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The effect of copper exposure on a simple aquatic food chain

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Abstract

The effect of copper (44 μg l^{-1}) on a simple food chain was studied using indoor experimental channels to identify the changes in periphyton community (metabolism, chlorophyll *a* content, abundance, composition and lipid and protein content) and in herbivore (*Stagnicola vulnerata*) growth rate and reproduction. The algal community was sensitive to copper at the beginning but differences between treatments were not significant during the experiment. However, copper affected growth rate, reproduction and embryo hatching on snails. These results indicate that the effects on snails are more sensitive endpoints in assessing sublethal copper toxicity than effects on periphyton.

Keywords: Copper; Grazing; Biofilm; Periphyton; Laboratory channels; *Stagnicola vulnerata*

1. Introduction

Copper is present in most European rivers and its widespread use has generated much research on its effect in aquatic systems. During 1997–1998, mean annual copper concentration in Catalan streams (NE Spain) ranged from 1.3 to 17.5 μg l^{-1} (Junta de Sanejament, 1998). Peak values (> 40 μg l^{-1}) were detected in highly polluted stream sites in agricultural and industrial catchments and in certain periods (mainly summer). Values of up to 100 μg l^{-1} have been occasionally detected in an extremely polluted stream in the same region (Armengol et al., 1993) before the implementation of the Catalan rivers Sanitation Plan (1992).

Several authors (Foster, 1982; Leland and Carter, 1984; Weber and McFarland, 1981) report changes in periphyton community composition after long-term exposure to copper. This effect was observed in a broad range of concentrations: from 5 μg l^{-1} (Leland, 1984) to 30 μg l^{-1} (Kaufman, 1982) and up to 120 μg l^{-1} (Weber and McFarland, 1981). Leland and Carter (1984) also reported inhibition of autotrophic periphyton production (between 57 and 87%) at 2.5 μg l^{-1} in an oligotrophic stream.

Biofilms are layers of polysaccharide-rich materials with active microbial components on the surface of inert materials and organic matter that modulate biological availability, hence the potential toxicity of heavy metals in the environment.

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The structure of biofilms consist of a package of cells in a dense mucous matrix which confers strong resistance to metal entrance (Starodub et al., 1987; Admiraal et al., 1999; Lehmann et al., 1999; Barranguet et al., 2000). Natural biofilms have a significant number of metal binding sites located either in the organic matrix, at the surface of the cells or in the particles trapped by the biofilm. With aging biofilm increases its density and complexity and offers more binding sites that immobilise copper and reduce metal diffusion and copper availability to the algae (Rose and Cushing, 1970; Ivorra et al., 2000). Grazers remove overlying and senescent cells from biofilms and maintain low levels of periphyton biomass (Steinman, 1996), and at the same time, their scraping mouth parts have strong impact on the cell membrane. Intensive grazing reduces the accumulation of algal extracellular products and may enhance the effect of a toxin, as was observed for a herbicide (Muñoz et al., 2001; Roses et al., 1999).

This study describes the effect of continuous addition of a concentration of copper (44 $\mu g\ l^{-1}$) to a simple aquatic food chain (periphyton + herbivores). We aimed to evaluate the changes in biofilm community metabolism, chlorophyll *a* content and periphyton composition and abundance after the introduction of a snail (*Stagnicola vulnerata* Küster) to experimental channels. Water and food chain transfer of metals may be the main routes of exposure for herbivores that are more sensitive than other functional feeding groups (Clements, 1991). The effects of copper on growth and reproduction in *S. vulnerata*, and the responses of the latter to these effects, are also discussed.

2. Materials and methods

2.1. Experimental design

Experiments were conducted in a set of indoors experimental channels during winter 1998. We used 14 identical U-shaped once-through perspex channels (170 cm long, 10 cm wide and 10 cm deep). The channels received a continuous flow of water from a well 50 m from the laboratory. Well water had high conductivity (1900 $\mu S\ cm^{-1}$, due to the high levels of sulphate and chloride) and pH ranged between 6.9 and 7.2 and alkalinity between 5.3 and 7.6 mEq l^{-1}. Nutrient concentration was relatively high for nitrate ($N–NO_3$: 14.5–16 mg l^{-1}) and low for phosphate ($P–PO_4$: 2.5–5 $\mu g\ l^{-1}$), while nitrite and ammonium were undetectable (< 0.01 mg l^{-1}). Copper content in the well water was 3.7 $\mu g\ l^{-1}$.

The physical and chemical characteristics of the water were maintained constant throughout the experiment. Water temperature and flow were monitored every 1–3 days. Water velocity in the channels was 1 cm s^{-1}, water depth was 2.5 cm and flow was 25.4 ± 0.2 S.D. $cm^3\ s^{-1}$. Light was provided by 500 W metal halide lamps mounted 1 m above the channels. Irradiance measured 16.5 cm above the water surface was 321 ± 45 S.D. $\mu E\ m^{-2}\ s^{-1}$. The photoperiod of the system was set at 9-h light: 15-h dark. Water temperature during the experiment ranged from a minimum of 18 °C in the dark period to a maximum of 23.5 °C in the light period.

2.1.1. Community collection and culture

In an unpolluted stream (Avencó, NE Spain, described in Guasch et al., 1997) periphyton communities were allowed to colonise the surface of sandblasted glass substrata for 16 days. Two sizes of artificial substrata were used: small glass squares (1.2×1.2 cm) and large glass plates (12×9 cm). The small glass substrata were glued to stones or placed in racks, and the large plates were placed vertically in open cages. Both types of substrata were left in shallow riffle areas with low canopy cover. After field colonisation the glass substrates were transported to the laboratory (within 1 h of sampling) in a tank filled with site water. The colonised substrata were placed in the artificial channels and allowed to develop in laboratory conditions for 27 days (the time required for algae to grow to guarantee enough biomass for grazing). Large glass plates were placed interspersed between several rows of small glass squares, the latter being used as sampling units for measuring chlorophyll *a* concentration and taxonomic composition.

S. vulnerata (Küster) snails were collected from the Francolí River (NE Spain). After collection, snails were maintained in several aquaria ($60 \times 32 \times 30$ cm) at room temperature of 18–20 °C and a photoperiod of 12-h light: 12-h dark. In each aquarium, a maximum of 250 snails were kept in shallow water (12 cm depth) with aeration using the same water as in the experiment. Their diet consisted of raw lettuce and algal periphyton on stones. The copper content of the lettuce was 6.7 ± 0.9 μg Cu g^{-1} DW.

On the basis of previous results (Muñoz et al., 2000) to maintain enough periphyton biomass and primary production available to herbivores ten animals were placed in each channel (tissue dry weight: 0.96 g m^{-2}), which accounted for 75% of the field density observed at the collection site. We used snails of 1.1–1.4 cm shell length.

2.1.2. Treatments

The following four treatments (with three channels as replicates) were assigned at random to the 12 channels used in the experiments: (i) control; (ii) snails; (iii) copper; (iv) snails + copper. The procedure in the experiment was firstly exposure of algae to herbivory for 9 days (for those channels in treatments ii and iv); and secondly, exposure of the community to a copper concentration of 44 μg l^{-1} for the following 14 days (for those channels in treatments iii and iv).

Copper solution was prepared from concentrated copper chloride (Merck©). This concentration was close to some peak values observed in polluted Catalan streams. Constant copper concentration was injected at the head of the channels by a peristaltic pump. Water samples were collected for copper analysis. These were fixed with 1% nitric acid and kept frozen until analysis. Copper was analysed using Inductivity Coupling Plasma (Optic Emission Spectroscopy).

To detect the effect of copper, snails and the interaction snails and copper, we monitored the following parameters: (a) in the algae: chlorophyll *a* concentration (on days 0, 1, 3, 7 and 14), composition and abundance of algal taxa (on days 0, 7 and 14); (b) in community metabolism: net oxygen production (on days 0, 1, 3, 5, 7, 10 and 14); and (c) in the herbivores: biomass changes, reproduction (number of egg masses and number of eggs per mass) and egg hatching at the end of the experiment.

2.2. Assessment of biological effects

Samples (five glass units) for measurements of chlorophyll content were frozen. Algal pigments were extracted in 90% acetone after sonication for 4 min.

Glass substrata for analysis of species composition were preserved in 4% formaldehyde. Colonised substrata were cleaned in a sonication bath as described in Guasch and Sabater (1998). The sonicated material was observed under a light microscope for the determination and quantification of non-diatom cells.

Net community metabolism (NCM) was measured in the channels using a modification (Guasch et al., 1995) of the open oxygen method (Odum, 1956). Metabolism was calculated as the difference between oxygen concentration at the outflows of the colonised channels and the channel covered with uncolonised glass substrata. In the colonised channels, changes in dissolved oxygen included changes resulting from gross primary production, respiration of bacteria, algae and invertebrates; and diffusion to or from the atmosphere. Since changes due to diffusion were controlled by the channel with uncolonised substrata, the resulting value estimates the NCM in the entire channel (1700 cm^2). This method is not destructive and as such allows repeated measurements.

2.2.1. Grazer analysis

Snails mortality was monitored daily. Dead snails were removed and replaced by snails of the same shell length from a bigger channel next to the experimental channels, which had been maintained under the same water, light and temperature conditions. A net was placed at the end of each channel to restrain the snails. Everyday individuals found in the net were counted and returned to the beginning of the channel. Weight gain was the difference in dry mass (70 °C to constant weight) at the beginning and at the end (all the snails in each channel) of the experiment (day 13). Initial snail biomass was calculated from the dry mass of

three subsamples of ten individuals each of the same size as those used in the experiment. Collected egg masses were examined under a stereomicroscope to count the number of eggs.

To detect effect of copper on the egg hatching, we first removed all the egg masses laid during the 9 days of pre-exposure to herbivores. After 14 days of copper exposure, we collected all the newly laid egg masses for that period and placed them in nurseries in two channels (three nurseries per channel) in the same conditions of flow, light and copper concentration for 23 more days. Every 2 days newly hatched juveniles were counted and removed. Hatching success was expressed as a cumulative percentage of newly hatched juveniles in relation to the total number of eggs counted from the egg masses laid during the experiment. At the end of the hatching experiment, all egg masses were observed under a stereoscopic microscope to determine whether the eggs or embryos were still alive. Well-preserved yolk eggs and developing embryos were considered alive.

2.2.2. Lipid and protein content

Percentage lipid and protein content was determined in biofilm and snails for each treatment at the end of the experiment. Samples were extracted for lipids using a hexane/isopropanol (2:1) procedure (method modified from Folch et al., 1957). Protein was extracted using 0.1 N NaOH and analysed by the protein–dye binding colorimetric method (Bradford, 1976).

2.3. Statistical analyses

Significant differences in protein and lipid content between treatments were determined using a one-way (for snails) or two-way (for algae) analysis of variance (ANOVA). Differences for chlorophyll *a* content and community metabolism were assessed throughout the experiment using a three way ANOVA on repeated measures (Winer, 1971). Between factor ANOVA of repeated-measures accounts for the temporal dependence of the observations that characterise a temporal sequence. The interaction (time × treatment) showed the differences over time caused by copper addition. Multiple comparisons between means were analysed in all the cases with a Tukey Honest significant difference (HSD) test (Winer, 1971). When necessary data were transformed to meet ANOVA assumptions of homogeneity of variances and normality of errors. Therefore, data referring to algae were log-transformed. As for grazer data, logarithmic transformation was applied to counts and arcsine transformation was applied to percentage data. Weight increase, number of eggs per egg mass, number of escaped individuals and cumulative hatching did not need transformation.

3. Results

Copper concentration recorded during random measurements carried out in the Cu exposed channels during the experiment was 44 $\mu g\ l^{-1}$ (± 1.4 S.E., 11 days sampled). According to pH and alkalinity values, only the 2.17% of total Cu content was in Cu^{2+} form, that is: 0.95 $\mu g\ l^{-1}$ (Stumm and Morgan, 1981). The mean value of routine measurements of copper in control channels (both control and snail treatments) was 3.7 $\mu g\ l^{-1}$ (± 1.2 S.E., 7 days sampled), which is the background copper concentration of the well water.

Chlorophyll *a* content (Fig. 1) increased significantly over time regardless of treatment (ANOVA, $P \leq 0.0002$). For instance, channels with grazers presented higher chlorophyll values on day 7 than those measured at the beginning of the experiment (Tukey test, $P < 0.05$). Grazers significantly decreased chlorophyll content in the channels (ANOVA, $P < 0.05$). Channels exposed to copper had a lower mean chlorophyll content than un-exposed channels but this difference was not significant.

Total algal density increased during the first 7 days of the experiment (Fig. 2) and decreased slightly on day 14 in all treatments, more in copper treatment (from 1100×10^6 to 600×10^6 cells m^{-2}). There were no significant differences between treatments in total algal abundance during the experiment. Herbivores controlled growth of the filamentous form with respect to control conditions and favoured the crustose and prostrate

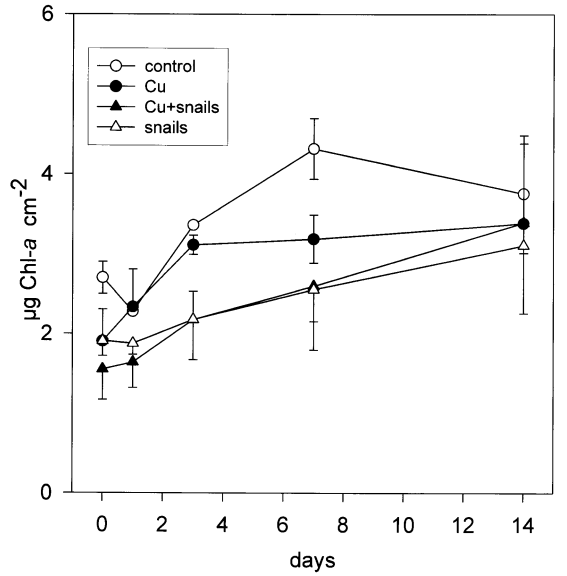

Fig. 1. Chlorophyll *a* concentration for each treatment over time. Data points are means from three channels of each treatment. Bars indicate standard error of mean.

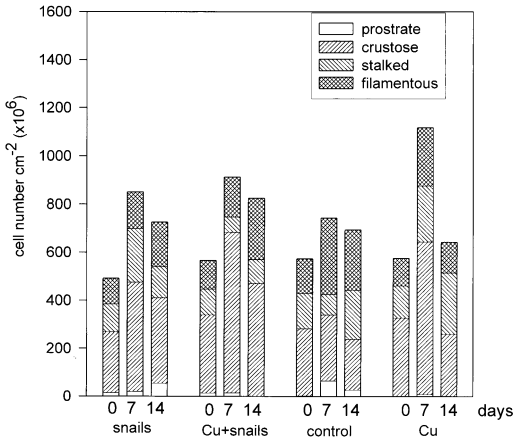

Fig. 2. Abundance of algal physiognomic classes for each treatment on three sampling dates (0, 7, 14 days). Data are means from three channels of each treatment.

growth forms. In the copper treatment, density of stalked growth form increased over time. Crustose and filamentous forms increased on day 7 but decreased at the end of the experiment. Prostrate density was low in this treatment. Treatment with copper and grazers did not show significantly different effects on algae growth forms with respect to the two factors separately.

There were significant differences over time in the NCM (ANOVA, $P < 0.0001$). There was a decrease in NCM until day 3 (Fig. 3), and a recovery and a clear increase afterwards. At the end of the experiment (days 10 and 12) NCM values in all treatments were significantly higher than at the beginning of the experiment (days 0–4) (Tukey test, $P < 0.05$). Grazers significantly reduced NCM (ANOVA, $P < 0.01$) mainly at the beginning of the experiment. Channels subjected to grazing presented significantly lower NCM values than the control (Tukey test, $P \leq 0.05$, on day 3) or the copper treatment (Tukey test, $P < 0.05$, on day 1). Channels exposed to copper had a mean lower NCM than un-exposed channels but this difference was not significant.

Over the experimental period, the grazer mortality remained at approximately 5%. Copper did not cause a significant effect on the number of escaping individuals: 2.8 ± 0.5 individuals escaped per channel during the pre-experimental period, but this number decreased after the addition of copper (1.5 ± 0.2 individuals).

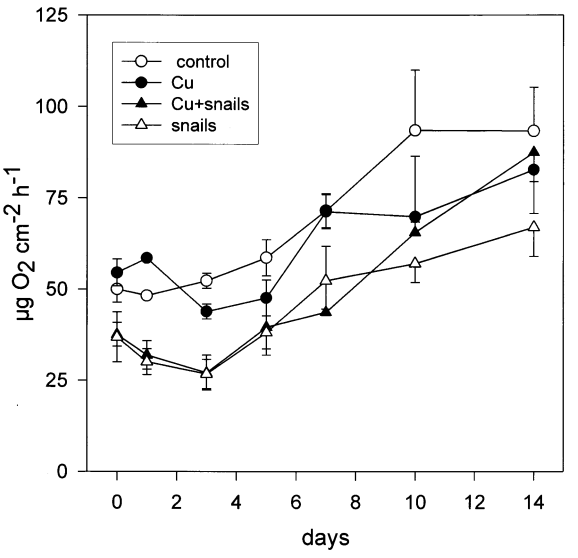

Fig. 3. NCM measured as oxygen production for each treatment over time. Data points are means from three channels of each treatment. Bars indicate standard error of mean.

Copper affected snail biomass and reproduction. Snails exposed to copper gained significantly less weight (Table 1) than control. The number of egg masses was significantly lower for those molluscs exposed to copper. The mean number of eggs per mass was also lower for those masses exposed to copper (Table 1). Copper also had a significant effect on hatching ($P < 0.01$). From day 21 on, cumulative hatching of the exposed eggs was significantly lower. After 36 days, only 42% of the eggs exposed to copper had hatched whereas 89% of the control eggs hatched. Thereafter, all the non-hatched egg masses were examined under the stereoscope microscope. Surprisingly, of the non-hatched eggs most were alive, i.e. growing into juveniles or still well preserved.

Snails from channels with copper had higher protein content (Fig. 4) than snails from channels without ($P < 0.05$). In contrast, copper did not affect their lipid content.

The effect of grazing significantly reduced ($P < 0.0001$) lipid content in algae, whereas copper increased it ($P < 0.0001$). Periphyton from channels with copper and snails showed intermediate lipid content between the two separate treatments. Copper ($P < 0.0001$) or snails ($P < 0.0001$) reduced protein content in algae with respect to control treatment. Interaction of toxicant and snails did not produce a significant difference with respect to the two factors separately.

4. Discussion

In this study, grazers gained less weight and laid fewer egg masses in the presence of copper. Less weight could mean a lower investment in reproduction and a physiological response to stress. Nevertheless, there was no immediate behavioural response to copper: the snails did not escape the copper treatment. The effects of diet and heavy metals on growth rate and fertility in snails are observed at relatively low concentrations. Arthur and Leonard (1970) found that the growth of *Physa integra* stopped at 28 μg Cu l^{-1}, while Dorgelo et al. (1995) reported that inhibition of growth rate in *Potamopyrgus jenkinsi* occurred at a lower Cu concentration (10 μg l^{-1}) than the inhibition of reproduction (30 μg l^{-1}).

Furthermore, we observed that embryo hatching success was reduced by copper. Cheung and Lam (1998) also reported that an increase of cadmium at sublethal levels reduced embryo growth rate and hatching from *Physa acuta*. However, in our experiment, after 36 days the non-hatched eggs that had been exposed to copper were mostly alive, that is, the yolk was not degraded. The same authors considered eggs that did not hatch after 28 days to be unhatchable. Nevertheless, these results indicate that reduction in growth rate, egg mass production and hatching success in *S. vulnerata* are sensitive markers of copper toxicity at sublethal concentration.

Grazers accounted for the differences between treatments since they significantly reduced chlorophyll and NCM in comparison to non-grazed channels. However, grazing and copper addition did not have a greater effects than the toxicant alone, in contrast with the results reported by Muñoz et al. (2001) following herbicide exposure. A consequence of biofilm biomass reduction by grazing pressure was lower lipid accumulation in biofilm (this experiment, Steinman, 1996). Lipids are critical factors in the transport and fate of many persistent lipophilic contaminants in the aquatic environment and biota (Napolitano and Richmond, 1995). In the present study the lipid content of biofilm exposed to copper increased over the 13 days. However, with the interaction of the two factors (copper and grazing) biofilm accumulated lipids in response to the toxicant, but not in the same proportion as in the absence of snails. Copper had no effect on the lipid content of the snails, perhaps because the experiment was too short to observe the lipid accumulation in a higher

Table 1
Changes in snails: dry weight increase, number of egg masses and number of eggs in each mass at the end of the experiment for each treatment

Treatment	mg DW ind^{-1}	Egg masses	Eggs $mass^{-1}$
Control	7.5 (0.9)	18.3 (6.1)	24.3 (1.0)
Copper	2.7 (1.0)	9.0 (2.6)	18.9 (1.4)
	$P < 0.05$	$P < 0.05$	$P < 0.01$

Values in parenthesis are standard error of mean.

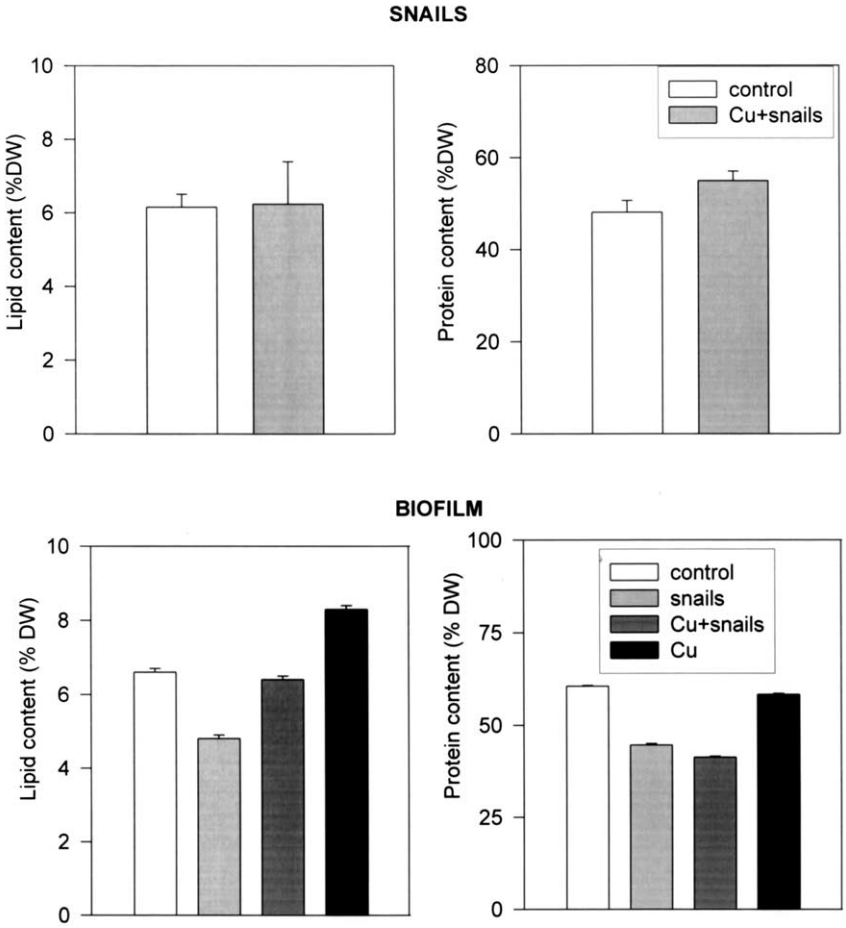

Fig. 4. Lipid and protein content (percentage with respect to dry weight) of snails and biofilm for each treatment. Bars indicate standard error of mean.

trophic level. Protein content in biofilm decreased with copper in our experiment. Similar decreases have also been reported in the literature (Pratt et al., 1987; Luderitz and Nicklisch, 1989). Control of biofilm biomass by grazing also resulted in a significant reduction of protein content as was observed for lipids. On the other hand, a significant increase was observed in protein content of snails exposed to copper that could be related to the induction of metalloprotein synthesis to bind heavy metals. Cu-metallothioneins induction was observed in other invertebrates: the common share crab (Hebel et al., 1997) and mussels (Bolognesi et al., 1999; Viarengo et al., 2000).

Chlorophyll content and NCM were not affected by copper. In previous experiments (Navarro, 2001) with similar conditions and copper exposure of 15 $\mu g\ l^{-1}$, with shorter exposure time (7 days instead of 14 days), copper had a significant effect on chlorophyll content (50% of reduction respect to control) and ^{14}C incorporation (47% of reduction). In the present experiment we exposed algal communities with longer period of pre-colonisation in the field and longer period of colonisation in the artificial channels. Community age is a factor that might modulate the ecotoxicological response of algae to toxicants (Ivorra et al., 2000; Guasch et al., 1998; Guasch

and Sabater, 1998). Biofilm accumulates extracellular products that can decrease toxicity. Such protection has been demonstrated for metals (Admiraal et al., 1999; Starodub et al., 1987) and herbicide (Muñoz et al., 2001), and may have reduced the sensitivity of biofilm to copper exposure. Furthermore, longer periods of colonisation favoured the filamentous forms, which accounted for 20–40% of the total abundance in our periphyton community. Filaments might form gradients in the biofilm that limit metal diffusion.

In this experiment we have did not observe a significant effect of copper on algal composition in contrast with the results reported by Leland and Carter (1984). These authors report a decrease in the abundance of most algal taxa at 5–10 $\mu g\, l^{-1}$ of copper in an oligotrophic stream. Kaufman (1982) working in stream mesocosms with higher copper concentration (30 $\mu g\ l^{-1}$) found a reduction in diatom species number and in global diversity. Crustose forms predominated in copper treatments: several authors (Deniseger et al., 1986; Leland and Carter, 1984; Ivorra et al., 2000) have reported a correlation between high number of species tightly attached to substrate in streams and increased concentrations of a variety of metals.

5. Conclusion

This study highlights the importance of the structure of biofilm and the sensitivity of snails to copper exposure in a simple experimental aquatic food chain. Primary producers growing in thick biofilms may escape from heavy metal toxicity by adsorption to the polysacharide matrix and limited metal diffusion (Sabater et al., 2002). However, aquatic snails are exposed to metal in their food and in the water for breathing. Therefore, snails may be a sensitive marker of sublethal metal doses.

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