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Treatment of recalcitrant wastewater from ethanol and citric acid production using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*

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Abstract

Laboratory-scale experiments were performed to develop a procedure for biological treatment of recalcitrant anaerobic industrial effluent (from ethanol and citric acid production) using first the microalga *Chlorella vulgaris* followed by the macrophyte *Lemna minuscula*. This recalcitrant dark-colored wastewater, containing high levels of organic matter and low pH, prevents the growth of microalgae and macrophytes, and therefore, could not be treated by them. Therefore, the wastewater was diluted to 10% of the original concentration with wash water from the production line. Within 4 days of incubation in the wastewater, *C. vulgaris* population grew from 5×10^5 to 2×10^6 cells/mL. This culture reduced ammonium ion (71.6%), phosphorus (28%), and chemical oxygen demand (COD) (61%), and dissolved a floating microbial biofilm after 5 days of incubation. Consequently, *L. minuscule* was able to grow in the treated wastewater (from 7 to 14 g/bioreactor after 6 days), precipitated the microalgal cells (by shading the culture), and reduced other organic matter and color (up to 52%) after an additional 6 days of incubation. However, *L. minuscula* did not improve removal of nutrients. This study demonstrates the feasibility of combining microalgae and macrophytes for bioremediation of recalcitrant industrial wastewater.

Keywords: Biotreatment; *Chlorella*; Duckweed; Industrial effluent; *Lemna*; Nutrient removal; Wastewater

1. Introduction

The composition of industrial wastewater varies greatly. Sometimes, several different industries discharge their wastewater into a large communal pool or lake [1], making the wastewater more difficult to treat. Each wastewater pond is therefore, unique and treatment first requires a survey of possible waste-degrading organisms. The industrial wastewater used in this study came from three different sources and its main characteristic was that it was recalcitrant because it inhibits the growth of both macrophytes and microalgae, even after anaerobic treatment by bacteria.

In the common procedure for wastewater treatment, after primary treatment to eliminate mainly suspended solids, the wastewater is treated microbiologically (stabilization ponds or activated sludge), to further eliminate solids and organic matter. Degradation of organic matter produces an excess of nutrients in the wastewater. These nutrients are usually treated chemically. In recent years, there has been a growing interest in developing biological systems based on microalgae and macrophytes that are less expensive and more "environmentally friendly" [2]. Bacteriological, microalgal, and macrophyte treatments are complementary.

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While the bacterial population initially degrades organic matter, macrophytes eliminate suspended organic matter by trapping, flocculating, and precipitating solid particles. The microalgae adsorb molecules (nutrients and toxins) released during the other processes. Thus, there is an advantage of combining several organisms, in tandem, to treat recalcitrant water. In this view, the aim of this study was to evaluate a sequential treatment of microalgae and macrophytes as potential treatments for recalcitrant anaerobic industrial effluent.

Macrophytes (aquatic floating or rooted plants growing in wetlands) are commonly used in artificial wetland constructions for treatment of wastewater [3], domestic sewage treatment [4], and also in treating industrial effluent [5]. Macrophytes are capable of removing many heavy metals [6], organic matter, and suspended solids [7], and to some extent, nutrients such as nitrogen and phosphorus [8] from wetland waters.

Many species of macrophytes are used for wastewater treatment. Species selection depends mainly on the availability of the species, their photosynthetic rate, and tolerance to the wastewater [5]. One of the most commonly used aquatic plant families is Lemnaceae (duckweed), particularly the edible, small, floating *Lemna gibba* [9]. *Lemna* species have a high capacity to adsorb nutrients from water and it is easy to manage in the ponds because of its small size [2].

Microalgae of various species are used for tertiary wastewater treatment [10], most often species of *Chlorella* (Chlorophyceae) [11]. *Chlorella* species can remove various phosphorus and nitrogen compounds [12,13], heavy metals [14], and toxic residues from wastewater [15]. A significant technical difficulty resulting from this procedure is the elimination of the large population of microalgae that develops in the water during the treatment. Such large amount of suspended solids limits reuse of the wastewater. A mixture of microalgae and macrophyes, as a single wastewater treatment, is normally unfavorable for the microalgae; the dense growth of the floating macrophyte on the wetland surface results in a sharp decrease in the penetration of light through the water [16], which potentially minimizes microalgal activities. Thus, a macrophyte treatment might eliminate the living population of microalgae from the wastewater.

All these features were used to develop and evaluate a sequential treatment for recalcitrant wastewater by two aquatic photosynthetic organisms.

2. Materials and methods

2.1. Industrial wastewater

The industrial wastewater used in this study was a mixture of two different effluents from a large sugar mill (Sucromiles S.A., Cali, Colombia) that processes molasses to produce citric acid, ethanol, acetic acid, and various acetates. The first effluent used (E1) was the untreated washing water from all the processes in the mill. This includes effluents from the ion-exchange equipment used for production of citric acid (COD, 1000–2000 mg/L; unstable pH of 2–4), which also has a significant floating layer composed of: (mg/L) lipids, 180, non-reducing sugars 2.36, oil 17, protein 180, and short chain aliphatic hydrocarbons (C-7 or C-8). The unidentified bacteria in the effluent exceeded 10^{11} cfu/g. The mill potentially produces 340 m^3/h of this wash water.

The second effluent (E2) had three different origins: (i) vinasse, a common by-product from the distillation of molasses into ethanol, acetic acid, and acetate, and the major pollutant of the liquor industries [17]. Vinasse wastewater from the mill under study had a high COD (80,000 mg/L) and low pH (3.7–4.5). During its anaerobic decomposition after discharge into rivers and streams, it causes massive die-out of organisms (plants, microfauna, and microflora) and unpleasant odors and gases. The common treatment of vinasse (by Sucromiles Industries, the source of the wastewater) is anaerobic digestion for 29.5 days with a flow of 800 m^3/day. (ii) Effluent produced during citric acid purification from sugar. The two effluents from this production line (one acidic and one basic) are combined (production capacity of 8–12 m^3/h). This effluent contains high levels of salts, low pH (3.5–4), and a COD of 10,000 mg/L. (iii) Domestic sewage from the plant. All three effluents are combined, and treated simultaneously by two consecutive anaerobic reactors at the plant. However, this dark-colored, anaerobically treated effluent (E2) still contains high level of contaminants; biological oxygen demand (BOD) 1393 mg/L, COD 13,471 mg/L, and total suspended solids (TSS) 1397 mg/L with low pH. The mill produces a total of 1780 m^3/day of effluent, which is currently discharged into Rio Palmira in Cali, Colombia without further treatment. Therefore, additional treatment is required to improve the quality of the discharged water.

2.2. Organisms

Microalgae: The microalgae *Chlorella vulgaris* (Beijerinck) (UTEX 2714) and *Scenedesmus acutus* (Meyen) Chodat were isolated from the secondary effluent of a wastewater treatment stabilization pond [12].

Macrophytes: Five free-floating aquatic plants were collected from unpolluted, natural ponds and streams around Santafe de Bogota, Colombia, and were evaluated for possible growth in the wastewater: *Eichhornia* crassipes (Mart) Solms (water hyacinth), *Azolla filiculoides* Lam (water fern), *Salvinia rotundifolia*

Willd (water fern), *Lemna minuscula* Herter (duckweed), and *Wolffia columbiana* Karsten (duckweed).

2.3. Culture media

The aquatic plants were routinely maintained for 15 days before experiments in 12 Plexiglass transparently covered, concrete tanks (200 L each, 360 m^2 surface area) at an ambient temperature of $23 \pm 1°C$, with 11 h of light per day (30 $\mu E\,m^{-2}$), in a commercial hydroponic solution (HidroCOLJAP®, Colombia). Growth media lost to evaporation was replaced every 7 days with distilled water to avoid changing the nutrient concentration of the solutions.

C. vulgaris cultures were stored and maintained in a synthetic mineral solution (C30) under illuminating conditions and *S. acutus* was grown on bold-mineral medium [12]. Before the experiments, the cultures were acclimatized in filtered (S&S 595 filter paper (0.45 μm), 185 mm in diameter, Schlicher and Schuell, Germany), sterile (autoclaved 20 min) wastewater (E2:E1, 1:10, v/v) in Erlenmeyer flasks for 15 days, at ambient temperature ($23 \pm 1°C$), under continuous fluorescent illumination (60 $\mu mol\,s^{-1}\,m^{-2}$), and were bubble-aerated using a small commercial aquarium pump. This allowed the microalgae to increase its population to the needed inoculation level by avoiding predation by protozoan always residing in the wastewater. The initial capacity of the two microalgal species to remove nutrients was first evaluated in round bioreactors (3 L volume, 2 L substrate [12]) in the mineral salt fraction of synthetic wastewater (composition (mg/L): NaCl, 7; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; KH_2PO_4, 21; K_2HPO_4, 8.5; Na_2HPO_4, 33.4; NH_4Cl, 10) before exposure to the wastewater used in this study.

2.4. Incubation of organisms in wastewater

Batch experiments were carried out in two different bioreactors. For microalgae, a 16.5 L, triangular, inverted, transparent, acrylic bioreactor ($60 \times 23.3 \times 33\,cm^3$, width/length/height, containing 5 L of solution) with a continuous aeration at the bottom tip (silicon tubing with holes connected to an ambient air supply). For macrophyte growth, microalgal treated wastewater was introduced to rectangular, glass bioreactors ($15 \times 30 \times 40\,cm^3$ width/length/height) with a capacity of 18 L (6 L of solution). The microalgal inoculum was 20% (v/v) of the wastewater with an approximate concentration of $0.5–1.0 \times 10^6$ cells/mL [12], and for macrophytes the initial inoculum was 7 g fresh weight per bioreactor [18]. The macrophytes and microalgae were incubated under the conditions described above.

2.5. Analytical determinations

For water analysis, 150 mL of wastewater was analyzed in the four replicates and control bioreactors. Ammonium ion and phosphorus concentrations, BOD, COD, TSS, and color of 100 mL wastewater samples were taken every 48 h and analyzed using standard methods [19]. The floating layer was collected and analyzed both as it was and after lyophylization by two independent analytical service laboratories (Department of Chemistry at Pontificia, Universidad Javeriana in Colombia and the Service Laboratories directorate of the Center for Biological Research, Mexico). The floating layer in effluent E1 was analyzed for reducing and non-reducing sugars, oil, lipids, and proteins by standard analytical methods. Hydrocarbons were evaluated by standard gas chromatography methods for these substances [19]. Evaluation of bacteria in the floating layer employed total cell counts and Gram staining by standard techniques.

2.6. Growth measurement of the organisms

C. vulgaris proliferation was measured by taking samples every 48 h and counting the number of cells by light microscopy using a Neubauer Hematocytometer [20]. Growth rate (K) was determined by the formula: $K = (\ln Nt_1 - \ln Nt_0)/t_1 - t_0$, where Nt_1 is the number of the cells at sampling and Nt_0 is the initial number of cells at the beginning of the experiment [21]. Macrophyte growth was determined by measuring fresh weight [22] and relative growth rate (g/g* day) by the formula: $\ln(P_1) - \ln(P_2)/t$, where P_1 and P_2 are the fresh weights of the plants at various times and t the time between sampling [23].

2.7. Statistical analysis

In each case, the results presented are from one representative experiment. Variations in growth and ion removal capacity between wastewater treatment experiments precluded combining the data. Non-homogeneous wastewater composition between samples taken over a 1 yr period is likely responsible for the variation. Data was analyzed by one-way analysis of variance (ANOVA) at $P < 0.05$ using the software package STATISTICA (StatSoft Inc.,Tulsa, OK, USA).

3. Results

3.1. Selection of plants and dilution of wastewater

Because large volumes of wastewater must be treated, using the lowest dilution of the wastewater is desirable for practical reasons. Undiluted effluent E1 inhibited the

growth of all macrophyte species tested (but not the growth of the microalgae), only when a thick surface floating layer was present. Effluent E2 also inhibited growth because of its high COD and dark color, which impaired photosynthesis of *C. vulgaris*. Plant growth in both effluents was inhibited within 48 h. When effluent E2 was diluted with effluent E1 (E2:E1, 1:10,1:20,1:30, v/v), of the five species of macrophytes tested separately in batch cultures for 4–20 days, only *L. minuscula* showed a positive relative growth rate (0.05 g/g* day) at 1:10 (E2:E1, v/v) dilution. *E. crassipes* survived in all dilutions, but did not grow and did not remove nutrients from the wastewater (data not shown). The other three species of macrophytes died in all dilutions tested. This procedure was repeated four times. The best wastewater dilution for growth of *L. minuscula* was 1:10; hence, this combination of macrophyte and wastewater dilution was used for the subsequent experiments. However, whenever the wastewater contained a surface film, *L. minuscula* plants did not grow, regardless of the dilution.

L. minuscula growth was slow and limited in wastewater compared to growth in a hydroponic solution (12 g vs. 32 g biomass per bioreactor after 20 days, and relative growth rate of 0.03 vs. 0.07 g/g* day). The quality of the water after the macrophyte treatment only marginally improved. Between the treated wastewater dilutions (1:10 vs. 1:30), COD differed only a little (from 1400 to 1000 mg/L after 20 days [1:10, 40% reduction] and from 700 to 500 mg/L [1:30, 40% reduction]). Suspended solids were similarly reduced (<40 mg/L after 20 days), and there was no effect on phosphorus removal, pH, dissolved organic matter, or water conductivity.

The microalgal species were selected after seven experiments (four with *S. acutus* and three with *C. vulgaris*). Batch cultures were done in five replicates (250 mL substrate, 10% inoculum volume, 8 days) under the conditions used for synthetic wastewater. Screening experiments on synthetic wastewater with both microalgae showed the growth of *C. vulgaris* was more consistent during 7 days of the experiment (growth rate of 0.51–0.53). Both microalgal species removed ammonium ions efficiently (from 3.5 to 0.04 mg/L, 96–99% removal). Nitrate and nitrite ions were not affected. Phosphate ions were removed only by *C. vulgaris* (from 3.5 to 2.5 mg/L, removal of up to 27%). Based on the above screening experiments, the organisms selected for the study in wastewater treatment were the microalga *C. vulgaris* and the macrophyte *L. minuscula* in dilutions similar to those used by the macrophyte.

Because wastewater samples (effluent E1) were usually covered with a floating film of lipids, oil, sugars, hydrocarbons, and large amounts of attached microorganisms (composition in materials and methods) that prevented the growth of the macrophyte, the wastewater was first treated with the microalga, which is not affected by the film. Then the macrophyte was used as a further wastewater treatment. At the end of the microalgal treatment, the surface film was completely dissolved into the bulk of the wastewater. Thus, it was impossible to analyze if its ingredients were metabolized. The wastewater contained abundant organic matter at the time of microalgal inoculation, and microalgal growth at the standard inoculum level of 10% [12] was insufficient. *C. vulgaris* started to grow only after 192 h of incubation, and it took an additional 30 h to double the population from 10^6 to 2×10^6 cells/mL. Therefore, the microalgae inoculum level was increased to 20% (v/v) of the treated wastewater.

3.2. Growth of *C. vulgaris* and *L. minuscula* in diluted industrial wastewater

Growth of *C. vulgaris* in diluted (1:10) wastewater (inoculum concentration at 20%) began almost immediately after inoculation and increased constantly for 4 days, reaching an average population density of 2×10^6 cells/mL (Fig. 1a). The floating layer on the surface of the wastewater was degraded by the microalgae. Therefore, *L. minuscula* could grow in the wastewater after microalgal treatment. The macrophyte grew continuously for 14 days, doubling its fresh weight after 8–10 days (Fig. 1c) with a relative growth rate of 0.07 g/g* day, which is equivalent to the growth rate of this species in hydroponic solutions. Using non-sterile wastewater as a substrate supported the growth of *C. vulgaris* to a lesser extent and was variable (Fig. 1b, two typical independent cycles are shown).

3.3. Removal of nutrients from diluted wastewater by *C. vulgaris* treatment

To determine the optimum incubation time (hydraulic retention time) for a two-phase wastewater treatment, macrophytes and microalgae were cultivated in the wastewater for 24 days in total (10 days for microalgae and 14 days for macrophytes). Later, most experiments were reduced to a total of 11 days because preliminary experiments (data not shown) showed no improvement in water quality after prolonged incubation. First, microalgae were grown in the wastewater for 5 days. Then the treated wastewater (including any microalgae that developed during this period) was transferred to bioreactors containing the macrophyte *L. minuscula* for an additional 6 days. Each experiment included four replicates, where one replicate consisted of one bioreactor inoculated with the organisms, and each experiment was repeated 4 times. Controls included bioreactors without microalgae, without macrophytes, and without any incorporated vegetation, and were maintained under the same incubation conditions as the wastewater treatments.

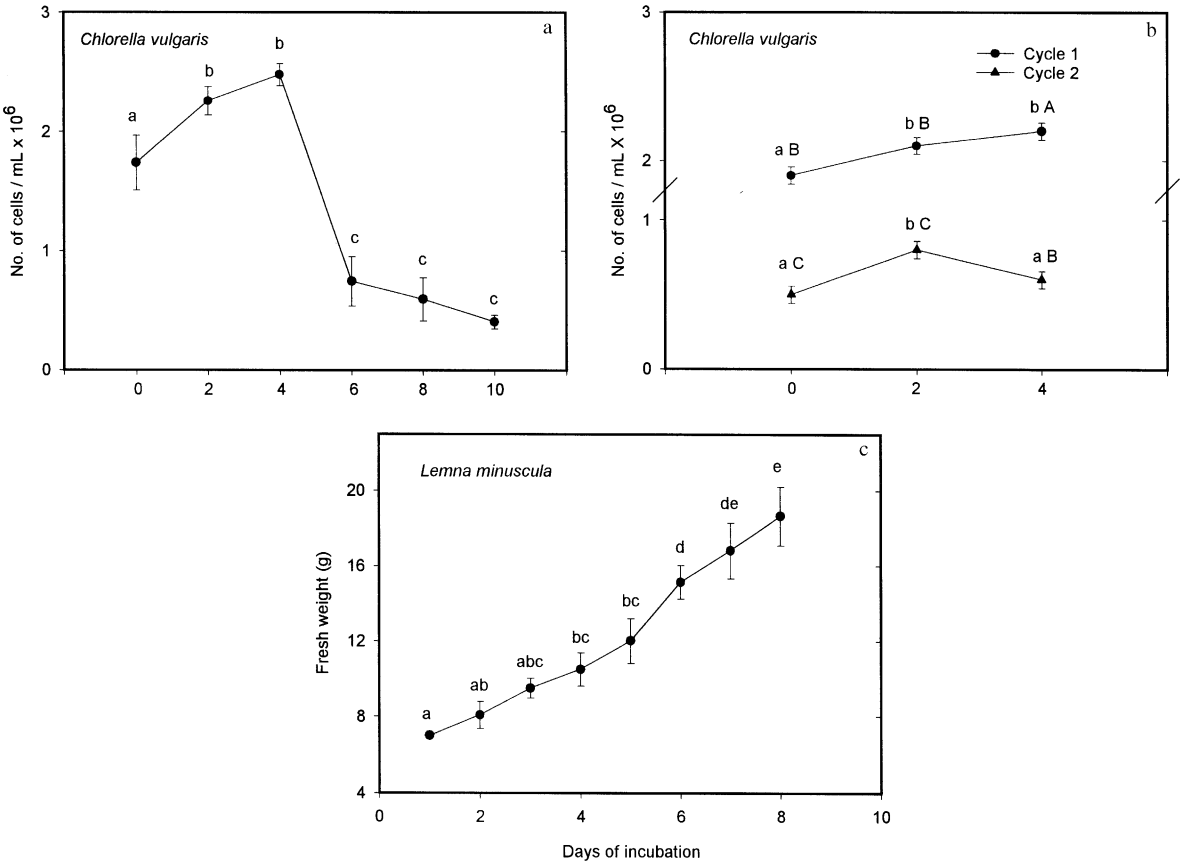

Fig. 1. Growth of *C. vulgaris* in (a) sterile and (b) non-sterile diluted (1:10) industrial wastewater and growth of *L. minuscula* (c) in diluted (1:10) industrial wastewater. In each subfigure, points denoted by different lower case letters differ significantly at $P<0.05$ by one-way ANOVA. In Fig. 1b, points at each sampling time denoted by a different capital letter differ significantly at $P<0.05$ by the Student's *t*-test. Bars represent SE. The absence of a bar means that the SE is smaller than the point.

Initially, the ammonium ion concentration in the wastewater was high (3–8 mg/L NH_4^+). Although incubation of the wastewater under aerated conditions removed ammonium ions, removal by *C. vulgaris* was significantly superior (Fig. 2A). After 5 days, the level of ammonium ion removal from the wastewater by *C. vulgaris* ranged from 60% to 78%, with an average of 72% (from four independent repetitions).

The initial concentration of phosphorus was also high (1.5–3.5 mg/L PO_4^{-3}). Treatment of the wastewater with *C. vulgaris* gradually decreased the phosphorus concentration, while phosphorus in the untreated aerated water varied greatly between sampling times (Fig. 2B). The level of phosphorus ion removal from the treated wastewater ranged from 0% to 51%, with an average of 28% (from four independent repetitions). *C. vulgaris* treatment lowered COD after 5 days of incubation by 61% (from 3100 to 1200 mg/L in one experiment and similarly in the other two repetitions).

3.4. Removal of *C. vulgaris* from the treated wastewater by *L. minuscula*

Addition of the macrophyte *L. minuscula* to treated wastewater containing a large population of *C. vulgaris* resulted in the dramatic elimination and sedimentation of the microalga to the bottom of the reactors (from 3.2×10^6 to 0.5×10^6 cells/mL), leaving the water transparent and light-brown in color after 6 days of incubation.

3.5. Effect of *L. minuscula* on organic matter, nutrients and color removal in post-microalgae treated wastewater

The macrophyte treatment had no additional effect on nutrient removal from the wastewater after the microalgae treatment (data not shown). Suspended solids (TSS) were reduced over time within the range of 26–56% of the initial TSS concentration, depending on the experiment (Fig. 3a). Although COD and BOD were

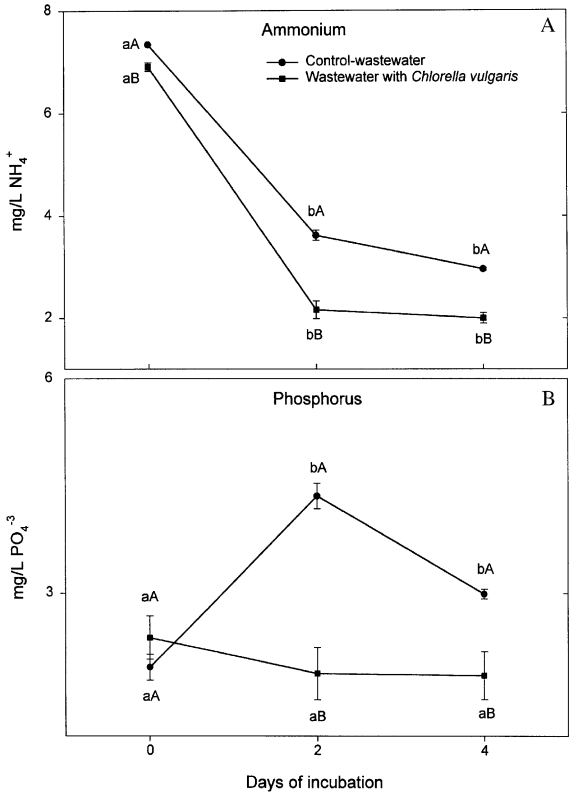

Fig. 2. Removal of (A) ammonium and (B) phosphorus from diluted (1:10) wastewater by C. *vulgaris* over a period of 4 days. In each subfigure, and in each treatment, points denoted by different lower case letters differ significantly at $P<0.05$ by one-way ANOVA. Points at each sampling time denoted by a different capital letter differ significantly at $P<0.05$ by Student's *t*-test. Bars represent SE. The absence of a bar means that the SE is smaller than the point.

reduced in untreated wastewater, perhaps by the indigenous microflora, the combined microalga-macrophyte treatment reduced these even further in four different independent repetitions (Fig. 3b,c). Macrophyte treatment without a microalgae pretreatment reduced the original color up to 10.1%. In 3 of the 4 experiments, the original color was reduced up to 52% in the combined treatment (Fig. 3d). The relative growth rate of the macrophytes was similar in all four experiments and was 0.03–0.05 g/g* day.

4. Discussion

Recalcitrant industrial wastewater is a serious threat to the operation of a contaminating industry and a challenge for cleanup. Wastewater heavily loaded with organic matter, such as the industrial wastewater used in this study, cannot be cleaned by biological treatment in artificial wetlands as the wastewater prevents aquatic plant growth [2]. Therefore, it was first necessary to dilute the wastewater, and then remove nutrients and organic matter using microalgae, before treating the wastewater with macrophytes. This procedure is contrary to the one more commonly used to cleanup domestic sewage [24]. Dilution of this recalcitrant industrial effluent with the washwater from the mill equipment was practical since the washwater is the most abundant effluent of the plant.

Removal of ammonium ions from the industrial wastewater in this study (73%) was lower than the removal of ammonium ions from agroindustrial water (97%), using the same microalga species [12] and lower than removal from domestic wastewater (91.5%) by *C. pyrenoidosa* [25]. The observed removal of ammonium ions from untreated wastewater is probably by an air-stripping mechanism [26], as our bioreactors were well-aerated. Removal of phosphorus from the industrial wastewater (up to 51%) was also slightly lower than from agroindustrial water (59%) [12] and from domestic wastewater (70–80%) [25]. We attributed the removal of phosphorus from the wastewater to phosphorus accumulation into the algal biomass [27].

The macrophyte used in this study, *L. minuscula*, was selected for its environmental fitness as the sole aquatic plant species that survived in the wastewater and for its contribution to the wastewater cleanup procedure, albeit to a lesser extent than the microalgae. In addition, it was beneficial as a final wastewater treatment because it eliminated the microalgal population from the water. The microalgal biomass produced during the earlier treatment is an obstacle for reuse of the water. Because the macrophyte grows in a dense layer on the water surface, it prevents light from penetrating to the deeper layers of the bioreactor and thereby prevents microalgal growth [2]. Other benefits of the macrophyte treatment include the reduction in water color, probably by direct adsorption of pigment particles by the plants [28], and production of a large plant biomass, a product valued by the wastewater-producing industry for its compost production.

The lower efficiency of *L. minuscula* in removing organic matter from wastewater in this study, compared to the removal levels reported in the literature [29,30], may be explained as follows: (i) The microalgal removal of organic matter is probably camouflaged by the growth of the microalgal cells. In an oxidation pond, most of the suspended solids are in fact microalgal communities [31]. (ii) Removal of contaminants by floating macrophytes can be increased by harvesting the plants periodically, thereby keeping the plant population in a continuous growth stage, which maximizes the rate of contaminant removal [29]. Because of the short duration of our treatment, this was not done in these

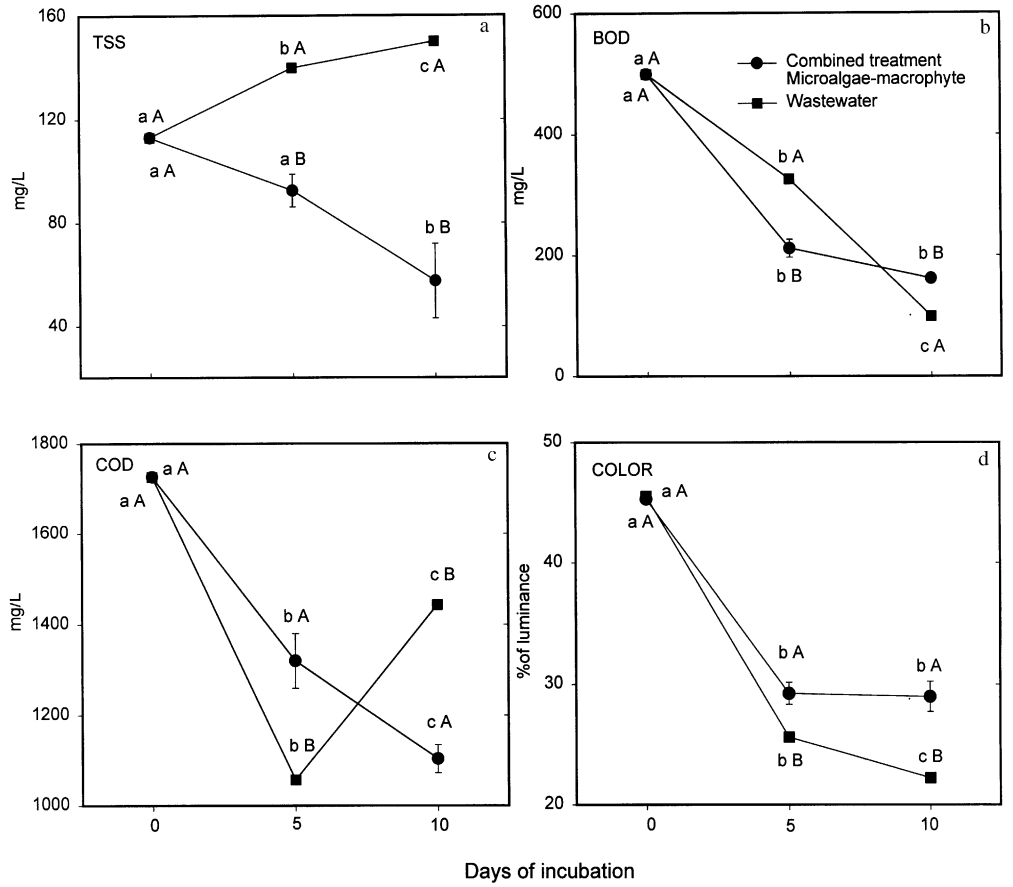

Fig. 3. Effect of a combined *C. vulgaris–L. minuscula* treatment on: (a) total suspended solids (TSS), (b) BOD, (c) COD, and (d) color of an industrial wastewater**.** The wastewater was analyzed before treatment, after microalgal treatment (5 days), and after macrophyte treatment (5 additional days). Points in each subfigure and in each treatment denoted by different lower case letters differ significantly at $P<0.05$ by one-way ANOVA. Points at sampling times denoted by a different capital letter differ significantly at $P<0.05$ by Student's *t*-test. Bars represent SE. The absence of a bar means that the SE is smaller than the point.

experiments and possibly precluded further growth. (iii) *Lemna* sp., as a small floating aquatic plant, stops accumulating contaminants when the concentration of organic material in the water are at levels up to 100 mg/L [2]. Industrial wastewater evaluated in this study had a much higher COD, up to 2500 mg/L.

5. Conclusions

We are proposing a sequential treatment process for recalcitrant industrial wastewater. First, a microalgal treatment removes nutrients and organic matter from wastewater and oxygen is produced for other organisms (fungi and invertebrates) that consume the organic matter. Secondly, macrophytes further remove organic matter and eliminate the microalgae from the treated wastewater.

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