Efectos directos e indirectos del cobre en una comunidad límnica experimental

Tesis

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por

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A mi familia

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Abstract

Pollutants are able to affect species within a community not only directly, but also through indirect paths. Indirect effects take place under a multispecies context, when organisms are affected through the effects that pollutants exert on other species that are ecologically related to the formers. Usually, the net effects of pollutant are assessed without considering the contribution of indirect effects, which could enhance, diminish or cancel out direct effects. The aim of this study is to comparatively assess the direction and magnitude of direct and indirect effects of copper on the components of an experimental planktonic assemblage. Specifically we evaluated the effects of copper on the population growth rate of the herbivores *Daphnia ambigua* and *Ceriodaphnia dubia* and the microalgae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*. Additionally, we decomposed the effects on herbivores into partial effects on their birth and death rates. Indirect effects on microalgae, on the other hand, were decomposed into partial indirect effects transmitted by each component species of the assemblage. The results indicated that in general the contributions of indirect effects to the net effects were equal or more relevant than direct effects. Our results also suggested that the effects exerted by copper on *D. ambigua* may be explained mainly by changes in birth rates while in *C. dubia* were associated to changes in death rates. Decomposition of indirect effects on microalgae showed that effects on *P. subcapitata* were transmitted principally by *D. ambigua*, whereas in the case of *C. vulgaris* the effects were driven by both herbivore species. This study presents new evidence about the responses to pollutants of natural inhabitants of Chilean freshwater systems, and also provides an advance in the understanding of the functioning of perturbed communities highlighting the crucial importance of indirect effects of stressors in ecosystems.

Keywords: Community interactions, metal, population growth rate, egg ratio, zooplankton, phytoplankton.

Resumen

Los contaminantes son capaces de afectar a las especies dentro de una comunidad no sólo directamente, sino que también a través de vías indirectas. Los efectos indirectos ocurren bajo un contexto multiespecífico, cuando los organismos son afectados a través de los efectos que los contaminantes ejercen sobre otras especies que están ecológicamente relacionadas con las primeras. Usualmente, los efectos netos de los contaminantes son evaluados sin considerar las contribuciones de los efectos indirectos, los cuales podrían aumentar, disminuir o anular los efectos directos. El objetivo de este estudio es evaluar comparativamente la dirección y magnitud de los efectos directos e indirectos del cobre, sobre las especies componentes de un ensamble planctónico experimental. Específicamente evaluamos los efectos del cobre sobre la tasa de crecimiento poblacional de los herbívoros *Daphnia ambigua* y *Ceriodaphnia dubia* y las microalgas *Pseudokirchneriella subcapitata* y *Chlorella vulgaris*. Adicionalmente, se descompusieron los efectos sobre los herbívoros en efectos parciales sobre sus tasas de natalidad y mortalidad. Los efectos indirectos sobre las microalgas, por otro lado, fueron descompuestos en efectos indirectos parciales transmitidos por cada una de las especies componentes del ensamble. En general, los resultados indicaron que las contribuciones de los efectos indirectos a los efectos netos, fueron tanto o más relevantes que las de los efectos directos. Nuestros resultados también sugieren que los efectos ejercidos por el cobre sobre *D. ambigua* podrían ser explicados principalmente por cambios en la tasa de natalidad, mientras que para *C. dubia* serían asociados a cambios en la tasa de mortalidad. La descomposición de los efectos indirectos sobre las microalgas mostró que los efectos sobre *P. subcapitata* fueron transmitidos principalmente por *D. ambigua*, mientras que en el caso de *C. vulgaris* los efectos fueron conducidos por ambas especies de herbívoros. Este estudio presenta nueva evidencia sobre las respuestas a los contaminantes de los habitantes naturales de sistemas de agua dulce chilenos, y también provee un avance en el entendimiento del funcionamiento de las comunidades perturbadas, destacando la importancia crucial de los efectos indirectos de los estresores en los ecosistemas.

Palabras clave: Interacciones comunitarias, metal, tasa de crecimiento poblacional, razón de huevo, zooplancton, fitoplancton.

1. Introduction

The release of pollutants to the environment represents one of the main current threats to biodiversity, affecting both richness and abundance of species within ecosystems (Millennium Ecosystem Assessment, 2005). However the understanding and assessment of actual effects of pollutants on natural communities may be difficult since they do not only affect species directly, but they could also exert effects through indirect routes. Direct effects of pollutants on a population arise when the pollutant itself affects some vital rates of the constituent organisms. Indirect effects, on the other hand, take place when the organisms are affected by the effects that the pollutant exerts on other -intermediate- populations that are ecologically related with the former, thus appearing only under a multispecies scenario (Wootton, 1994). Furthermore, indirect effects of pollutants could emerge either from changes in abundance of intermediate species ("numerical indirect interaction": Janssen et al., 1998; "density-mediated indirect interaction": Werner and Peacor, 2003) or through modifications of traits of the intermediate species ("interaction modification": Wootton, 1993; "trait-mediated indirect interaction": Werner and Peacor, 2003). These alterations of species' abundances or trait values produce chains of effects that could lead to positive or negative net effects of the pollutant on some species. The sign and strength of indirect effects will depend on the sign and strength of the ecological interactions among the species that compose the route stretched from the pollutant to the affected species. In other words, the properties of indirect effects of a contaminant will depend on the structure of communities (Fig. 1; see also Rohr et al., 2006; Clements and Rohr, 2009).

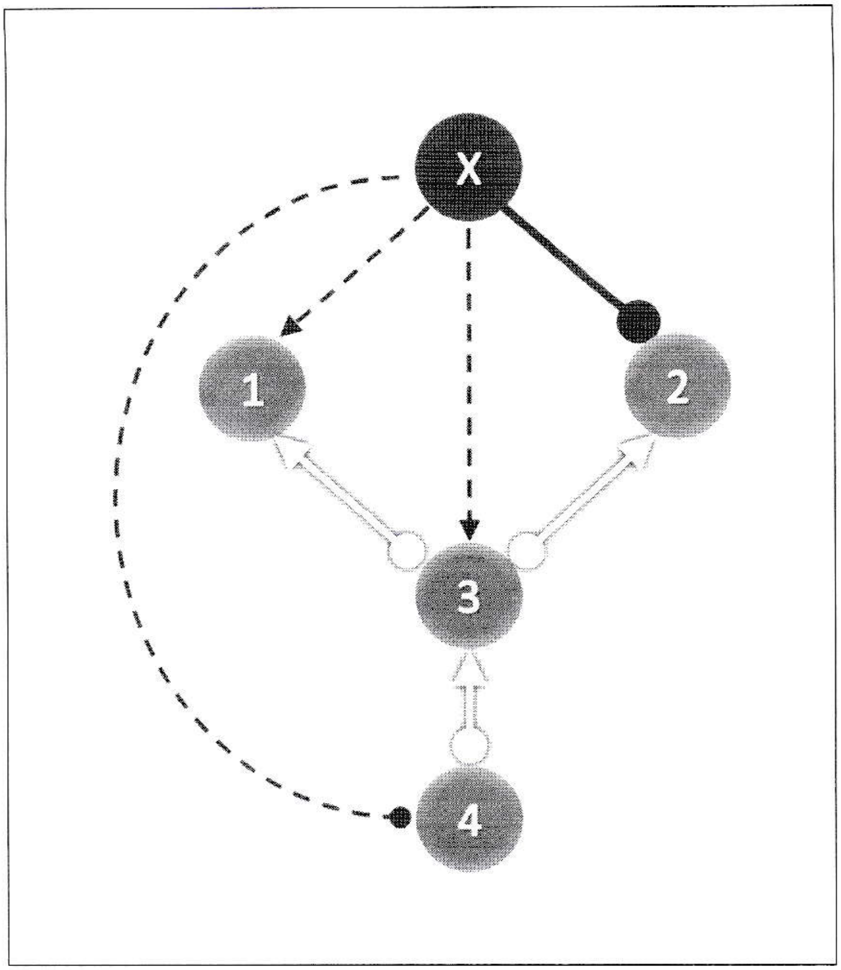

Figure 1. Graph representing effects (black lines) of pollutant X on the components (species 1-4) of a model community. Bright lines represent ecological interactions among species. Lines ending in arrowhead and circles represent positive and negative effects respectively. Solid lines denote direct effects and dashed lines denote indirect effects. Pollutant X exerts a negative direct effect on species 2 only. The direction, sign and magnitude of indirect effects generated on species 1, 3 and 4 are determined by species interactions.

Although the occurrence of effects transmitted by indirect paths had been reported for xenobiotics in terrestrial (e.g. Risch and Carroll, 1982; Sheffield and Lochmiller, 2001; Butler and Trumble, 2008) as well as aquatic communities (see Preston, 2002; Fleeger et al., 2003), it is not easy to determine the direction and strength of indirect effects in multispecies assemblages. This may lead to a wrong evaluation of the effects that pollutant release may exert on wild species, especially when those evaluations are based only on direct effects on isolated species. Standard ecotoxicological bioassays usually assess only direct effects of pollutant on species without addressing the contribution of indirect effects, which could enhance, diminish or even cancel out direct effects (Yodzis, 1988). Thus, in order to understand if net effects on species arise from pollutant toxicity (direct effects) or from chains of effects that depends on the community context (indirect effects), it is important to evaluate the magnitude and sign of both direct and indirect effects.

Among the elements that potentially affect Chilean ecosystems, copper is especially important. Copper represents the most important natural resource of Chile, and its exploitation is intensive along the country. The prevalence of copper industry in Chile and the intensity of their extraction processes makes this metal a threat to marine (Correa et al., 1996; Correa et al., 1999; Lee and Correa, 2004; Medina et al., 2005), terrestrial (Neaman et al., 2009) and freshwater systems (Vila and Pardo, 2003; Villavicencio et al., 2005; Narváez et al., 2007; von Gunten et al., 2009). Copper, between optimal concentrations, acts as a micronutrient participating in a wide range of biochemical redox reactions involving intracellular enzymes/proteins (Harris and Gitlin, 1996), thus being essential to processes such cellular respiration, protection from freeradicals, and cellular iron metabolism (Van Assche et al., 1997; Hopkin, 1989; Bossuyt and Janssen, 2003). However, above optimum concentrations, copper is highly toxic for individuals of many species, being able to produce oxidative stress (Barata et al., 2005), elevated oxygen consumption (Gerhardt, 1995), lowered feeding rate (Blockwell et al., 1998), altered locomotion (Untersteiner et al., 2003) and decreased individual growth rate (Winner, 1985). All of these effects are associated to detoxification and reparation mechanisms that require large amounts of energy, thus leaving few resources for growth and reproduction (Sibly, 1996; Kooijman et al., 2009). The final consequences of physiological copper effects are transmitted to a population level as reductions in population growth rate of exposed species (e.g. Winner, 1976; Ferrando et al., 1993; Wong, 1993; Franklin et al., 2002; Bossuyt and Janssen, 2004; Gama-Flores et al., 2007; Gama-Flores et al., 2009).

In this study our aim is to assess direct and indirect effects of copper exposure on species embedded in an experimental aquatic community. Specifically we estimate direction and strength of copper effects on an experimental assemblage composed by the microalgae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*, and the herbivore microcrustaceans *Daphnia ambigua* and *Ceriodaphnia dubia*. Selected microalgae and branchiopod species are used as a model because they are representatives of common inhabitants of fresh water systems of central Chile (Araya and Zúñiga, 1985; Vila et al., 1987; Ramos-Jiliberto et al., 1998; Ramos-Jiliberto et al., 2004; Montecino et al., 2011). This experimental assemblage was considered as being the minimal system able to resemble the diversity of natural communities, with more than one trophic level and more than one species per trophic level. This topological motif has been studied theoretically (Leon and Tumpson, 1975; Kirlinger 1989) as well as empirically (Tilman 1981; Dethier and Duggins, 1984). In addition, we decomposed indirect effects of copper on population growth rate of microalgae into the different paths by which they are transmitted. This allows the identification of the species within the community that are most important for the transmission of indirect effects towards microalgae. Finally, we also decomposed effects of copper on the population growth rate of herbivores into effects on the two underlying demographic processes: birth rate and death rate. This study contributes to increase our understanding about the mechanisms by which pollutants effects are transmitted, shaping biodiversity of natural communities.

2. Material and methods

2.1 Test species

2.1.1 Herbivores

The herbivore individuals utilized on this study belong to species *Daphnia ambigua* (Scourfield, 1947) and *Ceriodaphnia dubia* (Richard, 1894). They were collected from temperate lakes of central Chile. Clones were established from a single parthenogenetic female and maintained under standard laboratory conditions (pH 7.5 ± 0.1, temperature 20 ± 1 °C, photoperiod 14:10, L:D). Prior to each experimental test, individuals were fed with a mixture of *Pseudokirchneriella subcapitata* (Hindak, 1990) and *Chlorella vulgaris* (Beijerinck, 1890) in a 1:1 ratio, reaching a concentration of 10^6 cells mL^{-1}.

2.1.2 Microalgae

Strains of algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* were obtained from cultures maintained at the Biodiversity Laboratory of CENMA, Santiago, Chile. Prior to each test, cells from each algal species were cultured on EPA medium (USEPA, 1994) under standard conditions (pH 7.5 ± 0.1, temperature 23° ± 1 C, 2000 lux) following NCh 2706.

2.2 Single-species tests

With the aim of assessing the tolerance to copper exposure of the test species, we performed single-species bioassays. The information gathered allowed us to define the copper concentrations that exert small lethal effects ($<LC_{50}$) to perform the multispecies trials. For conducting single-species bioassays we used a moderately hard water medium (ASTM, 1980), at a pH of 7.5 ± 0.1 and 23.5° ± 1 C. In order to mimic natural conditions, nutrients and organic matter were added to the medium. Nutrients were added by mixing 1 volume of MBL medium (Stemberger, 1981) with 9 volumes of moderately hard water medium. Organic matter was supplied by adding 2.5 mL L^{-1} of nutritional supplement (Hayashi et al., 2008), composed of algal extract (Phyllum by ANASAC, Lampa, Chile). For estimating species sensitivities to copper, we used standard bioassay procedures for branchiopods (NCh 2083) and microalgae (NCh 2706). Branchiopod bioassays were performed at the following nominal copper concentrations: 0, 30, 60 and 120 μg Cu L^{-1}. On the other hand, microalgae trials were made with 0, 25, 50, 75, 100 and 200 μg Cu L^{-1}. The different copper concentrations were prepared by diluting a stock solution of $CuSO_4 \cdot 5H_2O$ (78.6 mg L^{-1}).

2.3 Multispecies tests

Multispecies tests were performed using the same medium and environmental conditions than the one used for single-species tests. The lighting conditions were a photoperiod of 14:10 (L:D) and a light intensity of 2000 lux (NCh 2706). Once the sensitivity of each single species to copper was assessed, we defined the pollutant concentration to be used in the multispecies tests, which were 0, 25, 50 and 75 μg Cu L^{-1}.

Effects of copper over population growth rate of the test species were estimated from the outcome of three multispecies bioassays composed of: (A) *P. subcapitata* + *C. vulgaris* + *D. ambigua* + copper, (B) *P. subcapitata* + *C. vulgaris* + *C. dubia* + copper, and (C) *P. subcapitata* + *C. vulgaris* + *D. ambigua* + *C. dubia* + copper. Experiments A and B were made in 500 mL jars with 300 mL of medium. Experimental trials were initiated with a density of 5 10^5 cells mL^{-1} of each algal species and, five mature branchiopod females (the species varied in each bioassay, see above). After initiated the experiment, the medium was not renewed but the pH was adjusted to 7.5 ± 0.1 every 24 hours. At all times the medium was gently homogenized in an orbital shaker for ensuring algae resuspension. Each 24 hours, a sample of 300 μL of was obtained from each jar for later counting of organisms. Population density of algae was determined on a hemocytometer. For branchiopods, censuses of eggs, juveniles and adults were made every 24 hours using a stereomicroscope. This procedure was applied to each replicate (16 for each bioassay). Experimental trials were ended at 144 hours for bioassay A and at 96 hours for bioassay B. The same experimental design was used for bioassay C but using 1000 mL jars with 600 mL of medium. This bioassay was stopped at 144 hours.

2.4 Assessing effects of copper on herbivore populations

2.4.1 Population growth rate

Population growth rate (*r*) of branchiopods was estimated by fitting temporal population trajectories of each replicate to an exponential curve. The strength of the indirect effect of copper on r of each species was determined assuming that net effects can be decomposed into the sum of direct and indirect effects (Damiani, 2005). Thus, net effects enclose all effects driven through direct and indirect ways. Then, indirect effects (IE_r) of the pollutant on r of each species were calculated as:

$$\mathrm{IE}_r = \mathrm{NE}_r - \mathrm{DE}_r \tag{1}$$

Net effects (NE_r) were obtained by contrasting population growth rate of each species with and without copper, when all other components of the assemblage were present. Thus,

$$\mathrm{NE}_r = r_{\mathrm{N,P}} - r_{\mathrm{N,C}} \tag{2}$$

where $r_{\mathrm{N,P}}$ and $r_{\mathrm{N,C}}$ are the population growth rates of a given herbivore species in presence (P: polluted) and absence (C: control) of the pollutant respectively, and sub index N stands for "net effect". On the other hand, direct effects (DE) were obtained by contrasting population growth rate of branchiopod species with and without copper, in absence of inter-specific competitors. Thus,

$$\mathrm{DE}_r = r_{\mathrm{D,P}} - r_{\mathrm{D,C}} \tag{3}$$

where $r_{\mathrm{D,P}}$ and $r_{\mathrm{D,C}}$ are population growth rates of a given branchiopod species, in presence and absence of the pollutant respectively, and sub index D stands for "direct effect".

2.4.2 Birth and death rates

With the aim of decompose mechanistically the effects of copper on branchiopods' growth rate we calculated the effects of copper on birth and death rates of herbivores. To accomplish this, we calculated birth rate at each time instant *t* by means of the modified egg ratio method (Paloheimo, 1974):

$$b_t = \frac{\ln\left(\frac{E_t}{N_t} + 1\right)}{D} \tag{4}$$

where E_t is the total number of eggs at time *t*, N_t is the population size at time *t* , and *D* is the egg development time. Daily censuses were carried out to estimate E_t and N_t, while *D* was set to 2 days, according to the temperature of the experiments (Lei and Armitag, 1980; Anderson and Benke, 1994). For obtaining a single birth rate value *b* for each replicate, we calculated the mean of b_t. Then, we calculated death rate (*d*) through

$$d = b - r \tag{5}$$

where *b* and *r* are estimates of birth rate and population growth rate of each replicate, respectively.

For the calculation of net, direct and indirect effects of copper over birth and death rates we used the same procedures done for calculating effects over the population growth rate (see equations 1 to 3), but replacing *r* by *b* or *d* as a response variable.

2.5 Assessing effects of copper on algal populations

2.5.1 *Effects on population growth rate*

Population growth rate (r) of microalgae was estimated by fitting temporal population trajectories to an exponential curve. The strength of indirect effects of copper over r of *P. subcapitata* and *C. vulgaris* were determined in a similar way than for branchiopods (equations 1 to 3). The only difference is that in this case we considered the data of isolated microalgae bioassays for estimating direct effects.

2.5.2 *Contribution of different paths on the strength of indirect effects of copper on algae*

With the purpose of assessing which of the potential paths were most relevant for the transmission of indirect effects on algae, we decomposed indirect effects on algae (IE_r) into paths passing through each of the accompanying species:

$$\mathrm{IE}_r = \mathrm{IE}_{r,\mathrm{DA}} + \mathrm{IE}_{r,\mathrm{CD}} + \mathrm{IE}_{r,\mathrm{MA}} \tag{6}$$

where $\mathrm{IE}_{r,\mathrm{DA}}$ and $\mathrm{IE}_{r,\mathrm{CD}}$ are the indirect effects of copper on the growth rate of algae, passing through *D. ambigua*, and *C. dubia* respectively. $\mathrm{IE}_{r,\mathrm{MA}}$ is the indirect effect of copper on the growth rate of one species of algae, passing through the other microalgal species. For the assessment of these parameters we used the results of bioassays where one of the herbivores was absent. In bioassay A, the herbivore *C. dubia* was absent, while in bioassay B, *D. ambigua* was absent. Thus we defined a net effect of copper in absence of *C. dubia* ($\mathrm{NE}_{r,\mathrm{A}}$) and a net effect of copper in absence of *D. ambigua* ($\mathrm{NE}_{r,\mathrm{B}}$). These partial net effects on each microalgae species were calculated through the following equations:

$$\mathrm{NE}_{r,\mathrm{A}} = r_{\mathrm{N,P,A}} - r_{\mathrm{N,C,A}} \tag{7}$$

$$\mathrm{NE}_{r,\mathrm{B}} = r_{\mathrm{N,P,B}} - r_{\mathrm{N,C,B}} \tag{8}$$

where $r_{\mathrm{N,P,A}}$ and $r_{\mathrm{N,P,B}}$ are population growth rates of a species of microalgae in presence of copper and in absence of *C. dubia* and *D. ambigua* respectively. On the other hand, $r_{\mathrm{N,C,A}}$ and $r_{\mathrm{N,C,B}}$ are population growth rates of a species of microalgae in absence of copper and one of the herbivores. Partial indirect effects ($\mathrm{IE}_{r,\mathrm{A}}$ and $\mathrm{IE}_{r,\mathrm{B}}$) generated in absence of each herbivore were obtained through

$$\mathrm{IE}_{r,\mathrm{A}} = \mathrm{NE}_{r,\mathrm{A}} - \mathrm{DE}_{r} \tag{9}$$

$$\mathrm{IE}_{r,\mathrm{B}} = \mathrm{NE}_{r,\mathrm{B}} - \mathrm{DE}_{r} \tag{10}$$

where DE_{r} is the direct effect of copper on population growth rate of a microalgal species. Finally, we estimated indirect effects transmitted to each microalgal species through each of the accompanying species. This was done by subtracting the partial indirect effects ($\mathrm{EI}_{r,\mathrm{A}}$ and $\mathrm{EI}_{r,\mathrm{B}}$) from total indirect effect (EI_{r}):

$$\mathrm{IE}_{r,\mathrm{CD}} = \mathrm{IE}_{r} - \mathrm{IE}_{r,\mathrm{A}} \tag{11}$$

$$\mathrm{IE}_{r,\mathrm{DA}} = \mathrm{IE}_{r} - \mathrm{IE}_{r,\mathrm{B}} \tag{12}$$

$$\mathrm{IE}_{r,\mathrm{MA}} = \mathrm{IE}_{r} - \mathrm{IE}_{r,\mathrm{CD}} - \mathrm{IE}_{r,\mathrm{DA}} \tag{13}$$

2.6 Statistics

The calculation of the value of the indirect effect on population growth rate (IE_r) and its respective error (δQ) was made combining equations 1 to 3 through the following equation:

$$\mathrm{IE}_{r} \pm \left(\delta Q\right) = \bar{r}_{\mathrm{N,P}} \pm \left(\delta q_{\mathrm{N,P}}\right) - \bar{r}_{\mathrm{N,C}} \pm \left(\delta q_{\mathrm{N,C}}\right) - \bar{r}_{\mathrm{D,P}} \pm \left(\delta q_{\mathrm{D,P}}\right) + \bar{r}_{\mathrm{D,C}} \pm \left(\delta q_{\mathrm{D,C}}\right) \tag{14}$$

where $\bar{r}_{i,j}$ are the mean population growth rates estimated from replicates (N=4) for each particular treatment and $\delta q_{i,j}$ are their respective error. The value of the indirect effect error (δQ) was calculated by "error propagation" following the relation:

$$\delta Q = \sqrt{\left(\delta q_{\mathrm{N,P}}\right)^2 + \left(\delta q_{\mathrm{N,C}}\right)^2 + \left(\delta q_{\mathrm{D,P}}\right)^2 + \left(\delta q_{\mathrm{D,C}}\right)^2} \tag{15}$$

Each error value ($\delta q_{i,j}$) was obtained from its respective 95% confidence intervals. On the other hand, confidence intervals were estimated by bootstrap, using the percentile method with a bootstrap sample size of 1000. Similar procedures were realized for the calculation of all net, direct and indirect effects and their respective uncertainties. All calculations were made on MATLAB (The Mathworks, Inc.).

3. Results

3.1 Single-species bioassays

The assessed 48-hour LC_{50} value for *C. dubia* and *D. ambigua* were 61.24 µg Cu L^{-1} and 114.38 µg Cu L^{-1} respectively. Likewise, we found a 72-hour EC_{50} value of 97.37 µg Cu L^{-1} for *P. subcapitata* and 177.83 µg Cu L^{-1} for *C. vulgaris*.

3.2 Multispecies tests: effects of copper on herbivores

3.2.1 Population growth rate

There were not significant net effects of copper on population growth rate of *D. ambigua*, over the tested gradient of copper concentrations (Fig. 2A left). Nevertheless, at the highest copper level of 75 µg Cu L^{-1} it was found a negative direct effect and a positive indirect effect, which cancelled out generating a null net effect on this species (Fig. 2B-C left). On the other hand, it was observed that copper exerted a weak but significant negative net effect on *C. dubia* growth rate at low concentrations (Fig. 2A right). Direct effects on this species were negative at all copper levels, being most pronounced at a concentration of 75 µg Cu L^{-1} (Fig. 2B right). Conversely, a significant positive indirect effect of copper on population growth rate of *C. dubia* was found at a concentration of 75 µg Cu L^{-1} (Fig. 2C right).

3.2.2 Birth rate

For *D. ambigua*, it was found a significant (positive) net effect of copper on birth rate (*b*) only at the highest (75 μg Cu L^{-1}) tested concentration (Fig. 3A left). In contrast, the direct effects on *b* were significantly negative under the low and the high copper concentrations (Fig. 3B left). On the other hand, indirect effects were significantly positive both at 25 and 75 μg Cu L^{-1} (Fig. 3C left). In the case of *C. dubia*, negative net effects were found at all tested concentration although stronger at medium and high concentrations (Fig. 3A right). Direct effects were significant only at high concentration on this species, while indirect effects were negative at lower and middle copper concentrations (Fig. B-C right).

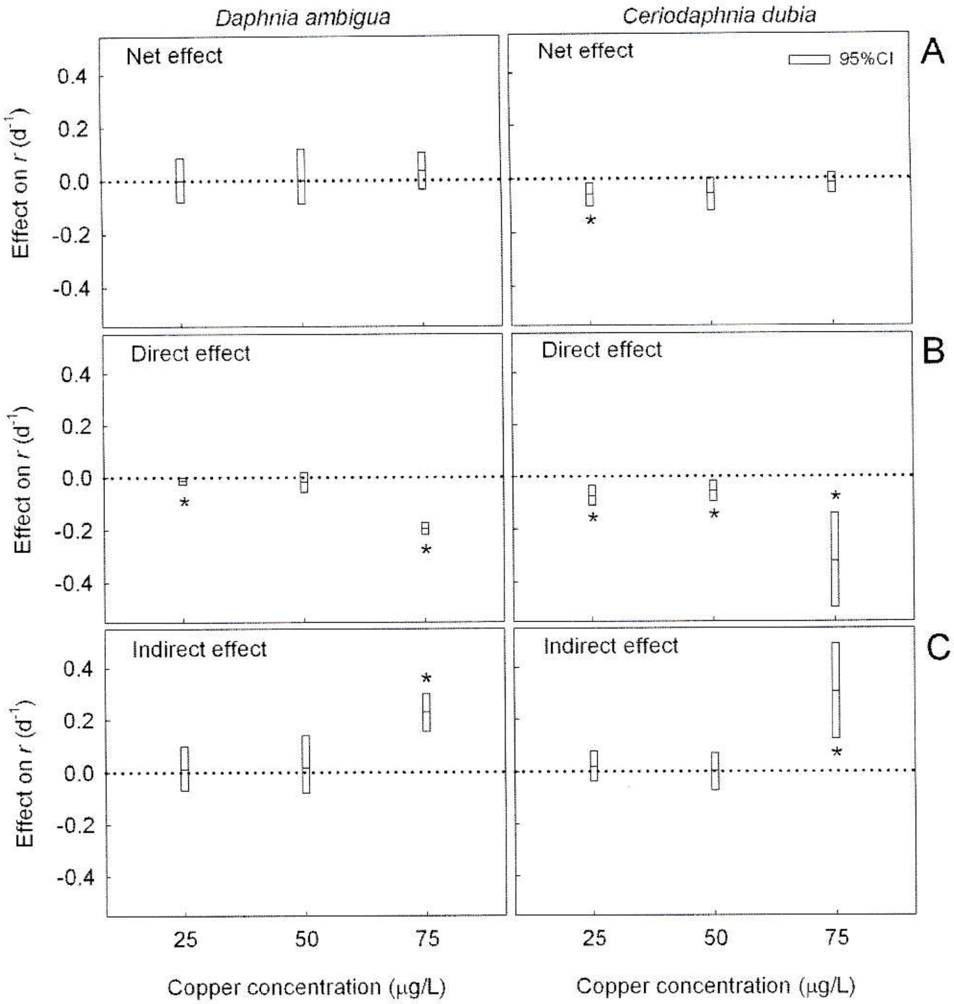

Figure 2. Net, direct and indirect effects of copper on population growth rate (*r*) of *Daphnia ambigua* (left) and *Ceriodaphnia dubia* (right). Each bar shows the mean and bootstrapped 95% confidence limits. Effects significantly different from zero are indicated by an asterisk.

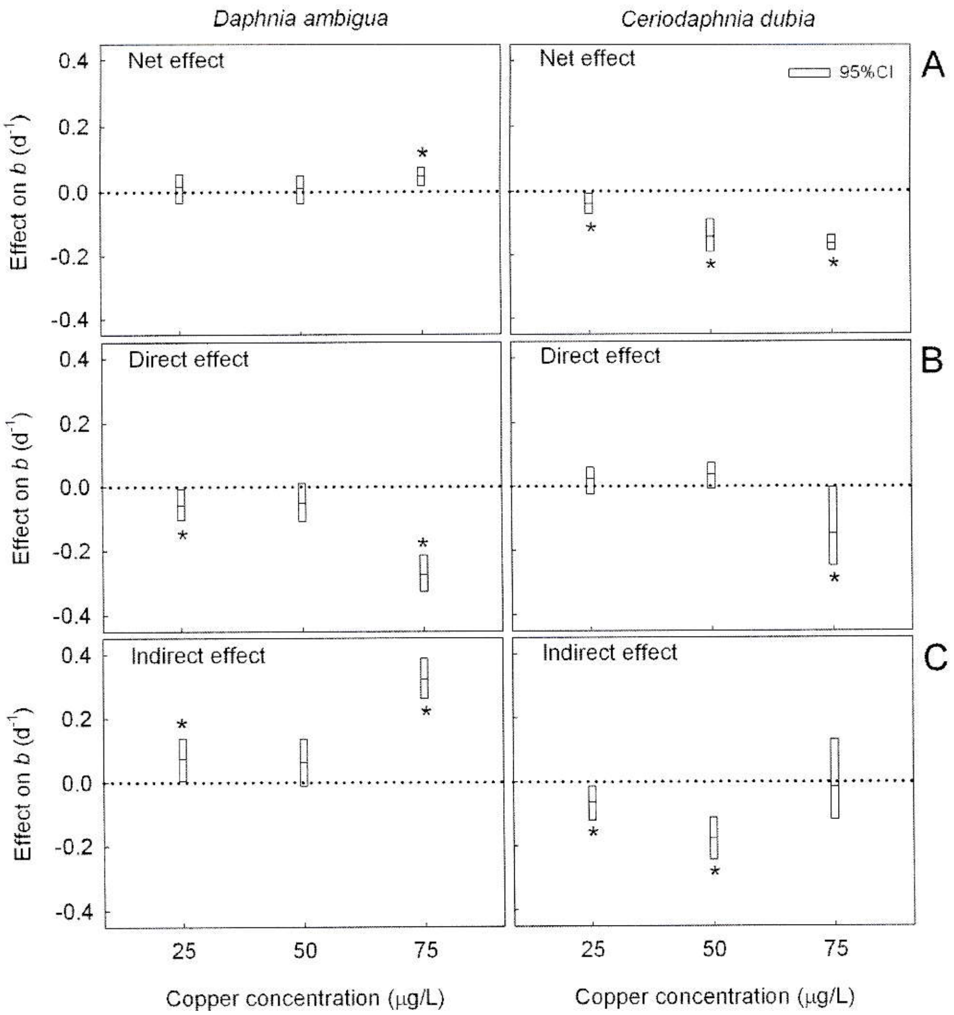

Figure 3. Net, direct and indirect effects of copper on birth rate (b) of *Daphnia ambigua* (left) and *Ceriodaphnia dubia* (right). Each bar shows the mean and bootstrapped 95% confidence limits. Effects significantly different from zero are indicated by an asterisk.

3.2.3 Death rate

We found negative direct effects together with positive indirect effects of copper on death rate (*d*) of *D. ambigua* at high concentration (75 μg Cu L^{-1}). Nevertheless, these effects cancelled out to render null net effects at all copper concentrations (Fig. 4 left). For *C. dubia,* direct effects on *d* were positive while indirect effects were negative, all increasingly stronger as copper concentrations were higher. This resulted in that net effects on *d* of *C. dubia* were null except at high concentration where the effect was negative (Fig. 4 right).

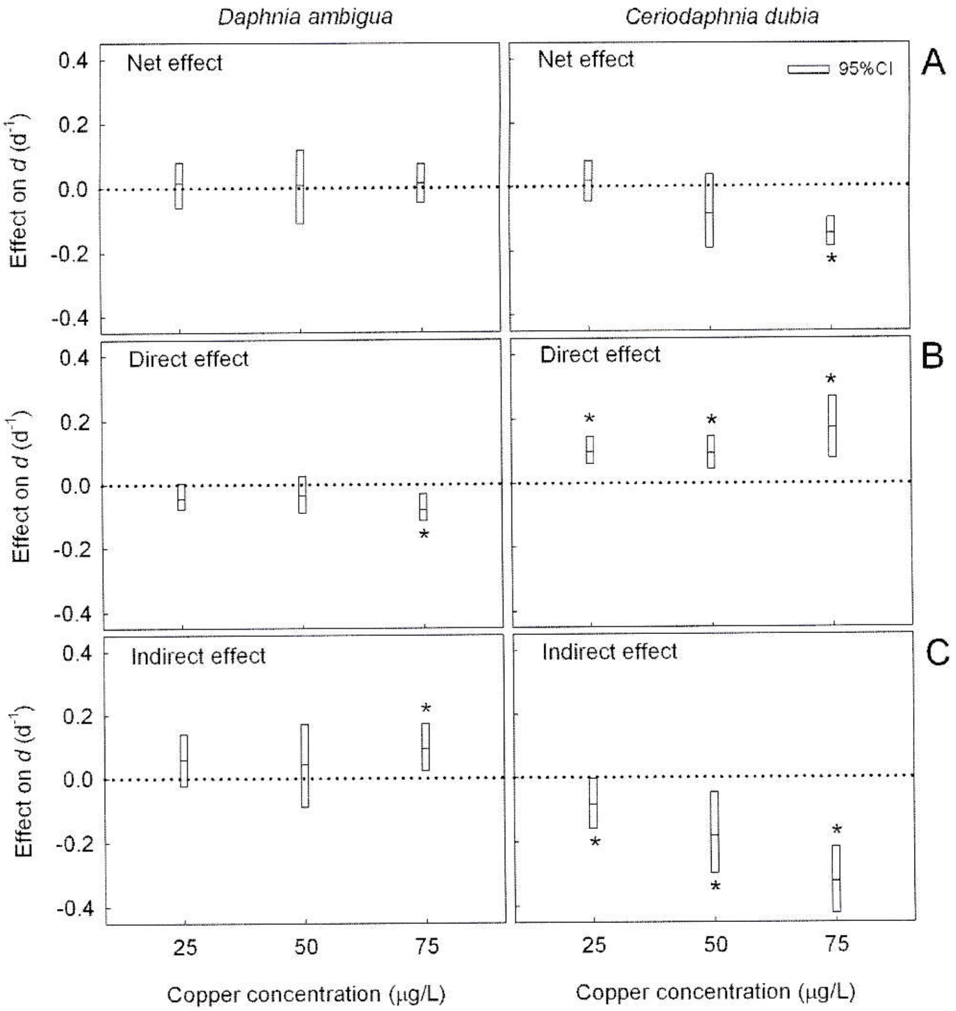

Figure 4. Net, direct and indirect effects of copper on death rate (*d*) of *Daphnia ambigua* (left) and *Ceriodaphnia dubia* (right). Each bar shows the mean and bootstrapped 95% confidence limits. Effects significantly different from zero are indicated by an asterisk.

3.3 Multispecies tests: effects of copper on microalgae

3.3.1 Population growth rate

Copper exerted a negative net effect on population growth rate of *P. subcapitata*, excepting at 75 μg Cu L^{-1} where the effect was null (Fig. 5A left). Direct effects were significant (positive) only at 75 μg Cu L^{-1}, and negative indirect effects were detected at all tested concentrations of copper (Fig 5B-C left). Net effects of copper on the population growth rate of *C. vulgaris* were significantly negative at 75 μg Cu L^{-1}, and also it was observed a negative direct effect at the lowest copper level (Fig. 5A-B right). Indirect effects on growth rate of *C. vulgaris* varied from positive to negative as copper concentration rose (Fig. 5C right).

3.3.2 Contribution of different paths on the strength of indirect effects of copper on microalgae

The effects of copper on growth rate of microalgae effectively were transmitted by the other interacting species (Fig. 6). Specifically, *D. ambigua* transmitted a significant negative effect on *P. subcapitata* exposed to the lowest copper concentration (Fig 6A left). On the other hand, we found that branchiopods mainly transmitted the effects of copper on *C. vulgaris*. In particular, negative effects transferred by *D. ambigua* were significant at the highest copper concentration (Fig. 6A right). Finally, at 25 and 50 μg Cu L^{-1} *C. dubia* transmitted a positive indirect effect of copper on *C. vulgaris* (Fig. 6B right).

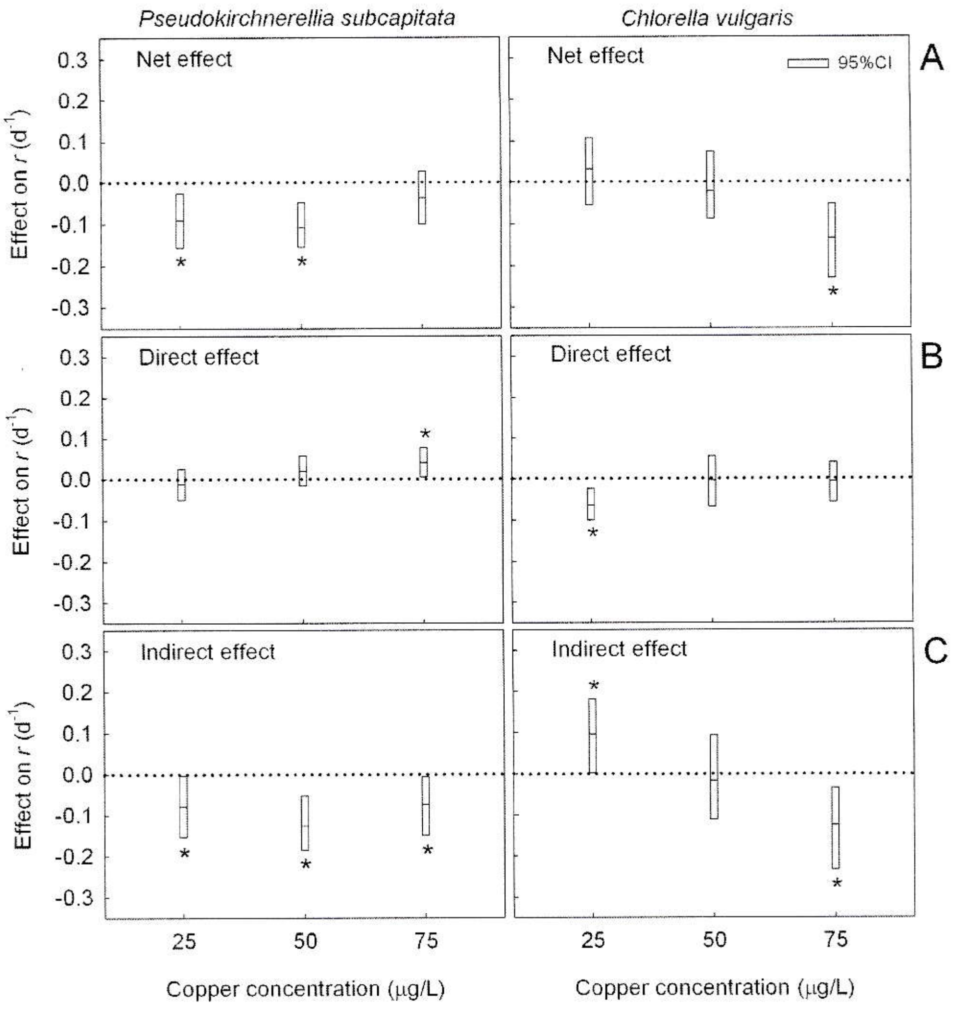

Figure 5. Net, direct and indirect effects of copper on population growth rate of *Pseudokirchneriella subcapitata* (left) and *Chlorella vulgaris* (right). Each bar shows the mean and bootstrapped 95% confidence limits. Effects significantly different from zero are indicated by an asterisk.

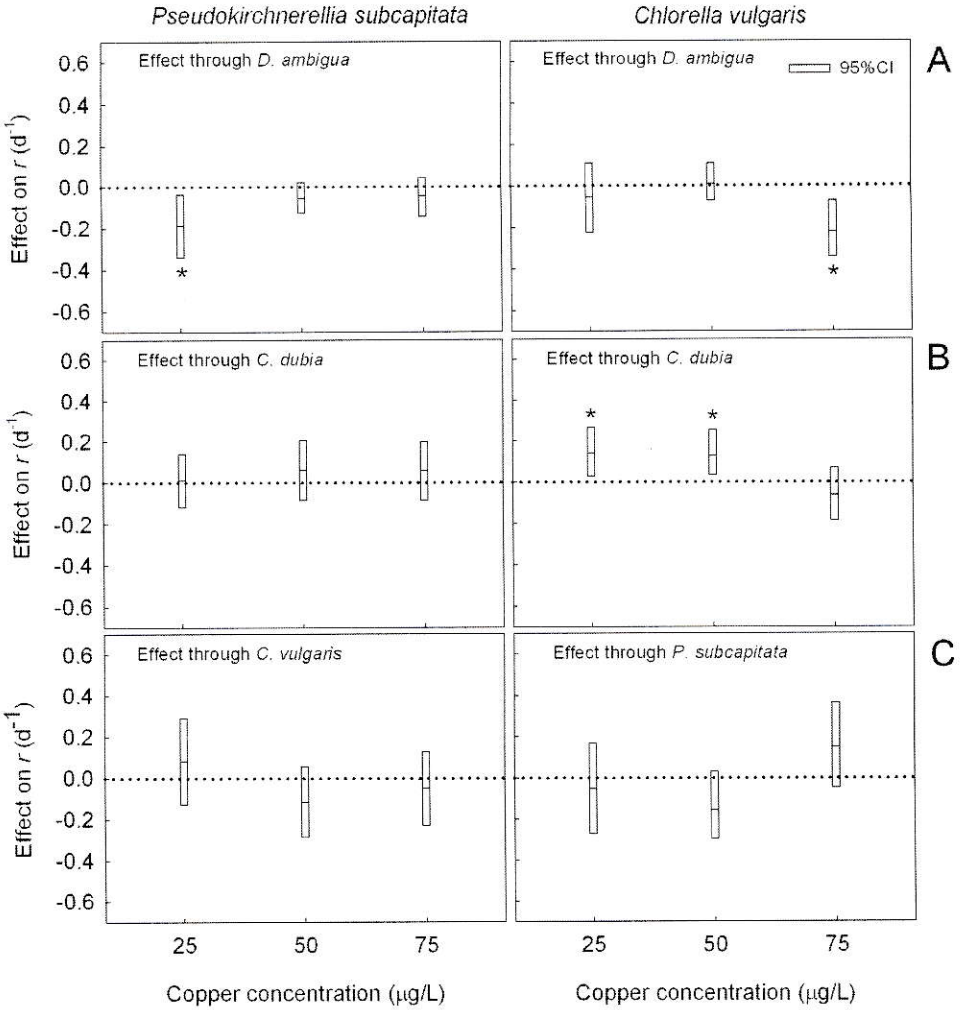

Figure 6. Indirect effects of copper on microalgae transmitted through species within the assemblage. Effects on *Pseudokirchneriella subcapitata* (left) and *Chlorella vulgaris* (right). Each bar shows the mean and bootstrapped 95% confidence limits. Effects significantly different from zero are indicated by an asterisk.

4. Discussion

4.1 Herbivores

Our results showed that copper caused effects over population growth rate, birth rate and death rate of branchiopods. Although net effects over population growth rate of both species appeared to be almost null, copper actually exerted a negative effect on *C. dubia* at lowest concentration. This small effect could be explained mainly by the direct effects of copper, that in the case of *C. dubia* were particularly more harmful than the direct effects expressed by *D. ambigua*. However, this explanation may not be applied for the highest concentration, where indirect effects counteracted the direct effects yielding a null net result.

Direct effects over each species were consistent with the responses obtained on acute bioassays, where *C. dubia* exhibited higher sensitivity to copper than *D. ambigua*. Previous studies evidencing differences on sensitivities between these species are not conclusive due the high variety of exposure concentrations and exposure times (USEPA, 2007). However, studies at the population level have found negative effects on *r* of *C. dubia* and *D. ambigua* at concentrations of 20 μg Cu L^{-1} and 60 μg Cu L^{-1}, respectively (Gama-Flores et al., 2007; Winner, 1976); values which are consistent with our results.

Population level effects are of course species-specific, and are consequence of a balance between birth and death rates. Thus, the mechanisms underlying the expression of both direct and indirect effects may illustrate the different ways by which each species faces pollution in a multispecies context. Our results showed that for *D.*

ambigua, negative direct effects were the result of decreasing birth rate, while for *C. dubia* these effects are explained by an increasing in death rate. On the other hand, positive indirect effects on these species are the product of the inverse phenomenon, suggesting that effects on *D. ambigua* were governed mainly by changes in birth (reproductive) rate and for *C. dubia* by changes in death rate. There are no clear evidences of direct effects of copper on birth and death rates of freshwater plankton, but information about the sublethal and lethal effects could elucidate the basis of these responses. Chronic exposure to copper on these branchiopods has shown that *D. ambigua* is slightly more sensitive than *C. dubia*, when the effects on their fertilities are compared (Harmon et al., 2003). Moreover, similar copper concentrations exerted negative effects on reproduction of *D. ambigua* and positive over-compensatory effects on *C. dubia* (Winner, 1976; Gama-Flores et al., 2007). On the other hand comparative studies on micronutrients like zinc and copper have revealed that *C. dubia* presents an equivalent or higher acute lethality relative to *D. ambigua* (Harmon et al., 2003; Shaw et al., 2006).

Conversely, indirect effects have not a simple explanation since they emerge from complex interactions between copper and the component species of the system. Nevertheless, our results could guide us to hypothesize some indirect ways of copper action. Positive indirect effects over birth rate of *D. ambigua* could be the result of the transformation of resources into newborn, supporting in this way an explanation based on a chain of effects transmitted through microalgae species. In an unpolluted community context, *D. ambigua* is outcompeted by *C. dubia* when they share *Chlorella* *vulgaris* as a food source (Martinez and Montecino, 2000). Over this scenario copper could act as a keystone species (Rohr et al., 2006), altering interaction between branchiopods, thus relaxing the competitive exclusion to finally favoring *D. ambigua*. Negative direct effects of pollutants on large zooplankton have generated similar positive indirect effects on insect larvae (Foekema et al., 1998), protozoan (Schauerte et al., 1982), some branchiopods (Barry and Logan, 1998) and rotifers (Papst and Boyer, 1980; Day et al., 1987; Brock et al., 1992; Hanazato and Kasai, 1995; Webber et al., 1992; Yasuno et al., 1993; van Donk et al., 1995; Jak et al., 1996; Jak et al., 1998; van den Brink et al., 2000; Sanderson et al., 2002). However, positive indirect effects on *C. dubia* could not be easily explained based on our findings since they probably may be a result of other kinds of interactions (for example, physical and/or chemical interactions) not experimentally assessed here.

Although all our explanations are based on projections from relatively short term assays and therefore dynamics eventually may drift to a reestablishment of normal interactions between species (LeBlanc, 1985), the positive effect of initial copper pulse on abundance could effectively affect negatively other zooplankton in nature if the zooplanktivorous abundance is increased.

4.2 Microalgae

The results revealed effects over population growth rate of *P. subcapitata* and *C. vulgaris*. Copper exerted a negative net effect over both species being more injurious for

P. subcapitata at low concentrations and for *C. vulgaris* at higher concentrations. These patterns were largely a consequence from indirect negative effects instead of direct effects since the latter showed to be almost null.

Direct effects over both species of microalgae were almost null, being *P. subcapitata* slightly favored over *C. vulgaris* under equal copper concentrations. The single species bioassays developed also demonstrated that both species had a relatively low sensitivity to copper (higher EC_{50} than branchiopods) at least under the particular experimental conditions imposed.

Copper indirect effects were the most important sort of perturbation over microalgae population growth within our experimental community. The nature of these effects could be explained by variations in grazing pressure of herbivores on microalgae. Therefore, negative indirect effects on *P. subcapitata* would be a result of an increase on foraging performed by branchiopods due to copper exposure. The subsequent analysis of indirect effects indicated that *D. ambigua*, rather than *C. dubia*, was the main responsible of these changes at least at low copper concentrations. On the other hand, decomposition of indirect effects on *C. vulgaris* indicated that they followed different routes depending on the copper concentration. At low levels of copper, positive indirect effects are transmitted through *C. dubia*, which would be negatively affected by the metal thus reducing its grazing pressure on its resource. Conversely, negative indirect effect generated by high metal concentrations were transmitted by *D. ambigua*, which would have taken advantage of the resource released by the copper-affected *C. dubia*, thus increasing its grazing on *C. vulgaris*.

Although our data are insufficient to estimate functional responses of each herbivore species under a copper-enriched environment, there are some previous evidences that could support our explanations. Martinez and Montecino (2000), working on a copper-free system, found that *C. dubia* outcompeted *D. ambigua* when *Oocystis* and *Chlorella* were the food resources. The smaller size of *C. dubia* would allow it to benefit preferably from *Chlorella vulgaris*, while the larger *D. ambigua* could profit from both microalgae species (Martinez, 2000; Brito et al., 2006). On the other hand, the comparable size ratio between microalgae (*Oocystis* / *Chlorella* ≈ 1.4 and *Pseudokirchneriella* / *Chlorella* ≈ 1.6) could lead to similar selectivities when they are grazed, thus making our system analogous to the previously reported.

4.3 Final remarks

In summary, copper exerted direct and indirect effects on the population growth rates of the herbivores and microalgae species of the assemblage. Direct effects over *C. dubia* were the initiators of a chain of effects within the system, presumably because population growth rate of this species was more affected by copper compared to *D. ambigua*, as previously found by Gama-Flores et al. (2007) and Winner (1976). However, indirect effects of copper played a primary role, affecting particularly the dynamics of microalgae and the interaction between the herbivores, which finally shaped the community outcome. The contribution of indirect effects to the net effects exerted by copper on herbivores was at least as important as the contribution of direct effects. Moreover, the magnitude of indirect effects of copper on microalgae far exceeded direct effects, thus demonstrating the main importance of indirect paths when pollutant effects are transmitted.

Interestingly, the results obtained from standard acute individual species bioassays performed on herbivores (see section 3.1) rendered a higher sensitivity to copper as compared to the sensitivity of the same species embedded in the experimental community. Conversely, microalgae species showed to be more tolerant to pollution when they were isolated than when they were immersed within the community. Current environmental policy and regulations used in Chile and many other countries rest on the use of single species bioassays as the primary tool for evaluating the effect of pollutants on ecological systems. Our study highlights that if we only consider the results of single species bioassays for assessing copper effects on biota, we would overestimate the effects on herbivores and underestimate the effects on microalgae. This illustrates the importance of understanding and considering indirect effects of pollutants for environmental protection actions.

Although the methodology utilized in this work allowed us to assess direct and indirect effects of pollutants within an experimental assemblage, we have to point out some limitations. The small number of component species selected for our trials could limits the extrapolations of our results to natural communities. Nevertheless, increasing the number of species imposes serious limits to experimental manipulations. On the other hand, the use of nominal copper concentrations unable us to know which were the actual amounts of bioavailable copper that exerted the observed effects, which difficult the quantitative comparison of our results with previous work. Despite of this, nominal concentrations are acceptable from a qualitative point of view, as used here.

Finally, this study provides an alternative way for assessing the magnitude and sign of the effects of stressors on the component species in an aquatic community. Once recognized the importance of indirect effects of the focal stressor, we identified the main paths by which indirect effects are transmitted, and decomposed population level effects into effects on the underlying demographic processes. This represents our main contribution towards understanding and predicting the functioning of natural communities subjected to current biodiversity threats.

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