 12 day bioassay was carried out using polypropylene reactors with continuous recirculating water. In order to evaluate the role of *H. ranunculoides* in removing contaminants from wastewater, the design of the trial consisted of three treatments in triplicate: presence of *H. ranunculoides* (Hr treatment), absence of the species in lighting conditions (Ai treatment) and absence of the species in dark conditions (Ao treatment). The photoperiod was 16:8 (light/dark) using natural light and, complementarily, artificial light. In the Hr treatment, the initial biomass used was 37.1 ± 5.1 g fresh weight (FW) per reactor, equivalent to 40 leaves per reactor. This test and the previous cultivation of the plants were carried out at the Argentine Museum of Natural Sciences "Bernardino Rivadavia" greenhouse. The initial volume of water in each reactor was 5 liters and the recirculation rate was 34 ± 1 ml/s in order to recreate the low current velocities of constructed wetlands for effluent treatment. The dimensions of the reactors were: 0.500 m x 0.155 m x 0.140 m (length x width x depth; volume: 10.85 l).

### Analytical determinations

For the characterization of the mixed wastewater from site T used in the bioassay, the physical and chemical variables listed in **Table 1** were determined by means of standardized methodologies. The water samples were filtered through a 0.45 mm Whatman GF membrane to determine the ammonium, nitrite, nitrate, soluble reactive phosphorus and total and dissolved organic carbon.

Considering the effects of evaporation and evapotranspiration (Tuttolomondo et al. 2016) and since the water losses were not compensate, the removal percentage of each analyte was calculated according to **Eq. 1**:

$$R\% = 100 \times \frac{v_i \times c_i - v_f \times c_f}{v_i \times c_i} \qquad \textbf{Eq. 1}$$

where $R\%$ is the removal percentage of the variable considered, $v_i$ and $c_i$ are the initial volume and concentration in each reactor, $v_f$ and $c_f$ the final ones, and the product of $v \times c$ is the load or mass of the analyte in each reactor ($L$).

### Indicators of faecal contamination

A subsample was taken from water collected at site T for microbiological analysis. The plate count technique was used for counting the faecal contamination indicator bacteria. One milliliter of serial decimal dilutions of the sample was inoculated in selective and differential medium in Slanetz-Bartley© agar and CHRO ECC agar (CHROMagar©) for enterococci counts, and total and faecal coliforms, respectively. The plates were incubated at 35 °C for 48 h, except for the faecal coliforms at 44 °C for 48 h (APHA et al. 2012).

### Statistical analysis

The existence of statistically significant differences ($p < 0.05$) between the values of the final loads of variables in the different treatments was examined by one-way ANOVA with *post hoc* Tukey comparisons. Moreover, Dunnet's *post hoc* comparisons between the initial and final concentrations of each treatment were made. Non-normal and/or heteroskedastic variables were previously transformed. In the case of nitrate concentrations, the transformation was not feasible, so a nonparametric Mann-Whitney *U* test to compare initial and final values was performed (Zar 1996). Genotoxicity data from the *A. cepa* test were analyzed using the Kruskal-Wallis test (Matsumoto et al. 2006).

**Table 1** Methodologies followed in the determinations made in water samples

| **Variable** | **Methodology** | **Reference** |
|---|---|---|
| Temperature (T) | Alcohol thermometer | - |
| pH | Hanna® meter | - |
| Electrical conductivity (EC) | Hanna® meter | - |
| Total suspended solids (TSS) | Gravimetry | APHA et al. 2012 |
| Dissolved oxygen (DO) | Hanna® meter | - |
| Ammonium nitrogen ($NH_4^+$-N) | Blue indophenol | Mackereth et al. 1989 |
| Nitrite ($NO_2^-$-N) | Diazotization | Strickland and Parsons 1972 |
| Nitrate ($NO_3^-$-N) | Diazotization with previous reduction with hydrazine sulphate and $Cu^{2+}$ and $Zn^{2+}$ ions | Downes 1978 |
| Dissolved inorganic nitrogen (DIN) | Sum of ammonium, nitrite and nitrate | - |
| Soluble reactive phosphorus (SRP) | Ascorbic molybdate | Strickland and Parsons 1972 |
| Total phosphorus (TP) | Ascorbic molybdate with previous digestion with $H_2SO_4$ and potassium persulphate | Strickland and Parsons 1972 |
| Total and dissolved organic carbon (TOC, DOC) | Oxidation in acidic medium | Golterman et al. 1978 |
| Particulate organic carbon (POC) | TOC - DOC substraction | - |

## Results and discussion

### Genotoxicity in *A. cepa*

Higher plants present characteristics that make them excellent genetic models to assess environmental pollutants, being frequently used in monitoring studies (Leme and Marin-Morales 2009). The main feature of *A. cepa* is their sensitivity to detecting mutagens in different environments, and the possibility of assessing several endpoints, such as cytotoxicity, by the increase or decrease in the MI, and genotoxicity by the high frequencies of MN and CA (Leme and Marin-Morales 2009). The results of *A. cepa* test show significant differences ($p < 0.05$) in the MI, frequency of CA and frequency of MN between the positive control and the negative control, and between mixed wastewater from site T and the negative control (**Table 2**), indicating the wastewater cytotoxicity and genotoxicity. Sewage effluents do not only contain organic matter, salts, nutrients and microorganisms, but also genotoxic compounds such as metals and emerging contaminants (Heberer et al. 2002). There are very few studies on the influence of discharges of municipal wastewater on genotoxicity in biota (Talapatra and Banerjee 2007). The main compounds of the sewage are carbohydrates (33-55 mg/L); free and bound amino acids (27.5-36 mg/L); higher fatty acids (71-74 mg/L); soluble acids (21-34 mg/L); esters (28.2-37.2 mg/L) and trace amounts of amino sugars, amide and creatinine are not considered to be genotoxic. However, there are inhibiting compounds of mutagenicity in human faeces (Jha et al. 1997), which could mask the toxic effects of the compounds mentioned. In recent years the presence of emerging compounds in wastewater is becoming more important, coming from pharmaceuticals used by the population (Heberer et al. 2002) and their metabolites (la Farre et al. 2008) which have genotoxic effects that could be causing the effect found in polluted sewage water.

Bioassay

The presence of characteristic bacterial periphyton in water chronically contaminated with organic matter was observed at the sampling site of water used in the bioassay. The pH of the wastewater increased over time in all treatments, reaching a maximum average of 9.17 in the treatment with the absence of *H. ranunculoides*, in lighting conditions (Ai treatment, **Fig. 2**). The marked increase in algal biomass on the reactor walls in all treatments, but especially in Ai, result in a significant $CO_2$ consumption by photosynthesis and a consequent increase in pH. The EC showed little variation over time in the Ao treatment and it increased in the remaining treatments, reaching a maximum average of 1359 µS/cm in the treatment with *H. ranunculoides* (Hr, **Fig. 2**). The similar values of water evaporation/evapotranspiration in Hr and Ai treatments (~21%) explain the higher EC in these treatments in relation to Ao treatment (evaporation of ~13%).

The dissolved oxygen (DO) values were similar for all treatments during the first 10 days, and then higher values were observed in the Ai treatment, highlighting the impact of recirculating water and phytoplankton and periphyton algae in the oxygenation of water. In all cases, DO concentrations were higher than 6.57 mg/L (**Fig. 2**). Increases of DO reaching values of supersaturation at the end of the test were associated in part with oxygen exchange between aerial tissues and roots (Reddy et al. 1990) as macrophytes carry about 90% of oxygen to the rhizosphere (Srivastava et al. 2008), as well as the photosynthetic activity of phytoplankton and periphyton. TSS concentration was reduced from 20 mg/L to between 3 mg/L (Ao treatment) and 6 mg/L (Hr treatment) (**Fig. 2**). In this case, the absence of plants and the limited presence of algae due to the absence of light resulted in a lower concentration of suspended solids.

**Table 2** Mitotic index (MI), frequency of chromosomal aberrations (CA), frequency of micronuclei (MN) in 5000 analyzed meristem cells of *Allium cepa* exposed to the sample of sewage wastewater, to the negative control and the methyl methanesulphonate solution (MMS, positive control). Mean ± standard deviation

| Treatment | MI (%) | CA (%) | MN (%) |
|---|---|---|---|
| Negative control | 58.96 ± 5.06 | 0.02 ± 0.06 | 0.60 ± 0.24 |
| Wastewater | 73.69 ± 4.16* | 0.16 ± 0.08* | 4.05 ± 1.44* |
| MMS | 61.80 ± 3.78* | 0.97 ± 0.70* | 6.25 ± 2.92* |

* Significant differences ($p < 0.05$) with respect to negative control according to Kruskal-Wallis test.

The mean loads of TSS, $NH_4^+$-N, DIN, TP, TOC, DOC and POC decreased throughout the test. There were significant ($p < 0.05$) or highly significant differences ($p < 0.01$) between treatments for most variables, except for DOC, POC and TOC. According to the Tukey *post hoc* comparisons for variables $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$-N, DIN, SRP and TP there were two homogeneous groups, one formed by the treatments in the presence of light and vegetation (Ai and Hr) and another for the treatment in darkness (Ao) (**Table 3**). The lowest load of suspended material at the end of the test was found in the Ao treatment, with a removal percentage of 84.9 ± 0.9% (**Table 3**). The main chemical species of dissolved nitrogen was ammonium. Removal of $NH_4^+$-N and DIN was complete in treatments with macrophytes and algae (Hr and Ai). Therefore, it can be inferred that the removal of ammonium is mainly due to the activity of the photosynthetic periphyton and phytoplankton organisms and, to a lesser extent, to the presence of *H. ranunculoides*.

At the end of the bioassay nitrites and nitrates increased in treatments without macrophytes, with significantly higher final loads of both nitrogen species over the initial loads in the Ao treatment. The presence of periphyton but mainly macrophytes may promote absorption of nitrates. Although ammonium is the main species of nitrogen absorbed by aquatic plants (Caicedo et al. 2000), plant preference for different forms of N is affected by temperature, pH and element composition of the solution as well as plant growth stage (Fang et al. 2007). In the subfamily Lemnoideae (duckweeds), differences in $NH_4^+$-N uptake might depend on plant requirements (Fang et al. 2007). A rise in external concentration from 50 to 250 µm $NH_4NO_3$ significantly decreased the uptake of both N forms by roots and increased the uptake of $NO_3^-$-N by fronds (Cedergreen and Madsen 2002).

The removal of $NH_4^+$-N and DIN was almost complete in the Hr and Ai treatments (**Table 3**). These removals were higher than those achieved in a phytoremediation assay of poultry wastewater in similar experimental conditions with the macrophytes *S. intermedia* (Basílico et al. 2016 a) and *Lemna gibba* + *H. ranunculoides* (Basílico et al. 2017), although the duration of these assays was half (6 day). After 12 days very significant reductions were observed in the concentrations of N-$NH_4^+$ and NID in the Ai and Hr treatments with respect to the initial concentrations of 19.71 and 19.79 mg/L, respectively (**Fig. 3**). In the treatment without *H. ranunculoides*, in darkness (Ao treatment), lower reduction in the concentrations of both variables was observed. In the Hr and Ai treatments, the presence of *H. ranunculoides* and algae favored nitrification of ammonium and the subsequent absorption of nitrates. In the Ao treatment nitrification was lower due to the lower level of dissolved oxygen, although an increase in the concentrations of $NO_2^-$-N and $NO_3^-$-N was observed (**Fig. 3**) presumably because there were fewer organisms capable of uptake these forms of N.

The presence of *H. ranunculoides* favored the removal of SRP and TP, which decreased from the baseline values of 2.02 mg/L and 2.48 mg/L, respectively (**Fig. 3**). In the other treatments, the final concentration of SRP was higher than the initial, reaching an average value of 2.53 mg/L in the Ao treatment. Total phosphorus concentration decreased in the Ai treatment and increased slightly in the Ao treatment (**Fig. 3**). A low removal of SRP was obtained in the Hr and Ai treatments and an increase in SRP load in Ao. Total phosphorus removal was higher in the Hr and Ai treatments (40.2 ± 5.4% and 33.9 ± 2.7% respectively) compared to Ao (11.8 ± 1.0%) (**Table 3**). A similar removal of TP was achieved by Basílico et al. (2017) in poultry wastewater treatment with *H. ranunculoides* (~45%), although the volume used in that case was 9 L. Moreover, an increase was observed in the load of SRP in the Ao treatment. It should be noted that the rapid turnover of phosphorus determines that while its absorption and deposition at the bottom of the containers occurs, release by changes in the redox potential or by decomposition can compensate for losses. It also has been detected in *Pistia* sp. and *Hydrocotyle* sp., among others, that the assimilation of phosphorus is short term as it is quickly released back into the environment by the decomposition of tissues (Reddy et al. 1999). Another factor influencing nutrient uptake is the N:P ratio in the water. *Eicchornia crassipes* had the highest uptake of N and P for the ratio N:P between 2.3 and 5 (Reddy and Tucker 1983). In this paper, the initial N:P ratio was 10:1, which could have a negative influence on the uptake of nutrients.

Mean loads of TOC per reactor calculated at the end of bioassay were lower than the initial (77.4 mg) with no significant difference between treatments ($p > 0.05$), reaching average values of 51.5 mg (Hr treatment), 49.2 mg (Ai treatment) and 46.3 mg (treatment Ao) (**Table 3**). Greater removal of the carbonaceous particulate fraction (POC) with respect to the dissolved fraction (DOC) was obtained; therefore the TOC removal was primarily associated with the deposition of the particulate material (**Table 3**). As it was found in previous phytoremediation bioassay (Basílico et al. 2016 a), the contribution of macrophytes to the removal of organic carbon seems to be of little importance (**Fig. 3**).

The initial biomass of plants was 37.1 ± 5.1 g DW. The RGR calculated from biomass was very low (0.007 g/g d), although in terms of the quantity of leaves it was higher (0.021 $d^{-1}$). The low growth rate observed could be because above certain levels of nutrients no increase in the productivity of aquatic plants occurs. For concentrations above 5.5 mg N/L, the growth of *E. crassipes* did not increase to the same extent, and the same was true when the levels of P exceeded 1.06 mg/L (Reddy et al. 1990). In this study, the nutrient levels far exceeded the values cited. *H. ranunculoides* absorbed nutrients resulting in a complete removal in some cases (ammonium), without substantially increasing their biomass, denoting a luxuriant consumption of N and P. Likewise, concentrations of nutrient elements in the assay exceed those in conventional cultures (Mkandawire and Dudel 2005). However the growth rates in the assay were much lower than growth in the culture media.  The effect probably resulted from the gradient change occurred in the recirculating mesocosm, which might induce stress in plants (Mkandawire and Dudel 2005). In the natural environment or in constructed wetlands would be expected higher rates of growth, because in those cases the supply of dissolved nutrients is constant (Basílico et al. 2017).

The conservation of riparian vegetation helps to reduce nutrient inputs from agriculture and livestock production reaching streams waters by runoff. Furthermore, the existence of marsh species, such as *H. ranunculoides*, on the banks and in streams and rivers can contribute to the recycling of nitrogen and phosphorus. The removal of floating and rooted aquatic plants may be justified in some cases, but only for hydraulic considerations, in order to speed up the flow during flooding. However, from a broader point of view, the environmental services provided by the presence of macrophytes, such as creating habitat for other aquatic species, nesting sites for birds and of course as biological filters of a large number of pollutants (Basílico et al. 2016 b), must be considered. Additionally, it should be noted that the presence of several species could result in a more efficient treatment of residual water, compared to monospecific cultures (Basílico et al. 2017).

**Table 3** Loads of suspended solids and nutrients per reactor in treatments with individuals of *H. ranunculoides* (Hr), in the absence of the species, in lighting conditions (Ai) and in the absence of the species, in dark conditions (Ao), detailing the *p*-values (ANOVA) and removal percentages (R%) per variable

| **Variable** | **Treatment** | **Initial load (mg)** | **Final load (mg)** | ***p*-value** | **R%** |
|---|---|---|---|---|---|
| TSS | Hr | 100 ± 17 | 25 ± 4 a | 0.017* | 75.3 ± 3.6 |
| | Ai | | 19 ± 3 ab | | 81.1 ± 2.8 |
| | Ao | | 15 ± 1 b | | 84.9 ± 0.9 |
| $NH_4^+$-N | Hr | 98.56 ± 4.63 | 0.05 ± 0.03 a | 0.000** | 100.0 ± 0.0 |
| | Ai | | 0.04 ± 0.00 a | | 100.0 ± 0.0 |
| | Ao | | 18.51 ± 2.13 b | | 81.2 ± 2.2 |
| $NO_2^-$-N | Hr | 0.11 ± 0.00 | 0.01 ± 0.00 a | 0.000** | 86.9 ± 2.6 |
| | Ai | | 0.54 ± 0.40 a | | + |
| | Ao | | 6.47 ± 0.33 b | | + |
| $NO_3^-$-N | Hr | 0.27 ± 0.02 | 0.24 ± 0.13 a | 0.000** | 7.7 ± 50.0 |
| | Ai | | 0.95 ± 0.52 a | | + |
| | Ao | | 9.88 ± 3.28 b | | + |
| DIN | Hr | 98.94 ± 4.64 | 0.31 ± 0.14 a | 0.000** | 99.7 ± 0.1 |
| | Ai | | 1.53 ± 0.91 a | | 98.5 ± 0.9 |
| | Ao | | 34.86 ± 5.54 b | | 64.8 ± 5.6 |
| SRP | Hr | 10.11 ± 0.37 | 7.61 ± 0.87 a | 0.001** | 24.8 ± 8.6 |
| | Ai | | 8.28 ± 0.33 a | | 18.2 ± 3.2 |
| | Ao | | 11.00 ± 0.39 b | | + |
| TP | Hr | 12.41± 0.47 | 7.43 ± 0.67 a | 0.000** | 40.2 ± 5.4 |
| | Ai | | 8.21 ± 0.34 a | | 33.9 ± 2.7 |
| | Ao | | 10.94 ± 0.13 b | | 11.8 ± 1.0 |
| TOC | Hr | 77.4 ± 2.8 | 51.5 ± 3.0 a | 0.464 | 33.4 ± 3.9 |
| | Ai | | 49.2 ± 7.7 a | | 36.4 ± 10.0 |
| | Ao | | 46.3 ± 1.1 a | | 40.1 ± 1.4 |
| DOC | Hr | 35.3 ± 2.7 | 31.6 ± 2.9 a | 0.636 | 10.6 ± 8.3 |
| | Ai | | 31.6 ± 1.4 a | | 10.5 ± 4.1 |
| | Ao | | 29.3 ± 4.6 a | | 17.1 ± 13.1 |
| POC | Hr | 42.1 ± 3.8 | 19.9 ± 5.3 a | 0.793 | 52.6 ± 12.5 |
| | Ai | | 17.6 ± 7.2 a | | 58.2 ± 17.0 |
| | Ao | | 17.0 ± 3.5 a | | 59.5 ± 8.4 |

The asterisk (*) indicates statistically significant ($p < 0.05$) or highly significant difference (**) ($p < 0.01$) between the final loads of Hr, Ai and Ao treatments. The same letters (a and b) indicate membership in homogeneous groups according to Tukey *post hoc* comparison ($p < 0.05$). The + sign indicates a load increase throughout the bioassay.

## Indicators of faecal contamination

A gradual decrease in microbial loads was observed in the control without water recirculation and in the water recirculating reactors, with mean values in the range of 16-146 CFU/mL for coliforms and 3-597 CFU/mL for enterococci (**Fig. 4**). In both indicators of faecal contamination, the largest reduction was observed in the early days, being more important in the case of coliforms. The level of enterococci was adjusted to 1st order kinetics, in the case of treatment plants (**Fig. 4**). Brix (1993) notes that the deposition and subsequent death of microorganisms and the excretion of antibiotics by the roots of aquatic plants growing in constructed wetlands are some of the mechanisms involved in the removal of pathogens from domestic wastewater.

## Conclusions

Sewage-polluted water poured into La Choza stream was genotoxic and characterized by high concentration of nutrients and indicator of faecal contamination, highlighting the need for tertiary treatment and remediation of polluted surficial waters. During the remediation bioassay, a significant decrease in the concentrations of ammonium, DIN and POC in respect to the initial values was observed in the wastewater in all treatments. The ammonium, DIN, SRP and TP loads per reactor was significantly lower in treatments in lighting conditions at the end of the trial. The removal of these nutrients was associated with absorption and adsorption by periphyton, and secondarily with the action of *H. ranunculoides*. On the other hand, the nitrite and nitrate loads increased in the treatments without plants, although this increase was much higher in dark conditions. The removal of microbial indicators of faecal contamination was higher during the first days and was similar in all treatments, attributed mainly to sedimentation and death throughout the trial.

The results of this work demonstrate the luxuriant consumption of N and P by *H. ranunculoides*, which favors nutrient removal without increasing its biomass. The information obtained in this study and other cited works (Basílico et al. 2016 a; 2017) added to the mentioned ecosystem services provided by macrophytes, enable us to recommend the maintenance and even the planting of macrophytes in streams in the upper basin of the Reconquista River and other lowland streams with similar characteristics.

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## Figure captions

**Figure 1** Study area

**Figure 2** Values of pH (above, left), EC (above, right), DO (below, left) and TSS (below, right) during the bioassay, in the treatment with *H. ranunculoides* (Hr) in the absence of the species in lighting conditions (Ai) and in the absence of the species in dark conditions (Ao)

**Figure 3** Concentrations of $NH_4^+$-N-and DIN (above, left), $NO_2^-$-N and $NO_3^-$-N (above, right), SRP and TP (below, left) and TOC, DOC and POC (down, right) in wastewater treatment in *H. ranunculoides* (Hr) in the absence of the species in lighting conditions (Ai) and in the absence of the species in dark conditions (Ao). The asterisk (*) indicates significant differences ($p \leq 0.05$) between the initial and final concentrations, according to Dunnett's *post hoc* comparisons or Mann-Whitney *U* test (nitrates)

**Figure 4** Mean values of faecal coliforms at 44 °C (left) and enterococci (right) in the wastewater over time, in *H. ranunculoides* treatment (Hr) in the absence of the species in lighting conditions (Ai) and in the absence of the species in dark conditions (Ao). The plotted curve (right) corresponds to a 1st order kinetics for treatment Hr data. Control corresponds to wastewater without recirculation