



Removal of nutrients from Organic Liquid Agricultural Waste using filamentous algae.

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Abstract

A feasibility analysis of tertiary treatment for Organic Liquid Agricultural Waste is presented using filamentous algae belonging to the genus *Cladophora* sp. as an alternative to chemical tertiary treatment. The main advantages of tertiary treatments that use biological systems are the low cost investment and the minimal dependence on environmental variables. In this work we demonstrate that filamentous algae reduces the nutrient load of nitrate (*circa* 75%) and phosphate (*circa* 86%) from the organic waste effluents coming from dairy farms after nine days of culture, with the added advantage being that after the treatment period, algae removal can be achieved by simple procedures. Currently, the organic wastewater is discarded into fields and local streams. However, the algae can acquire value as a by-product since it has various uses as compost, cellulose, and biogas. A disadvantage of this system is that clean water must be used to achieve enough water transparency to allow algae growth. Even so, the nutrient reduction system of the organic effluents proposed is friendly to the ecosystem, compared to tertiary treatments that use chemicals to precipitate and collect nutrients such as nitrates and phosphates.

Keywords: nutrient removal, phycoremediation, dairy wastewater.

Remoção de nutrientes de Resíduos Agrícolas Líquidos Orgânicos utilizando algas filamentosas.

Resumo

Uma análise de viabilidade do tratamento terciário para Resíduos Agrícola Líquidos Orgânicos é apresentada usando algas filamentosas pertencentes ao gênero *Cladophora* sp. como alternativa ao tratamento químico terciário. Os tratamentos terciários que utilizam sistemas biológicos têm baixo custo de investimento e a dependência de variáveis ambientais é mínima. Neste trabalho, é demonstrado que essas algas filamentosas reduzem a carga nutricional de nitrato (*circa* 75%) e fósforo (*circa* 86%) dos efluentes de resíduos orgânicos provenientes de fazendas de leite em nove dias de cultura e tem a vantagem de que as algas podem ser facilmente coletadas posteriormente. Atualmente, as águas residuais orgânicas são descartadas nos campos e córregos locais. Posteriormente, as algas podem ser consideradas como matéria prima, uma vez que possuem várias utilidades como composto, celulose e biogás. Uma desvantagem desse sistema é que água limpa deve ser usada para obter transparência de água suficiente para permitir o crescimento de algas. Mesmo assim, o sistema de redução de nutrientes dos efluentes orgânicos propostos é amigável ao ecossistema, comparado aos tratamentos terciários que utilizam produtos químicos para precipitar e coletar nutrientes como nitratos e fosfatos.

Palavras-chave: remoção de nutrientes, ficorremediação, águas residuais de laticínios.

1. Introduction

Removal of nutrients from wastewater is very important to maintain the environmental health of aquatic systems. Heavy loads of nutrients to lacustrine systems may accelerate eutrophication and cause economic losses and sickness to animal and human communities (Aslan and Kapdan, 2006). Chemical removal of nutrients such as nitrate and phosphate is costly and not environmentally friendly because it requires additional chemicals that generate secondary pollution (Shin and Lee, 1998). Therefore, a biological tertiary process would be most welcome since it allows the reuse of water and the utilization of the generated biomass.

Chile has 373 sewage treatment plants but only 8% of the plants have some type of tertiary treatment (Baraño and Tapia, 2004). Plants with this type of treatment are divided into two groups: 65% use biological technologies to remove nitrogen and phosphorus, while 30% use chemicals to remove phosphorus (Baraño and Tapia, 2004).

The green filamentous algae *Cladophora* sp. grows in almost all aquatic systems worldwide (Whitton, 1970; Dodds and Gudder, 1992; Higgins et al., 2008; Graham et al., 2018). Light intensity determines the depth at which *Cladophora* can develop its biomass. In Lake Huron, *Cladophora glomerata* needed a minimum irradiance of 30 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the temperature range of 5-20 °C, to have a positive net photosynthesis (Graham et al., 1982). *Cladophora* sp. has been reported to grow at temperatures in the range of 13-35 °C (Graham et al., 1982; Lester et al., 1988; Higgins et al., 2005) and at the optimal light intensity of 200-300 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Graham et al., 1982; Canale et al., 1982). Such a broad tolerance to irradiance and temperature has contributed to its invasive behavior in some habitats (Zulkifly et al., 2013). As with most other algae, freshwater *Cladophora* sp. removes nitrate and phosphate from water to make biomass and is primarily limited by phosphorous availability (Canale et al., 1982; Planas et al., 1996; Higgins et al., 2006, 2008; Malkin et al., 2009; Young et al., 2010; Liu and Vyverman, 2015). Furthermore, *Cladophora* sp. has been effective in heavy metal removal from industrial liquid wastewater (Lee and Chang, 2011; Ji et al., 2011; Abdel-Raouf et al., 2012). Although, a Chilean environmental law regulates the discharge of organic wastewater into the environment, there are still an important number of small dairy industries that do not comply with the law, discharging wastewater directly into the environment (Baraño and Tapia, 2004). Law enforcement is still soft, especially for small farmers, mainly because water treatments require the use of costly chemicals or advanced technology, which are difficult to afford (Dirección General de Aguas, (Chile, 2004)). If the law is not enforced, the environment is subject to being damaged every day, and if it is enforced, small farmers might be fined. Therefore, the filamentous algae *Cladophora* sp. is a suitable organism to be used as a low-cost technology for the removal of organic nutrients from agricultural liquid waste from small dairy farms, primarily because

positive net photosynthesis can be achieved at very low irradiance (Graham et al., 1982). Although the biological removal of nutrients is efficient and considered a low-cost technology/method, the use of filamentous algae has its limitations. One of these limitations is the amount of clean water needed for the dilution of the dark organic wastewater in order to allow the filamentous algae to grow. Nevertheless, filamentous algae like *Cladophora* sp. can be harvested and used as a secondary product for compost, biofertilizer, bioestimulants or as a good source of cellulose fibers, helping farmers obtain alternative resources (Wilkie and Mulbry, 2002; Ross et al., 2018; Sucaldito and Camacho 2017).

2. Materials and Methods

2.1. Sample collection

Filamentous algae samples were collected within the urban perimeter of Chillán from a small pond located in the Ñuble Region, Chile. The filamentous algae are part of the metaphyton, therefore, it was collected manually with prehensile tools and by dragging it from the shore (Collins and Weber, 1978). The samples were transported to the laboratory in hermetically plastic bags where they were cleaned of leaf detritus and observed under an Olympus CX31 optical microscope for identification. The algae were identified to the genus level using taxonomic keys for Chlorophycean algae (Graham et al., 2018). Additionally, pictures of the algae were sent to phycology expert Dr. Linda Graham (UW-Madison, USA) for further identification confirmation.

2.2. Filtered pool water (FW)

Water from the pond where algae thrived naturally was sampled and filtered through a Whatman® filter N°1 to take out coarse leaf and insect detritus. Inorganic nutrients or bacterial consortium were not eliminated because it was desirable to preserve the natural habitat of the algae. Filtered water was used to make enough algal biomass and was also used as control.

2.3. Industrial Liquid Wastewater (ILW)

Wastewater was obtained at the end of summer 20 km north of Chillán, from an experimental dairy farm located on property owned by the University of Concepción for veterinary research. The milking procedure is rudimentary: untreated wastewater runs off into the fields and a nearby stream. The small farm has 40 milking cows and the entire milk yield is sold to a dairy company. Wastewater was collected in plastic bottles, carried immediately to the laboratory, and kept refrigerated at 4°C in the dark until it was ready to use the following day.

2.4. Sample treatment

Algae filaments were cleaned from leaf detritus and cultured at 18 °C in filtrated pond water (FW) to achieve greater algal biomass and preserve fresh samples by simulating its natural habitat. In addition, the algal filaments were cultivated in liquid agricultural wastewater

(ILW) to verify their reproductive success and prove their effectiveness in the reduction of PO_4^{3-} and NO_3^- .

2.5. Algae incubation

The initial inoculum was prepared by separating the algal mats using a sterile scalpel to obtain an even mix of algal filaments that were diluted with 100 mL of FW. ILW was diluted with FW at five different incremental ratios (Table 1). Each ILW:FW dilution was dispensed in 1000-mL flasks and inoculated with 1 mL of the solution of algal filaments, starting with an initial concentration of $1.0 \text{ mg} \cdot \text{L}^{-1}$ of Chlorophyll-a in all treatments in triplicates. The algal filaments grew for at least three days in every treatment for acclimatization. Chlorophyll-a was used to know the optimal ILW: FW dilution at which the algae could still thrive under minimum light intensity. The incubation period would be finished when chlorophyll-a would reach a similar or higher concentration value to that obtained in FW as the control.

The flasks were illuminated with fluorescent white light at an intensity of $1667 \text{ } \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ Photosynthetically Active Radiation (PAR), with a photoperiod of 16:8 (L:D) and at a temperature of $18 \text{ } ^\circ\text{C}$ during the whole incubation period. PAR light intensity was measured at every ILW:FW dilution using a Quantometer/Photometer LiCOR. The pH of the culture medium was 6.9, which matched the pH of

the collected wastewater. The pH level was continuously monitored and did not change during the incubation period. A schematic figure of the system is shown (Figure 1).

Chlorophyll-a was measured every day in triplicate during the whole incubation period in all treatments. Measurements were done by spectrophotometry using the following equation: $\text{mg} \cdot \text{L}^{-1} = 11.6 (\text{D.O. } 665) - 1.31 (\text{D.O. } 645) - 0.14 (\text{D.O. } 630)$ (Hansmann, 1973).

2.6. Nutrient analyses

Nutrients such as PO_4^{3-} and NO_3^- were measured in triplicate on days one and nine, following the methodology suggested by APHA (Eaton and Franson, 2005). Statistical analyses were done using Duncan Test at the significant level of $p \leq 0,05$. All analyses were performed with Statistica 10.0.

3. Results

Filamentous algae collected from a freshwater pond were identified as belonging to the Division: Chlorophyta, Family: Cladophoraceae, Order: Cladophorales and Genus: *Cladophora* (Kützing, 1843). The filaments are septated and branched, chloroplasts are reticular with numerous pyrenoids. Morphological features and habitat are evidence that the algae belong to the genus *Cladophora*.

Table 1. Levels of chlorophyll-a ($\text{mg} \cdot \text{L}^{-1}$) (n=3) after 9 days of cultivation and light intensity that reached the algae at the culture solution. FW (filtered water), ILW (agricultural liquid waste).

Culture solutions ILW:FW	Chlorophyll "a" ($\text{mg} \cdot \text{L}^{-1}$)	SD	Light intensity ($\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)
0:100	2.634	0.319	1600
2.5:97.5	2.160	0.385	1580
5.0:95.0	2.199	0.180	1350
10.0:90.0	2.999	0.460	300
*25.0:75.0	3.250	0.608	200
50.0:50.0	0.657	0.102	80

* (ILW: FW) at 25:75 ratio was used for culturing algae to remove nutrients.

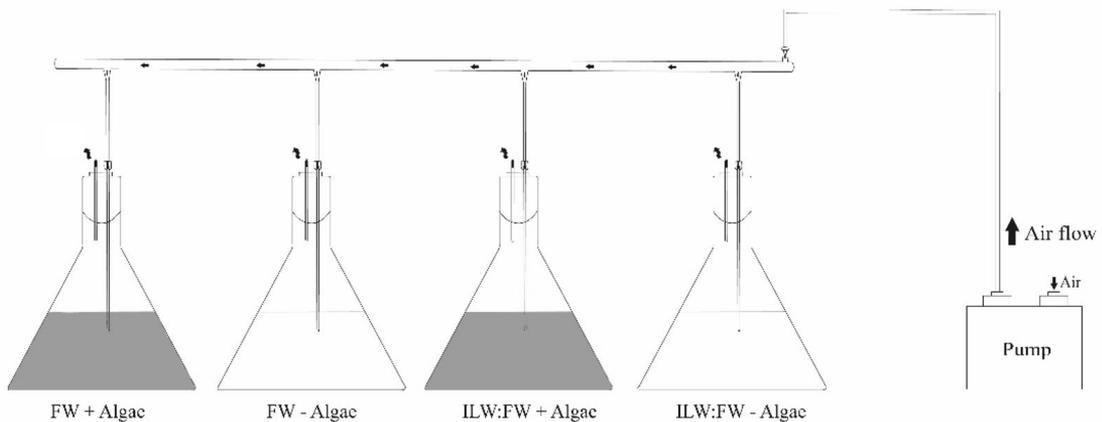


Figure 1. A schematic figure of the treatments at the laboratory-scale experiments for nutrient removal using filamentous algae *Cladophora*.

3.1. Chlorophyll-a

Chlorophyll-a measurements after 9 days of cultivation had a peak of $3.25 \text{ mg} \cdot \text{L}^{-1}$ at the ILW:FW (25.0:75.0) ratio when light intensity illuminating algae

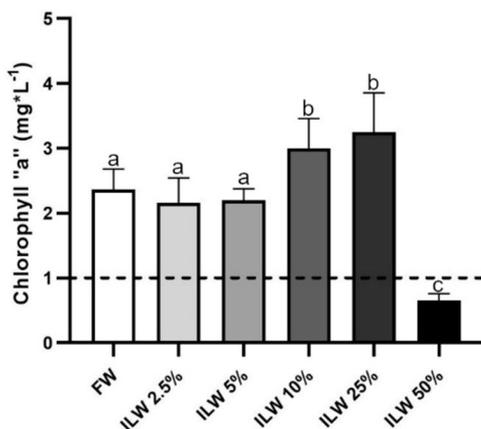


Figure 2. Concentration of chlorophyll "a" measured in filtered water (FW), and in a ILW:FW 2.5%, 5.0%, 10.0%, 25.0% and 50.0% ratios after nine days of cultivation. The punctuated line shows the initial concentration of chlorophyll-a in all treatments. Different letters indicate statistical differences among averages; $p < 0.05$.

was $200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Meanwhile at the positive control (FW) the chlorophyll-a reached $2.63 \text{ mg} \cdot \text{L}^{-1}$ (Figure 2; Table 1) at day 9.

The algae grew in dilutions of ILW:FW 2.5%, 5.0% reaching levels of chlorophyll-a of $2.16, 2.19 \text{ mg} \cdot \text{L}^{-1}$ respectively, which is below the level of chlorophyll-a ($2.63 \text{ mg} \cdot \text{L}^{-1}$) measured at the positive control. The ILW:FW 10.0% dilution rate had a final chlorophyll-a level of $2.99 \text{ mg} \cdot \text{L}^{-1}$ when light intensity measured inside each algal culture flask diminished to $300 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The ILW:FW of 25% reached chlorophyll-a levels of $3.25 \text{ mg} \cdot \text{L}^{-1}$ when light intensity diminished to $200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Finally, at the 50% dilution, chlorophyll-a dropped to $0.65 \text{ mg} \cdot \text{L}^{-1}$ when light intensity dropped to $80 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Table 1).

3.2. Phosphate

The phosphate content of filtered water during the first incubation day was $21.2 \mu\text{g} \cdot \text{L}^{-1}$. When algae were added, phosphate concentration dropped to $3 \mu\text{g} \cdot \text{L}^{-1}$ on the ninth day, while treatment without algae dropped to $16 \mu\text{g} \cdot \text{L}^{-1}$. In ILW:FW (25:75) ratio plus algae, the initial phosphate content on day one was $78.5 \mu\text{g} \cdot \text{L}^{-1}$. When algae were added, phosphate concentration dropped to $9.7 \mu\text{g} \cdot \text{L}^{-1}$ after nine days, while in the ILF:FW (25:75) treatment without algae phosphate concentration dropped to $70 \mu\text{g} \cdot \text{L}^{-1}$. (Figure 3a, Table 2).

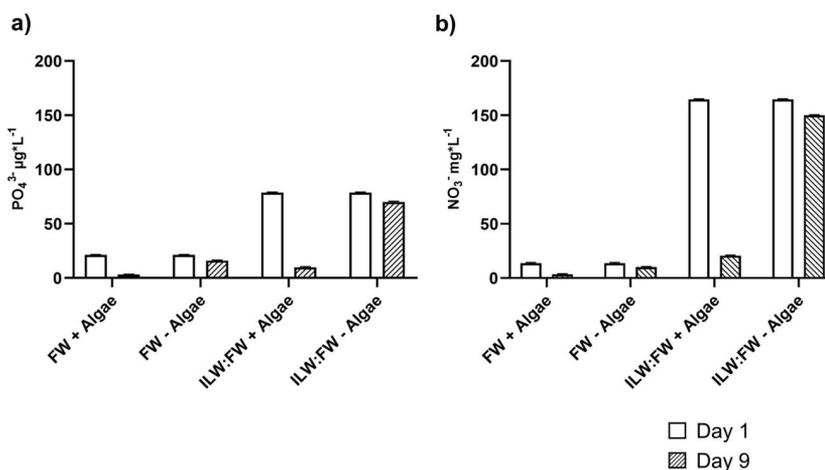


Figure 3. (a) initial and final (after 9 days of cultivation) concentration of phosphate in filtered water (FW) with (+) and without (-) *Cladophora* sp. and agricultural liquid waste (ILW:FW) at 25:75 ratio with (+) and without (-) *Cladophora* sp. (b) initial and final (after 9 days of cultivation) concentration of nitrate in filtered water (FW) with and without *Cladophora* sp. and agricultural liquid waste (ILW:FW) at 25:75 ratio with (+) and without (-) *Cladophora* sp.

Table 2. Concentration levels of phosphate ($\mu\text{g} \cdot \text{L}^{-1}$): Initial and final (after 9 days of cultivation) concentration of phosphate in filtered water (FW) with (+) and without (-) *Cladophora* sp. and agricultural liquid waste (ILW:FW) at 25:75 ratio with (+) and without (-) *Cladophora* sp. $n=3$. All values are significant $p < 0.05$.

	Day 1	SD	Day 9	SD
FW + Algae	21.2	0.556	3	0.501
FW - Algae	21.2	0.632	16	0.556
ILW + Algae	78.5	0.756	9,7	0.771
ILW - Algae	78.5	0.733	70	0.809

Table 3. Concentration levels of nitrate ($\text{mg}\cdot\text{L}^{-1}$): Initial and final (after 9 days of cultivation) concentration of nitrate in filtered water (FW) with and without *Cladophora* sp. and agricultural liquid waste (ILW:FW) at 25:75 ratio with (+) and without (-) *Cladophora* sp. $n=3$. All values are significant $p<0.05$.

	Day 1	SD	Day 9	SD
FW + Algae	13.7	0.721	3.4	0.740
FW - Algae	13.7	0.634	10.1	0.765
ILW + Algae	164.8	0.401	20.7	0.675
ILW - Algae	164.8	0.407	150.0	0.683

3.3. Nitrate

The initial nitrate content of filtered water was $13.7 \text{ mg}\cdot\text{L}^{-1}$. After nine days of incubation with algae, nitrate concentration dropped to $3.4 \text{ mg}\cdot\text{L}^{-1}$. However, with the FW treatment without algae, the nitrate concentration dropped to $10.1 \text{ mg}\cdot\text{L}^{-1}$ after nine days of incubation. In the treatment ILW:FW (25:75) ratio plus the algae inoculum, the initial nitrate content at day one was $164.8 \text{ mg}\cdot\text{L}^{-1}$, and after nine days dropped to $20.7 \text{ mg}\cdot\text{L}^{-1}$. Filtered water and ILW:FW (25:75) dilution rate minus algae treatments dropped to $150.0 \text{ mg}\cdot\text{L}^{-1}$ (Figure 3b, Table 3).

4. Discussion

The use of algae for wastewater treatment has been shown an effective and efficient method to reduce nutrient loads and production costs (Kube et al., 2018). The filamentous algae *Cladophora* sp. have very low nutritional and environmental requirements, which may result in a cost and productivity benefit (Sturm and Lamer, 2011; Cydzik-Kwiatkowska and Zielińska, 2016).

The results indicate the optimal dilution rate for the efficient removal of nutrients by *Cladophora* is ILW:FW (25:75) ratio, given environmental parameters such as the ambient temperature of 18°C and PAR irradiance of $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Chlorophyll-a level showed that algae could thrive at $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which is high in comparison with the reported range between $6\text{-}44 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Graham et al., 1982). Even though the flasks were illuminated with a constant PAR of $1667 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the dilutions were dark enough to reduce the PAR intensity as low as $80 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the (ILW:FW) 50:50 ratio, when chlorophyll-a reached the lowest value ($0.657 \text{ mg}\cdot\text{L}^{-1}$). At that point, removal of nutrients also dropped in *circa* 80% of the samples. Light intensity and chlorophyll-a are interlinked in a way that can be used as indicators of the point at which algae will remove enough nitrate and phosphate to be discharged into the environment without exceeding the levels set in Chilean laws (Chile 2001, Law N° 19.300. Decree 90/00). In addition to nutrient removal from ILW, the possibility of separating and extracting the algal biomass opens the door to a world of opportunities from its use, with the potential to create a self-sufficient energy plant with recovery of nutrients such as nitrogen or phosphorus, decreasing the ecological debt (Santos and Pires, 2018).

Given the results of this research, agricultural wastewater may be treated in suitable mass waters like lagoons and

wetlands using *Cladophora* sp. to remove organic phosphorus and nitrate (Zhu et al., 2018). Since *Cladophora* sp. can thrive under low light intensities, lagoons should be no more than one meter deep to avoid anoxic bottom. Thus, it is suggested that freshwater should be introduced into the ILW until reaching a minimum light intensity of $200 \mu\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. According to Kube et al. (2018), algae may continue removing nitrogen and phosphorus as long as phosphorus is not totally depleted, which makes algae a good candidate for removing both nutrients efficiently.

Some members of the genus *Cladophora* sp. must be anchored to a surface in their initial growth stages (Graham et al., 1982), needing only small water motion to thrive. The fact that *Cladophora* sp. grows in filaments, makes the task of harvesting easier by physical mechanisms such as hand-pulling. The harvested algae could then become valuable as compost in the same farm. *Cladophora* sp. requires a hard surface for initial attachment, warm water, some motion, and nutrients from the effluent from the milk processing site. In natural habitats *Cladophora* sp. is a foul-smelling nuisance algae that harbors many human pathogens (Ishii et al., 2006). Therefore, it is suggested that *Cladophora* sp. must be handled carefully when extracting it from nearby ponds, including cleaning it from detritus while bacteria remains in place. The bacteria consortium plus the algae must be taken into consideration for the removal of nutrients as a whole, since they were never removed when the experiments were set up.

In Chile, the law to discharge effluents in lakes and streams states that the total phosphorous maximum allowed is $10 \text{ mg}\cdot\text{L}^{-1}$ without dilution, and $15 \text{ mg}\cdot\text{L}^{-1}$ with dilution. For nitrogen, the maximum allowed is $50 \text{ mg}\cdot\text{L}^{-1}$ without dilution, and $75 \text{ mg}\cdot\text{L}^{-1}$ with dilution (Chile 2001, Law N° 19.300. Decree 90/00). Filamentous algae can be collected and should be spread on large surfaces for subsequent drying to avoid decomposition.

Although this study is considered preliminary because it reports on a simple laboratory-scale experiment on the possibility of treating dairy processing effluents, it shows the feasibility of nutrient removal using the filamentous algae *Cladophora* sp.

5. Conclusion

The freshwater filamentous algae *Cladophora* sp. reduces phosphorus and nitrate concentration levels at laboratory-scale. Thus, it is possible to use the algae for the treatment of dairy processing effluents. Nonetheless,

pilot and real-scale experiments are needed to validate this biological wastewater treatment method. Consequently, the use of algae for wastewater treatment in small milk farms could help the farmers to comply with Chilean law and at the same time help to reduce eutrophication in nearby lakes and streams. Lastly, a recommendation to reassure ideal conditions of the treatment is intense monitoring of pathogens that can be present in the algal mats.

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