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Optimization of a Bioremediation Strategy for an Urban Stream of Matanza-Riachuelo Basin

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***Abstract*—**In the present work, a remediation bioprocess based on the use of a local isolate of the microalgae *Chlorella vulgaris* immobilized in alginate beads is proposed. This process was shown to be effective for the reduction of several chemical and microbial contaminants present in Cildáñez stream, a water course that is part of the Matanza-Riachuelo Basin (Buenos Aires, Argentina). The bioprocess, involving the culture of the microalga in autotrophic conditions in a stirred-tank bioreactor supplied with a marine propeller for 6 days, allowed a significant reduction of *Escherichia coli* and total coliform numbers (over 95%), as well as of ammoniacal nitrogen (96%), nitrates (86%), nitrites (98%), and total phosphorus (53%) contents. Pb content was also significantly diminished after the bioprocess (95%). Standardized cytotoxicity tests using *Allium cepa* seeds and Cildáñez water pre- and post-remediation were also performed. Germination rate and mitotic index of onion seeds imbibed in Cildáñez water subjected to the bioprocess was similar to that observed in seeds imbibed in distilled water and significantly superior to that registered when untreated Cildáñez water was used for imbibition. Our results demonstrate the potential of this simple and cost-effective technology to remove urban-water contaminants, offering as an additional advantage the possibility of an easy biomass recovery, which may become a source of alternative energy.

***Keywords*—**Bioreactor, bioremediation, *Chlorella vulgaris*, Matanza-Riachuelo basin, microalgae.

I. Introduction

ANTHROPOGENIC activity originates considerable amounts of non-biodegradable inorganic and organic compounds, which are often released to the environment without any treatment. The Municipal Environmental Protection Agency (APRA, Spanish acronym) of Buenos Aires City reported the presence of waste materials coming from industries settled in the surroundings of the Cildáñez stream, a creek belonging to Matanza-Riachuelo Basin, as well as the existence of sewer discharges. These discharges include inorganic compounds that negatively affect the ecosystem. In this sense, it has been well established that ammoniacal nitrogen and phosphorus excess often result in water eutrophication and ecosystemic imbalances [1]. On another hand, pathogenic microorganisms coming from sewer discharges and the presence of heavy metal ions turn this urban water body a serious threat for the vast population settled at its nearby [2].

Cildáñez is an encased stream except at its last segment, before pouring its water in the Riachuelo, near Lugano Lake. From colonial times, this watercourse has been recognized as a hot-spot of contamination in Buenos Aires City, due to the presence of slaughterhouses wastes installed in the nearby. Because of this feature, Cildáñez stream received the colloquial name of “blood stream”. Today, this watercourse is characterized by a prominent fluctuation in the levels of biological and chemical contaminants along the year.

In recent decades, increasing the protection of natural resources and remediating contaminated sites have become a priority in many countries. Among other decontamination strategies, the use of microalgae as effective bioremediation agents has been repeatedly documented [3]-[6]. The immobilization of a native isolate of the microalgae *Chlorella vulgaris* inside an alginate matrix rendered encouraging results in a previous work performed by our group [7]. This technology has gained acceptance as an alternative process for water decontamination and is potentially applicable at open spaces. Besides, it offers the advantage of an easy recovery of the biomass involved, avoiding eutrophication and adding the possibility of obtaining by-products of economic value [8], [9].

Microalgae can be immobilized in different materials such as alginate, agar-agar, cellulose, and silica gel, among others. Alginate is a natural matrix of polysaccharides, especially useful for the manufacture of spherical capsules commonly called "beads". The immobilization in alginate beads protects the microalgae cells from the toxic effects of numerous substances and extreme pH and temperatures, allowing better survival and greater efficiency in the production of biomass [9], [10]. In addition, beads are easy to remove from the treated water.

Pilot-scale biomass production systems usually comprise stirred-tank bioreactors with aeration. These systems allow rapid growth of the microalgae biomass, which results in faster removal of contaminants. Growth systems may be classified as mixotrophic (supplemented with a carbon source and with photosynthetically active radiation ─PAR─), autotrophic (PAR illumination without the addition of a carbon source), and heterotrophic (only fed with a carbon source; non-PAR illumination), being the first the system which provides the highest productivity rate [9], [11].

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The selection of the stirrer propeller is a relevant point for the bioprocess efficiency since this choice largely determines fluid fluxes inside bioreactors. Classic Rushton turbines produce radial flows and considerable cutting forces, while marine style propellers produce axial and radial flows, with the highest flow rate for the lowest peripheral speed and are, therefore, recommended for organisms that are sensitive to shear stress [12]. On another hand, biological tests are often used to assess environmental quality and to detect contaminants of water supplies. These tests allow the simultaneous assessment of cytotoxic, genotoxic, and mutagenic effects of environmental samples [13]. The *Allium cepa* test is an economic and classic test that evaluates cytostatic effects, DNA instability, and inhibition of cell division caused by xenobiotics. The reduced number of chromosomes (2n = 16) of this plant species and their large size constitute a practical advantage, which may be complemented by the direct observation of onion roots. It may be reminded that plant root is the first plant structure exposed to soluble contaminants [14].

The objective of the present work was to evaluate through multiple approaches the efficiency of a bioprocess based on the immobilization of a local isolate of *C. vulgaris* in alginate beads, to bioremediate Cildáñez stream waters.

II. Methods

A. Microorganisms and Water Samples

A unialgal culture of *Chlorella vulgaris* previously isolated from the Atlantic Coast, near Chubut city, was obtained from Universidad Nacional de la Patagonia San Juan Bosco (UNSJB) Culture Collection. This culture was maintained on MS synthetic culture media supplemented with sucrose (3% w/v) and indolacetic acid (1 mg/L) as growth regulator [15], and kept at 24 ± 2ºC in mixotrophic conditions, with a photoperiod of 16 h PAR (400 µmol photon m^{-2} s^{-1}).

Water samples were obtained at spring and summer from Cildáñez stream (34°67′60.00″S; 58°44′37.06″W) and analyzed at the laboratory of APRA (Agencia de Protección Ambiental, Gobierno de la Ciudad Autónoma de Buenos Aires). The choice of these sampling periods was based on the analysis of 4-years historical records (2013-2017).

B. Immobilization of Microalgae Cells in Alginate Beads

An alginate solution (2% w/v) was mixed with a cellular suspension of *C. vulgaris* grown in MS medium with sucrose (2 x 10^6 cells mL^{-1} alginate). The mixture obtained was dropped on a solution of $CaCl_2$ (0.1 M) using a 50 mL syringe, with a 2 mm diameter outlet. The beads thus obtained were incubated for 1 h, and then washed with saline solution (NaCl 0.9% w/v).

C. Bioreactor Process Optimization

C. vulgaris cells entrapped in alginate beads (obtained as previously described) were placed in a stirred-tank bioreactor (Minifors, Infors HT®, Switzerland) vessel, fed with MS culture media, with mechanical agitation provided by a marine or a Rushton propeller (150 rpm), and a bubble aeration system given by a porous metal sparger. The working volume (1.5 L) was inoculated with 2 x 10^6 cells mL^{-1} alginate in MS.

The microalgal growth rate was assessed by counting *C. vulgaris* cells in a Neubauer chamber [4]. The specific growth rate (µ) and the duplication time were calculated using the software Fermenter Tool [16].

D. Bioremediation Process

Once optimized, the bioprocess was carried out with water from Cildáñez stream using a marine propeller at 24±2 ºC in autotrophic conditions, with a photoperiod of 16 h PAR (400 µmol photons m^{-2} s^{-1}). Water samples were obtained during the summer/spring of 2017/2018. In each assay, 100 beads prepared with a culture of 2 x 10^6 cells mL^{-1} were used; the bioprocess lasted 6 days. Before and after the bioprocess, total numbers of coliforms and *Escherichia coli* were determined according to APHA [17].

Using standard techniques established by APHA [17], the following physicochemical parameters were determined before and after the bioprocess: turbidity (NTU), nitrites (mg L^{-1}), nitrates (mg L^{-1}), ammoniacal nitrogen (mg L^{-1}), total phosphorus (µg L^{-1}), electrical conductivity (µS cm^{-1}), and pH.

Heavy metals were also assessed by atomic absorption spectrometry (Analyst 800, Perkin Elmer, USA) using nitric acid-digested samples. Lead (Pb), cadmium (Cd), and arsenic (As) were determined using an electrothermal procedure (graphite oven). Chromium, (Cr), copper (Cu), and zinc (Zn) were analyzed by flame spectrometry.

E. Bioreactor Operative Conditions

The bioreactor was set at 24 ± 2 ºC, with a photoperiod of 16 h PAR (400 µmol photons m^{-2} s^{-1}) and aeration of 0.1 vvm. The relative partial O_2 pressure (Oxyferm 225, Hamilton®) and pH (Mettler Toledo®) were monitored on-line by autoclavable electrodes; O_2-electrodes calibration was performed with pure N_2. The whole process was monitored by the Iris Explorer software version 5.2. Oxygen transfer coefficient (KLa) and oxygen uptake rate (OUR) were estimated by the dynamic method [18].

F. Cytotoxicity Assay

A cytotoxicity test was performed using seeds of *Allium cepa* following the methodology described by [19]. A total of 300 seeds were placed in Petri plates containing a filter paper imbibed with: i- 20 mL of water samples of Cildáñez stream before the bioprocess (untreated water, UW), ii- 20 mL of water samples of Cildáñez stream after the bioprocess (treated water, TW); iii- 20 mL of distilled water (DW). The plates with the seeds were kept at 24 ± 2 °C for 48 h. The germination index (GI) was calculated at 48 hours and 7 days as:

GI= nº of seed with root tips/total seeds

Root tips were fixed for 24 h in acetic Carnoy. Chromosomes of the meristematic root cells were stained with Orcein in 2% acetic acid. The mitotic index (MI) was calculated by counting the cells undergoing mitotic stages, and

then dividing this value by the total number of cells, using a microscope Olympus BX40 (Olympus ®).

G. Statistical Analysis

Six independent experiments were carried out in the bioreactor: 3 using MS culture media (for the optimization of *C. vulgaris* growth) and 3 using Cildáñez water samples obtained at summer or spring (for the remediation bioprocess). Physicochemical and microbiological analyses were performed at the beginning and the end of the bioprocess (day 6). Analytical determinations were performed by triplicate. Cytotoxicity test was carried out using onion seeds (10 independent experiments, 10 replicates each). Results were evaluated by ANOVA with post-hoc Tukey test for multiple comparisons, or by Kruskal-Wallis test for non-normal variables, using Infostat software [20], [21].

III. Results and Discussion

Fig. 1 shows the growth kinetics of immobilized *C. vulgaris* cultures (batch system) under mixotrophic conditions, using a well-known synthetic medium (MS) supplemented with sucrose and two mixing systems, classic propellers (double blades Rushton turbines) or marine style propeller blades. The growth kinetics of *C. vulgaris* showed a two days-lag period followed by an exponential growth period, which was similar for both types of propellers until day 4. After that, only the culture supplied with a marine propeller kept on growing until day 8, with a specific growth rate (μ) of 0.672 d-1 and a duplication time of 1.03 d. With the Rushton propeller, a lower growth speed (μ= 0.319 d-1) and a higher duplication time (2.17 d) were observed. Microalgae growth was 11-fold greater in the bioprocess carried out with marine propellers (respect to Rushton propeller) after 8 days in culture. According to these data, we decided to use marine propellers in the bioprocess performed to decontaminate the water from the Cildáñez stream, and the batch system was maintained for 6 days.

The growth kinetics of our isolate of *C. vulgaris* in Cildáñez water under autotrophic conditions was characterized by a 3-fold lower specific growth rate (μ= 0.263 d-1) and a higher duplication time (2.6 d) compared to the results obtained in MS medium supplemented with sucrose (mixotrophic conditions). Similar observations regarding substrate composition and *C. vulgaris* growth were already reported by our group [7]. Consistent with the historical record of Cildáñez stream covering the last 4 years (2013-2017), total bacterial counts (data not shown), total coliforms (Fig. 2 (A)), and *E. coli* (Fig. 2 (C)) are usually lower in the winter months.

Based on this information, it was decided to evaluate the efficiency of the bioprocesses using water samples taken in the warmer months (spring or summer).

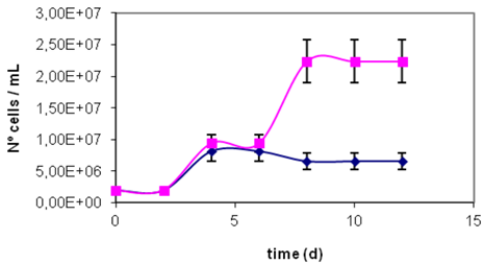

Fig. 1 Time course of *C. vulgaris* growth immobilized in alginate beads. Blue line: Rushton propeller; pink line: marine propeller (mean ± standard deviation)

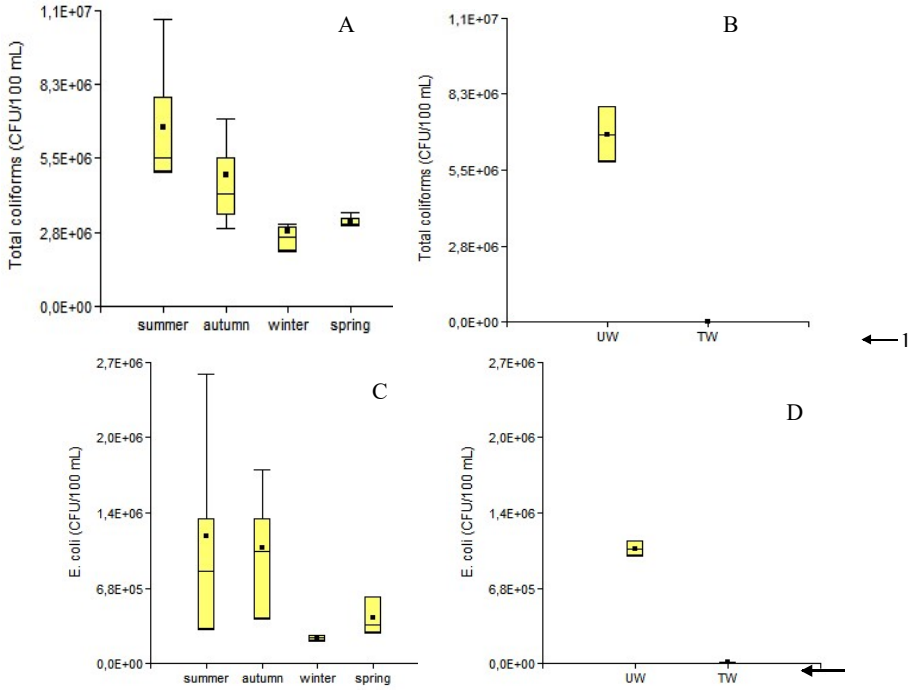

Fig. 2 Bacteriological analyses of Cildáñez stream water. Total coliforms, A and B; *E. coli*, C and D. A and C: historical records of the last 4 years; B and D: experimental results. UW: untreated water; TW: treated water. Arrow shows reference value, according to [28]

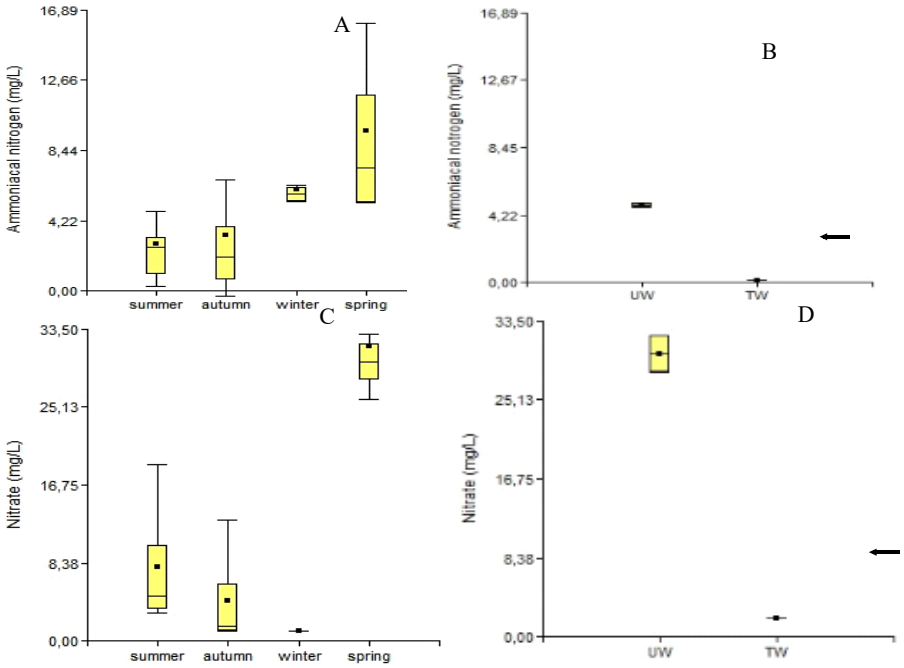

Fig. 3 Chemical analysis of Cildáñez stream water. Ammoniacal nitrogen (A, B); nitrates (C, D). A and C: historical records of the last 4 years; B and D: experimental results. UW: untreated water; TW: treated water. Arrows show reference values, according to [26] and [27], respectively

The results showed that total coliforms (Fig. 2 (B)) and *E. coli* colony-forming units were significantly reduced after 6 days of treatment (Fig. 2 (D)), with a population reduction of about 99%.

Sforza and co-workers [22] have reported a competition between microalgae and the microbiota of watercourses and observed that microalgae could grow and displace these populations of microorganisms. Under our bioprocess conditions, the pH decreased from an average of 7.8 to 6.9. In this sense, the semi-decomposition of carbonaceous sources has been implicated in pH decreases in similar experiments [23].

The last 4-years historical record shows that, unlike microbiological dynamics, the concentration of inorganic compounds including ammoniacal nitrogen (Fig. 3 (A)), nitrates (Fig. 3 (C)), nitrites (Fig. 4 (A)), and total phosphorus (Fig. 4 (C)) tends to be lower in summer. The bioprocess based on the multiplication of *C. vulgaris* cells immobilized in alginate beads allowed a high percentage of removal of these compounds: 96% for ammoniacal nitrogen (Fig. 3 (B)), 86% for nitrates (Fig. 3 (D)), 98% for nitrites (Fig. 4 (B)), and 53% for total phosphorus (Fig. 4 (D)). These results are coincident with our own previous results [7] and with those reported by El-Sheekh and co-workers [10].

According to historical record of the last 4-years, heavy metals concentration remains within the admissible levels throughout the year (data not shown) except for lead, which exceeded the admissible limits proposed by the American Public Health Association [17] in winter and spring (Fig. 5 (A)).

Lead is a persistent, toxic metal that can be harmful to human health even at low exposure levels, and tends to bioaccumulate in the body over time [24]. A significant lead removal (95%) was observed under our experimental conditions (Fig. 5 (B)), in concordance with previous results [7]. Regaldo and co-workers [25] reported that Pb removal efficiency of *Chlorella vulgaris* cells was noticeable but negatively correlated with the increase of metal concentration.

Through a classic biological test using seeds of *Allium cepa*, we corroborated the cytotoxicity of untreated Cildáñez stream water, revealed by a lower germination index at 48 h (Table I) and a decreased mitotic index (Fig. 6) as compared with seeds imbibed in distilled water. When the water was subjected to the bioprocess involving immobilized microalgae, a significant increase in the GI was observed (Table I). Thus, the lowest GI was observed with the untreated water (55%), and the bioprocess applied allowed the elevation of this index to 74%, reaching in this way similar values to those observed with distilled water. After 7 days, almost all the seeds were already germinated when using for imbibition the bioremediated water, while only about 70% of them were germinated when imbibed with untreated water. Additionally, this result indicates that the cytotoxicity of Cildáñez water does not attenuate with time. On another hand, it was observed that while seeds exposed to distilled water had mycelial development (characteristic of the germination of environmental fungal spores), none of the seeds germinated in the presence of untreated Cildáñez water had mycelia, and seeds exposed to treated water had 40% of the seeds with mycelial growth (data not shown). This finding would be corroborating the toxic effect of Cildáñez stream water, which also prevents the germination of fungal spores.

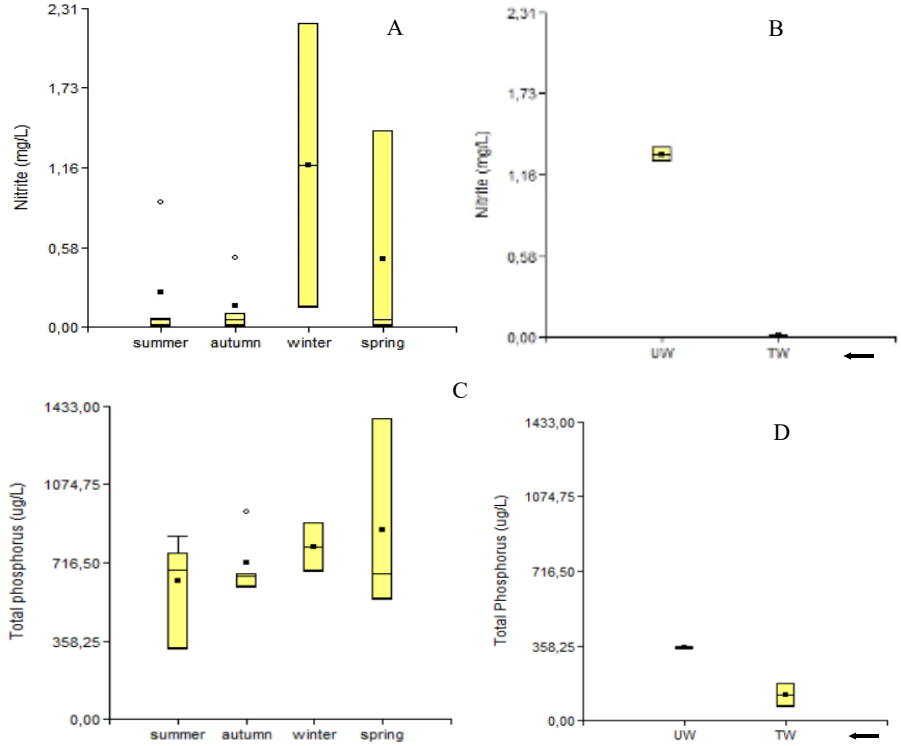

Fig. 4 Chemical analysis of Cildáñez stream water. Nitrites (A), (B); total phosphorus (C), (D). A and C: historical records of the last 4 years; B and D: experimental results. UW: untreated water; TW: treated water. Arrows show reference values, according to [28]

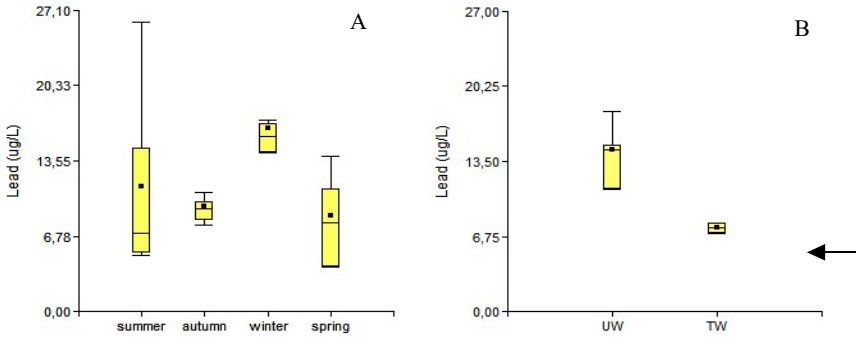

Fig. 5 Lead content of Cildáñez stream water. A: historical records of the last 4 years; B: experimental results. UW: untreated water; TW: treated water. Arrow key shows reference value, according to [28]

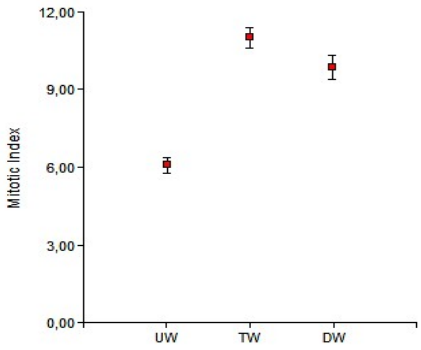

Fig. 6 Bioprocess effects on water cytotoxicity. Mitotic index of *Allium cepa* seeds. UW: untreated Cildáñez water, TW: treated Cildáñez water; DW: distilled water. Data shows mean values ± 95% confidence interval of three independent experiments

TABLE I
CYTOTOXICITY EVALUATION USING *ALLIUM CEPA* SEEDS

	GI (%) 48 h	GI (%) 7 d
UW	55b	70b
TW	74a	93a
DW	86a	96a

UW: untreated Cildáñez water, TW: treated Cildáñez water; DW: distilled water. Bioprocesses were carried out in triplicate. Letters indicate significant differences after ANOVA followed by Tukey test ($p<0.05$).

Mitotic index was also affected (Fig. 6) and in a similar way than GI. The lower MI was observed in seeds exposed to untreated water. According to Leme and co-workers [14], lower mitotic indexes indicate a negative action of xenobiotics on the growth and development of the root apex. This result would explain the delay observed in the germination of onion seeds. Additionally, the detoxifying effect of the treatment

with immobilized *Chlorella* cells was corroborated.

IV. Conclusion

Cildáñez stream, an urban watercourse at Buenos Aires City, contains both sewer and industrial contaminants. Different nitrogenated compounds, phosphorus, metals, as well as saprophytic and pathogenic bacteria, are found. This watercourse exhibits a wide range of contaminants levels along the year, mainly depending on the seasonal regime. At certain periods of the year, these levels usually exceed the limits established by international standards of water quality [26]-[28].

Our native isolate of *Chlorella vulgaris* could grow inside alginate beads based on the consumption of several of the above-mentioned contaminants such as phosphorus and nitrogenated ions. The bioreactor supplied with the marine propeller seems to be the most effective in this sense, probably due to allowing better control of water oxygenation and less shear stress, as it was previously reported [29], [30].

Among the various metals assessed, lead was particularly diminished with the bioprocess here described. On the other hand, the cytotoxicity assays performed corroborated that the bioprocess using the microalga *C. vulgaris* effectively improved the water quality of Cildáñez stream. The efficacy of *C. vulgaris* to remove nitrogen and phosphorus from residual waters has been verified before by other authors [31]-[33]. Biosorption of metals in microalgal biomass, however, is scarcely documented [25], arising in recent years as an inexpensive, rapid, and efficient technology to reduce metal concentration in water. The bioprocess here proposed not only allows the improvement of water quality in an economic and environmental-friendly manner, but it also opens the possibility of using the microalgae biomass produced along the process to obtain alternative energies, such as biodiesel.

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