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Research Article

Control of *Branchionus* sp. and *Amoeba* sp. in cultures of *Arthrospira* sp.

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ABSTRACT. Cultivation of cyanobacterium *Arthrospira* sp. has been developed in many countries for the production of proteins, pigments and other compounds. Outdoor mass cultures are often affected by biological contamination, drastically reducing productivity as far as bringing death. This study evaluates the control of *Branchionus* sp. and *Amoeba* sp. with two chemical compounds: urea (U) and ammonium bicarbonate (AB), in laboratory conditions and outdoor mass culture of *Arthrospira* sp. The lethal concentration 100 (LC100) at 24 h for *Branchionus* sp. and *Amoeba* sp. determined was of 60-80 mg L^{-1} (U) and 100-150 mg L^{-1} (AB). The average effective inhibition concentration for 50% of the population (IC50) in *Arthrospira* sp., after 72 h, was 80 mg L^{-1} (U) and 150 mg L^{-1} (AB). The application of doses of 60 mg L^{-1} (U) or 100 mg L^{-1} (AB) in the outdoor mass culture of this contaminated microalga, completely inhibited grazing and did not affect the growth of *Arthrospira* sp. but rather promoted rapid recovery of algal density at levels prior to infestation. These compounds provided an economical and effective control of predators in cultures of *Arthrospira* sp.

Keywords: *Arthrospira*, *Branchionus*, *Amoeba*, predator control, ammonium, bicarbonate, urea, Chile.

Control de *Branchionus* sp. y *Amoeba* sp. en cultivos de *Arthrospira* sp.

RESUMEN. El cultivo de la cianobacteria *Arthrospira* sp. ha sido desarrollado en muchos países para la obtención de proteínas, pigmentos y otros compuestos. Cultivo que a nivel industrial se ve afectado frecuentemente por contaminación biológica, reduciendo drásticamente la productividad hasta causar la muerte. Este estudio evalúa el control de *Branchionus* sp. y de *Amoeba* sp. con dos compuestos químicos, la urea (U) y bicarbonato de amonio (AB) en cultivos de *Arthrospira* sp. La concentración letal 100 (LC100) determinada a las 24 h para *Branchionus* sp. y *Amoeba* sp. fue de 60-80 mg L^{-1} (U) y 100-150 mg L^{-1} (AB). La concentración media de inhibición efectiva, después de 72 h, para el 50% de la población (IC50) en *Arthrospira* fue de 80 mg L^{-1} (U) y 150 mg L^{-1} (AB). La aplicación de dosis de 60 mg L^{-1} (U) ó 100 mg L^{-1} (AB) en los cultivos abiertos contaminados, inhibió completamente el pastoreo y no afectó el crecimiento de *Arthrospira* sp., permitiendo una rápida recuperación de la densidad algal a niveles anteriores a la infestación. Estos compuestos proporcionan un medio económico y efectivo de control de estos depredadores en cultivos de *Arthrospira* sp.

Palabras clave: *Arthrospira*, *Branchionus*, *Amoeba*, control depredadores, bicarbonato de amonio, urea, Chile.

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INTRODUCTION

The microalgae in their natural habitat, such as extensive and intensive cultures developed by aquaculture, are affected by different types of biological contaminants, among which are viruses, bacteria, protists, fungi and several predators (Hoffman *et al.*, 2008). Microorganisms influence negatively the microalgal growth and the quality of the product (Richmond, 2000), being most significant in intensive outdoor mass cultures, where the microorganisms can compete for nutrients, and graze

upon the microalgae, reducing drastically the commercial production (Richmond & Becker, 1986; Contreras *et al.*, 2003; Ugwu *et al.*, 2008).

The colonizing organisms of the microalgae culture, can reach very high densities, generating operation problems, increasing the costs of production as well as inputs and workforce. This can reduce the production up 90% and in some cases the total loss of the culture (Lincoln *et al.*, 1983; Richmond, 2004). For these reasons, it is important to identify the type of contamination in order to implement control measures to mitigate or eliminate the contaminant, to maintain a healthy culture, highly productive, to obtain specific products such as vitamins, proteins, fatty acids, polyunsaturated, pigments, antibiotics, hydrogen, hydrocarbons, and bioactive compounds (Borowitzka, 1992; Metting, 1996; Cohen, 1999; Pulz *et al.*, 2001; Morales *et al.*, 2002; Domínguez *et al.*, 2003).

The culture of *Arthrospira* sp. outdoors can be contaminated with microalgae, as *Chlorella* sp., *Oocystis* sp. or by other species of cyanobacteria and protozoans such as *Amoeba* sp. and *Paramecium* sp. (Vonshak *et al.*, 1983; Vonshak & Richmond, 1988; Pedraza, 1989; Belay, 1997; Vonshak, 1997). In some cases the *Chlorella* spp. can even displace the *Arthrospira* sp. and turn rapidly into a dominant algae in the culture if there is not a suitable control of it (Richmond *et al.*, 1990). The estimated annual losses caused by the biological contamination in the culture of *Arthrospira* sp. is around 15 to 20% (Belay, 1997). The outdoor mass cultures of *Arthospira* sp. conducted in the north of Chile are being contaminated with amoebae (*Amoeba* sp.) and rotifers (*Branchionus* sp.). These organisms generate stress, cellular breaking, detriment and death of the culture. Described contaminations by amoebae suggest that cultures die within days if invasion is not treated quickly (Wagener & de Luca, 1987; Duerr *et al.*, 1997). Rotifers have always been seen as very useful organisms in aquaculture as live food (Planas & Cunha, 1999), however these organisms can cause significant biological contamination in algal cultures (Oswald, 1980). Currently there are no mass cultures of *Arthrospira* sp. reports of contamination by species of this genus; therefore, this one would be the first case of infestation control in culture of *Arthrospira* sp. in Chile.

A number of methods have been investigated for predator control in the cultures of microalgae, with limited success. Early attempts of control have been performed by means of heating, centrifugation, changes in pH, salinity and the use of chemicals such as chlorine, formaldehyde, hydrogen peroxide, ivermectin, metronidazole, quinine sulfate, ammonium and ammonia (Lincoln *et al.*, 1983; Vonshak *et al.*, 1983; Vonshak & Richmond, 1988; Borowitzka, 1999; Moreno-Garrido & Cañavate, 2001; Richmond, 2004; Zmora & Richmond, 2004, Garric *et al*., 2007).

This study was aimed to control the biological contamination of *Amoeba* sp. and *Branchionus* sp. present in the culture of *Arthrospira* sp. using two chemical agents such as urea and ammonium bicarbonate. These compounds can reduce to ammonium and ammonia (Trenkel, 1997; Carpio & Moran, 2005), which are toxic for aquatic organisms (Zhao *et al.*, 1997). Ammonium toxicity for aquatic organisms is related to the non-ionized form (ammonia), mainly due to its high lipid solubility, which facilitates their passage through biological membranes, causing damage to the respiratory structures (Jensen, 2003). The urea and ammonia have been used to control a type of amoeba in the culture of *Arthrospira* sp., giving good results (Vonshak & Richmond, 1988). As for rotifers, it is known that ammonia is one of the main compounds that inhibit growth and cause death of these organisms (Yoshimura *et al.*, 1996).

From the economic point of view, the use of ammonium compounds and/or urea is particularly attractive, since they are inexpensive (Corverti *et al.*, 2006). The urea and some ammonium have been used as sources of nitrogen in culture of *Arthrospira* sp. (Stanca & Popovici, 1996; Vieira *et al.*, 2001; Danesi *et al.*, 2002; Costa *et al.*, 2004; Rangel-Yagui *et al.*, 2004; Sanchez-Luna *et al.*, 2004; Soletto *et al.*, 2005; Sassano *et al.*, 2007, 2010), however, there are no reports on the use of ammonium bicarbonate in the culture of this microalgae.

MATERIALS AND METHODS

Microorganism

Arthrospira sp., inoculums were grown at 30 ± 1ºC and fluorescent light intensity of 117 µmol m^2 s^{-1} in 250 mL Erlenmeyer flasks containing the medium Zarrouk (1966). The work was developed in several phases. The first phase in the laboratory involved toxic tests in *Arthrospira* sp., *Amoebas* sp., and *Branchionus* sp. with different concentrations of urea [$CO(NH_2)_2$], and ammonium bicarbonate [NH_4HCO_3] (industrial grade) to determine the average concentration of inhibition (IC50) and lethal concentration (LC100) for microalgae and predators respectively. The second phase was performed outdoors, using the

outcome of the toxicity test in microalgae and predators in series of 1,000 m^2 raceway contaminated.

Toxicity test in *Arthrospira* sp.

The bioassays were carried out in 5 L plastic bottles with a final volume of 4 L using medium SSM (Ayala & Bravo, 1982) modified by Ayala & Ayala (2009), which was maintained through the daily addition of distilled water to replace lost by evaporation. Cultures were performed in triplicate with constant aeration and a photoperiod of 12:12 illumination with white fluorescent light intensity of 117 µmol m^2 s^{-1}. In the experiments, two different concentrations of ammonium bicarbonate (mg L^{-1}) 0, 50, 100, 150 and of urea (mg L^{-1}) were used, respectively. Evaluations were performed at 24, 48, 72 y 96 h, to observe the effect on microalgae growth. Biomass of *Arthrospira* sp. was determined daily by sample optical density at 560 nm in a Smart LMC202 colorimeter. These values were compared with previously prepared calibration curves of optical density versus biomass dry weight as described by Leduy & Therien (1977) and applied by Vonshak *et al.* (1988). The specific growth rate (µ) was calculated by the method of Leduy & Zajic (1973).

Toxicity test in *Branchionus* sp. and of *Amoeba* sp.

For toxicity experiments, microorganisms were isolated from contaminated outdoor cultures. They were carried out in 5 L plastic bottles with volume of 4 L, with an initial density of 400 rotifers mL^{-1} and 600 amoeba mL^{-1} in each of the bottles. The culture conditions of these microorganisms were the same used for toxicity tests with microalgae *Arthrospira* sp.: concentrations of ammonium bicarbonate (mg L^{-1}) 0, 50, 100, 150 and of urea (mg L^{-1}) 0, 40, 60, 80. Predator's mortality was counted by Olympus CX21 light microscopy at 20x magnification, with a Sedgewick-Rafter counting chamber. In every culture there were 10 subsamples of 1 mL; each concentration experiment was performed in triplicate. After 24 h, survival of predators was checked. Death of *Amoeba* sp. was defined as breaking of the cytoplasm and for *Branchionus* sp. when the lorica was empty.

After the toxicity test in microalgae and predators, results were applied in 1,000 m^2 outdoor contaminated mass cultures of *Amoeba* sp. and *Branchionus* sp.

Data analysis

Prior to statistical analysis, data was tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene test). Comparison between concentrations of urea and ammonium in specific growth rate and algae biomass was done by one-way ANOVA. Where significant main effects were found, multiple comparisons Dunnett test was used to determine significant differences among means (Zar, 1984). All statistical analyses were performed with Statistica 10 at a significance level of $P < 0.05$.

The effective inhibition concentration for 50% of the population (IC50) in *Arthrospira* sp., after 72 h and lethal concentration 100 (LC100) at 24 h for *Branchionus* sp. and *Amoeba* sp. were calculated according to protocols for toxicity testing in microorganisms OECD (1984), USEPA (1996), and ASTM (2004).

RESULTS

The biomass curves of *Arthrospira* sp. under different concentrations of ammonium bicarbonate and urea in laboratory are shown in Fig. 1. *Arthrospira* sp. showed a maximum dry weight biomass of 1339 ± 9.815 mg L^{-1} and 1255 ± 9.238 mg L^{-1} after 72 h, with a concentration of 100 mg L^{-1} ammonium bicarbonate and 60 mg L^{-1} urea, respectively. At 80 mg L^{-1} urea and 150 mg L^{-1} ammonium bicarbonate showed decreasing of biomass with increasing incubation time. Statistical analysis revealed that there was significant difference in the growth of this microalga ($P < 0.05$). The curves of Figure 1 with ammonium bicarbonate 0, 50, 100 mg L^{-1} and of urea 0, 40, 60 mg L^{-1} did not show any lag phase, notwithstanding the use of a different nitrogen source. The specific growth rates responses at each of the selected chemicals are presented in Table 1.

The outcomes of the laboratory tests of toxicity for urea and ammonium bicarbonate for *Amoeba* sp. and *Branchionus* sp., (24 h LC100) are shown in Table 2. IC50 values and LC100 for *Arthrospira* sp. and predators for each of the compounds used are shown in Table 2. In toxicity tests for predators of the culture, it was observed that concentrations of 60 and 80 mg L^{-1} and 100 and 150 mg L^{-1} urea and ammonium bicarbonate, respectively, exterminated between 99 and 100% of these microorganisms in 24 h. These results suggest that urea and ammonium bicarbonate could be highly toxic to *Amoeba* sp. and *Branchionus* sp. and slightly toxic to *Arthrospira* sp.

Considering the laboratory results, the selected dose of urea was 60 mg L^{-1} and 100 mg L^{-1} ammonium bicarbonate for predator control in the culture of *Arthrospira* sp. open reactor of 1,000 m^2. The effect of the application of these compounds in the reactors contaminated with *Amoeba* sp. and *Branchionus* sp. is described in Figures 2a and 2b,

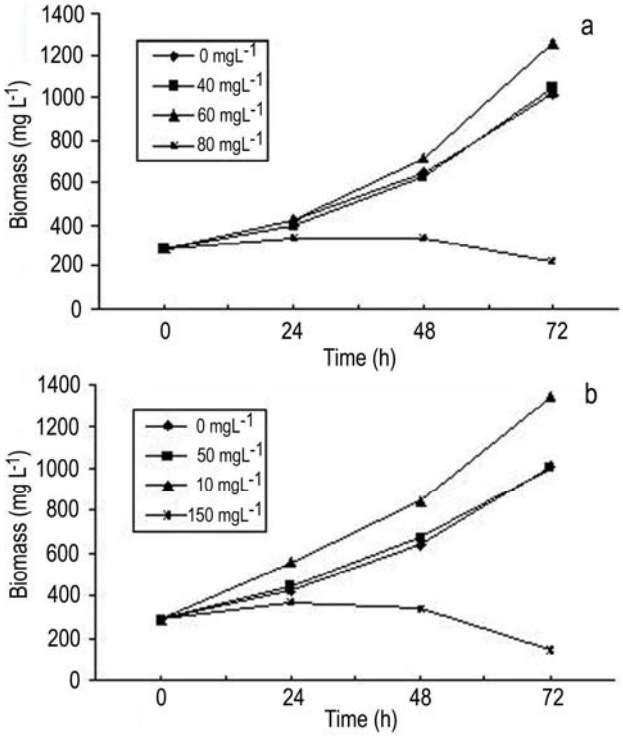

Figure 1. a) Effect of different concentrations of urea, and b) ammonium bicarbonate, on biomass of *Arthrospira* sp. Values are given as means (n = 3).

Figura 1. a) Efecto de diferentes concentraciones de urea, y b) de bicarbonato de amonio, sobre la biomasa de *Arthrospira* sp. Los valores se expresan como medias (n = 3).

showing that the cultures of these microalgae have a fast recovery.

DISCUSSION

Recommendations by different authors for the treatment against grazers' contamination in micro algal cultures are limited to maintaining optimal values of pH, light and temperature, in order to increase algal growth, and therefore surpass the damage caused by grazing (Richmond & Becker, 1986). Such equilibrium was not possible in the present case, since the high grazing capacity of the rotifer and amoebae involved leads to a complete elimination of the micro algal population within two days. Upon adding urea or ammonium bicarbonate to culture, a fast increment is produced in the concentration of ammonium and ammonia which is regulated by pH (Alonso, 2006). These compounds themselves become the main factors that inhibit or limit the growth of the rotifer (Yoshimura *et al.*, 1996). In the culture of *Arthrospira* sp. the pH is maintained in a range of 9.5 to 10.5, that favors the predominance of NH_3 over NH_4 (Laliberté *et al.*, 1997). The rotifers have been more affected with the high levels of NH_3 than other groups of aquatic invertebrates (Arauzo, 2003). It has been reported that these compounds affect other rotifers like *Branchionus rotundiformis* and *B. plicatilis* causing their collapse in the culture, diminishing swimming and causing death of the culture (Snell *et al.*, 1987; De Araujo *et al.*, 2000, 2001; Yoshimura *et al.*, 2003). In natural populations *B. calyciflorus* and *B. rubens* are inhibited with concentrations of around 2.5 mg L^{-1} (Arauzo, 2003). In a study carried out by Lincoln *et al.* (1983), they utilized ammonium hydroxide of to control the contamination by the rotifer (*Brachionus rubens*) in 24 h. LC100 with an approximate concentration of 17 mg L^{-1} of ammonium without inhibiting the algal growth. Duerr *et al.* (1997) supplied urea in a daily concentration of 50 mg L^{-1} to the culture of *Arthrospira* sp., contaminated with *Amoeba* sp., giving as a result the control of these protozoa, besides providing an entrance of nitrogen to the culture. With similar concentrations used in this work (60 mg L^{-1} of urea and 100 mg L^{-1} ammonium bicarbonate) this microorganism was controlled.

As it can be seen, the inhibition concentrations of these reduced nitrogen compounds is lower for *Branchionus* sp., and *Amoeba* sp., than to inhibit the growth of these microalgae, which corroborates the results of this study. The nitrogen source has a great importance because it is the second most abundant element of *Arthrospira* sp. biomass, representing up to about 10% of its total content (Soletto *et al.*, 2005). After carbon, nitrogen is the nutrient which provides more algal biomass (Grobbelaar, 2004). The conventional nitrogen source for *Arthrospira* sp. is nitrate (Zarrouk, 1966). The microalgae having used nitrate needs to reduce it to nitrite and later to ammonia (Hatori & Myers, 1966). Since this process needs energy, the algae prefer using reduced nitrogen in the forms of ammonium and urea that are toxic in high concentrations (Belkin & Boussiba, 1991). Nevertheless, urea and ammonium bicarbonate are easily assimilated by *Arthrospira* sp. probably due to its spontaneous hydrolysis to ammonia under alkaline conditions, which is the preferential form of nitrogen uptake by this cyanobacterium (Danesi *et al.*, 2002; Coverti *et al.*, 2006). This process of assimilation is realized by the stirrup-strap of the ureasa and by the glutamine synthetase (Carvajal *et al.*, 1980; Herrero *et al.*, 2001). Stanca & Popovici (1996) observed that using reduced nitrogen improves the production of biomass of *Arthrospira* sp., a result obtained in the present work with concentrations of 60 mg L^{-1} urea

Table 1. Specific growth rate (μ) of *Arthrospira* sp. at different concentrations of urea and ammonium bicarbonate.

Tabla 1. Tasa especifica de crecimiento (μ) de *Arthrospira* sp. a diferentes concentraciones de urea y bicarbonato de amonio.

Chemicals	Concentration (mg L^{-1})	Time (h)		
		24	48	72
Urea	0	0.089 ± 0.001	0.098 ± 0.002	0.107 ± 0.001
	40	0.085 ± 0.002	0.098 ± 0.001	0.109 ± 0.001
	60	0.089 ± 0.001	0.103 ± 0.001	0.114 ± 0.001
	80	0.072 ± 0.001	-0.001 ± 1.2x10^{-5}	-0.665 ± 0.001
Ammonium bicarbonate	0	0.089 ± 0.001	0.098 ± 0.002	0.107 ± 0.001
	50	0.092 ± 0.001	0.098 ± 0.001	0.105 ± 0.003
	100	0.101 ± 0.001	0.102 ± 0.001	0.112 ± 0.002
	150	0.081 ± 0.002	-0.138 ± 0.001	-0.906 ± 0.003

Table 2. Toxicity of chemicals agents on *Arthrospira* sp. and the grazer predators.

Tabla 2. Toxicidad de los agentes químicos en *Arthrospira* sp. y en los depredadores.

Chemicals	LC50 (mg L^{-1} 72 h) *Arthrospira* sp.	LC100 (mg L^{-1} 24 h) *Amoeba* sp.	LC100 (mg L^{-1} 24 h) *Branchionus* sp.
Urea	80	60-80	60-80
Ammonium bicarbonate	150	100-150	100-150

and 100 mg L^{-1} ammonium bicarbonate. On the contrary, in the processing of 80 mg L^{-1} urea and of 150 mg L^{-1} ammonium bicarbonate, a decrease in the biomass of this microalgae was observed, this observation is in agreement with the results reported by Sassano *et al*. (2004) that also determined that the excess of urea or ammonium may cause the inhibition of the growth of *Arthrospira* sp. Belkin & Boussiba (1991), indicate that ammonia in high concentrations inhibits the growth and is responsible for cell death of this microalga. The ammonia present in the cultivations could enter the cells without energy expenditure, according to an extracellular/intracellular gradient of pH ((Boussiba, 1989). The pH in culture is among 9.5 to 10.5 and the cell cytoplasm is 8.5, this difference of pH suggests that there exists a movement of ammonia towards inside the cell with an intracellular accumulation that can arrive to a toxic level. The inhibition levels of ammonium are among 30-36 mg L^{-1}, and toxic 180 mg L^{-1} for this microalga (Carvalho *et al.*, 2004; Soleto *et al.*, 2005; Coverti *et al.*, 2006). These authors concentrations are high compared to given compounds in the present study.

The *Arthrospira* sp. used in the present study achieved higher biomass concentrations compared to previous studies concerning *Arthrospira* sp. (Lodi *et al*., 2005; Narayan *et al.*, 2005; Radmann *et al.*, 2007). This species had higher growth rate (μ) than in previous studies for *Arthrospira* sp. (Lodi *et al*., 2005; Andrade & Costa, 2007; Colla *et al*., 2007). It is a consequence of species having different response to different environmental conditions and demonstrates a quick adaptation to the new medium.

The production average of *Arthrospira* sp., in biomass drought of a reactor is of 9 kg m^{-2} day^{-1} and it drops drastically when the culture presents a biological contamination. If the necessary controls to the culture are not applied, a strong economic loss is presented since, upon trying a new culture, this will begin its production after 15 to 20 days, that costs around US$ 1,500 by reactor and a business handles near 20 reactors; the total loss would be of US$ 30,000 without including the nutrients. Besides the economic losses, also a delay in the planning and the goals of production of the business is generated.

The nutrients in the culture of *Arthrospira* sp., have a cost about 15 to 20% (Vonshak, 1997). It is therefore important to use low-cost nutrients to reduce the production costs. The use of urea and/or ammonium bicarbonate as nutrients in the culture of these microalgae offers great advantages, since the cost of these inputs in comparison with other nutrients

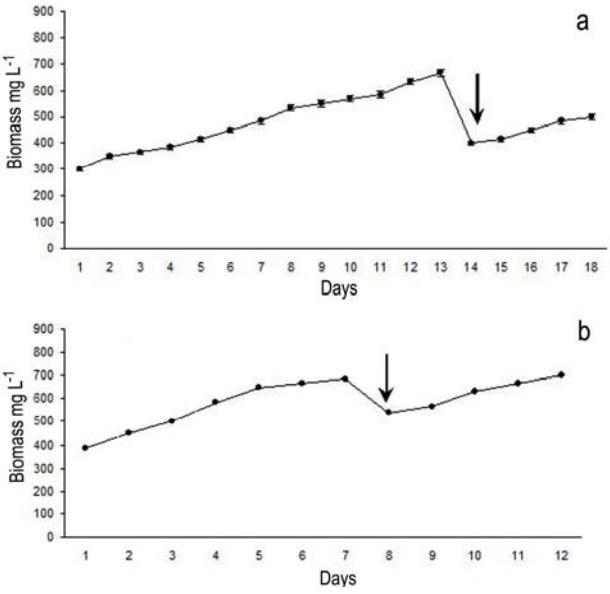

Figure 2. Infection occurred in outdoor mass culture of *Arthrospira* sp. of 100 m^2. a) *Branchionus* sp. Arrow indicates the addition 100 mg L^{-1} ammonium bicarbonate, b) *Amoeba* sp. Arrow indicates the addition 60 mg L^{-1} urea. The presence of predators coincides with a decrease in microalgae biomass.

Figura 2. Infección ocurrida en reactores abiertos de *Arthrospira* sp. de 1000 m^2. a) *Branchionus* sp. La flecha indica la adición de 100 mg L^{-1} de bicarbonato de amonio, b) *Amoeba* sp. La flecha indica la adición de 60 mg L^{-1} de urea. La presencia de depredadores coincide con la disminución de la biomasa de la microalga.

is cheaper. The urea has a cost of approx. US$ 0.25 kg, the ammonium bicarbonate approx. US$ 0.2 kg, in comparison with the main source of nitrogen, the sodium nitrate, which is approx. US$ 0.5 kg (these are prices in Chile). The decrease of costs is reflected in industrial cultures, in which large quantities of nutrients are employed, in the case of a reactor of 1,000 m^2 an approximate quantity of 600 kg of sodium nitrate is used for the preparation of the initial broth. If using urea as nitrogen source, the company would save US$ 20, this reduction is reflected as more culture reactors are used, considering a company that has 20 reactors it will be saving US$ 400 only at the planting and this reduction in the costs of the nutrients would be of US$ 28,000, besides a control on the present predators is generated and finally would be done an entrance of carbon to the culture, because a mol of urea contributes a mol of carbon (Levasseur *et al*., 1993).

The application of urea as of ammonium bicarbonate in the selected concentrations eliminates these two grazing organisms as *Branchionus* sp. and *Amoeba* sp. in a short period of time (24 h), helping to recover the cultures with the supply of nutrients and thus avoiding economic losses for the companies that cultivate *Arthrospira* sp.

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