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FATE AND EFFECTS OF COPPER IN FLUVIAL ECOSYSTEMS: THE ROLE OF PERIPHYTON

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Fate and effects of copper in fluvial ecosystems: the role of periphyton

Memòria presentada per Alexandra Serra Gasa per optar al grau de Doctor per la Universitat de Girona

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CONTENTS

SUMMARY

Human activity is one of the major causes of elevated concentrations of nutrients and toxic substances in fluvial ecosystems. A wide range of organic and inorganic pollutants from industrial, urban, mining and agricultural activities in the catchments eventually reach streams and rivers through diverse surface and subsurface flowpaths. Among the many ecosystem stressors, eutrophication and metal pollution are the two major environmental problems in many developed and developing countries. Copper (Cu) is the most commonly used toxic heavy metal for industrial purposes and its presence in aquatic systems arises from both naturally occurring and manmade origin.

Periphyton, communities colonizing the illuminated surface of shallow aquatic systems, are the first to interact with dissolved substances such as nutrients, organic matter, and toxicants, they can actively influence the sorption, desorption and decomposition of pollutants and they integrate the effects of environmental conditions over extended periods of time. For all these reasons, fluvial biofilms (periphyton communities) are a useful tool for monitoring the effects of pollutants (e.g. nutrients and metals) into aquatic ecosytems.

The present study aims to investigate the fate and effects of Cu in fluvial ecosystems focusing on periphyton communities. Different methodologies have been developed and/or adapted to specifically investigate the dynamics of Cu, its toxicity and bioaccumulation on natural periphyton communities, and the interaction between eutrophication and Cu toxicity in these ecosystems.

CHAPTER II: Measuring in-stream retention of copper by means of constant-rate additions. This chapter aimed to study the dynamics of Cu at sub-toxic concentrations using the nutrient spiraling theoretical and methodological framework. The influence of hydrologic and biological factors (water discharge and algal biomass of the biofilm communities colonizing the channels bed) on Cu retention were evaluated in an indoor channel system. Furthermore, the dynamics of Cu was compared to the dynamics of phosphate (PO_4^{3-}), which has been widely studied within the context of the nutrient spiraling concept. The methodology used allowed a successful quantification of Cu and PO_4^{3-} retention. The results showed higher retention efficiency for PO_4^{3-} than for Cu. The biofilm played a key role in retaining both solutes. Although

retention efficiency for both solutes was higher in the experiments with colonized substrata compared to uncolonized substrata, we found a positive relationship between uptake rate and chlorophyll-*a* only for PO_4^{3-}. Finally, retention efficiency for both solutes was influenced by water discharge, showing lower retention efficiencies under higher flow conditions. These results suggest that the fate and toxic effects of Cu on stream biota may be strongly influenced by the prevailing environmental conditions. Our results indicate that the experimental approach considered can provide new insights into the investigation of retention of toxic compounds in fluvial systems and their controlling mechanisms.

CHAPTER III: Effects of chronic copper exposure on fluvial systems: linking structural and physiological changes of fluvial biofilms with the dynamics of toxicants. This study aimed to link the changes that chronic Cu exposure cause on the structure and physiology of fluvial biofilms with the efficiency of the river systems in retaining phosphate and Cu. The effects of a chronic Cu exposure on the structure; physiology and induction of Cu-tolerance of the community were evaluated by comparing this community with a non exposed one. Results showed that periphyton chronically exposed to Cu had lower algal biomass, higher proportion of green algae, lower proportion of brown algae, and higher EPS content per unit of biomass than the un-exposed community. In addition, the chronically exposed community showed a Cu content (both total and intracellular Cu content) ten times higher than the un-exposed community. While in-stream phosphate retention was not influenced by chronic Cu exposure; Cu retention was clearly reduced, as was shown by a reduction in Cu retention efficiency ($Cu\text{-}S_w$) and demand (Cu-Vf). The chronically exposed periphyton, in spite of having high intracellular Cu concentration, showed similar photosynthetic efficiency than the un-exposed community and showed a higher Cu tolerance. It indicated that this community was adapted to Cu exposure and that this adaptation was probably linked to the ability to immobilize the metal in a detoxified form. These observations suggest that the fate of Cu in fluvial ecosystems will be influenced by the exposure history of the system. Metals will travel longer distances in metal polluted

streams compared to pristine systems having effects on water quality farther downstream.

CHAPTER IV: Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history. This study aimed to analyze the effect of different Cu exposure episodes on the structure (community composition) and functioning (including toxicity and Cu accumulation kinetics) of fluvial periphyton communities. In this chapter, three different exposure episodes were performed in the artificial river system. In the first case, the periphyton community was not exposed to the metal (No-Cu); in the second case it was acutely exposed (through three pulses) to a low Cu concentration (Cu-Pulsed exposure); and in the third case, the community was chronically exposed to Cu (Cu-Continuous exposure). We hypothesize that metal exposures differing in the time scale (duration of exposure), will have a different impact on the structure and functioning of periphyton. Pulsed Cu exposure did not affect the community structure but influenced the accumulation kinetics (decreasing intracellular Cu uptake). On the other hand, continuous Cu exposure caused a huge increase in metal content (both total and intracellular) and modified the structure of the community (increasing the percentage of cyanobacteria, decreasing the percentage of diatoms and increasing the diatom diversity). Both pulsed and continuous periphyton metal exposure may have negative repercussions for the fluvial ecosystem. While Cu pulsed exposure may be toxic to periphyton communities, continuous exposures may lead to community adaptation, which is often related to changes in species composition and higher metal contents being transferred to higher trophic levels of the stream food chain.

CHAPTER V: Influence of phosphorus on copper sensitivity of fluvial periphyton: the role of chemical, physiological and community related factors. The main goal of this study was to explore the influence of eutrophication of fluvial ecosystems (caused by increased phosphorus concentration) on periphyton Cu sensitivity. We hypothesized that eutrophication will lead to a reduction of Cu sensitivity of natural periphyton communities due to variations in metal bioavailability as well as the phosphate

regime during growth. The study design included three tiers: a field study including the characterization of land use and the ecological state of the corresponding river sections in the Fluvià River watershed, an experimental investigation performed with natural periphyton from the previously studied stream sites in indoor channels, and finally a culture study in the laboratory. Results showed that differences in Cu sensitivity of natural periphyton communities followed the gradient of nutrient concentration found in the field. Results from the culture experiments, which allowed isolating the effects of phosphorus on Cu toxicity, demonstrated that both, P-conditions during growth and P-content in the media are important factors modulating the toxicological response of algae to Cu. From these observations, it is expected that point-source or accidental metal pollution in the fluvial systems will have great negative effects in slightly human impacted sites, where the presence of inorganic and organic ligands in water is low and the phosphorus concentration as well. On the other hand, Cu toxicity is expected to be lower in impacted sites where water eutrophication is higher. The observations from this study indicate that the ecological effects of metal pollution in rivers might be obscured by eutrophication.

RESUM

L'activitat humana és una de les majors causes d'elevades concentracions de nutrients i substàncies tòxiques en els ecosistemes fluvials. Un ampli ventall de contaminants orgànics i inorgànics provinents d'activitats industrials, urbanes, mineres i agrícoles que es desenvolupen a les conques fluvials, arriben finalment als rius per diferents vies superficials i subsuperficials. Entre la gran varietat de factors que alteren aquests ecosistemes, l'eutrofització i la contaminació per metalls pesants són els dos principals problemes ambientals en països desenvolupats i en vies de desenvolupament. El coure (Cu) és un dels metalls pesants potencialment tòxic més utilitzat en processos industrials. La seva presència en els ecosistemes aquàtics prové tan de fonts naturals com antropogèniques.

Els biofilms fluvials (perifíton), comunitats que colonitzen la superfície il·luminada d'ecosistemes aquàtics somers, són els primers d'interaccionar amb les substancies dissoltes de l'aigua com són els nutrients, matèria orgànica o les substàncies tòxiques. Aquestes comunitats poden influenciar activament l'adsorció i la descomposició del contaminants i integren els efectes de les condicions ambientals al llarg de perllongats períodes de temps. Per totes aquestes raons, els biofilms fluvials (també anomenats comunitats perifítiques) representen una eina valuosa per avaluar els efectes dels contaminants (ex. nutrients i metalls) en els ecosistemes aquàtics.

Aquest treball pretén investigar el destí i els efectes del Cu en els ecosistemes fluvials centrant-se en les comunitats perifítiques. Diferents metodologies han estat desenvolupades i/o adaptades per investigar específicament la dinàmica del Cu, la seva toxicitat i bioacumulació en comunitats perifítiques naturals, i la interacció entre l'eutrofització i la toxicitat del Cu en aquests ecosistemes.

CAPÍTOL II: Mesura de la retenció de coure en rius mitjançant adicions de flux constant. Aquest capítol pretenia estudiar la dinàmica del Cu a concentracions sub-tòxiques, utilitzant el marc teòric i metodològic que ofereix el concepte de "nutrient spiraling". La influència de factors hidrològics i biològics (cabal i biomassa del biofilm que colonitza el sistema) en la retenció del Cu ha

estat avaluada en un sistema de canals experimentals. A més, la dinàmica del Cu ha estat comparada amb la dinàmica del fosfat, un nutrient àmpliament estudiat en el context del "nutrient spiraling". La metodologia emprada ha permès aconseguir la quantificació de la retenció de Cu i fosfat. Els resultats d'aquest estudi mostren una major eficiència de retenció pel fosfat comparat amb el Cu. El biofilm ha jugat un paper decisiu en la retenció d'ambdós soluts. Malgrat que l'eficiència de retenció dels dos soluts va resultar major en els experiments realitzats amb substrats colonitzats, es va trobar una relació positiva entre la taxa d'assimilació i la clorofil·la *-a* només en el cas del fosfat i no en el Cu. Finalment, l'eficiència de retenció dels dos soluts va resultar influenciada per el cabal, mostrant menor eficiències de retenció en condicions d'alt cabal. Aquests resultats suggereixen que el destí i els efectes del Cu en la biota fluvial es veuran fortament influenciats per les condicions ambientals predominants. A més, els nostres resultats indiquen que l'aproximació experimental considerada en aquest estudi pot proporcionar nous elements per entendre la retenció dels compostos tòxics en els ecosistemes fluvials així com els mecanismes que la controlen.

CAPÍTOL III: Efectes de l'exposició crònica de coure en els ecosistemes fluvials: relació dels canvis estructurals i fisiològics dels biofilms fluvials amb la dinàmica de tòxics. Aquest estudi tenia com a objectiu principal relacionar els canvis que una exposició crònica de Cu provoca en l'estructura i la fisiologia dels biofilms fluvials amb l'eficiència dels ecosistemes fluvials retenint fosfat i Cu. Els efectes d'una exposició crònica de Cu sobre l'estructura; fisiologia i la inducció de tolerància al Cu de la comunitat han estat avaluats comparant aquesta comunitat amb una comunitat no exposada. Els resultats d'aquesta investigació mostren que la comunitat perifítica crònicament exposada al Cu conté una menor biomassa algal, una major proporció d'algues verdes, una menor proporció de diatomees i un major contingut d'EPS per unitat de biomassa que la comunitat que no ha estat exposada al metall. A més, la comunitat crònicament exposada mostra un contingut de Cu (total i intracel·lular) deu vegades superior al de la comunitat no exposada. Mentre que la retenció de fosfat no s'ha vist influenciada per l'exposició crònica al Cu; la retenció de Cu s'ha vist clarament reduïda,

mostrant una reducció en l'eficiència de retenció (Cu-S_w) i la demanda de Cu (Cu-Vf). La comunitat perifítica que ha estat crònicament exposada, malgrat contenir elevades concentracions de Cu intracel·lular, mostrava una eficiència fotosintètica similar a la de la comunitat no exposada, i una major tolerància al Cu. Aquests resultats indiquen que aquesta comunitat estava adaptada a l'exposició de Cu i que aquesta adaptació estava probablement relacionada amb l'habilitat d'immobilitzar el metall intracel·lularment d'una forma no tòxica. Aquestes observacions suggereixen que el destí del Cu en els ecosistemes fluvials estarà influenciat per la història d'exposició del sistema. Els metalls viatjaran distancies més llargues en els sistemes fluvials més contaminats comparat amb els sistemes pristins, repercutint en la seva qualitat aigües avall.

CAPÍTOL IV: Acumulació i toxicitat del coure en comunitats perifítiques fluvials: la influència de l'historia d'exposició. L'objectiu d'aquest estudi consistia en analitzar l'efecte de diferents episodis d'exposició al Cu en l'estructura (composició de la comunitat) i el funcionament (incloent la toxicitat i la cinètica d'acumulació del Cu) de les comunitats perifítiques fluvials. En aquest capítol es van realitzar tres episodis diferents d'exposició en el sistema de canals experimentals. En el primer cas, la comunitat perifítica no va ser exposada al Cu (No-Cu); en el segon cas, la comunitat va ser exposada de manera aguda (mitjançant tres pulsos) a una concentració relativament baixa de Cu (Cu-Pulsed); i en el tercer cas, la comunitat va ser crònicament exposada al Cu (Cu-Continuous). La hipòtesi d'aquest estudi era que les exposicions al metall diferint en la seva durada tindran un impacte diferent tan en l'estructura com en el funcionament del perifíton. Els resultats d'aquesta investigació mostren que l'exposició aguda (Cu-Pulsed) no va afectar l'estructura de la comunitat però va influenciar la cinètica d'acumulació de Cu (consistint en una reducció de l'assimilació intracel·lular del metall). Per altra banda, l'exposició crònica (Cu-Continuous) va provocar un notable increment del contingut de metall de la comunitat (tan total com intracel·lular) i va modificar l'estructura de la comunitat (provocant un increment en el percentatge de cianobacteris, una disminució del percentatge de diatomees i un increment en la diversitat de diatomees). Aquest estudi conclou que ambdues exposicions, aguda i crònica, poden tenir efectes negatius en l'ecosistema

fluvial. Mentre que les exposicions agudes poden causar efectes tòxics en les comunitats perifítiques, les exposicions cròniques poden derivar en una adaptació de les comunitats al metall, sovint relacionat amb un canvi d'espècies i elevada acumulació de metalls que podran ser transferits a nivells superiors dins la cadena tròfica de l'ecosistema fluvial.

CAPÍTOL V: La influència del fòsfor en la sensibilitat al coure de les comunitats perifítiques fluvials: el rol del factors químics, fisiològics i factors relacionats amb la comunitat. L'objectiu principal d'aquest estudi consistia en explorar la influència de l'eutrofització dels ecosistemes fluvials (causada per un increment de la concentració de fòsfor) en la sensibilitat al Cu de les comunitats perifítiques. En aquest estudi s'hipotetitzava que l'eutrofització reduiria la toxicitat del Cu en les comunitats perifítiques degut tan a variacions en la disponibilitat del metall com a variacions en el regim de fòsfor durant el creixement. El disseny experimental va incloure tres nivells: un estudi de camp incloent la caracterització dels usos del sòl i l'estat ecològic de diferents trams de la conca del riu Fluvià, un experiment realitzat amb comunitats perifítiques naturals procedents de diferents punts prèviament estudiats en un sistema de canals experimentals, i finalment un estudi utilitzant cultius algals al laboratori. Els resultats varen mostrar que les diferencies en la sensibilitat al Cu de les comunitats perifítiques seguien el mateix gradient de concentració de nutrients trobat en el camp. Els resultats dels experiments realitzats amb cultius algals demostren que ambdós, les condicions de fòsfor durant el creixement i el contingut de fòsfor en el medi, juguen un paper important modulant la resposta toxicològica de les algues al Cu. Tenint en compte aquestes observacions, és d'esperar que entrades puntuals o accidentals de metalls en l'ecosistema fluvial tindran efectes negatius més severs en llocs menys impactats per l'activitat humana, on la presència de lligands orgànics i inorgànics és baixa i la concentració de fòsfor també. Per altra banda, la toxicitat del Cu és d'esperar que sigui menor en llocs més afectats per l'eutrofització. Les observacions d'aquest estudi indiquen que els efectes ecològics de la contaminació per metalls en rius es poden veure emmascarats per l'eutrofització.

Chapter I: General introduction

In this introductory chapter, an overview of the influence of human activity on fluvial ecosystems, focusing on the periphyton responses to land derived substances is given. Ecosystem-scale effects such as eutrophication, and the potential impact of other sources of pollution like heavy metals are elucidated. Knowledge about copper (Cu) toxicity and accumulation in algae is reviewed as well as a framework for the more specific study of Cu toxicity on periphyton.

This background provides the rationale of the dissertation presented. The main objectives and hypotheses are also listed at the end of this introductory section.

Influence of human activity on fluvial ecosystems

Throughout history, humans have purposely, accidentally or unknowingly forced substantial changes on rivers. Modifications made anywhere in the catchments have implications downstream and may compromise the viability of ecosystems (Norris et al., 2007). As a result of these human activities, the conditions of many aquatic environments have been degraded. Therefore, human activities at the landscape scale are a principal threat to the ecological integrity of river ecosystems, impacting habitat, water quality, and the biota via numerous and complex pathways (Allan et al., 1997; Strayer et al., 2003; Townsend et al., 2003).

River regulation by dams, diversions, channelization and other physical controls over natural flow regimes have substantially affected the majority of rivers in developed countries causing critical effects to the river ecosystem, such as a reduction in natural variability in discharge and temperature; shifts in species composition due to altered habitat conditions, or interference with fish migration. Human activities have also profoundly altered water chemistry and particularly nutrient levels in many of the world's surface waters from both, agricultural and urban sources. Agricultural runoff is a major source of nutrients to aquatic habitats. Agriculture increases nutrient levels due to fertilizers and animal wastes, and also by increasing soil erosion, which particularly affects the transport of phosphorus (Allan, 1995). Municipal wastes and fertilizers are significant nutrient sources from urban areas. In addition to nutrients, as a

consequence of human activity and the associated change in watershed land uses, rivers receive high amounts of pesticides, heavy metals, organic compounds, and other contaminants leading to a degradation of the fluvial systems and the aquatic biota associated to these habitats (e.g. Paul and Meyer, 2001).

Water pollution

The primary sources of water pollution are generally grouped into two categories based on their point of origin. ***Point source*** pollution occurs when harmful substances are emitted directly into a body of water, for instance, discharges of urban and industrial wastewater effluents. Alternatively, ***Non-point source*** pollution is often a cumulative effect of small amounts of contaminants gathered from a large area. Nutrient runoff from agricultural fields, or metals and hydrocarbons from an area with high impervious surfaces and vehicular traffic are examples of non-point source pollution (Fig. 1). The relative contribution of these two types of sources can differ substantially from watershed to watershed, depending upon local human population densities and land uses (Carpenter et al., 1998; Smith et al., 1999).

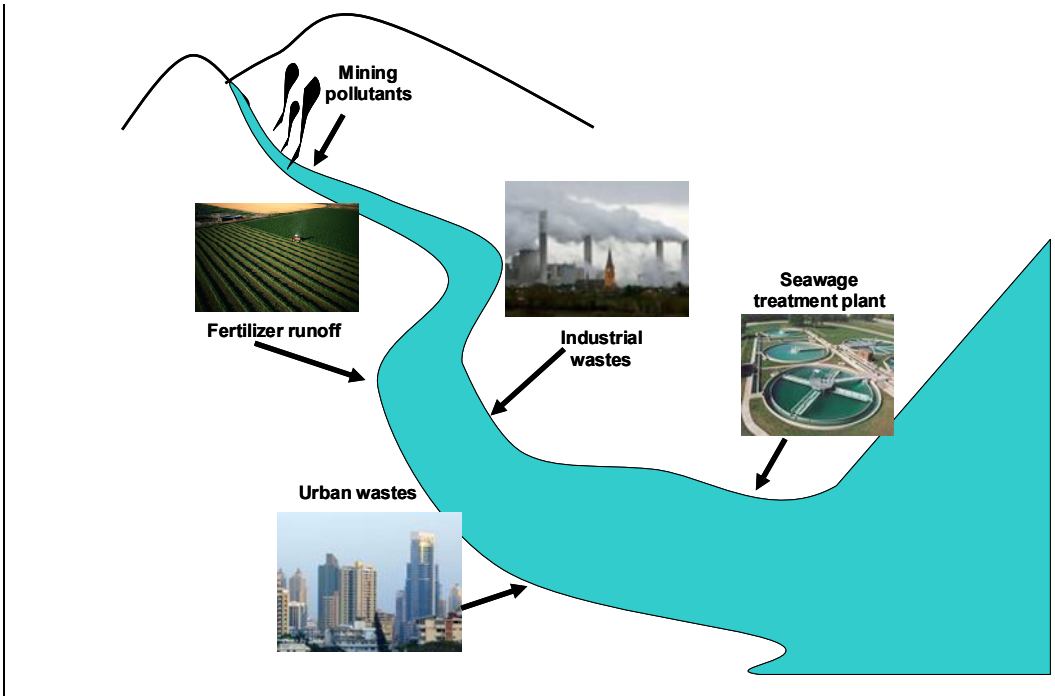


Figure 1. Scheme representing different types of river pollution.

Periphyton as a biological interface for studying effects of pollutants in rivers

The effect of pollutants in aquatic ecosystems can be assessed at several scales (Fig. 2). Classical toxicology has been one of the primary approaches by which the effects of environmental pollutants on aquatic organisms have been assessed. A variety of standardized laboratory toxicology studies have been conducted where contaminant dose, for example, has been related to responses of individual organisms particularly at biochemical or physiological levels (Adams and Greeley, 2000). Most ecotoxicological tests are performed in the laboratory, on small populations of certain species and, although they provide useful information on the effects of these toxicants, they are not fully reliable to forecast effects in natural systems (Cairns and Niederkenher, 1995; Navarro et al., 2002). Testing on single species does not enable the effects of toxicants at community level to be understood (Sabater et al., 2007) and lacks ecological realism (NRCC, 1985; Lagadic et al., 1994; Adams et al., 2000). On the other hand, tests using natural communities appropriately reflect the ecological reality of a natural system (Cairns and Niederkenher, 1987).

Fluvial biofilms (also known as phytobenthos or periphyton) are attached communities consisting of bacteria, algae and fungi embedded within a polysaccharide matrix (Lock, 1993). In rivers, these communities are the first to interact with dissolved substances such as nutrients, organic matter, and toxicants (Sabater et al., 2007). Biofilms can actively influence the sorption, desorption and decomposition of pollutants (Schorer and Eisele, 1997). They integrate the effects of environmental conditions over extended periods of time, mainly because of their small size and rapid growth, species richness, and the physiological variety of the organisms of which they are formed (Sabater et al., 2007). For all these reasons, biofilms are a useful tool for monitoring specific types of pollution, such as nutrient enrichment (Kelly and Whitton, 1995), as well as for assessing the effects of metal toxicity on fluvial ecosystems (e.g. Ivorra et al., 2002; Guasch et al., 2009).

In order to evaluate the potential impact of pollutants such as metals on freshwater aquatic populations and communities, the use of controlled exposures, like micro/mesocosms, provides an excellent basis (Graney et al., 1994).

Although smaller and less complex than real-world ecosystems, experimental channels provide the opportunity to perform ecosystem-level research in replicated test systems under conditions that are manageable in terms of costs and logistics (Roussel et al., 2007.) Small scale laboratory experiments where algal cultures are exposed for short-time to different environmental conditions have low realism but are easily replicated and provide tight control over experimental variables. Experiments conducted at larger temporal and spatial scales (i.e. exposing natural biofilms in experimental streams) have greater ecological relevance but lack rigorous control and are difficult to replicate as the system increases in dimension and complexity (Fig. 2).

An investigation that integrates experimental approaches at different scales is optimal for determining causation. The underlying mechanisms responsible for changes in the structure and functioning of ecosystems after disturbance can be examined in less complex communities at smaller temporal and spatial scales.

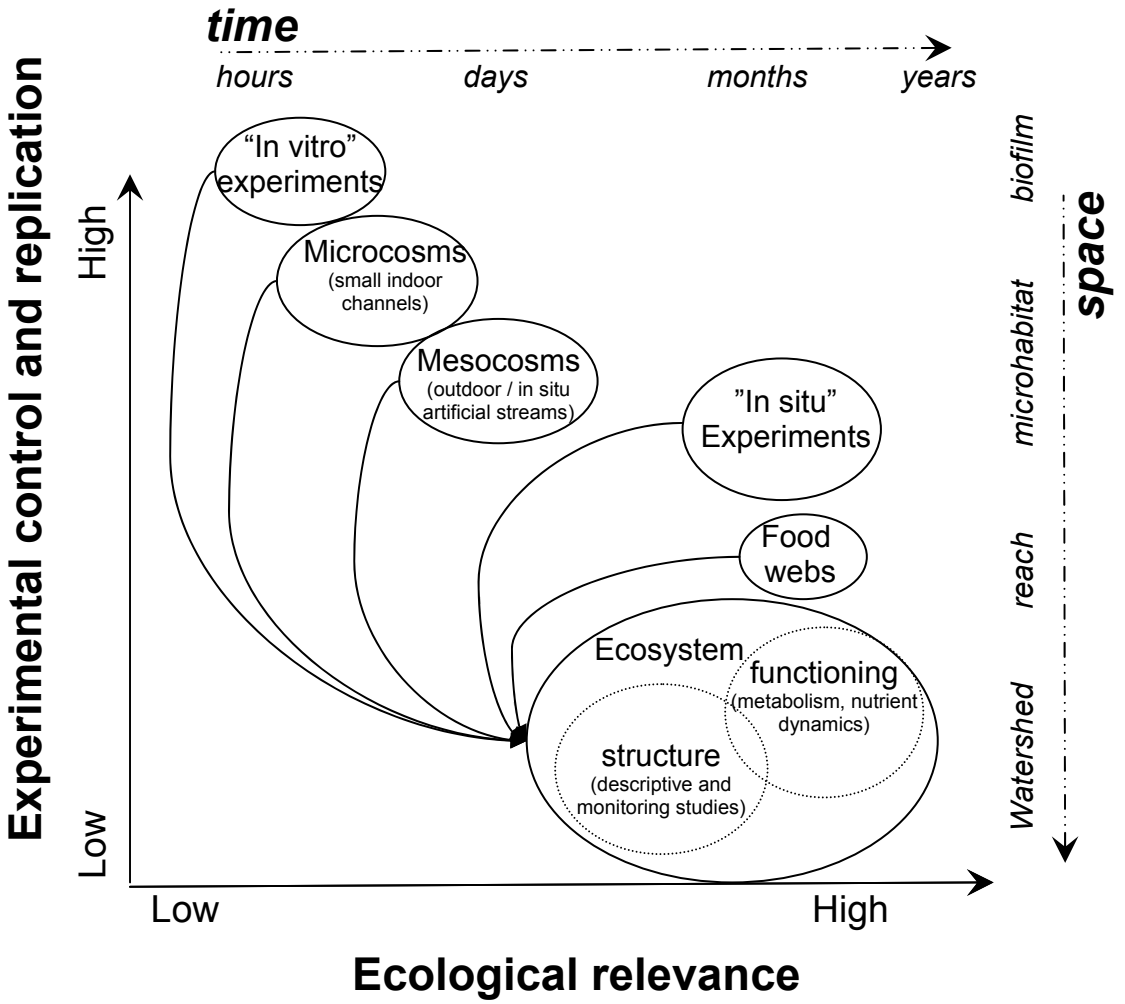


Figure 2. The relationship between ecological relevance, experimental control and replication in stream ecology based on the structure and function of benthic autotrophic organisms. (Modified after Clements and Newman, 2002).

The use of artificial streams or microcosms and mesocosms, in which biofilm communities can develop, may achieve the necessary compromise between simplification and standardisation of the natural system and the required replicability and repeatability (Sabater et al., 2007). This system is especially appropriated in ecotoxicological studies due to the impossibility of experimentally releasing toxicants to the natural environment. The use of artificial channels has also previously been shown to be ideal for examining particular mechanisms influencing nutrient dynamics under controlled conditions (e.g. D'Angelo et al., 1991; Mulholland et al., 1991).

Nutrients in rivers: nutrient dynamics and eutrophication

Cycling of limiting nutrients is an important property of ecosystems (Essington and Carpenter, 2000). For many ecosystems, internal nutrient cycling is the dominant source of nutrients for primary producers (e.g. Levine and Schindler, 1980; Tripathi and Singh, 1994), so there is tight coupling between the rate of nutrient cycling and primary productivity. Consumers thereby can control primary productivity by altering the rate of nutrient remineralization and subsequent uptake (Kitchell et al., 1979). Furthermore, the magnitude of nutrient cycling has important effects on ecosystem resistance and recovery from disturbance (DeAngelis et al., 1989). For these reasons, processes governing the rate of cycling have received considerable attention from ecologists (Essington and Carpenter, 2000).

Knowledge of the processes contributing to in-stream nutrient retention is largely attributable to the concept of nutrient spiraling, a conceptual and empirical model of cycling in fluvial ecosystems (Ensign and Doyle, 2006). Nutrient retention in stream ecosystems is a combination of hydrologic, biological, and chemical retention (Valett et al., 1996). Biotic uptake of nutrients can take place by macrophytes or riparian vegetation (Hearne and Howard-Williams, 1988), by phytoplankton or by biofilms. In streams with few macrophytes, nutrient uptake mainly takes place in the biofilm that covers the river bottom. Biota removes dissolved nutrients from the water, converts them into particulate forms and then returns them to the water when the organism dies (Nijboer and Verdonschot, 2004). Biofilms function as a link between dissolved nutrients in the water column and higher animals and plants in the system (Hynes, 1970) as nutrients are stored via the biofilm as well as passed to other organisms (Costerton et al., 1987; Freeman et al., 1995).

Dynamics of non-conservative solutes (which are affected by biotic activity), especially those that regulate metabolic processes, may be significantly influenced by both abiotic and biotic processes (Allan, 1995). Because of the importance of hydrologic processes in stream ecosystems, a conceptual model of nutrient cycling known as nutrient spiraling has been developed (Webster and Patten, 1979; Newbold et al., 1981). This model was developed to integrate a traditional ecological focus on nutrient cycling in fluvial

systems which are dominated by downstream fluxes. Downstream movement effectively displaces nutrient cycles along a longitudinal axis, converting them into spirals. The nutrient spiraling theory developed from work on biogeochemistry of stream ecosystems and recognizes the importance of understanding transport balance (i.e. hydrology) and retention processes (i.e. uptake of nutrients by plants, algae or microbes) in determining the rate of flux of materials to downstream ecosystems (Newbold et al.,1983; Newbold, 1992).

Over the past three decades, the nutrient spiraling concept has provided an excellent framework to advance research on nutrient retention in streams. Numerous studies have used this concept to evaluate the variation of nutrient retention among different streams and to examine controlling factors (Ensign and Doyle, 2006). A quantitative framework for the study of nutrient spiraling has been developed (Stream Solute Workshop, 1990), in which spiraling length is described. Spiraling length is defined as the sum of uptake length (S_w) and turnover length (S_b). Under this definition, spiraling length represents the distance an average nutrient atom travels downstream in dissolved form (S_w) and within the biotic compartment (S_b) until its eventual release back into the water column (Fig. 3). Low values (short distances) for S_w reflect greater demand for the nutrient in question and more frequent cycling of each atom over a given length of stream (for a review see Allan, 1995).

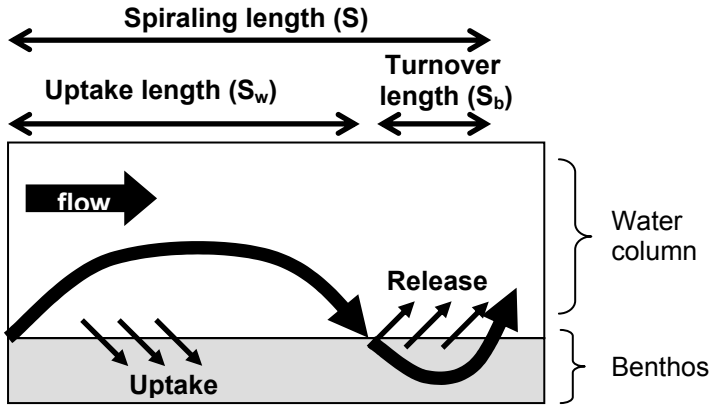


Figure 3. Scheme of two-compartment nutrient spiraling model. (Modified from Newbold, 1992).

The majority of nutrient spiraling studies have been conducted using additions of inorganic nutrients to the stream which allows the measurement of

nutrient uptake along a stream reach. However, other methods have been developed to measure nutrient uptake length in streams. The use of stable and radio isotope tracer experiments has been an extremely informative tool for studying in-stream nutrient processes (e.g. Elwood and Nelson, 1972; Newbold et al., 1983; Mulholland et al., 1985; Gregory, 1978; Hart et al., 1991; Mulholland et al., 1997; Peterson et al., 1997). However, the high cost of isotopically enriched material and analysis and health and safety concerns have made it impractical to use this technology in most streams (From Payn et al., 2005). Despite some recent advances in the study of nutrient spiraling (Mulholland et al., 2002; Thomas et al., 2003; Payn et al., 2005; Doyle, 2005), the basic approach has been essentially unchanged over the last three decades.

Much attention has been given to the importance of headwater streams in nutrient spiraling research (Alexander et al., 2000; Peterson et al., 2001; Ensign and Doyle 2006). High efficiency of first- to third-order streams in nutrient retention has been largely reported (e.g. Mulholland et al., 1985; Martí and Sabater, 1996; Valett et al., 1996; Peterson et al., 1997; Mulholland and DeAngelis, 2000; Peterson et al., 2001). Most existing data on nutrient retention efficiency has been obtained from nearly pristine streams, and there is limited study on retention under polluted conditions (Haggard et al., 2001; Martí et al., 2004). Few studies of nutrient retention performed under “polluted conditions” showed decreased nutrient retention efficiencies in these nutrient-enriched systems compared to unpolluted ones (Martí et al., 2004; Haggart et al., 2005).

The problem of elevated nutrient emissions has been reported frequently worldwide. Eutrophication is a process whereby water bodies, such as lakes, estuaries, or streams receive excess nutrients that stimulate excessive plant growth (algae, periphyton attached algae, and nuisance plants/weeds). In some water body types, this can be a natural process, but in the vast majority of instances, eutrophication is brought about by human inputs of nutrients and is termed “cultural eutrophication” (Dodds et al., 2002; Dodds, 2006). Its consequences are increased frequency of algal blooms, increased water turbidity, and oxygen depletion among others.

Although point sources such as sewage discharges may contribute significantly to nutrient enrichment in some regions, diffuse sources – particularly agriculture – are the major contributors. At watershed scale, excessive input of phosphorus derived from agricultural practices is the most common cause of eutrophication in freshwater lakes, reservoirs, streams, and in the headwaters of estuarine systems (Johnson et al., 1997; Correll, 1998).

Even minimal phosphorus content (some tens of µg/L) can constitute a dangerous pollutant. Thus, according to the UN-ECE classification of surface water, water is considered eutrophic at 25 µg/L (total phosphorus). Dissolved inorganic phosphorus, also referred to as soluble reactive phosphorus (SRP), averages 10 µg/L worldwide among unpolluted rivers (Meybeck, 1982 and 1993).

Nutrient dynamics and eutrophication studies are examples of ecosystem approaches which upscale nutrient uptake and their consequences at reach scale. Considering that human activities cause the input of many other non-conservative solutes (e.g. heavy metals), it is of interest to take this approach to understand better the dynamics and effects of these solutes entering the fluvial ecosystem.

Among the many ecosystem stressors, eutrophication and metal pollution are the two major environmental problems in many developed and developing countries (Wang and Dei, 2006).

Metals in rivers

The aquatic environment is highly affected by metal pollution, as it is an ultimate receptor of urban wastewater, industrial and mine effluents, agriculture runoff and atmospheric deposition (Nriagu, 1979). Some heavy metals are well-known as freshwater and marine pollutants, and much interest has been dedicated to elucidate their toxic effects on aquatic organisms and on algae in particular (Reed and Gadd, 1990; De Filippis and Pallaghy, 1992). The most important heavy metals from the point of view of water pollution are Zn, Cu, Pb, Cd, Hg, Ni and Cr.

One of the major sources of heavy metal pollution is the mining and smelting of metalliferous ores (Li and Thornton, 2001). Concentration of

dissolved metals in rivers impacted by mining activities may fairly exceed the water quality criteria. For example, in the Eagle River, a tributary of the Colorado River, USA which is highly impacted by abandoned mines; concentrations of >700 µg Zn/L and >18 µg Cu/L were reported (Hill et al., 2000). Another area impacted by abandoned mines, the Río Tinto, (South West Spain), reported levels of dissolved metals in water of 61.8 mg Zn/L; 27.2 mg Cu/L; 0.19 mg Pb/L; 0.25 mg Cd/L; 75 mg Al/L (Olías et al., 2006). In addition to mining activity, metals in streams can also derive from urban wastewater or agricultural discharge which contains residuals of pesticides and fertilizers containing metals.

Heavy metal concentrations in unpolluted streams are much lower. For example, in the headwater of the Fluvià watershed (Catalonia, NE Spain), metal concentration dissolved in water is 5.54-16.58 µg Zn/L; 0.65-1.44 µg Cu/L; 0.37-0.59 µg Pb/L; 0.05-0.06 µg Cd/L; 1.18-19.54µg Ni/L (Guasch et al. 2009). These values are in the range of reference values fixed by environmental agencies. For instance, the National Institute of Public Health and the Environment in The Netherlands, established the Maximum Permissible Concentrations (MPC) of metals in freshwater at: 9.4 µg Zn/L; 1.5 µg Cu/L; 11 µg Pb/L; 0.42 µg Cd/L; 0.24 µg Hg/L; 5.1 µg Ni/L; 8.7 µg Cr/L (Crommentjuijn et al., 1997).

Some of the trace metals, such as Fe, Cu, Zn, Mn, Co, Ni and Mo, are essential micronutrients for algal communities, whereas others like Pb, Cd, Ag and Hg are not required for algal growth. The need for trace metals is due to their function in organisms; they are essential for the catalysis of redox reactions, electron transport, structural functions in nucleic acid metabolism and as a cofactor of various enzymes (Lehninger, 1982). Each algal species shows specific requirements in terms of trace metal concentration for optimal development. Nevertheless, at high concentrations these trace metals are becoming toxic for algae (Sunda, 1989; Sunda and Huntsman, 1998).

Many factors including alkalinity, hardness, pH, redox state, and complexation to organic ligands are important for determining the speciation, bioavailability, and toxicity of metals. In freshwater, pH and organic complexation, are particularly significant (Wood, 1983; Meador, 1991; Welsh et al., 1993; Meador, 1998).

The chemical speciation of the metal is defined as its distribution among different phases and among different dissolved forms. When heavy metals enter aquatic systems, they can stay in several phases; in solution as free ions, soluble salt, associated with dissolved inorganic or organic ligands, or they can be bound to particulate matter. All these metal species are following chemical equilibria that regulate the concentration of the free metal ion as well as all the metal complexes (Meylan et al., 2003). Metal speciation is of great interest since bioavailability and toxicity of trace metals to aquatic microorganisms are dependent on metal speciation (Morel and Hering, 1993; Tessier and Turner, 1995; Meylan et al., 2004)

Trace metals can be accumulated by organisms living in metal contaminated areas. Once accumulated, metals can cause different metabolic alterations and potentially can interfere with the organisms' biochemical machinery thereby causing toxic effects on several organismal levels. Once entering the cell, the heavy metal ions may either be detoxified or adversely affect cell processes such as photosynthesis and cell division (Stauber and Davies, 2000). Moreover, heavy metal accumulation not only can exert numerous toxic effects on the individual organism itself but may also give rise to populations or community-wide problems. Through the accumulation of metals in periphyton, metals may be transferred to higher trophic levels of the fluvial food webs (Fisher and Reinfelder, 1995).

Copper occurrence, toxicity, accumulation and tolerance

Copper is the most commonly used toxic heavy metal for industrial purposes and its presence in aquatic systems arises from both naturally occurring and manmade origin. Various sources of Cu, including industrial and domestic wastes, agricultural practices, copper mine drainage, copper-based pesticides, and antifouling paints, have contributed to a progressive increase in Cu concentrations in aquatic environments (Ma et al., 2003; Andrade et al., 2004).

Background Cu concentrations can vary considerably with geographical area. According to Bossuyt and Janssen (2004), the 5th and 95th percentile for dissolved copper in unpolluted European waters is 0.6–10.9 µg Cu/L, respectively (based on data from The Netherlands, Germany and the UK).

However, copper concentrations can reach values as high as 870 µg Cu/L in rivers near a copper mine (ATSDR, 1990; Roussel, 2007). In moderated polluted sites, copper concentrations in water reported in the literature are between 30 and 60 µg Cu/L (Armengol, 1993). And in unpolluted areas, dissolved copper concentration in water does not exceed 5 µg Cu/L (Guasch et al., 2009).

Copper is an essential micronutrient which is needed for algal growth in low concentrations, participating in important biological reactions as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes (Andrade et al., 2004) but, when in excess, it becomes highly toxic (Dewez et al., 2005).

High copper concentration reduces growth as well as photosynthetic and respiratory activities (Nalewajko and Olaveson, 1994). The photosynthetic apparatus is particularly susceptible to this metal, resulting in a decrease in the activity of photosystem II and electron transfer rates (Fernades and Henriques, 1991; Mallick and Mohn, 2003). Toxicity of Cu might also result from the oxidation of sulphydryl groups of enzymes leading to their inhibition (Teisseire and Guy, 2000) and from the induction of oxidative stress (for a review see Pinto et al., 2003), by generating reactive oxygen species like superoxide and hydroxyl radicals (Fig. 4).

Copper has been shown in short-term toxicity tests performed at neutral pH (7-7.5), to have negative effect on Chlorophyta (*Chlorella sp.*) at 4.6 µg/L (Charles, 2000), on Cladocera (*Ceriodaphnia dubia*) at 19-41 µg/L; on amphipod (*Hyalella azteca*) at 4.9-120 µg/L; on Cyprinidae (*Pimephales promelas*) at 36-53 µg/L and on Oligochaeta (*Lumbiculus variegates*) at 200-360 µg/L (Schubauer-Berigan et al., 1993). In the long term, copper can cause structural effects on the communities by replacing the sensitive species by the more tolerant ones (Blanck and Wängberg, 1988). Other microcosm studies showed negative effects of Cu on phytoplankton communities at 20 µg/L (Winner et al., 1990) and on periphyton at 2.5 µg/L (Leland and Carter, 1985) or 10 µg/L (Guasch et al. 2002), which are levels commonly found in unpolluted rivers.

Periphyton sensitivity to copper is shown to vary in a wide range depending on the environmental conditions (water pH, algal biomass, water

phosphorus concentration, etc.) Data concerning Cu toxicity on fluvial biofilms found in different microcosms experiments are summarized in Table 1.

Algae have a high capacity to accumulate heavy metals from the surrounding environment (Newman and McIntosh, 1989, Mehta and Gaur, 2005). Metal accumulation has frequently been used as a biological endpoint to measure metal bioavailability as, in most cases, metals need to be internalized to show an effect (Campbell et al., 2002; Meylan et al., 2003). This capacity of the algae for removing metals from water also provides a valuable tool for waste water treatment (for a review, see Mehta and Gaur, 2005).

In general, kinetics of metal accumulation in algae involves two main processes: a rapid adsorption of the metal ions to the surface of the alga, which is a metabolism independent step, and slower intracellular uptake, which depends on the metabolism of the organism (Crist et al., 1994; Knauer et al., 1997; Vasconcelos and Leal, 2001). After entering the cell, the metal ions are compartmentalized into different subcellular organelles (Arunakumara and Xuecheng, 2008).

Several factors can influence the uptake of heavy metals by stream autotrophic biofilms. These include chemical factors (pH, salinity, phosphate concentration) which affect metal bioavailability by either altering the speciation of the metal or by complexing it at the surface of the organism (e.g. Mason and Jenkins, 1995; Guasch et al., 2004), but also other biological and physical factors. Among the biological factors, the thickness and nature of the biological layer (biomass accumulation, exopolymer abundance) is of considerable significance (Loaec et al., 1997; Admiraal et al., 1999) for metal toxicity. Among the physical factors, current velocity has a special significance for benthic biofilms because it can modulate the uptake of metals and their effects (Hill et al., 2000; Sabater et al., 2002).

Table 1. Summary of copper toxicity on natural biofilms obtained from different experiments performed with natural biofilms.

EC_{50} Cu (µg/L)	End-point	Duration exposure	Experimental conditions
[a] 57.15-88.9	Fo	2 weeks	High phosphate concentration and high pH.
[a] 292.1-393.7	Y	2 weeks	High phosphate concentration and high pH.
[b] 45	Fo	3 days	Oligotrophic stream
[b] 935	Y	3 days	Oligotrophic stream
[b] 18	Fo	16 days	Oligotrophic stream
[b] 64	Y	16 days	Oligotrophic stream
[b] 230	Y	Short-term toxicity test	Low P conc. (1 µg/L P-SRP)
[b] 718	Y	Short-term toxicity test	High P conc. (17.5 µg/L P-SRP)
[c] 20-50	Y	Short-term toxicity test	Spring (lower algal biomass)
[c] 100-350	Y	Short-term toxicity test	Summer (higher algal biomass)

[a] Barranguet et al. 2002; [b] Guasch et al. 2002; [c] Navarro et al. 2002

Some algae are able to survive under metal-rich conditions (Perales-Vela et al., 2006). Mechanisms that may explain the differential ability of algae to survive and reproduce in metal-polluted environments range from control of interspecific competitive interactions to the cellular attributes of individual populations. Control of metal uptake, excretion of accumulated metals and intracellular or extracellular immobilization of accumulated metals are the main mechanisms that may operate alone or in concert to protect algae from toxic effects (Brown et al., 1988; Gerringa et al., 1995; Mason and Jenkins, 1995; Gonzalez- Davila et al., 1995; Moffett and Brand, 1996; Knauer, 1997; Gledhill et al., 1999; Leal et al., 1999; Croot et al., 2000; Soldo et al. 2005; García-Meza et al., 2005). Metals, once inside the cells, may be detoxified by accumulation in polyphosphate bodies and in intracellular metal-binding proteins (Zhang and

Majidi, 1994), and within the vacuoles of some eukaryotic algae (Gadd, 1988; Garnham et al., 1992; reviewed in Mehta and Gaur, 2005) (Fig. 4).

There is considerable need to understand the interactions of multi-stressors in aquatic ecosystems. Several investigations have focused on the interrelationships between trace metals and nutrients in phytoplankton and algal biofilms (Wang and Dei, 2001; Interlandi, 2002; Ivorra et al., 2002; Riedel and Sanders, 2003; Guasch et al., 2004). However, most of the research in this topic has been developed in the laboratory using mono-specific algal cultures. Field testing and studies at community level remain scarce (e.g. Ivorra et al., 2002; Guasch et al., 2004).

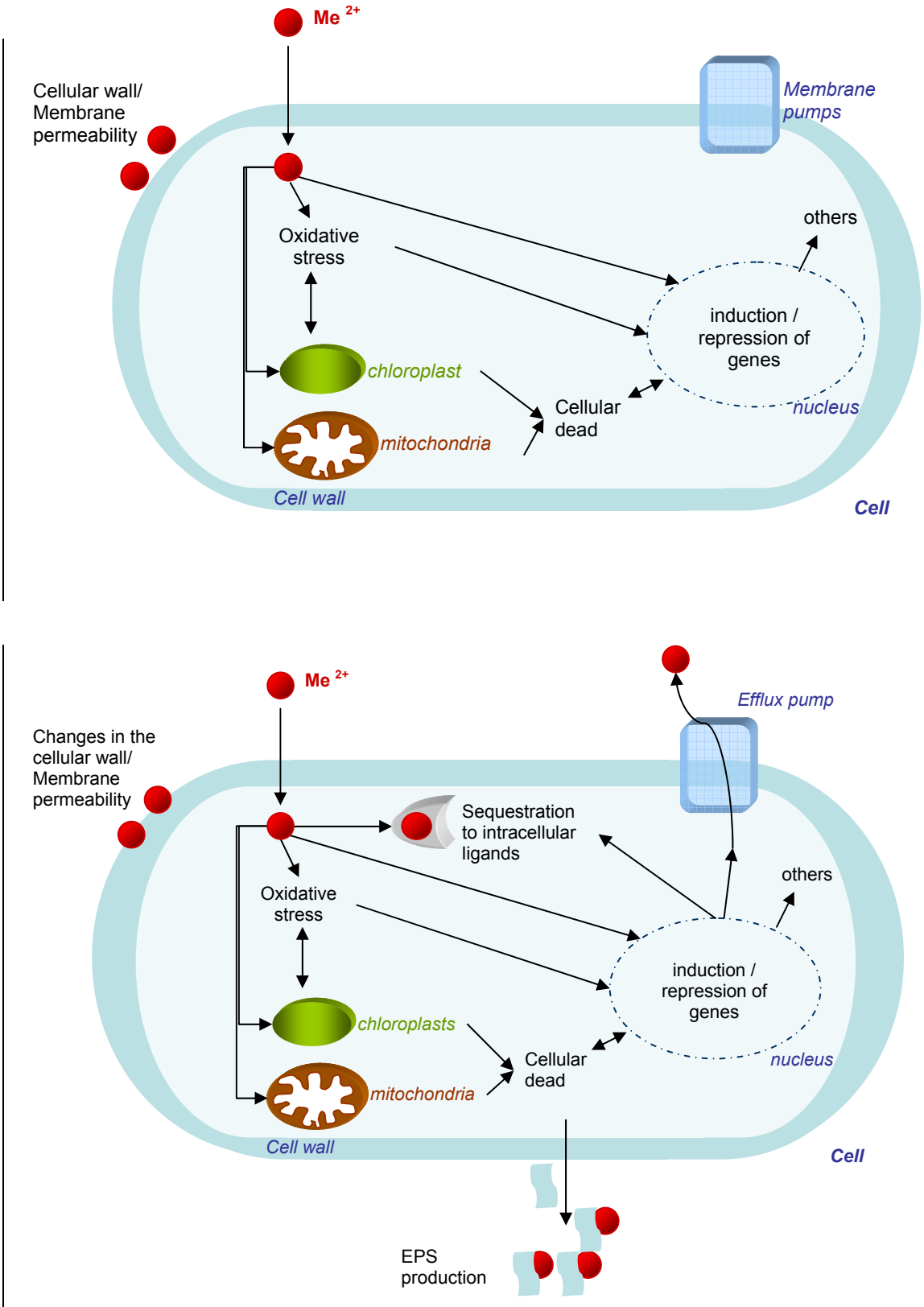


Figure 4. Scheme representing the variety of metal effects to phototrophic organisms at cellular level (upper panel) and detoxification mechanisms (lower panel). Once entering the cell, metals can alter the membrane permeability, may induce oxidative

stress and may affect the photosynthetic apparatus causing damages in the cells that can derive in cellular death. Photosynthetic cells have developed several tolerance mechanisms against the toxic effects of metals including changes in membrane permeability, metal binding in intracellular ligands or the production of extrapolisaccharide (EPS) which can extracellularly bind the metal. Modified from Morin (2006).

Objectives

The present study aims to investigate the fate and effects of copper in fluvial ecosystems focusing on periphyton communities. Different methodologies have been developed and/or adapted to specifically investigate the dynamics of copper, its toxicity and bioaccumulation on natural periphyton communities, and the interaction between eutrophication and Cu toxicity in these ecosystems.

This main objective is approached by the following specific objectives:

- To set up a specific methodology for the study of in-stream copper retention at reach scale, and apply this methodology to investigate the influence of hydrology (water discharge); biomass accrual and Cu pre-exposure on periphyton Cu retention.

- To elucidate the transfer of this toxicant between different compartments of the fluvial ecosystems, from the water column to different fractions of the periphyton (intra and extracellular).

- To explore the effects of copper on fluvial periphyton at different time scales of exposure (chronic versus acute exposures).

- To examine the significance of the exposure time (chronic versus acute) on the copper accumulation kinetics of periphyton.

- To study the interaction between phosphorus and copper at different experimental scales: using natural periphyton communities and using algal cultures.

Hypotheses

Based on the current knowledge and methods developed so far in the fields of periphyton ecology, nutrient dynamics and metal ecotoxicology, the following hypotheses are formulated:

- The application of the nutrient spiraling concept and methodologies associated will allow the estimation of the in-stream copper retention including its downstream transport (developed in chapters II and III).

- Water discharge, algal biomass and Cu pre-exposure will influence Cu dynamics in fluvial systems (studied in chapter II).

- Temporal scale of copper exposure will influence its retention and bioaccumulation kinetics in periphyton as well as its downstream transport, leading to different effects on the fluvial ecosystem (addressed in chapters III and IV).

- Temporal scale of copper exposure will determine its toxic effects on fluvial periphyton. Short-term metal exposure will not allow the adaptation of the community to the metal. On the other hand, long-term exposure will produce structural changes leading to the adaptation of the community. This Cu-adapted community will be less sensitive to Cu than the non-adapted one (investigated in chapters III and IV).

- Eutrophication of the stream ecosystem will influence the toxicological response of periphyton to copper due to the interaction between P and Cu in the water and/or into the cell. Particularly, phosphorus enrichment will lead to a reduced Cu toxicity on periphyton (studied in chapter V).

Chapter II: Measuring in-stream retention of copper by means of constant-rate additions

Introduction

Human activity is one of the major causes of elevated concentrations of nutrients and toxic substances in fluvial ecosystems (Cairns, 1993; Pereira and Hostettler, 1993; Ekholm et al., 2000). A wide range of organic and inorganic pollutants from industrial, urban, mining and agricultural activities in the catchments eventually reach streams and rivers through diverse surface and subsurface flowpaths (Foster and Charlesworth, 1996). Because these pollutants are transported along the fluvial networks in both particulate and dissolved forms, their effect on aquatic communities can be traced even at far distances from their input source (Johnson et al., 2005). Heavy metals are one of the most common inorganic pollutants in aquatic ecosystems (Genter, 1996). Many of the heavy metals in aquatic ecosystems, such as Cu, Mn, Fe and Zn, are essential micronutrients; therefore, their presence in water at trace concentrations is required for algal growth. However, at elevated concentrations these elements can be toxic to algae as well as to other aquatic organisms (Hall et al., 1989; Nies, 1999).

Several attempts have been made to develop mathematical models to describe the transport of toxic substances, such as heavy metals, in fluvial ecosystems. Those models are mainly based on hydrology and on rates of sorption and desorption of metals onto sediment and suspended matter particles (Ciffroy et al., 2000; Jackman et al., 2001; Owens et al., 2001; Johnson et al., 2005). In those models, the role of biotic processes on heavy metal transport and the potential implications of toxics for biota are miss-considered. Nevertheless, several field and laboratory studies have revealed the important role of freshwater and marine algae in removing heavy metals from the water column through uptake and accumulation processes (Sunda and Huntsman, 1998; Vasconcelos and Leal, 2001; Wang and Dei, 2001; Campbell et al., 2002; Meylan et al., 2003). To our knowledge, the dynamics of heavy metals in fluvial ecosystems considering both hydrologic transport and biological processes has hardly been addressed and it is still unclear how microbenthic communities influence the downstream transport of these solutes. To fill up this gap, the conceptual context of the nutrient spiraling (Webster and Patten, 1979) and the mathematical and methodological approaches associated

to it (Newbold et al., 1981; Webster and Valett, 2006) have been used in this study.

The nutrient spiraling concept was developed to describe the simultaneous processes of nutrient cycling and downstream transport, and it has significantly contributed to increase our knowledge of nutrient dynamics in stream ecosystems (Triska et al., 1983; Mulholland et al., 1985; Munn and Meyer, 1990; Martí and Sabater, 1996). This theoretical framework and all the empirical studies based on it have evidenced that streams are not just transport systems, but they also have the capacity to use, transform, and retain nutrients (Bernhardt et al., 2005). Microbial communities developed on benthic stream substrata (usually referred to as biofilms) are mostly responsible for nutrient retention and transformation processes in streams (Allan, 1995). Recent research has shown the influence of physical factors such as temperature, discharge and transient storage (Valett et al., 1996; Butturini and Sabater, 1998; Peterson et al., 2001) as well as biological factors such as algal biomass (Mulholland, 1994; Sabater el al., 2000) on the transformation and retention of nutrients during downstream transport. These factors may likely affect downstream transport of heavy metals as well, but their relative effect is expected to differ from that on nutrient dynamics. For instance biological demand of a micronutrient as Cu is expected to be lower than the demand of a macronutrient. Some authors indicate that physical and biological factors such as water velocity and biomass accrual may influence Cu toxicity (Sabater et al., 2002) and metal sorption (Hill et al., 2000). Total metal removal is expected to increase at higher biomass concentration (Mehta and Gaur, 2005) although reduced metal sorption per unit of biomass is also expected due to decreased sorption capacity of the biofilm (Hamdy, 2000; Gong et al., 2005). However, studies focusing on the influence of physical and biological factors on the downstream transport of metals at sub-toxic concentrations are scarce.

The objectives of this study were to i) quantify the retention of dissolved copper (Cu), a potentially toxic compound, using the nutrient spiraling theoretical and methodological framework and ii) examine the influence of algal biomass and water discharge on Cu retention.

To avoid negative/lethal effects on biota, Cu retention was studied at a concentration <40µg Cu/L, which is shown to be sub-toxic for periphytic biofilms

under acute exposures (i.e. Navarro et al., 2002; Guasch et al., 2004). Cu retention was compared with retention estimates of a macronutrient, phosphate (PO_4^{3-}), which has been widely studied within the context of the nutrient spiraling concept. The study was conducted in an indoor artificial channel system containing colonized substrata as the biotic compartment. This system is especially appropriated in ecotoxicological studies due to the impossibility of experimentally releasing toxicants to the natural environment. The use of artificial channels has previously been shown to be ideal to examine particular mechanisms influencing nutrient dynamics under controlled conditions (e.g., D'Angelo et al., 1991; Mulholland et al., 1991). This approach at micro/mesocosm level contributes to increase our understanding of the fate of toxicants delivered to fluvial ecosystems.

Materials and methods

Experimental setup

To conduct this study, we constructed an indoor experimental system consisting of ten connected Perspex channels (each 170 cm long and 9 cm wide) (Fig. 1).

Each channel unit ended with a Perspex piece to keep the water column depth around 1.5 cm. The system was supplied with dechlorinated tap water filtered through an active carbon (AC) filter. Water input at the head of the first channel unit was provided from a 10L carboy using a centrifuge pump. The water outflow of this channel was subsequently directed to the next 10L carboy to feed the contiguous channel units. All the carboys were placed in a water bath for water temperature control (Fig. 1). A tap located at the head of each channel allowed flow regulation to keep similar discharge among channel units. Ambient light was provided by halogen lamps (80-100 µmol photons/m^2 s) following a 12h/12h light and dark cycle. This experimental set-up corresponds to a simplified system which includes two main components, the water column and the benthos colonized with periphyton. Despite being a simple system it is complex enough to allow the study at community level to be performed, including different species embedded in a polysaccharide matrix which is of high ecological relevance.

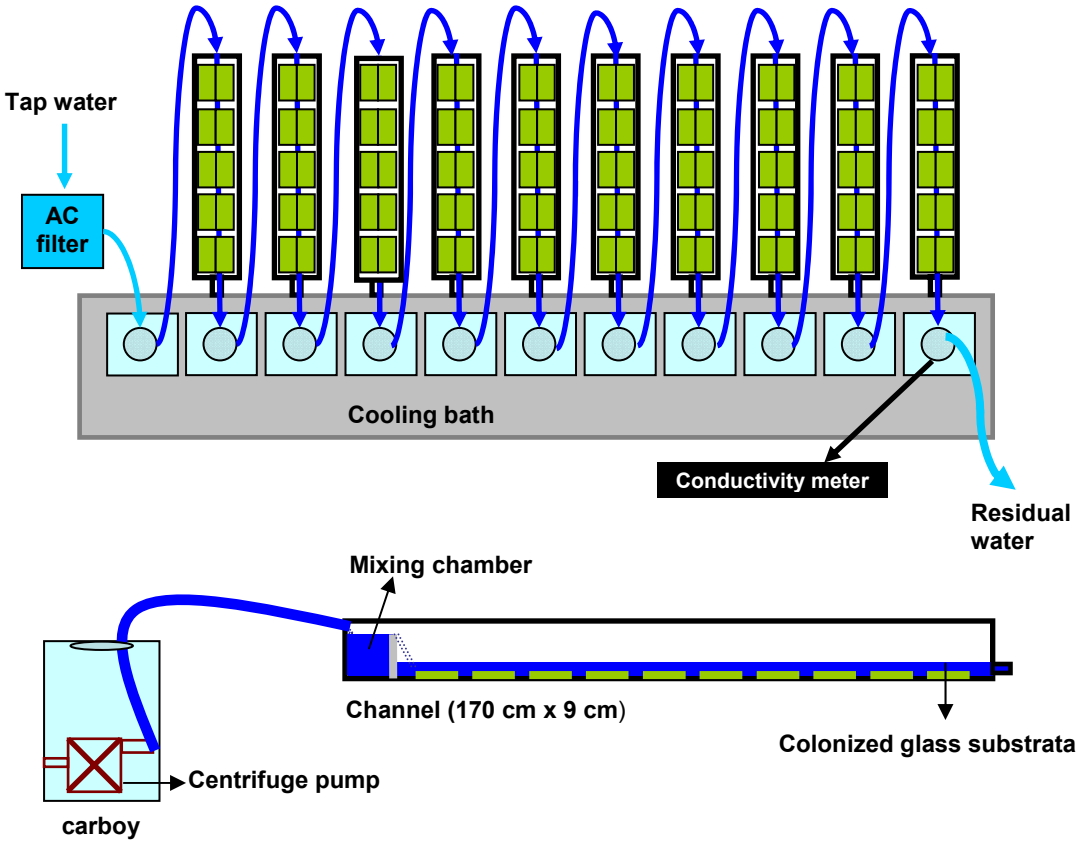


Figure 1. Scheme of the experimental channel system showing an aerial view of the water flow connection among channel units (upper panel) and a detailed scheme of the longitudinal section of a channel unit (lower panel).

To test the influence of the stream biofilm on solute retention, the experiments in the system were conducted both with and without a biotic component. In the experiments without biota (considered as controls) 10 acid-rinsed etched glass substrata (8.5x12 cm) were introduced into each channel unit (Fig. 2). These experiments were used to quantify the solute retention due to physical adsorption onto the channel and carboy walls, the tubing system and the artificial substrata. In the experiments with biota, the artificial substrata introduced into the channels (i.e., 10 etched glasses in each channel unit) were colonized. For the colonized substrata, etched glasses were placed in an aquarium during eight weeks to allow a mature biofilm community to be developed on them. The aquarium was filled with water from the Llémena stream, a small tributary of the River Ter (NE Spain), and contained a centrifuge pump to simulate turbulent water flow. One aliquot of the natural biofilm

community scraped from the stream cobbles was used as an inoculum for the artificial substrata. Once a week, water in the aquarium was replaced with new water from the stream and reinoculated. A total of three colonizations were done over the course of the study to obtain colonized substrata for the different experiments. During the colonizations the average (± SEM) water temperature in the aquarium was 16.4 ± 0.7 ºC, pH was 8.2 ± 0.1, the concentration of soluble reactive phosphorus (SRP) was 36.6 ± 6.1 µg/L, and conductivity was 504.3 ± 28.9 µS/cm (n = 17).

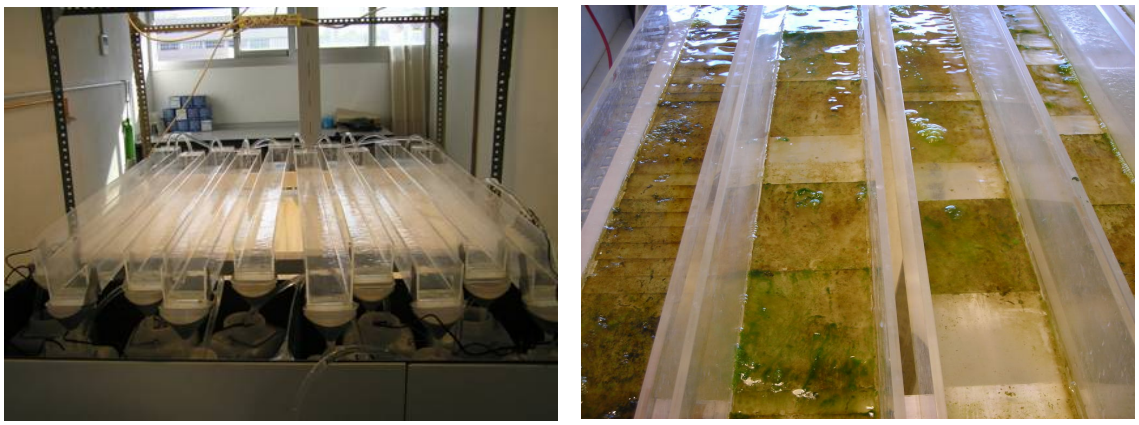


Figure 2. Picture of the experimental channel system without colonized substrata (left panel) and a detail of periphyton colonized substrata (right panel).

Constant-rate solute additions

To quantify solute retention, we conducted constant-rate additions using either PO_4^{3-} or Cu in the channel system following the method described by Webster and Valett (2006). Cu retention was studied at a concentration <40µg Cu/L, which is shown to be sub-toxic for periphytic biofilms under acute exposures (i.e. Navarro et al., 2002; Guasch et al., 2004). This method consists of adding a stock solution of the target compound at constant-rate at the head of the channel system to raise its concentration above ambient levels and using the downstream disappearance of the added solute to quantify the retention parameters (Stream Solute Workshop, 1990).

In our experiments, phosphate was added as KH_2PO_4 and copper as $CuCl_2$ (copper titrisol, Merck, Darmstadt, Germany). Chloride, as NaCl, was added with both PO_4^{3-} and Cu as a conservative tracer to correct the downstream changes in the concentration of these solutes for water advection,

dilution and dispersion. The nominal concentrations of the stock solution were 1663 and 618 µg/L for PO_4^{3-} and 1000 and 415 µg/L for Cu at high and low flow conditions respectively to achieve the desired increase in concentration. In all cases, additions of PO_4^{3-} and Cu were conducted separately to avoid possible interactive effects. The stock solution containing Cu was left to equilibrate for 24 hours at room temperature prior to the additions and was set to result in sub-lethal concentrations at plateau conditions. The stock solution for both PO_4^{3-} and Cu was added at a constant-rate (0.1 L/min) at the carboy of the first channel unit using a peristaltic pump. A conductivity meter with internal data logger (WTW 340i) was placed at the end of the channel system to continuously record changes in conductivity during the addition (Fig. 1). The solute addition lasted until the conductivity reached plateau conditions. This indicated that the solute added was completely mixed through the system. On average, the duration of each addition was between 1.5 and 2.5 hours, depending on the water flow of each experiment.

Ten sampling points were defined along the channel system. Each point was located at the end of each channel unit, corresponding to every 1.7 m. At these points, three water sample replicates were collected prior to each constant-rate addition for background concentrations and once the addition reached plateau conditions. Water samples were collected with 20 mL polyethylene syringes and immediately filtered with 0.2 µm nylon filters. Samples were analyzed for PO_4^{3-} and total dissolved Cu concentrations. In each experiment, water temperature, pH and light intensity (LiCor quantum sensor, LI-192B) were measured at the beginning and at the end of the addition. In three experiments, additional water samples were taken at all sampling points before the addition in order to measure the background concentration of dissolved organic and inorganic carbon (DOC and DIC, respectively), and major cations and anions.

A total of nine additions of PO_4^{3-} and nine additions of Cu were conducted in this study. For each solute, the additions were done with and without biofilm communities (referred to as B and C, respectively) and at a low (1 L/min) and high (2 L/min) flow (referred to as L and H, respectively). The combination of the two factors resulted in four different treatments: LC, LB, HC, and HB. The additions with colonized substrata started after allowing the biofilm

communities to adapt to the channel conditions for two days. Each set of colonized substrata received a maximum of four constant-rate additions, which were separated by a 24h period. These four additions followed the methodical sequence: first the high flow phosphate addition, followed by the low flow phosphate addition, the high flow copper addition and finally the low flow copper addition. This sequence was set to account for the negative effect of discharge on retention efficiency described for nutrients (Butturini and Sabater, 1998; Peterson et al., 2001) and to minimize the possible influence of previous additions on biofilm responses. Each constant-rate addition, a periphyton sample was taken to assess their physiology by measuring photosynthesis efficiency (Yield).

Biofilm measurements

Photosynthetic activity of the algal component of the biofilm communities and algal biomass were measured during the experiments. In each experiment, three colonized substrata were collected from three different channel units before the solute addition and at the plateau conditions for photosynthesis measurements. Comparison between background and plateau biofilm samples allowed examining possible effects of the solute additions on the physiologic status of the biofilm communities. We used the Pulse of Amplitude Modulated fluorometry (PHYTO-PAM Heinz Walz GmbH, Effeltrich, Germany) to measure the effective quantum yield of illuminated cells (Yield), which is an indicator of the photosynthetic efficiency of algae (see fluorescence measurements section in Material and Methods of Chapter III for more details). As this technique is non-destructive, the same colonised substrata used for PAM fluorometry measurements were also used to measure the algal biomass, estimated as the chlorophyll-*a* concentration (chl-*a*), after extraction with 90% acetone and spectrophotometric measurements (Jeffrey and Humphrey, 1975). In the experiments performed without biofilm (referred to as C in the text), the algal biomass was not measured as empty glass substrata were used.

Observations of dominant algal groups of mature biofilms were conducted under light microscopy.

In two different Cu addition experiments (at high flow and low flow conditions), colonized substrata were also collected to measure the total Cu

content in the biofilms. Three samples were collected before the addition for background Cu content and three other samples were collected at the plateau conditions. Biofilm samples were scraped from the glass substrata with a microscope slide, lyophilized and weighed. Dry samples were digested with 4 ml of concentrated nitric acid (suprapure, Merck) and 1 mL of hydrogen peroxide (30%, suprapure, Merck) in a high performance microwave (Milestone, Ethos sel) and were thereafter diluted to 25 mL with milli-Q water before analysis. The concentration factor (CF) was calculated as the ratio between the increase of Cu content in the biofilm and the increase of Cu in water.

Laboratory analyses

The concentration of SRP (soluble reactive phosphorus) in water samples was analysed by the Murphy and Riley (1962) molybdenum blue colorimetric method following APHA (1989). Concentrations of major cations and anions dissolved in water were analysed by ion-chromatography (Metrohm Ltd., Herisau Switzerland). Anions were measured using a METROSEP A SUPP 5 column and $NaHCO_3$ (84 mg/L) and Na_2CO_3 (339 mg/L) as eluents. Cations were measured using a METROSEP C 2 column and tartaric acid (2,3-dihydroxybutanedioic acid; 4mM) and dipicolinic acid (pyridine-2,6-dicarboxylic acid; 0.75 mM) as eluents.

The filtered samples for Cu analysis were acidified with 1% of nitric acid (suprapure, Merck) and stored in the refrigerator at 4ºC until analysis. Dissolved Cu concentration was analyzed using inductively coupled plasma mass spectrometry (7500c ICP-MS Agilent Technologies, Inc., Wilmington, DE). Cu content of digested biofilm samples was analysed using the same methodology. Considering that trace metal bioavailability to aquatic micro-organisms is dependent on metal speciation (Morel and Hering, 1993), the inorganic Cu-complexes and their solubility in the media were estimated using the chemical-equilibrium-diagram-tool Hydra/Medusa (Puigdomenech, 2002), which takes into account the concentration of the major ions in water.

Hydrology and solute retention calculations

Discharge (Q, L/min) into the channel system was determined by a mass balance approach (Gordon et al., 1992) using the conductivity data measured at the bottom of the last channel unit in the following equation (1):

$$Q = \frac{Q_a (T_a - T_p)}{T_p - T_b} \quad (1)$$

Where Q_a is the addition flow rate (L/min), and T_a, T_b and T_p are the values of the hydrological tracer (i.e., conductivity, µS/cm) in the added solution and in the water at background and plateau conditions, respectively.

Average water velocity (vel) in cm/s, along the channel system was calculated based on the total length of the system and the time required for the conductivity to reach one half of plateau value.

Removal of PO_4^{3-} and Cu from the water column was quantified using the retention metrics derived from the spiraling concept and widely used in studies of nutrient dynamics in stream ecosystems (Stream Solute Workshop, 1990; Webster and Valett, 2006). The uptake rate coefficient per unit length (K_c; m^{-1}) was measured following the equation (2):

$$\mathrm{Ln}\left[\frac{C_p - C_b}{T_p - T_b}\right]_x = - K_c x \quad (2)$$

Where C_b and C_p are the concentrations of the solute (Cu or PO_4^{3-}) at background and plateau conditions, respectively, and x is the distance between the addition point and each sampling point. Solute uptake length (Sw; m), the negative inverse of K_c, is an indicator of the in-stream solute retention efficiency (Newbold et al., 1981). Shorter S_w indicates higher retention efficiency than longer values.

We also calculated the areal uptake rate at background concentrations (U; µg/m^2 min) for the two solutes, following equation (3) (Stream Solute Workshop, 1990):

$$U = \frac{C_b Q}{S_w w} \quad (3)$$

Where w is the width of the channel. U is the mass of a solute that is removed from the water column per unit time and benthic area. Several biotic and abiotic processes, such as biological uptake, adsorption, precipitation and

complexation, contribute to this removal (e.g., Triska et al., 2006). As this parameter corrects S_w for hydrology and solute concentration, we use it in this study to explore the influence of algal biomass on PO_4^{3-} and Cu retention including data from all the additions with biota together.

Statistical analyses

Possible effects of solute additions, especially those of Cu, on the photosynthetic activity of the biofilm were evaluated using paired T-test (StatSoft, Inc., 1999) on Yield values measured before the additions and at plateau. The influence of water discharge and algal biomass on PO_4^{3-} and Cu retention was examined by means of linear regression analyses. The relationship between solute retention and discharge was examined using data from additions with and without biota separately. For algal biomass, this relationship was examined using only data from additions with biota.

Results

Characterization of the experimental conditions

During the constant-rate solute additions, average ± SEM discharge and water velocity of the experimental system were 0.83 ± 0.05 L/min and 0.59 ± 0.08 cm/s, respectively, at low flow conditions (n = 10), and 2.11 ± 0.05 L/min and 0.89 ± 0.03 cm/s, respectively, at high flow conditions (n = 8). Water column depth of the experimental system was on average 2.83 ± 0.21 cm under low flow conditions (n = 10) and 4.45 ± 0.20 cm under high flow conditions (n = 8). Water temperature, pH and conductivity varied in a narrow range on all experiments and averaged 15.3 ± 0.6 ºC, 8.04 ± 0.37 and 484 ± 2 µS/cm, respectively (n = 18). A more detailed description of the average water chemical composition for all the experiments is summarized in Table 1.

Table 1. Background concentration of nitrogen compounds (nitrate, nitrite and ammonium); major cations and anions. and dissolved inorganic and organic carbon (DIC and DOC, respectively) in the water of the channel system.

Parameter	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	Ca^{2+}	Mg^{2+}	Na^+	Cl^-	DIC	DOC
Concentration (mg/L)	1.68	0.07	<0.1	38.33	54.32	7.93	21.89	23.84	26.8	2.9
SEM	0.14	0.01		3.52	0.83	0.10	0.33	2.36	0.4	0.1

Values are means and standard error of the mean (SEM) of twenty samples taken before the solute additions. For DIC and DOC, values are means and standard error of the mean of three samples.

The average background PO_4^{3-} and Cu concentrations were 18.1 ± 0.6 µg/L (C.V. = 10.61%) and 1.2 ± 0.5 µg/L (C.V. = 113.19%), respectively. At plateau conditions, the concentrations were increased up to 96.4 ± 8.7 µg/L (C.V. = 27.18%) for PO_4^{3-} and 34.8 ± 2.3 µg/L (C.V. = 19.69%) for Cu (n = 9 for each solute). Inorganic Cu speciation measured for the pH range of the experiments showed that dissolved $Cu(OH)_2$ was the predominant Cu-complex (>90% of the total copper) in the water during the experiments. The results presented correspond always to total dissolved Cu concentration.

The algal component of the biofilm was dominated by diatoms and filamentous green algae (*Cladophora sp*. and *Spirogyra sp*.). Biofilm chlorophyll-*a* content varied one order of magnitude among the different experiments and ranged from 1.62 to 14.1 µg chl-*a*/cm^2 (Table 2). The average ± SEM (n = 5) photosynthetic yield (Yield) of the biofilm before the solute additions was 0.51 ± 0.08 after one day, 0.52 ± 0.03 after two days and 0.51 ± 0.08 after three days in the channels system, indicating that the physiological state of the community was not changing in the course of the experiments. In addition, it did not change during the course of the additions. It was 0.51 ± 0.03 and 0.50 ± 0.02 before the Cu additions and at plateau conditions, respectively For PO_4^{3-} additions, Yield average values were 0.54 ± 0.03 and 0.52 ± 0.02 before the additions and at plateau conditions, respectively. Results from the T-test analysis did not show any significant difference in Yield between the two sampling times on each of the additions performed.

Table 2. Discharge (Q), chlorophyll content (Chl-*a*), uptake length (S_w) and areal uptake rate (U) obtained for each PO_4^{3-} and Cu addition experiment conducted at high (H) and low (L) flow, and with (B) and without (C) biofilm.

	Cu ADDITIONS				**PO_4^{3-} ADDITIONS**			
Treatment	**Q**	**Chl-*a***	**S_w-Cu**	**U-Cu**	**Q**	**Chl-*a***	**S_w-PO_4^{3-}**	**U-PO_4^{3-}**
	L/min	µg/cm^2	m	µgCu/ m^2 min	L/min	µg/cm^2	m	µgPO_4^{3-}/ m^2 min
LC	0.68	ND	89.3	0.01	0.82	ND	NS	NS
	0.73	ND	91.7	0.02	0.72	ND	NS	NS
HC	2.27	ND	163.9	0.04	2.13	ND	NS	NS
	2.36	ND	161.3	0.23	1.91	ND	NS	NS
LB	1.13	1.6 ± 0.7	15.2	0.29	1.02	2.6± 0.1	18.0	10.38
	0.79	11.8±1.5	21.3	1.51	0.7	7.0± 2.3	13.3	12.13
	0.76	14.1±1.6	19.3	0.34	0.95	12.8±1.7	8.5	21.28
HB	1.97	9.8 ± 1.4	67.6	1.11	2.05	4.5 ± 1.0	30.8	13.54
	2.09	11.9±4.7	39.4	0.41	2.12	7.0 ± 1.0	25.6	14.71

Values of chl-*a* are means ± standard error of the mean (SEM) of three replicates taken at plateau conditions. NS: not significant. ND: no data.

Copper and phosphate retention metrics

In the addition experiments with uncolonized substrata (i.e. control experiments), Cu concentration showed a significant decline along the channels at plateau conditions, whereas PO_4^{3-} concentration did not show any significant decline (Fig. 3). All the addition experiments performed in channels with colonized substrata showed a significant longitudinal decline for both PO_4^{3-} and Cu concentrations at plateau conditions (Fig. 3).

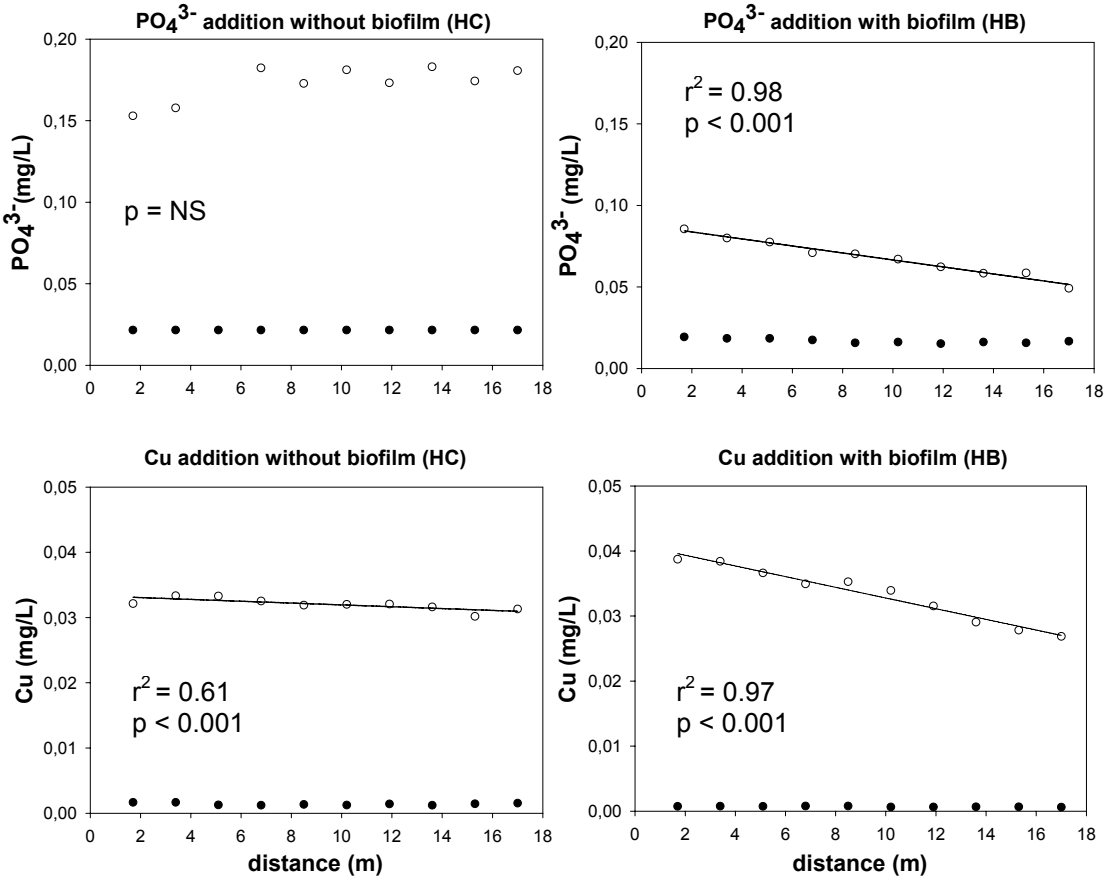


Figure 3. Illustrative examples of results from solute additions showing longitudinal variation of Cu and PO_4^{3-} concentrations along the channel system at background (solid circles) and plateau (open circles) conditions. Adjust (r^2) and significance (p) of the linear regression is also indicated.

Retention metrics for Cu and PO_4^{3-} obtained from the addition experiments are summarized in Table 2. Uptake length of Cu (S_w-Cu) ranged from 89.3 to 163.9 m in control experiments and from 15.2 to 67.6 m in channels with colonized substrata. Therefore, S_w-Cu was from 2 to 6 times shorter in the experiments with colonized substrata. Uptake length of PO_4^{3-} (S_w-PO_4^{3-}) was measurable only in channels with colonized substrata where it ranged from 8.5 to 30.8 m. S_w-Cu from control experiments was positively related to water discharge ($r^2 = 0.996$, $p < 0.01$; $n = 4$). Although a positive trend was observed between S_w-Cu and discharge in the experiments with colonized substrata, the relationship was not statistically significant ($r^2 = 0.65$, $p = 0.1$, $n = 5$). S_w-PO_4^{3-} from experiments with colonized substrata were positively related to water discharge ($r^2 = 0.80$, $p < 0.05$, $n = 5$).

In the experiments performed with biofilm, U-Cu ranged from 0.29 to 1.5 μgCu/m^2 min and U-PO_4^{3-} from 10.3 to 21.3 μgPO_4^{3-}/m^2 min. U-Cu in control experiments ranged from 0.01 to 0.23 and was on average c.a. 10 times lower than in experiments with colonized substrata. The highest U-Cu value measured in control experiments coincided with a 5 times increase in ambient Cu concentration (i.e., from < 0.27 to 1.4 μgCu/L). U-Cu did not show any significant relationship with chl-*a* content, whereas U-PO_4^{3-} was positively related with this parameter (r^2 = 0.87; $p < 0.05$; n = 5). Measurements of Cu accumulation in the biofilm showed higher concentration factors in the experiments performed under low flow (CF = 4.48 ± 0.20) than in those under high flow conditions (CF = 1.28 ± 2.23) (average ± SEM; n = 3).

Discussion

The experimental design used in the present study was addressed to quantify copper retention at microcosm scale considering both, downstream transport and the influence of fluvial communities on the retention of this element. To our knowledge, this ecological perspective has not been previously considered in copper retention studies.

Retention of copper was quantified by applying the concepts and methodologies comprised in the nutrient spiraling theory (Newbold et al., 1981; Stream Solute Workshop, 1990; Webster and Valett, 2006), and results were compared to retention measurements of phosphate, a macronutrient used as a reference. The concentrations of both solutes resulting from the constant-rate additions were high enough to detect longitudinal changes along the channel distance, but sufficiently low to avoid either positive or negative effects on the biota as indicated by the lack of significant effects on the photosynthetic activity of the biofilms during the additions.

The indoor channel system was sensitive enough to measure retention of the two solutes and to detect differences in their retention according to slight variations in hydrological and biological conditions. Phosphate uptake length was only measurable in experiments with colonized substrata evidencing the relevance of biofilms in controlling retention of this element. Values of phosphate uptake length were short and within the range of other phosphate

dynamics studies performed in artificial channels (D'Angelo et al., 1991). This indicates that the system was highly efficient retaining phosphate. We were also able to quantify copper retention, the main aim of this investigation. In contrast to phosphate, copper uptake lengths were measurable in experiments performed with and without biota. However, values of copper uptake length were shorter in the presence of biofilms, indicating that retention efficiency for this element was enhanced by the presence of stream-bed biota (microbenthic biofilms). This increase may be related to a greater availability of metal binding sites and also to the indirect effects of biotic activity (photosynthesis), since photosynthesis causes an increase in the local pH which can modify metal solubility and bioavailability (Guasch et al., 2002). The relatively low DOC concentration of the water used for the experiments, suggests that precipitation of Cu-organic complexes was unlikely under our experimental conditions (Breault et al., 1996) thus it is not expected to contribute in the Cu removal from the water column.

The abiotic retention differences between Cu and phosphate may be due a) to their anionic and cationic behaviour, b) to specific binding sites for Cu in the channel system, or a combination of both. The role of the cationic/anionic behaviour can not be derived from our results. This could be addressed with abiotic experiments using other metallic anions (chromium).

In fact, using results of copper uptake rates from experiments with and without biota we estimated that 89.8% of the retention was driven by the biofilm while the remaining 10.2% could be attributed to physical adsorption onto the walls of the channels, carboys and tubes. Peña-Castro et al. (2004) measured the capacity of heavy metal removal by the microalga *Scenedesmus incrassatulus* in continuous culture assays and found similar results relative to biotic copper removal in their experiments, accounting for 72% of the total copper removal contrasting with the 28% attributed to abiotic factors. These results together with ours indicate the relevance of the biotic control on dynamics of copper under sub-lethal concentrations (<40µg Cu/L) in aquatic systems. Nevertheless, retention efficiency for copper was 1.7 times lower than that for phosphate in the experiments with colonized substrata. A different demand of the stream-bed biota for the two solutes may explain this difference. Phosphate is a macronutrient, and it is well known that algae have mechanisms

to retain it very efficiently (Doods, 2003), whereas copper is a micronutrient. Therefore, biological requirements for phosphorus are expected to be higher than those for copper. Although differences between Cu and P demand have not been directly assessed in periphyton, marine plankton studies reported average Cu:P ratio between 0.4 and 0.5 mmol/mol (Martin et al., 1976; Ho et al., 2003). This low value of the ratio supports our statement since Cu requirement of algae is about 1000 times lower than the requirements for phosphorus.

The experimental setup also allowed examining the influence of hydrologic (water discharge) and biological factors (algal biomass) on the retention of the studied solutes. Retention efficiencies for both solutes were influenced by water discharge, being 2 and 3 times lower for phosphate and copper respectively, in the experiments conducted under higher flow conditions. These results agree with previous findings from stream nutrient retention studies (Butturini and Sabater, 1998; Peterson et al., 2001; Hall et al., 2002). Most biological activity in small streams is associated to the stream benthos where a higher discharge reduces the surface-volume ratio and decreases nutrient exchange with benthic microbial communities; thus, reducing nutrient retention efficiencies (Martí et al., 2004). Our results indicate that copper retention may also be subjected to this hydrological control in streams. The lack of relationship found between U-Cu and algal biomass combined with the lower Cu retention found under high flow conditions and the higher accumulation of copper (as measured by the concentration factor) found in the biofilm exposed to lower flow conditions supports this hypothesis.

In the range of algal biomass measured in the experiments, phosphate uptake rates increased with increasing chl-*a* content. This finding agrees with previous studies and demonstrates the important role of algal activity in controlling nutrient dynamics (Grimm et al., 1981; Triska et al., 1983; Tate, 1990, Grimm, 1992). As it is reviewed in Doods (2003), phosphate uptake is influenced by periphyton biomass and metabolic activity, and advective transport of phosphorus to periphyton assemblages. Sabater et al. (2000) found that phosphate mass-transfer velocity (which also indicates nutrient retention efficiency) was positively correlated with primary production in a forested Mediterranean stream. In addition, in calcareous streams periphyton activity can

enhance phosphate retention by increasing pH and thus inducing phosphate co-precipitation with calcium carbonate (Martí and Sabater, 1996). Finally, Mulholland et al. (1994) documented that thick periphyton masses increased six times phosphate retention compared to thin biofilms by increasing the relative area of dead zones and enhancing internal cycling and retention. All these above mentioned processes contribute to an increase of the short-term phosphorus retention; and thus, it is not surprising that phosphate uptake from our microcosm experiments gradually increased with algal biomass.

Despite the difference in copper retention efficiency between the colonized and uncolonized experiments, we did not find any relationship between copper uptake rates and algal biomass.

Some studies report the influence of biomass concentration on metal removal, however no consistent results are found in this issue (Mehta and Gaur, 2005). Reduced metal adsorption per unit of biomass was found when algal biomass was higher, and was related to a decreased sorption capacity of the biofilm (Hamdy, 2000; Gong et al., 2005). However other studies reported increased metal removal at higher biomass concentration (Hill et al., 2000; Mehta and Gaur, 2001).

The duration of the copper additions in our experiments may have been too short to observe the diffusion effect, limiting copper uptake mostly to the surface layer of the biofilm regardless of its thickness. This phenomenon has been described in other studies (Barranguet et al., 2002) and would explain the lack of relationship between copper uptake rate and algal biomass in our experiments.

The use of biofilm communities in our study offers a more realistic view of the processes involved in the downstream transport of heavy metals compared to the commonly used laboratory experiments focused on estimations of metal uptake rates or bioaccumulation kinetics by algal cultures and periphyton (e.g. Campbell, 1995; Vigneault et al., 2000; Meylan et al., 2003; Morin et al., 2008). The results obtained evidence that this experimental design provides a relatively simple but ecologically relevant approach to investigate dynamics of toxicants in fluvial systems and the influence of biofilm as well as other ecologically relevant factors on them.

The quantification of biotic and abiotic metal retention in a simplified river system, including its downstream transport, provides an ecological perspective based on fluvial ecology concepts. This approach complements the information obtained from existing studies addressing the transport of metals in fluvial systems (e.g. Runkel, 1995; Ciffroy et al., 2000) and the retention of metals by algae (e.g. Knauer et al., 1997; Sunda and Huntsman, 1998). We foresee future applications of the conceptual and experimental approach considered to further investigate the influence of different environmental factors on copper dynamics, the effects on the benthic microbial communities and the dynamics of other toxic compounds.

Having into account the observations of this study, which have been done in an artificial river, we can tentatively extrapolate them to a real field situation. Overall it is expected that biofilms will have an important role on Cu retention and that this will be increased under low flow conditions. This approach may contribute notably to increase our understanding on the fate of toxicants entering the stream ecosystem.

Chapter III: Effects of chronic copper exposure on fluvial systems: linking structural and physiological changes of fluvial biofilms with the dynamics of toxicants

Introduction

The aquatic environment is highly affected by metal pollution, as it is an ultimate receptor of urban wastewater, industrial and mine effluents, agriculture runoff and atmospheric deposition (Nriagu, 1979). Copper is one of the most important and potentially toxic metallic pollutants (Chang and Sibley, 1993; De Oliveira-Filho et al., 2004).

Periphyton communities, also called phototrophic biofims, are the main primary producers of most of the fluvial ecosystems and they are the first to interact with dissolved substances such as nutrients, organic matter, and toxicants (Sabater et al., 2007).

Long-term metal exposure, and copper in particular, is known to be responsible for a large variety of structural and functional changes in periphyton communities (e.g. Soldo and Behra, 2000; Guasch et al., 2002, Barranguet et al., 2002). A frequent consequence of chronic chemical exposure is the replacement of sensitive individuals or species by tolerant ones (Foster, 1982; Deniseger et al., 1986; Lindstrom and Rorslett, 1991). Thus, the tolerance of a community previously exposed to a toxicant for the long term is expected to be greater than that of a community that has never been exposed (Soldo and Behra, 2000).

Several mechanisms have been described as responsible for algal survival in metal-polluted environments; these mechanisms include the increased production of extracellular metal chelators (Gerringa et al., 1995; Moffett and Brand, 1996; Gledhill et al., 1999; Leal et al., 1999; Croot et al., 2000), immobilization of metals through binding at the cell surface or to mucilage (Brown et al., 1988; Gonzalez- Davila et al., 1995; García-Meza et al., 2005). In other cases, tolerance to copper is effective through internal mechanisms of storage and detoxification (Soldo et al., 2005).

The importance of periphyton and other algae in retaining heavy metals from the water column have been extensively described in several studies (Newman and McIntosh, 1989; Liehr et al., 1994; Farag et al., 1998; Wright and Mason, 1999; Behra et al., 2002; Meylan et al., 2003). Most of the studies focusing on the accumulation of pollutants by biofilms are available mainly from laboratory studies with defined conditions. Investigations on natural river

ecosystems or at a micro/mesocosm scale are scarce; however, they may be an important key to the understanding of loadings, transport and sink of pollutants in the aquatic system (Schorer and Eisele, 1997).

In a previous study (Chapter II), the transport and retention of copper in an artificial river system was explored by applying the concepts and methodologies described for the study of nutrient dynamics in streams (Newbold et al., 1981; Stream Solute Workshop, 1990). That study highlighted the relevance of the biofilm in retaining the metal from the water column as well as the influence of the water flow and algal biomass on it. It was conducted using communities that had never been pre-exposed to copper.

The present investigation aims to elucidate if changes in the periphyton community derived from chronic copper exposure will have repercussions on the copper retention efficiency of the system.

In order to achieve this goal, periphyton communities were chronically exposed to copper in an artificial river system. Structural and functional parameters of the biofilm were compared to a non-exposed community in order to detect the effects of chronic copper exposure. The artificial river system used allows replicated treatments (in recirculating mode) to be performed and also the toxicant dynamics (using the system in one-through flow mode) to be studied, enabling us to evaluate in the same study the effects of chronic copper exposure on periphyton structure as well as their influence on the dynamics of the toxicant in a water flowing system. Furthermore, periphyton communities were also exposed to a higher (potentially toxic) copper concentration to investigate tolerance induction after chronic exposure.

Since copper retention depends on the concentration gradient between the overlaying water and the sediment (Sloof et al., 1989) it is expected that in-stream Cu retention efficiency might be reduced after chronic Cu exposure of the system. On the other hand, it is also expected that chronic Cu exposure will cause changes at the community level and/or the activation of detoxification mechanisms which may influence the retention efficiency of the system. Chronic metal exposure may cause an increase in the metal binding sites of the biofilm linked to the enhancement of EPS production (García-Meza et al., 2005) as a detoxification mechanism. If this is the case, an increase in Cu retention efficiency could easily occur.

Several studies described that metal exposure can suppress algal macronutrient assimilation (Singh and Yadava, 1983; Rai et al., 1998; Barranguet et al., 2002; Mosulén et al., 2003; Kaneko et al., 2004; Miao and Wang, 2006). Thus, phosphate in-stream retention was also evaluated in this study since it might be directly affected by Cu pre-exposure resulting in a decreased P-uptake. It may also cause indirect effects since chronic Cu exposure is expected to decrease algal biomass (Barranguet et al., 2002) which can result in a decreased P-demand.

Materials and methods

Experimental setup

Two consecutive sets of experiments were performed in indoor experimental channels in order to measure the efficiency of phosphate and copper retention and Cu sensitivity of different periphyton communities. These communities differed in their Cu exposure during growth.

The experiments were carried out in the same artificial river system described in Chapter II, using two different setups (Fig. 1). In the present study, experimental channels were used in recirculating mode for the colonization of periphyton under different Cu conditions, in one-through flow mode for studying PO_4^{3-} and Cu retention, and changed again to recirculating mode for short-term exposure to higher Cu concentration (Fig. 2).

In the recirculating mode, 10L of carbon-dechlorinated tap water were recirculated at a rate of 1L/min from a carboy located at the end of each channel through centrifuge pumps. In the one-through flow mode, the tap water was directed from one carboy to the next channel through a centrifuge pump at a rate of 1L/min using tubes which connected each channel unit with the following one, as described in the previous chapter (see Figs. 1 and 2 in Chapter II).

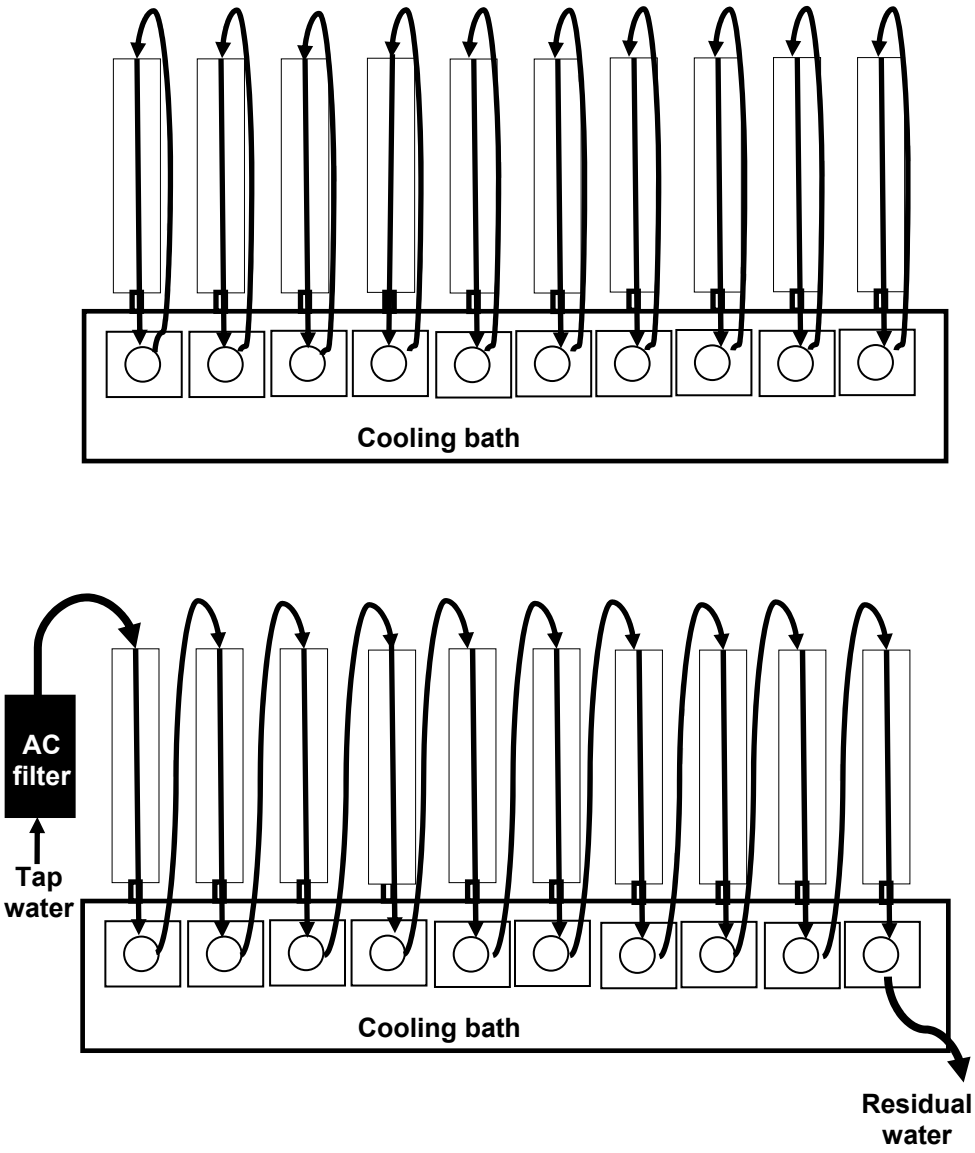


Figure 1. Scheme of the experimental channels in recirculating mode used during the colonization of periphyton and short-term Cu toxicity experiment (upper panel) and one-flow through mode used during the Cu and PO_4^{3-} retention experiments (lower panel).

Ten channels were used for the colonization of periphyton that lasted for four weeks. This experimental setup facilitated the control of water chemistry, the periodic renewal of water and the addition of new nutrient and Cu (in the +Cu growth treatment) to the carboys.

At the end of colonization, several Cu and PO_4^{3-} retention experiments were done. For this purpose, the ten channels were interconnected. Three constant-rate additions of PO_4^{3-} and Cu were consecutively performed for each

treatment (-Cu growth and +Cu growth). Before each constant-rate addition, dechlorinated tap water (without Cu) was circulated through the channels during three days to allow the remaining Cu from previous additions to be cleaned.

After the retention experiments, the colonized substrata were distributed in six channels for short-term exposure to higher Cu concentration (100 µg/L, nominal concentration) using the recirculating mode. This short-term exposure experiment was also used to investigate Cu-accumulation kinetics as described in Chapter IV. Three channels were used as controls and three with high Cu concentration.

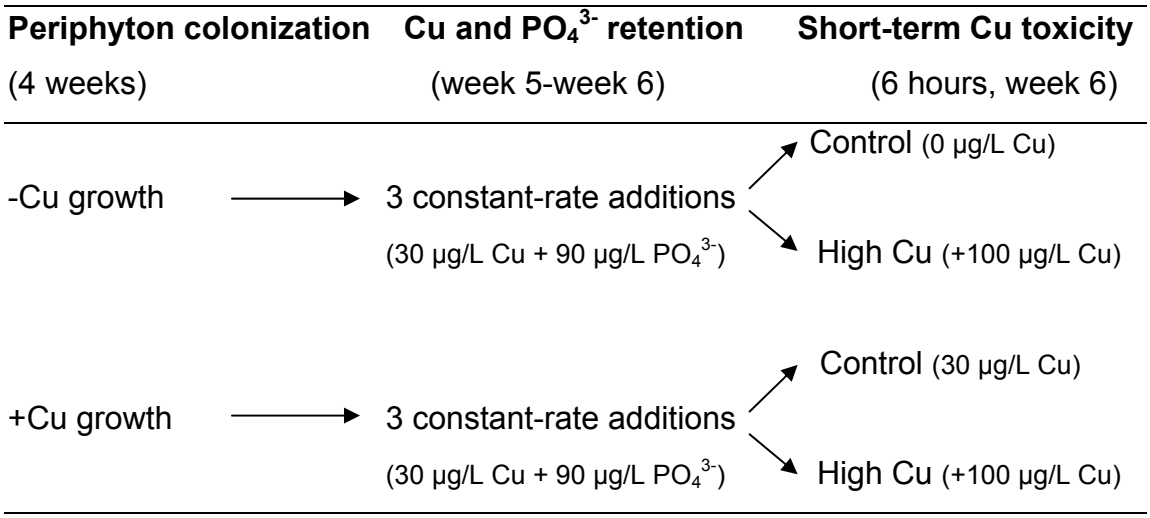


Figure 2. Scheme of the experimental setup including the duration and conditions (nominal concentrations) of each experiment

Periphyton colonization

Periphytic communities were allowed to colonize the surface of etched glass substrata (8.5x12 cm) placed at the bottom of each channel for four weeks in order to obtain mature periphyton communities. Laboratory periphyton communities were obtained from field biofilm inocula. The inocula were always taken from the Llémena River, a small calcareous tributary of the Ter River (North-East Spain), after scraping three cobbles. New inocula were provided weekly to each channel during the four weeks of colonization.

Two colonizations were conducted: one with no addition of copper and referred to as –Cu growth, and the other with a continuous supply of 30 µgCu/L (nominal concentration) and referred to as +Cu growth.

During the two colonization periods, water from the channels was completely renewed three times a week, and 30 µg/L of phosphate (nominal concentration) was added as KH_2PO_4 (Merck, Darmstadt, Germany) to avoid nutrient depletion. In the second colonization, additionally, 30 µg/L of copper (nominal concentration) was added as $CuCl_2$ (copper titrisol, Merck, Darmstadt, Germany). Before and after each water renewal, water samples were taken to determine phosphate (SRP) and dissolved copper (Cu) concentrations, and physicochemical parameters (temperature, pH, dissolved oxygen and conductivity) were measured.

Biofilm measurements

At the end of each colonization period, three colonized substrata were taken randomly for fluorescence measurements. The percentage of the different algal classes from each mature community was estimated as the chlorophyll fluorescence (Fo) measured at different wave lengths using a Pulse of Amplitude Modulated fluorometer (PHYTO-PAM Heinz Walz GmbH, Effeltrich, Germany) see “fluorescence measurements” section.

As PAM fluorometry is a nondestructive technique, the same periphyton samples were used for algal biomass determination which was estimated as the chlorophyll-*a* concentration (chl-*a*) after extraction with 90% acetone and spectrophotometric measurements (Jeffrey and Humphrey, 1975).

Cu and PO_4^{3-} retention experiments

After the colonization period, the 10 channels were interconnected obtaining a one-flow through system to conduct the retention experiments (Fig. 1) following the method described in Chapter II (Constant-rate solute additions section) with small differences. On this occasion, copper and phosphate were added together in order to investigate the dynamics of both solutes at the same time. In a preliminary investigation, copper and phosphate were added separately and the retention parameters were compared with additions done with both solutes added together. Results from this preliminary study confirmed

that Cu and PO_4^{3-} retention did not differ between single and combined phosphate and copper additions at the conditions tested. PO_4^{3-} uptake rate (U-PO_4^{3-}) resulted in 14.41 ± 4.17 μgPO_4^{3-}/m^2 min and 15.69 ± 11.31 μgPO_4^{3-}/m^2 min (AVG ± SEM) in single and combined additions respectively; and Cu uptake rate (U-Cu) resulted in 0.73 ± 0.55 μgCu/m^2 min and 0.27 ± 0.11 μgCu/m^2 min. The calculation procedure and meaning of this parameter is detailed in Chapter II (Hydrology and solute retention calculations section).

This previous study also showed that constant-rate additions of the concentrations of Cu and PO_4^{3-} used did not have negative effects on periphyton, as photosynthetic activity of periphyton measured after solute additions did not differ from the measurements done before the additions.

A total of six constant-rate Cu-PO_4^{3-} additions were performed: three at the end of –Cu growth colonization and three at the end of the +Cu growth colonization.

Nutrient uptake length (S_w), the average distance travelled by an element before it is removed from the stream water column as a result of benthic abiotic or biotic processes (Stream Solute Workshop, 1990; Newbold, 1992), was used as an index of the stream nutrient and copper retention efficiency as described in Chapter II. Shorter uptake lengths indicate higher retention efficiencies than longer distances.

Another useful retention metrics used in the present study was the mass transfer coefficient (Vf) which was calculated as follows (see equation1):

$$Vf = Q / (S_w . w) \quad (1)$$

where *Q* is the average discharge (m^3/s), and *w* is the channel width (m). This parameter indicates the vertical velocity at which a nutrient molecule travels from the water column to the benthos, and thus, represents the demand of nutrients relative to the concentration in the water column (Stream Solute Workshop, 1990). Vf is a useful measure for isolating and comparing the effect of biochemical processes on streams (Wollheim et al., 2001; Hall et al., 2002) because this parameter removes the effects of discharge (Stream Solute Workshop, 1990).

Biofilm measurements

Once each solute addition was finished, three colonized glass substrata from three different channels were removed to determine total Cu content in biofilms and intracellular Cu concentration. For this purpose, periphyton was scratched from the glass substrata with a cell scraper (Fig. 3) and suspended in 150 mL of dechlorinated tap water previously filtered using a 0.2 µM filter. This suspension was then divided into two fractions. One fraction was treated for 10 minutes with 4.0 mM EDTA (final concentration) in order to measure intracellular Cu content, and the other fraction was used to measure the total amount of Cu accumulated in the periphyton. Each fraction was kept in continuous shaking in order to allow a homogenous periphyton suspension.

Three aliquots (10 - 15 mL) exceeding the 0.04 mg/L from each fraction were taken and filtered on a previously acid-washed and weighted filter membrane (cellulose nitrate 0.45 µm, Whatman). After 15 h drying at 50 °C, the periphytic material left on the filter was weighted and the dry weight (DW) was deduced, following Meylan et al. (2003). Filters containing dry periphyton were digested with 4 mL of concentrated nitric acid (suprapure) and 1 mL of hydrogen peroxide (31%, suprapure) in a high-performance microwave digestion unit (Milestone, Ethos Sel) for Cu analysis. After dilution to 25 mL with MiliQ water, samples were stored in the fridge until they were analysed following the same procedure as for total dissolved Cu concentration in water samples.

The accuracy of the analytical methods was checked periodically using two certified reference materials (Riverine Water Reference Material for Trace Metals; NRC-CNRC, Ottawa, Canada, and Trace Elements in Plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

Three more colonized glass substrata were collected for extraction of extracellular polymeric substances (EPS) using a cation exchange resin following Romaní et al. (2008).

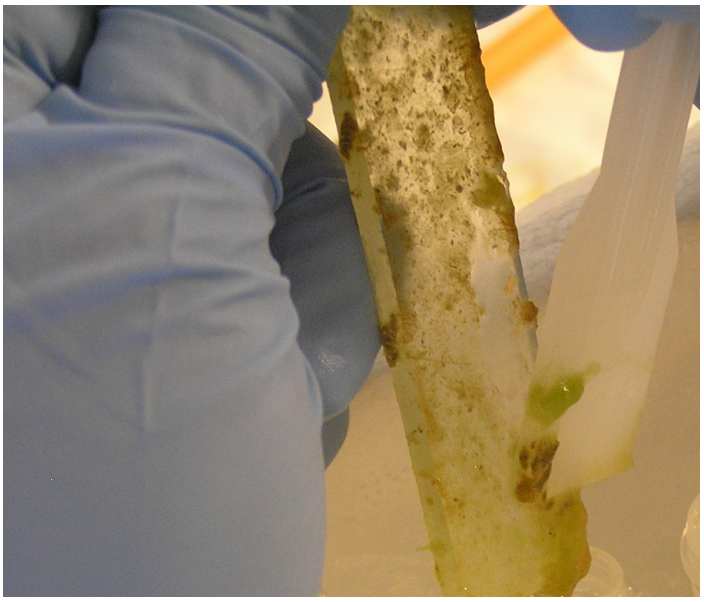


Figure 3. Detail of the periphyton scratching for Cu analysis.

Laboratory analyses

Phosphate concentration, anions, cations, chloride and dissolved Cu were analysed following the procedures described in Chapter II (laboratory analyses section).

Short-term Cu toxicity

Both periphyton communities (-Cu growth and +Cu growth) were exposed to 100 µgCu/L (nominal concentration) during 6 hours in order to explore possible changes in their Cu sensitivity due to their Cu conditions during growth (Figure 2). This concentration is close to the measured EC_{50} for periphyton (Navarro et al., 2002; Guasch et al., 2004) thus, it is expected to cause toxicity, at least to the –Cu growth community.

The effects of the acute Cu exposure on the algae were evaluated measuring the effective quantum yield (Y) as a measure of the photosynthetic efficiency using the phytoPAM fluorometer (see Fluorescence measurements section).

Fluorescence measurements

Chlorophyll fluorescence of the different algal classes

Chlorophyll fluorescence emission was measured using the Phyto-PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). This pulse-amplitude-modulated fluorometer is based on a fluorescence method described by Schreiber (1998). It employs an array of light-emitting diodes (LED) to excite chlorophyll fluorescence at different measuring lights (470, 520, 645 and 665 nm), and to illuminate samples with actinic light and saturation pulses. The deconvolution of the overall fluorescence signal into the contributions of three algal groups is based on the internal 'reference excitation spectra' of a pure culture (Schmitt-Jansen and Altenburger, 2008). The differences in pigment composition of the antenna complexes of photosystem II can be determined because the shapes of the excitation spectra depend on the spectra of three algal groups (Ruser et al., 1999). Reference spectra which have previously been validated for periphyon communities were used (Schmitt-Jansen and Altenburger, 2008). The fluorescence linked to cyanobacteria class, referred to as F(Bl), the fluorescence linked to green algae class, referred to as F(Gr) and the fluorescence linked to diatom class, referred to as F(Br), were used for evaluating the relative contribution (in percentage) of each algal class to the whole community.

Effective quantum yield (Y)

The measurements of in vivo chlorophyll fluorescence of PSII were used to estimate F, which corresponds to the steady-state fluorescence in the given actinic irradiance, and F'm, which refers to the maximum fluorescence yield of an actinic-adapted sample. These two parameters were used to calculate the effective quantum yield (Y) according to Genty et al. (1989) (see equation 2).

$$Y = \frac{(F'm - F)}{F'm} \qquad (2)$$

In our study, Y was based on the fluorescence obtained with 665 nm light-emitting diode (F4). Y measurements were used to follow changes in the photosynthetic efficiency of the communities after the short-term Cu exposure.

The measurements were performed at room temperature (20 ºC). Saturation pulses were applied in the same actinic light conditions as the ones used for periphyton colonization (90-115 µmols photons/m^2 s).

Statistical analyses

Differences in water physicochemical parameters between treatments were examined using one-way ANOVA by means of SPSS software. One-way ANOVA was also used to examine differences in algal composition, algal biomass, and EPS production, as well as to detect differences in the retention parameters of phosphate and copper between the two treatments. Differences in copper tolerance of both communities were analysed using two-way ANOVA. When significant interaction ($p < 0.05$) was found, data were analysed by a Tuckey-Honest significant difference test (Winer, 1971).

Results

Physicochemical conditions during colonization

Chemical composition of the dechlorinated tap water used in the experimental system is summarized in Table 1.

Table 1. Concentration of the major anions and cations, dissolved inorganic and organic carbon (DIC and DOC) in the water before entering the channels system.

Parameter	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	Ca^{2+}	Mg^{2+}	Na^+	Cl^-	DIC	DOC
Concentration (mg/L)	12.73	<0.01	<0.1	43.74	33.38	8.43	27.12	46.64	27.83	2.81
SD	3.58			1.03	1.27	0.35	1.70	0.73	0.18	0.13

Values are average and standard deviation (SD) of twenty samples.

Physicochemical parameters measured during the colonization of periphyton are summarized in Table 2. In both experiments, the temperature was kept around 19 ºC, pH ranged between 7.8 and 8.4, conductivity was between 409 and 468 µS/cm, oxygen near saturation and phosphate concentration around 35 µg/L. The average of initial and final values illustrates

changes in water chemistry after three days of recirculation in the channels: the temperature slightly decreased (1ºC), phosphate decreased and pH increased. On the other hand, conductivity and dissolved oxygen did not show any significant variation between water renewals. While temperature and phosphate concentration were similar between the control and Cu treatments, pH, conductivity and dissolved oxygen were, on average, slightly higher in the control but differences were always below 20% (Table 2).

Table 2. Average ± standard deviation of physicochemical parameters measured during the two colonization periods before (final) and after (initial) water renewals (n = 11 for the –Cu growth colonization and n = 10 for the +Cu growth colonization). Significance of ANOVA comparing physicochemical parameters of water between treatments is also presented in the table.

Treatment	Time	Temp (ºC)	pH	Cond (µS/cm)	oxy (mg/L)	conc. PO_4^{3-} (µg/L)	Conc. Cu (µg/L)
-Cu growth	Initial	19.28±0.49	8.25±0.14	468±17	9.74±0.28	35.32±21.14	0.88±0.16
	Final	18.95±0.61	8.38±0.17	451±33	9.83±0.23	23.05±10.17	N.M.
+Cu growth	Initial	19.46±0.64	7.80±0.59	409±6	9.45±0.24	36.08±20.30	32.50±36.36
	Final	18.84±1.43	8.34±0.33	416±12	9.50±0.43	11.41±7.29	13.52±14.86
ANOVA	Treatment	N.S.	<0.005	<0.005	<0.005	N.S.	

N.S.: not significant. N.M.: not measured.

Periphyton community characterization

At the end of each colonization period, the community chronically exposed to Cu had a lower algal biomass than the unexposed one ($F_{1,16}$ = 6.839; $p < 0.05$). It was 0.80 ± 0.17 µg chl-*a*/cm^2; (AVG ± SE; n = 9), a 42% lower than the control community (1.37 ± 0.14 µg chl-*a*/cm^2).

The fluorescence signals of the periphyton community also showed differences between colonization treatments. The +Cu growth community showed a higher percentage of fluorescence linked to green algae ($F_{1,5}$ = 50.29; $p < 0.005$) and a lower percentage of fluorescence linked to brown algae ($F_{1,5}$ = 86.99; $p < 0.001$) than the control community. On the other hand, the blue

green algae fluorescence signal did not differ between colonization treatments (Fig. 4).

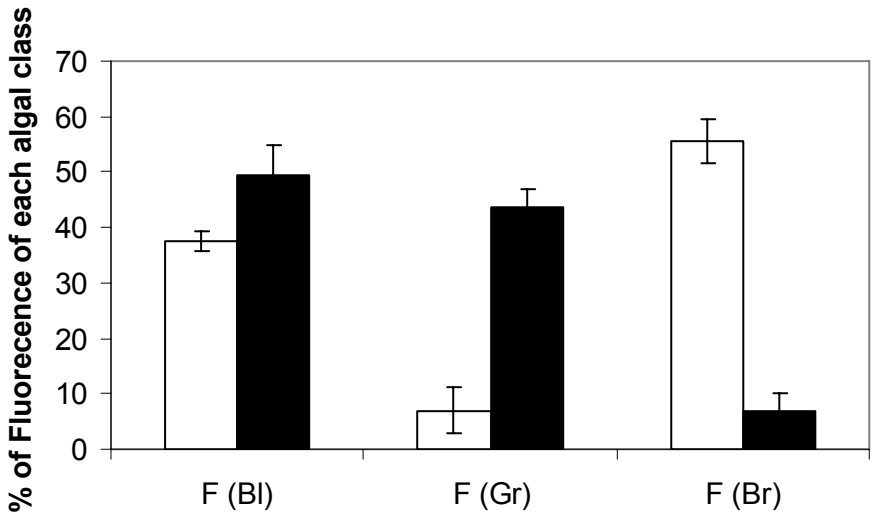


Figure 4. Mean and standard error of the percentage of fluorescence (F) corresponding to each algal class (Bl, blue-green algae; Br, green algae; Br, brown algae) of the -Cu growth community (white bars) and +Cu growth community (black bars) (n = 3).

EPS content of the +Cu growth community was 251.57 ± 59.70 µg gluc-eq/cm^2 being 1.7 times higher than in the unexposed community which was 143.71 ± 23.98 µg gluc-eq/cm^2 (AVG ± SE; n = 9). These differences were not statistically significant ($F_{1,16}$ = 2.789, p = 0.114).

Both communities also differed in their copper content (Fig. 5). Copper concentration of periphyton after the +Cu growth colonization treatment was almost ten-fold higher than the –Cu growth treatment. The percentage of the intracellular fraction of copper in relation to the total copper content was similar in both communities: 74 % in the –Cu growth and 77% in the +Cu growth community.

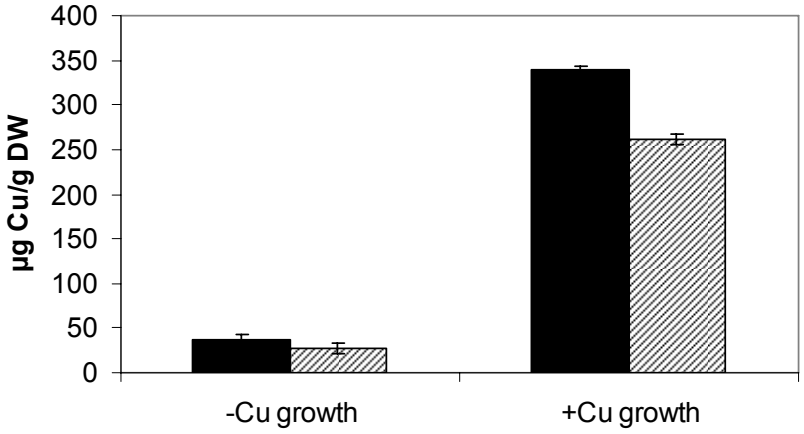


Figure 5. Mean and standard error of the total (black bars) and intracellular Cu content (dashed bars) of the periphyton communities after five weeks in the channels (n = 9).

Copper and phosphate retention

The solute additions performed during the experiments resulted in an increase up to 88.83 ± 26.11 µg/L PO_4^{3-} and up to 29.30 ± 10.39 µg/L Cu (AVG ± SD; n = 6) at plateau.

Cu uptake lengths (S_w-Cu) of the chronically exposed community were longer than Cu uptake length of the unexposed community ($F_{1,4}$ = 123.01; $p < 0.001$). PO_4^{3-} uptake lengths (S_w-PO_4^{3-}) were similar among treatments ($F_{1,4}$ = 1.53; p = 0.28) (Table 3).

The mass transfer velocity followed the same pattern as uptake lengths. Vf-PO_4^{3-} were similar comparing both treatments ($F_{1,4}$ = 2.12; p = 0.22), and, Vf-Cu was two-fold lower in the +Cu growth treatment than in the –Cu growth one ($F_{1,4}$ = 17.06; $p < 0.05$) (Table 3).

Table 3. Summary of hydrologic and retention parameters for phosphate (PO_4^{3-}) and copper (Cu) measured in the -Cu growth and +Cu growth treatments. Q (discharge), Vel (average water velocity), Cb (background concentration), S_w (uptake length), Vf (mass transfer velocity).

Treatment	Q (L/min)	Vel (cm/s)	Cb-PO_4^{3-} (µg/L)	S_w-PO_4^{3-} (m)	Vf-PO_4^{3-} (m/s)	Cb-Cu (µg/L)	S_w-Cu (m)	Vf-Cu (m/s)
-Cu growth	0.85	0.73	27.37	6.55	3.18E-05	1.73	13.19	1.20E-05
	(0.12)	(0.06)	(6.37)	(4.37)	(2.02E-05)	(0.32)	(1.43)	(2.45E-06)
+Cu growth	0.76	0.83	11.32	10.26	1.44E-05	4.12	22.84	6.16E-06
	(0.05)	(0.06)	(4.07)	(2.78)	(3.51E-06)	(0.58)	(0.47)	(3.23E-07)

For each treatment, data are mean values and standard deviation (in brackets) of the three independent constant-rate additions.

Short-term Cu toxicity

Cu concentration during short-term Cu exposure experiments was close to nominal concentration. It was 95.30 ± 2.52 µg/L in the short-term exposure experiment carried out with the –Cu growth community and 92.07 ± 0.23 µg/L (AVG ± SD) in the experiment done with the +Cu growth community.

The photosynthetic efficiency (measured as photon yield, Y) of the –Cu growth periphyton community resulted significantly reduced after the short-term Cu exposure ($F_{1,16} = 13.275$, $p < 0.01$) . On the other hand, no significant effect of the short-term Cu exposure was observed in the +Cu growth community ($F_{1,16} = 0.477$, $p = 0.50$). Moreover, under control conditions, both communities showed similar photosynthetic efficiency values in spite of their different exposure conditions during colonization (Fig. 6).

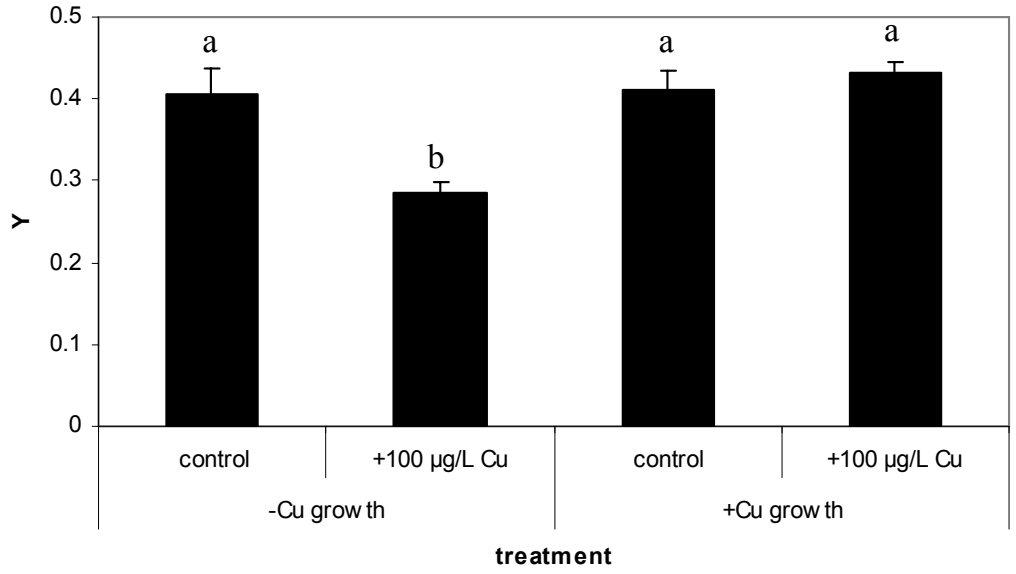


Figure 6. Mean Yield values (± SE) under four treatments (control and short-term Cu exposure for each experiment). Different lower-case letters above bars indicate significant differences among treatments (Tukey's HSD).

Discussion

In agreement with our hypothesis, chronic Cu exposure caused clear effects on the structure of periphyton, affecting the Cu retention capacity of the system.

Effects of chronic Cu exposure on periphyton

Chronic Cu exposure caused a clear algal growth reduction, leading to a lower accumulation of algal biomass in the exposed compared to the unexposed community after four weeks of colonization. A more drastic reduction in algal biomass due to chronic metal exposures has been reported in other studies at lower Cu concentrations compared to our findings. A reduction of 50% in algal biomass was observed under 10 µg/L of copper in periphyton communities from oligotrophic stream sites (Guasch et al., 2002). In the experiments reported here, a reduction of 42% in algal biomass occurred at higher copper concentrations (32.5 µg/L) indicating greater tolerance of the benthic communities compared to previous observations (Guasch et al., 2002).

In a more recent study, Guasch et al (2004) reported that fertilization (P-addition) previous to copper exposure was responsible for a marked reduction in copper toxicity indicating a negative relationship between copper toxicity and P-availability, as is also reported by Hall et al. (1989). They conclude that this interaction between P and Cu allows the growth of algae under high nutrient conditions in spite of the presence of potential copper concentrations. According to these findings, the P-rich conditions (higher than 30 µg/L PO_4^{3-}, considered above the oligotrophic boundary proposed by Dodds et al., 1998) in which our communities have been developed, might allow the development of mature periphytic communities, even though they have been chronically exposed to relatively high copper concentration.

Chronic Cu exposure also caused a shift in the dominant algal groups leading to a replacement of diatoms (brown algae) by green algae, while no changes were observed in the proportion of cyanobacteria of the community.

Changes in the community composition have been pointed out by several authors as a vital element in determining metal tolerance (Say and Whitton, 1981; Clements and Kiffney, 1994; Admiraal et al., 1999; Barranguet et al., 2000; Guasch et al., 2004). For example, Genter et al. (1987) observed a shift in community composition from diatoms to green algae and cyanobacteria, when exposed to zinc. Soldo and Behra (2000) reported a shift from a community dominated by cyanobacteria to one dominated by chlorophyta (Green algae). These findings, like the ones observed in our investigation, reflect the high tolerance of green algae to copper and other metals (Foster, 1982). However, the change in the structure of the community is not consistent between different studies reported in the literature. For instance, other studies reported changes in the periphyton community from diatoms to cyanobacteria after long-term copper exposure (Barranguet et al., 2000 and 2002; Roussel et al., 2007).

The –Cu growth and +Cu growth communities differed in terms of their composition (as discussed above) but had similar photon yield (see the controls in Fig. 6). This result indicates that the chronically-exposed community was able to cope with copper exposure during growth reaching a relatively high photosynthetic efficiency after long exposure due to adaptation. Our

observations, like the ones found in García-Meza al. (2005), support the hypothesis by Guasch et al. (2002) that structural changes are more prominent than physiological damage, such as a lowered photosynthetic efficiency, because of toxic metal exposure. According to this hypothesis, the biofilm will maintain certain photosynthetic activity, although a large part of the biomass (mostly composed of sensitive species) will be lost due to long-term metal exposure. Therefore, the biofilm is adapted to the prevailing high metal concentration. This adaptation was shown in the short-term test, since the chronically-exposed community was much more tolerant to a short-term copper exposure to high Cu concentration than the unexposed community, indicating an induction of copper tolerance after chronic exposure. All these studies, including our findings, are in agreement with the pollution-induced tolerance (PICT) concept (Blank et al., 1988) which implies an increase in tolerance under exposure of toxicants provoked by a shift of the community by an increasing abundance of tolerant species (Gustavson and Wängber, 1995).

Another effect of chronic Cu exposure found in the present investigation was the increase in EPS content of the benthic community. As described, extracellular polymeric substances (EPS) potentially act as detoxification agents against metals, because they contain high amounts of negatively charged functional groups such as carboxyl, phosphate, and sulphate groups, acting as metal-binding sites (Kaplan et al., 1987; García-Meza et al., 2005). The protective role of the matrix of the biofilm has been attributed to local pH and hypoxia conditions in the internal layers of the biofilm (Teissier and Torre, 2002), which modify the redox conditions and, possibly, cell bioaccumulation potentials. It is generally accepted that exopolysaccharides secreted by algal and bacterial communities (Pistocchi et al., 1997; Decho, 2000; Muller et al., 2005; Morin et al., 2008) also play a role in binding metals, thus reducing their bioavailability and toxicity toward live cells.

An increase in EPS production as a response to metal exposure has been described in various studies of phototrophic biofilms and algal cultures (Vymazal, 1984; Pistocchi et al., 1997; García–Meza et al., 2005). As found by Lombardi et al. (2002) EPS may enhance the extracellular metal accumulation and immobilization, even on the relatively short-term exposure of five days. García–Meza et al., (2005) observed an increase from 2- to 5.6-fold higher in

the EPS content after five days of copper exposure compared to the control. This increase in EPS was proportional to the concentration of metal exposed with the difference becoming maximal between controls and the highest metal concentrations. A much slighter increase in EPS content was found in our experiments (1.7-fold higher in pre-exposed than the control biofilms) and this was not statistically significant. However, taking into account that algal biomass was also reduced after chronic copper exposure; differences in EPS per unit of algal biomass were more evident, nearly three times higher in the chronically exposed than in the unexposed communities.

Overall, it is not expected that EPS production may act as the main detoxification mechanism in our study based on the results of internal and total metal bioaccumulation found. Intracellular Cu concentration of the chronically exposed community, measured after washing with EDTA, was 10 times higher than the –Cu growth community suggesting that the increase in the EPS content was not high enough to avoid the penetration of Cu into the cell. In addition, the fact that the proportion of intracellular Cu content in relation to total Cu content of the biofilm was similar in the two treatments indicate that Cu detoxification may also imply intracellular Cu immobilization. The lack of effect on the photosynthetic efficiency of the community after chronic Cu exposure, in spite of containing high levels of the metal in the intracellular compartment, supports this hypothesis. Cu might be sequestered by phytochelatins (Ahner and Morel, 1995) or polyphosphate bodies (Jensen et al., 1982; Wong et al., 1995; Hall et al., 1989) which can bind intracellularly the excess of metal in a detoxified form (Jensen et al., 1982; Twiss and Nalewajko, 1992; Soldo and Behra, 2000).

Effects of chronic Cu exposure on in-stream copper and phosphate retention

Significant differences in Cu dynamics were observed comparing both, control and Cu-exposed systems. Cu retention efficiency was reduced after chronic Cu exposure which is indicated by longer Cu uptake lengths (S_w-Cu) and lower mass transfer coefficients (Vf-Cu) obtained in the Cu-exposed system compared to the –Cu growth treatment. These results indicate lower Cu retention efficiency and lower Cu demand after chronic Cu exposure.

The increase in extracellular polysaccharide matrix (García-Meza et al., 2005) or the sequestration of the metal by cellular ligands, (Twiss and Nalewajko, 1992; Mason and Jenkins, 1995; Soldo et al., 2005), did not cause an increase in the efficiency of the system for removing the metal from the water column. In our study, these mechanisms, even though they probably allowed the adaptation of the community to metal polluted conditions, did not involve an increase in the metal removal efficiency of the whole ecosystem. According to the results from the previous study of Cu dynamics (Serra et al., 2009*b*), copper retention was not significantly related to algal biomass. Therefore, the reduced algal biomass found after the chronic copper exposure in the present study, is not expected to be responsible for the reduced copper retention capacity found in the pre-exposed system.

The results obtained suggest that the saturation of binding sites of the chronically-exposed periphyton had lowered the retention efficiency of the system after chronic exposure. It is described that copper uptake by algae involves a combination of biosorption and bioaccumulation (Xue and Sigg, 1990; Vasconcelos and Leal, 2001). The phenomenon is based on the assumption that equilibrium is attained between the metal in the bulk solution and the metal adsorbed to active sites on the cell surface (Sunda and Huntsman, 1998; Kola and Wilkinson, 2005). Under such steady-state conditions, the flux of metal into the cells follows the Michaelis–Menten kinetics for facilitated or active transport. Thus, in most natural waters the majority of metal transport sites will be available and the uptake rate should be directly proportional to either the concentration of free metal in solution or to the metal transport sites (Sunda and Huntsman, 1998; Kola and Wilkinson, 2005). However, in a polluted area with high dissolved copper levels, the active sites of the algal surface become saturated and the intracellular metal becomes independent of metal concentration (Andrade et al., 2006). Reduction in Cu uptake in polluted environments has been attributed to the possible saturation of the metal binding sites in the cells (Di Toro et al., 2001; Rijstenbil and Gerringa, 2002; Morin et al., 2008; Serra et al., 2009*a*).

Concerning phosphate retention, the structural changes in the biofilm observed after chronic Cu exposure, did not influence the stream phosphate retention efficiency as demonstrated by the similar retention parameters

obtained in both, -Cu growth and +Cu growth periphyton communities. Inhibition of N and P uptake by trace metals has been reported for plants and phytoplankton (Singh and Yadava, 1983; Rai et al., 1998; Gouia et al., 2000; Mosulén et al., 2003). Exposure to high metal concentrations may alter the plasma membrane permeability as a consequence of membrane functionality loss (Meharg, 1993; Hernandez et al., 1997) and lower nutrient uptake. In our study, however, no effect of Cu exposure on nutrient uptake was observed and this was probably due to the fact that the new Cu-adapted community reached similar phosphorus demand to the control community. These results are in agreement with the maintenance of the photosynthetic activity of the community after chronic Cu exposure which indicates no negative effects on the physiology of the communities from chronic Cu exposure.

It has been suggested that copper can induce the deficiency of phosphorus directly by the inhibition of phosphate uptake or indirectly by reducing the permeability of the cell membranes (Nalewajko and Olavenson, 1994) or by an extracellular copper-phosphorus interaction (Guasch et al., 2004). This interaction could, a priori, have repercussions on P retention. However, this effect was not expected in our experiment since P was always added when water was refreshed in order to avoid P-limitation. On the other hand, the slight increase in S_w-PO_4^{3-} observed in the pre-exposed system, although not being statistically significant, could be attributed to an indirect effect of the copper pre-exposure by reducing the algal biomass. As reported in another previous study of Cu and PO_4^{3-} dynamics (Serra et al., 2009*b*), PO_4^{3-} uptake rate was positively related to algal biomass. Therefore, the lower algal biomass found in the chronically exposed system could explain the reduced PO_4^{3-} retention efficiency of the system.

It is well described in the literature that the fate of copper in the aquatic environment is influenced by several processes such as complexation to inorganic and organic ligands, sorption to metal oxides, clays, and particulate organic matter, bioaccumulation and exchange between sediment and water (Stiff, 1971; Callahan et al., 1979; Roussel et al., 2007) as well as hydrologic factors (Serra et al., 2009*b*). In addition, results from the present investigation demonstrate that the fate of copper in streams will also be influenced by the exposure history of the system.

Our investigation has demonstrated that the efficiency of stream periphyton to remove heavy metals from the water column could be altered by previous exposures to metals. In this study we observed that metal-polluted systems will become less efficient in removing metals from the water compared to non-polluted ecosystems. It is expected, therefore, that metals will travel longer distances compared to pristine systems affecting water quality farther downstream.

As periphyton communities are on the basis of the trophic food web of the stream ecosystem and due to their high capacity to accumulate metals from the benthic environment (Newman and McIntosh, 1989), their transference to higher trophic levels is to be expected, being an environmental and human health concern (Chen et al., 2000).

This point illustrates a big difference between the ecological implications of nutrient enrichment versus other contaminants. While nutrient retained by periphyton will finally be consumed by heterotrophic organisms following the nutrient cycling described for lotic ecosystems (e.g. Mulholland et al., 1991), heavy metals such as copper will be transferred and accumulated in the higher trophic levels of the food web.

Chaper IV: Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history

Introduction

Some heavy metals are well-known as freshwater and marine pollutants, and much interest has been dedicated to elucidate their toxic effects on algae (Reed and Gadd, 1990; De Filippis and Pallaghy, 1992). Copper, in particular, plays a dual role in the metabolism of photosynthetic organisms. It is both a micronutrient and a toxicant depending on the dose. Cu is essential as a component of algal enzymes (e.g. oxidases) and the electron transport chain (e.g. plastocyanin) (Pinto et al., 2003). However, at high concentration and/or in prolonged exposures (Fernandes and Henriques, 1991; Guasch et al., 2002), it inhibits photosynthesis and affects metabolic processes related to growth (Maksymiec, 1997).

In the fluvial system it is difficult to relate biological effects (including functional and structural changes) to metal exposure. This difficulty is largely attributed to the complexity of fluvial systems and the short stage of the metal in the dissolved phase. Depending on the dose (concentration) and exposure time (chronic vs. acute), heavy metals, and copper, in particular, may produce changes in the structure (architecture and species composition) and function (inhibiting photosynthesis) (Soldo and Behra, 2000; Barranguet et al., 2002; Guasch et al., 2002; Massieux et al., 2004) of periphyton, or even induce an increase in tolerance at the community level (Blanck et al., 1988).

Natural periphyton communities, the main primary producers of fluvial ecosystems, have a complex structure and are composed of different species of algae and bacteria embedded in a polysaccharide matrix. Intrinsic characteristics of the biofilm can explain differences in sensitivity to pollutants from the media (Barranguet et al., 2000). The capacity of periphyton and other algae to take up heavy metal or organic pollutants from the water, producing an internal concentration greater than in their surroundings has been shown in several studies (Newman and McIntosh, 1989; Liehr et al., 1994; Farag et al., 1998; Wright and Mason, 1999; Behra et al., 2002; Meylan et al., 2003). As periphyton is in the interface between the overlaying water and the sediments in the fluvial systems, they provide an integrated representation of the accumulation of toxicants in the benthic environment (Newman and McIntosh, 1989; Lowe and Pan, 1996; Prygiel et al., 1999).

Although metal accumulation has been relatively well investigated in phytoplankton and macroalgae (i.e. Anderson and Morel, 1982; Crist et al., 1994; Sunda and Huntsman, 1998; Knauer et al., 1997; Vasconcelos and Leal, 2001; Hudson, 2005), metal accumulation in periphyton is currently poorly understood (i.e. Meylan et al., 2003; Morin et al., 2008). Periphyton accumulates heavy metals following three main mechanisms (Holding et al., 2003): adsorption in extracellular polymeric substances, cell surface adsorption, and intracellular uptake (or absorption). The two main processes of metal uptake in periphyton (adsorption and absorption) have been evaluated by measuring total and intracellular metal content in periphyton (Meylan et al., 2003).

It is reported that bioconcentration factors (ratio between metal concentration in the biota and in water) can differ by up to several orders of magnitude among different algal species and metals (Fisher, 1986; Fisher and Reinfelder, 1995). We hypothesize that metal exposures differing in the time scale (duration of exposure), will have different impacts on the structure and functioning of periphyton communities.

The present study aims to analyze the effect of different copper exposure episodes during the colonization of periphyton communities on their structure (algal classes) and functioning (including toxicity and copper accumulation kinetics). Three different Cu exposure episodes were performed in an artificial river system. In the first case a periphyton community was allowed to grow without any Cu exposure (No-Cu treatment); in the second case a periphyton community was acutely exposed to Cu (through three pulses) in the last week of colonization (Cu-Pulsed); and in the third case, a community was chronically exposed to similar Cu concentration during the whole colonization period (Cu-Continuous). We expected that the metal exposure history during the development of the communities would influence the structure of periphyton and their sensitivity to metals as well as their metal accumulation kinetics (viewed as accumulation of metal in the biota along the time period), and their accumulation potential (viewed as the amount of metal retained in the periphyton).

The Cu-Pulsed and the Cu-Continuous communities correspond to the -Cu growth and +Cu growth communities described in the previous Chapter. In

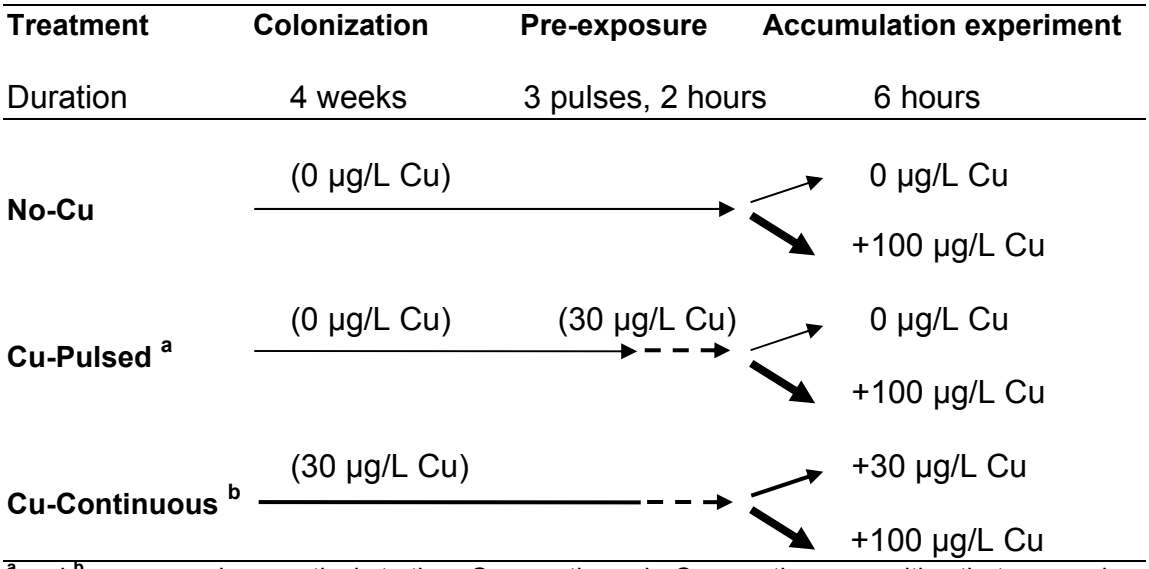
Chapter III, the results obtained from constant-rate Cu additions (carried during the last week of colonization) and a summary of the effects caused after 6h of exposure to higher Cu concentration (carried out after the constant-rate additions) were reported and discussed. In the present Chapter, the Cu-accumulation kinetics and the corresponding changes in photosynthesis (effective Yield) occurring during the last experiment (referred to as Cu short-term in Chapter III) are studied in detail. The –Cu growth community described in Chapter III which received three pulses of Cu (constant-rate additions) at week five, is now referred to as Cu-Pulsed community. This community was thereafter used to investigate Cu kinetics. In addition, a third colonization with no Cu addition (neither constant nor pulsed) is included and used to investigate Cu accumulation kinetics and toxicity as well. This community is referred to as No-Cu community since this community has never been exposed (Fig. 1).

Materials and methods

The experiments were carried using of six Perspex channels (each 170 cm long and 9 cm wide) in recirculating mode as it is described in the Material and Methods of Chapter III (experimental setup section).

Periphyton colonization and pre-exposure

Three consecutive colonization series were performed using the channel system described in Chapter III (in recirculating mode). The periphyton communities were allowed to colonize the glass substrata during four weeks without Cu in the No-Cu treatment and Cu-Pulsed treatment, and with 30 µg/L of Cu in the Cu-continuous treatment. During the fifth week the Cu-Pulsed and Cu-Continuous communities received three pulses of Cu (2h duration each one) which corresponded to the three constant-rate additions described in chapter III. The No-Cu community was not pre-exposed to Cu during the colonization and pre-exposure periods (Fig. 1).



[a] and [b] correspond respectively to the –Cu growth and +Cu growth communities that appear in Chapter III.

Figure 1. Scheme of the experimental setup including the duration and conditions (nominal concentrations) of each experiment. Arrows width is proportional to the Cu concentration used.

Water sampling

During the three colonization series (No-Cu, Cu-Pulsed and Cu-Continuous), the physicochemical parameters (temperature, pH, dissolved oxygen and conductivity) were measured using a multi-parametric probe (WWT) before and after each water renewal (three or four times per week in three different channels each time) and water samples were taken for the analysis of phosphate and copper following the procedure described in Chapter II (laboratory analysis section).

Periphyton sampling

In order to monitor changes in algal biomass during colonization and to ensure that accumulation experiments were applied on mature periphyton communities, colonized substrata were periodically sampled during the five weeks of colonization. For this purpose, one colonized glass substrata was randomly removed from each channel a minimum of four times during this period and used for chlorophyll fluorescence (Fo) measurements using Phyto-PAM fluorometer (see algal biomass and minimum fluorescence section in this

chapter). After these fluorescence measurements, the glasses were returned back to the channels as Phyto-PAM measurements are non-destructive.

At the end of each colonization period, three glass substrata were removed at random from three different channels to characterize the chlorophyll fluorescence of the different algal classes composing the periphyton communities by means of a Phyto-PAM instrument (see chlorophyll fluorescence of different algal classes section in Chapter III). Six glass substrata (one from each channel) were used for measuring the chlorophyll-*a* (chl-*a*) concentration as a measure of the algal biomass of the periphyton after acetone extraction following Jeffrey and Humphrey (1975). At the end of each colonization period, three colonized glasses from three different channels were randomly removed for diatom taxonomic analyses as is described below.

Copper accumulation experiments

Cu accumulation was investigated for each colonization experiment (No-Cu, Cu-Pulsed and Cu-Continuous). In each case, five weeks-old periphyton communities (including the colonization and pre-exposure periods), were randomly distributed in six channels: three channels were exposed for six hours to a high Cu concentration (100 µg/L, nominal concentration) referred to as High-Cu in the text; and the other three channels were used as controls (maintaining the colonization conditions) referred to as Control in the text (Fig. 1). Thus, the three control channels with No-Cu and the Cu-Pulsed communities were maintained without Cu in the water during the accumulation experiment and the Cu-Continuous community was maintained under the same Cu conditions as during colonization and pre-exposure (around 30 µg/L). The time of exposure lasted for only six hours in order to minimize changes in water chemistry (e.g. metal speciation) due to metal complexation with algal extracellular compounds (Wang and Dei, 2001).

For the accumulation experiment, carboys were replaced by new carboys containing Cu equilibrated solutions obtained after leaving Cu solutions for 24 h at room temperature.

During the accumulation, the effects of Cu on the photosynthetic efficiency of periphyton communities were evaluated by means of in vivo chlorophyll fluorescence measurements using Phyto-PAM

Water sampling

The physicochemical parameters of the water were measured at time 0 and then, 2, 4, and 6 h after short-term Cu exposure in one channel. At the beginning (time 0) of the short-term Cu exposure, 5 mL of water from the three Cu-enriched channels (High-Cu) were taken in triplicate to measure the total Cu concentration in the water. These samples were filtered, acidified and stored as described above.

Periphyton sampling

In order to follow the Cu accumulation kinetics in periphyton, three colonized glass substrata were removed at each sampling time (0, 2, 4 and 6 h) from the three Cu-enriched channels, and the total and intracellular Cu content of the periphyton was measured as is described in the Chapter III (biofilm measurements section).

Periphyton samples for Phyto-PAM measurements and Cu accumulation were taken at each sampling time. For the Phyto-PAM measurements, three glasses from each Cu-enriched channel and three from the three control channels were randomly sampled to measure effective quantum yield (Y).

Water analyses

The total dissolved Cu, phosphate concentration and chlorine, anions and cations were analysed following the procedure described in Chapter II (laboratory analyses section)

Measurements of in-vivo chlorophyll fluorescence

Chlorophyll fluorescence (F) of the different algal classes and effective quantum yield (Y) were measured using the Phyto-PAM following the procedures described in Chapter III (fluorescence measurements section). Effective quantum yield (Y) measurements were used to evaluate the effects of Cu on periphyton during the accumulation experiments.

Algal biomass and minimum fluorescence

Chlorophyll-*a* concentration was used as a measure of algal biomass following the procedure described in Chapter II (biofilm measurements section).

For the minimum fluorescence yield of dark-adapted cell (Fo) measurements the periphyton was incubated for 20 min in dark conditions in order to ensure that all reaction centers were open, and then, a weak measuring light was applied to obtain the Fo parameter using the Phyto-PAM chlorophyll fluorometer. Fo is correlated to the algal biomass (Serôdio et al., 1997; Rysgaard et al., 2001), thus, it was used to follow the growth of the periphyton during the colonization. Chl-*a* concentration ranging between 0 and 7.5 µg/cm^2, as a function of periphyton biomass, showed significant lineal regression with the Fo, according to Schmitt-Jansen and Altenburger (2008).

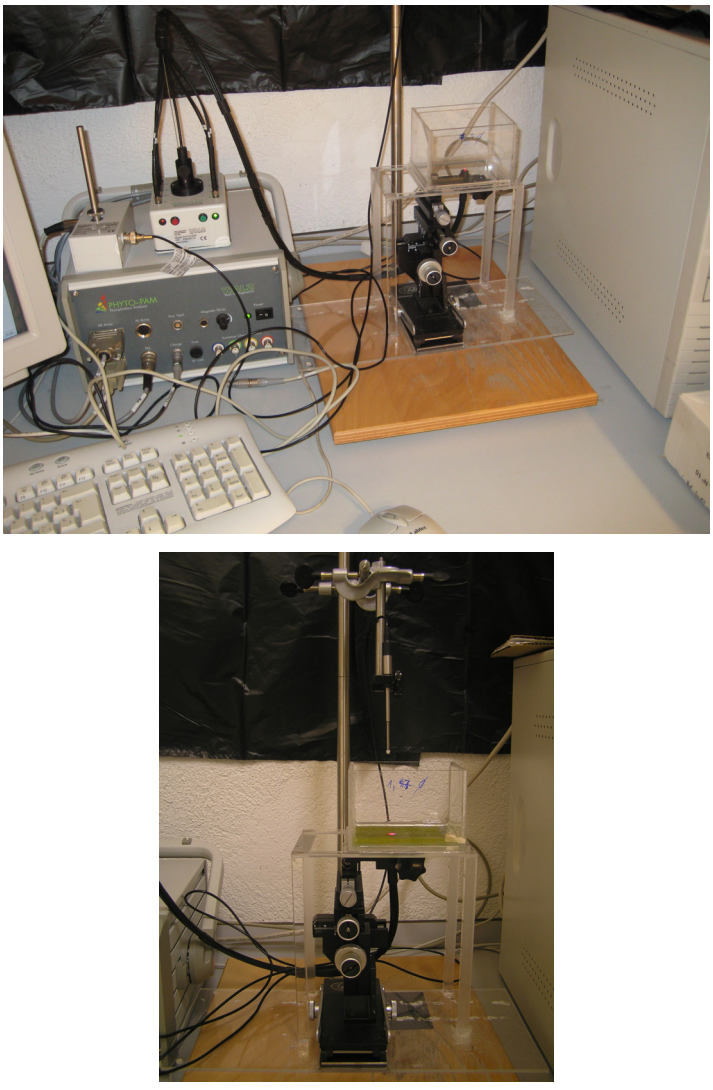


Figure 1. Detail of the phyto-PAM chlorophyll fluorometer (upper panel) and measurements on periphyton (lower panel).

Taxonomic analyses

Colonized substrata were scraped with a cell scraper and fixed with formaldehyde (final concentration 4%) until further preparation. A sub-sample was used for diatom identification. Diatoms were identified after acidic digestion with hydrochloric acid (37% HCl) and hydrogen peroxide (30% H_2O_2). Frustules were mounted on permanent slides using Naphrax (r.i. 1.74). At least 400 frustules of diatoms (following, Tornés et al., 2007) were counted on each slide in random transects under light microscopy using Nomarski differential interference contrast optics at a magnification of 1000x. The frustules of the taxa were identified mainly according to Lange-Bertalot (2001). The relative abundance of each species and the Shannon index of diversity (H') were calculated.

Data treatment

Changes in algal biomass (estimated as Fo) during the colonization were adjusted to growth curves. Data were fitted to a logistic model using the SIGMAPLOT software (see equation 1).

$$y = \frac{a}{\left(1 + \exp\left[-\frac{x - xo}{b}\right]\right)} \quad (1)$$

The *xo* parameter represents the time of response to reach the plateau. In our experiment the parameter *xo* was used to evaluate the growth phase of periphyton when accumulation experiments started.

A one-way ANOVA test was used to examine differences in the community structure (F(Bl), F(Gr), F(Br) and the diversity of the diatom assemblages) between treatments by means of SPSS software. To asses the relative contribution of the different diatom groups of each experiment, similarity analyses (BRAY-CURTIS coefficient, after square root transformation) was applied followed by cluster (average linkage method) and Multidimensional Scaling (MDS) techniques by means of PRIMER software.

An ANOVA of repeated measures was applied to examine the inhibition of effective quantum yield (Yield) during the Cu-accumulation experiments by means of the SPSS software.

A one-way ANOVA test was also used for testing differences in algal biomass (measured as chl-*a* concentration) among the different colonization treatments.

Results

Periphyton colonization

Physicochemical characteristics of the water from the artificial channel system

The physicochemical conditions of the water remained stable during each colonization period and were similar between colonization treatments. Table 1 summarizes the physicochemical parameters measured at the beginning of the water renewals. Cu concentration was <2 µg/L during the whole No-Cu colonization, as well as during the first four weeks of the Cu-Pulsed treatment. Cu concentration during the short-term pulses in the fifth week was 20.29 ± 2.07 µg/L (AVG ± SE). In the Cu-Continuous exposure, Cu concentration was, on average, 26.08 ± 4.84 µg/L (average of the initial and final values).

When comparing the initial physicochemical parameter values (at the beginning of water renewal) and the final values (after three days) for each treatment, we observed that water temperature, pH, conductivity and oxygen remained stable (varying in all cases between 1% and 3%). Phosphate concentration decreased between water renewals (every three days) in the three treatments due to periphyton uptake. In the No-Cu treatment the phosphate concentration decreased by 43%; in the Cu-Pulsed treatment the reduction was 37% and it was 48% in the Cu-Continuous treatment. In the Cu-Continuous exposure, Cu concentration decreased 48% during the period between the water renewals.

Periphyton characterization

After five weeks of colonization in the artificial channels, mature biofilms had developed on the artificial substrata. The growth curves of periphyton measured using the Fo as an indirect measure of algal biomass during the

periods of colonization showed a significant fit in the logistic model (r^2 = 0.478; p < 0.001 for the No-Cu treatment; r^2 = 0.515; p < 0.001 for the Cu-Pulsed treatment; and r^2 = 0.149; p < 0.001 in the Cu-Continuous treatment). All the three periphyton communities have reached a plateau state in the last week of colonization (*xo* = 27 days; p < 0.0001 for the No-Cu treatment; *xo* = 21 days; p < 0.0001 for the Cu-Pulsed treatment; and *xo* = 17 days; p < 0.0001 in the Cu-Continuous treatment).

Table 1. Summary of the physicochemical parameters measured after each water renewal (corresponding to initial conditions), during the three colonization periods: temperature (temp), pH, conductivity (cond), dissolved oxygen (oxy), phosphate concentration (PO_4^{3-}) and copper concentration (Cu).

TREATMENT	No - Cu			Cu – Pulsed			Cu – Continuous		
	AVG	SE	n	AVG	SE	N	AVG	SE	N
temp (˚C)	19.93	0.08	60	19.22	0.08	42	19.51	0.1	33
avg pH	8.32	0.01	60	8.18	0.03	42	8.08	0.09	33
cond (µS/cm)	493.1	2.09	60	472.3	2.88	42	422.8	4.7	33
oxy (mg/L)	8.98	0.02	60	9.68	0.05	42	9.32	0.07	33
PO_4^{3-} (µg/L)	21.16	2.6	49	34.55	3.29	40	46.55	5.42	33
Cu (µg/L)	1.62	0.13	35	0.87[a]	0.03	33	36.12	11.3	20

Values are average (AVG), standard error (SE) and number of samples (n) obtained throughout time until the Cu accumulation experiment begun (at week six).

[a] The Cu concentration of the three pulses was 20.29 ± 2.07 µg/L (AVG ± SE).

At the end of the colonization period, some differences in the fluorescence of algal classes were found when comparing the three communities (Fig. 2). In the Cu-Continuous colonization treatment, the percentage of fluorescence of cyanobacteria and green algae (F(Bl) and F(Gr)) was higher than in other colonization treatments ($F_{2,8}$ = 5.54; p = 0.04 and $F_{2,8}$ = 15.29; p = 0.004, respectively) and the percentage of diatoms (F(Br) very much lower ($F_{2,8}$ = 78.19; p < 0.001). There were no statistically significant differences among the No-Cu and Cu-Pulsed colonization treatments (Fig. 2).

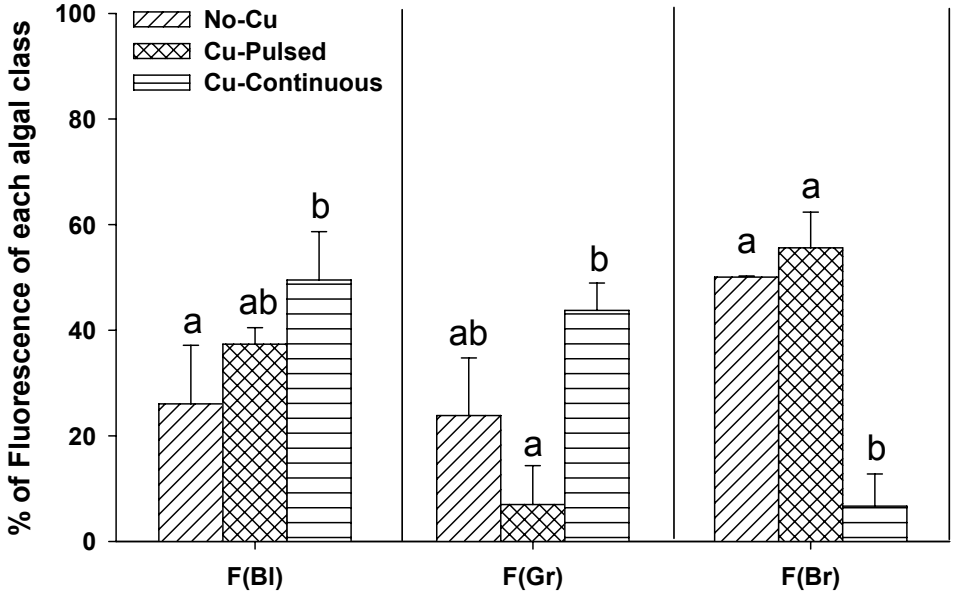


Figure 2. Characterization of the periphytic communities at the end of the respective colonization periods. Percentage of chlorophyll fluorescence (F) of each algal class (Bl, blue-green algae; Gr, green algae and Br, brown algae) from the three treatments (AVG ± S.E.; n = 3). Different lower-case letters next to the bars indicate significant differences ($p < 0.05$) among treatments (Tukey's HSD from ANOVA one way test).

The results of diatom counting also illustrate differences between colonization experiments and are presented as the AVG ± SD of six replicates. The number of diatom species in No-Cu and the Cu-Pulsed communities were similar (12 ± 3 and 12 ± 2, respectively) and were dominated by the taxa *Achanthes minutissima* (91.9 ± 3 % and 86.1 ± 4.8 %, respectively). The number of species was higher in the Cu-Continuous community (19 ± 1) and the dominant taxa *A. minutissima* accounted for 82.4 ± 3 % of cell counting.

In terms of diatom diversity, the Cu-Continuous community had higher diversity (measured using the Shannon index) ($F_{2,8} = 12.4$; $p < 0.05$) compared to the No-Cu and the Cu-Pulsed communities (Fig. 3). Cluster analysis separated the diatom communities in three different groups ($p < 0.05$): No-Cu, Cu-Pulsed and Cu-Continuous. 67% similarity was obtained between the Cu-Pulsed and No-Cu communities differing significantly ($p < 0.05$) from the Cu-Continuous community (with a 62% similarity) (Fig. 3).

Algal biomass, measured as chl-*a* concentration, at the end of the colonization period was always low: 1.25 ± 0.16 µg/cm^2 of chl-*a* in the No-Cu colonization; 2.91 ± 0.30 µg/cm^2 in the Cu-Pulsed and 1.17 ± 0.17 µg/cm in the Cu-Continuous colonization (AVG ± SE, n = 6).

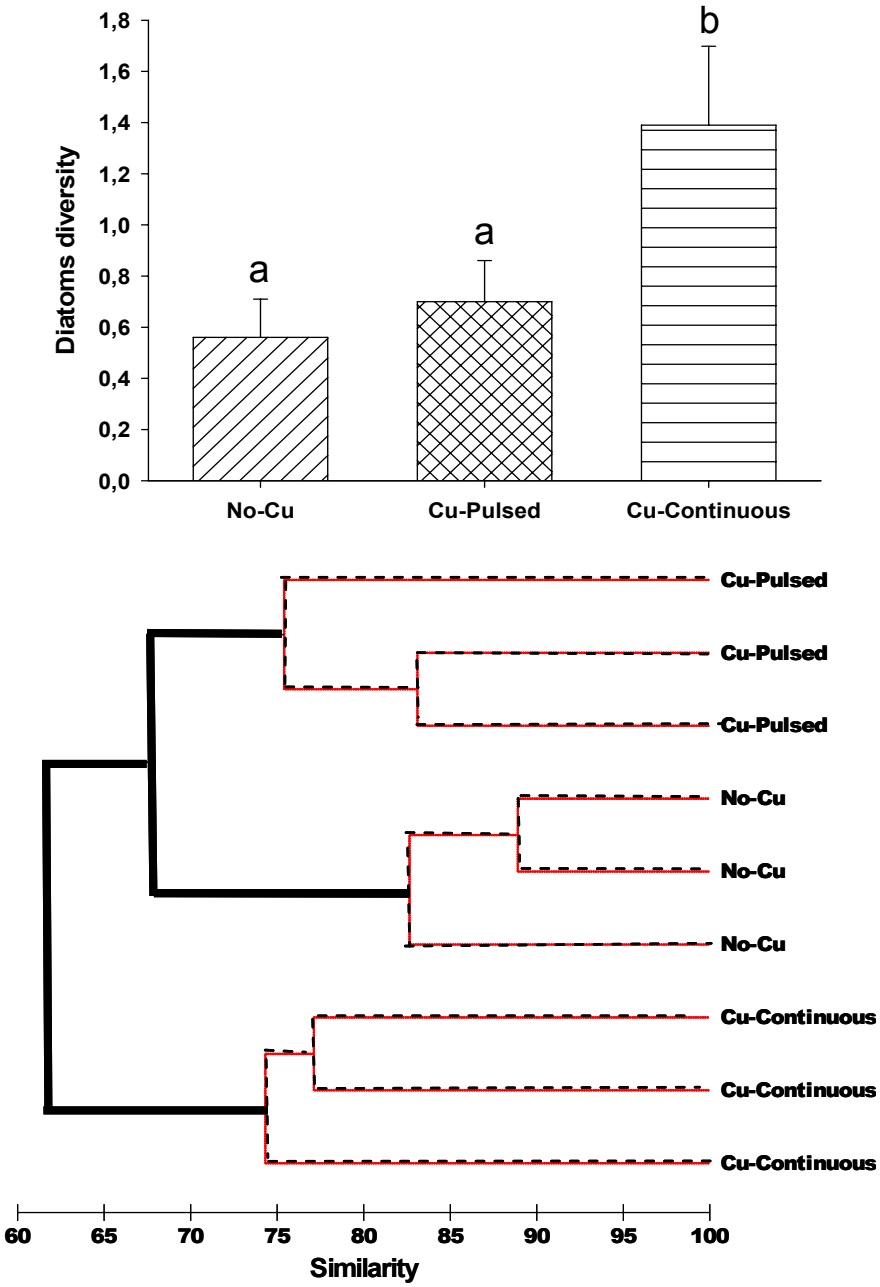


Figure 3. Shannon's diversity index of the diatom assemblages for each treatment (upper panel) (AVG ± S.E.; n = 3). Different lower-case letters next to the bars indicate significant differences ($p < 0.05$) among treatments (Tukey's HSD from ANOVA one way test). Cluster analysis (lower pannel) based on taxonomic diatoms composition where dashed lines denote no significantly different groups and continues lines denote significantly different groups.

Copper accumulation experiments

Physicochemical parameters

Cu concentration in water at the beginning of the accumulation experiments was 99.60 ± 3.09 µg/L (AVG ± SE; n = 9). It was 95.30 ± 1.46 µg/L in the No-Cu treatment 92.07 ± 0.71 µg/L in the Cu-Pulsed and 11.44 ± 2.13 µg/L in the Cu-Continuous treatment (AVG ± SE; n = 3)

During the different accumulation experiments (a total of three), the physicochemical parameters of water were similar and remained fairly constant over time. During the six hours of exposure, water temperature decreased slightly in the three experiments (between 1.5 and 2.2 ºC), dissolved oxygen increased between 0.2 and 0.5 mg/L, pH increased between 0.3 and 0.8 units and conductivity decreased between 6 and 42 µS/cm (Table 2).

Table 2. Summary of the physicochemical parameters measured during the Cu accumulation experiments: temperature (temp), pH, conductivity (cond), dissolved oxygen (oxy).

TREATMENT	No-Cu		Cu-Pulsed		Cu-Continuous	
	AVG	SE	AVG	SE	AVG	SE
temp (ºC)	19.95	0.32	19.73	0.52	19.08	0.60
oxy (mg/L)	8.98	0.04	9.90	0.10	9.52	0.11
pH	8.24	0.05	8.51	0.14	8.45	0.15
cond (µS/cm)	471.8	1.25	471.5	8.57	410.3	3.98

Values are average (AVG) and standard error (SE). n = 4 for each parameter.

Cu accumulation in periphyton

In the No-Cu community, the total Cu concentration (including both total and intracellular fractions) at the beginning of the accumulation was 2.84 µg Cu/gDW, and experimented a rapid increase reaching 28.37 µg Cu/gDW of Cu in two hours of Cu exposure, which was kept constant during the following four hours of the accumulation experiment (increasing 1.64 µg Cu/gDW during the last four hours of the experiment) (Fig. 4).

Intracellular Cu accumulation in this No-Cu community followed an exponential increase (r^2 = 0.80; p < 0.001), characterized by a slight increase in the first two hours (0.59 µg Cu/gDW h of Cu, on average) and a rapid increase

during the following period (4.85 µg Cu/gDW h of Cu on average during the following four hours).

In contrast with the unexposed periphyton, total Cu content in the Cu-Pulsed community was, at the beginning of the accumulation experiment, above 30 µg Cu/gDW (36.53 µg Cu/gDW on average). In this treatment, the total Cu accumulation also reached a steady state after two hours of Cu exposure. During this period (the first two hours of Cu exposure), the total amount of Cu in the periphyton increased up to 71.65 µg Cu/gDW, almost doubling the initial concentration, and in the next four hours the total Cu concentration in the periphyton remained constant (Fig. 4). Intracellular Cu concentration for the Cu-Pulsed treatment at the beginning of the accumulation experiment (time 0) was 27.18 µg Cu/gDW, corresponding to 74% of the total Cu accumulated in periphyton, and increased 7.5 µg Cu/gDW during the first two hours. Intracellular Cu concentration remained constant for the next four hours of the experiment (Fig. 4).

In the continuously exposed community, a large amount of Cu was accumulated during the colonization period. At the beginning of the accumulation experiment (time 0) this periphyton had almost 10 times higher total Cu content than the Cu-Pulsed community, of which 77% corresponded to intracellular Cu (Fig. 4). During the accumulation experiment, in the Cu-Continuous community, the total Cu concentration increased during the first two hours (33%), decreased later on (19%) and increased again during the last two hours (32%). Intracellular Cu followed the same pattern of variation but the magnitude of the changes was smaller (from 10% to 14%).

Effects of Cu during the accumulation experiment on the effective quantum yield (Y)

The effective quantum yield (Y) (Fig. 5) of the periphyton measured in Cu-Continuous community was not affected during the 6 h that the accumulation experiment lasted ($F_{1,4}$ = 0.915; p = 0.39, respectively). In contrast, the No-Cu and the Cu-Pulsed communities showed a significant reduction of the Y during the experiment ($F_{1,4}$ = 8.66; $p < 0.05$ and $F_{1,4}$ = 42.36; $p < 0.005$, respectively) reaching values up to 32% lower than the controls (Fig. 5).

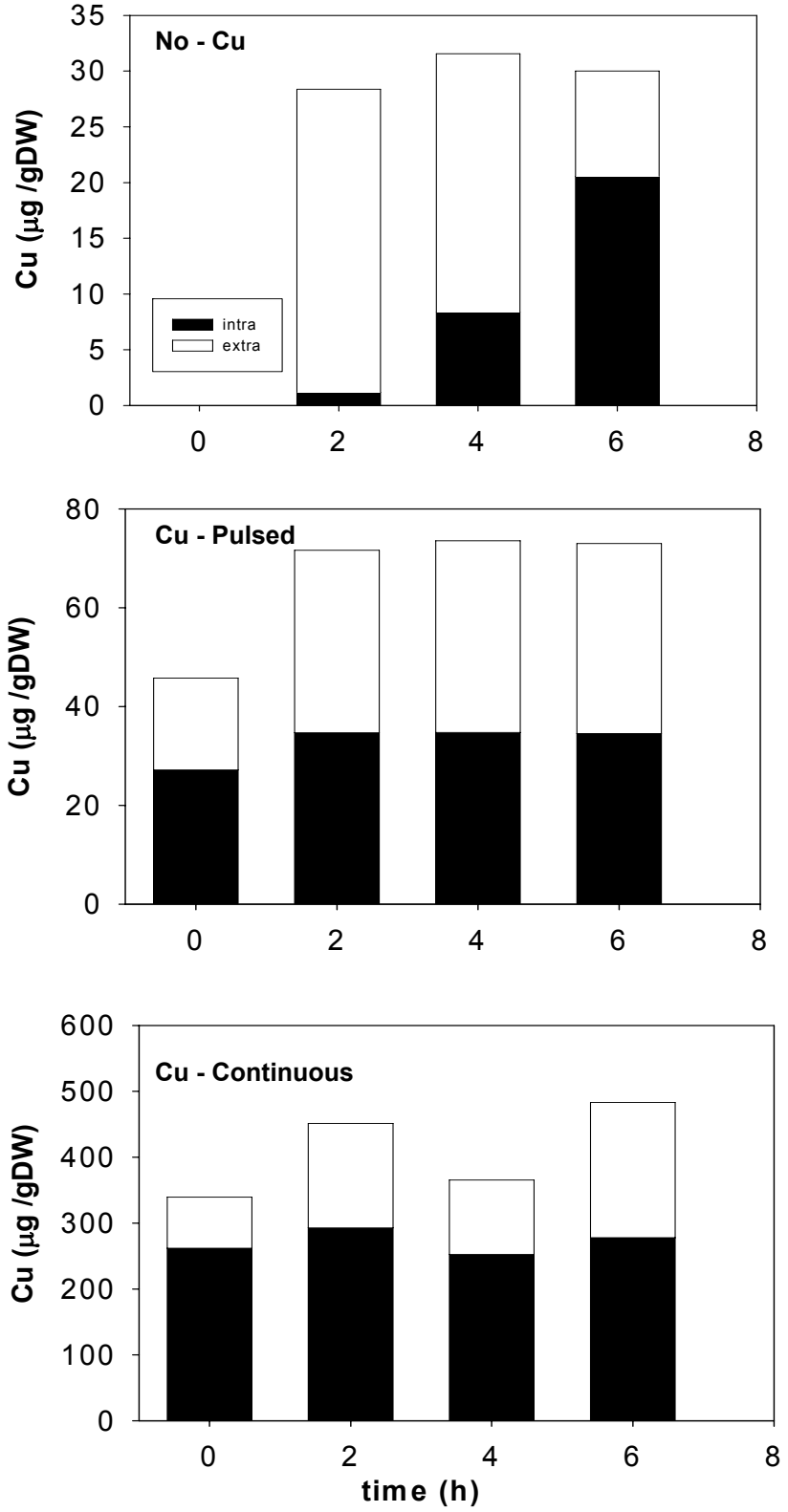


Figure 4. Time-dependent intra (black bars) and extracellular (white bars) copper concentration in periphyton.

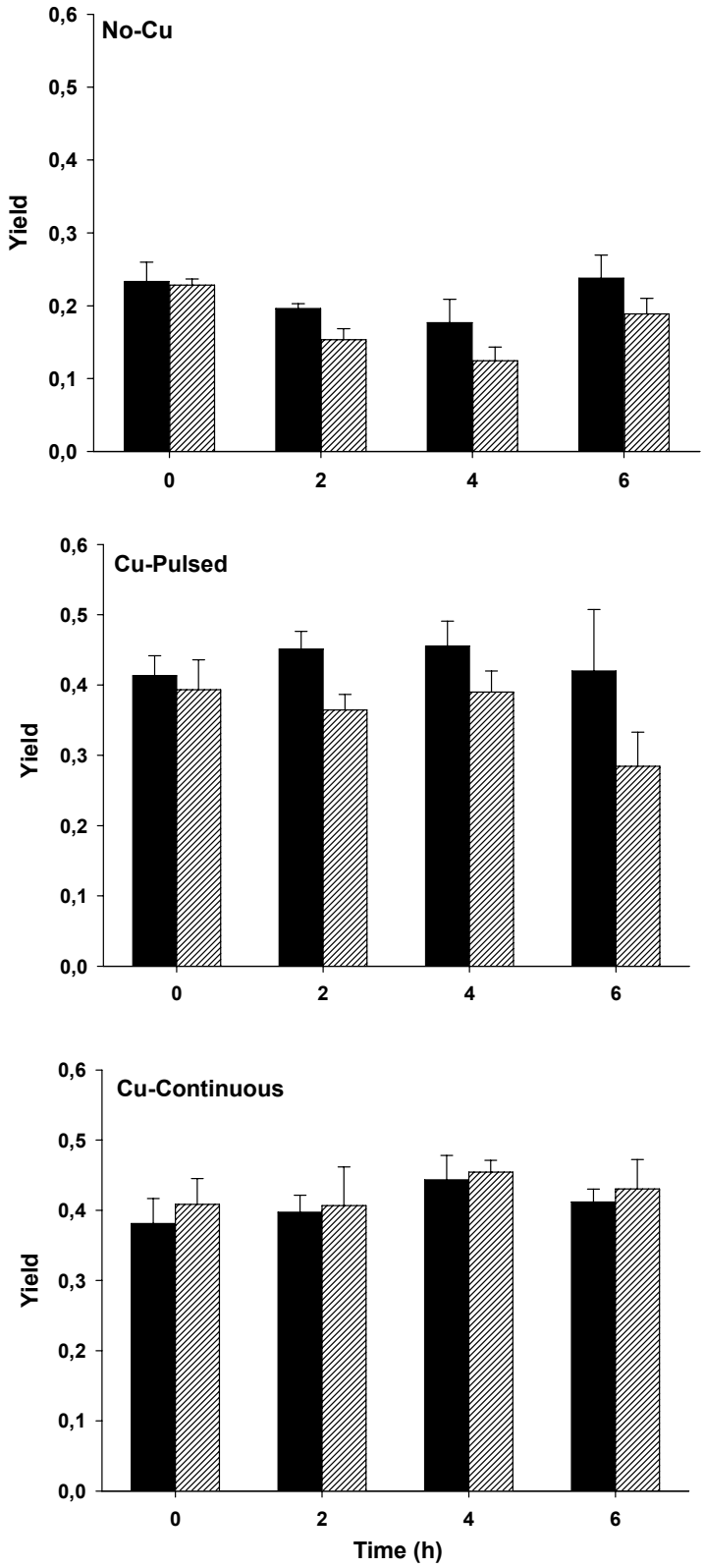


Figure 5. Photosynthetic efficiency (Yield) of the periphyton communities in the Control (black bars) and in the High-Cu (dashed bars) treatments, during the Cu accumulation experiment (AVG ± S.D.; n = 3)

Discussion

As described in the literature, the effects of Cu on periphyton communities may differ in their temporal pattern: short-term physiological alterations and long-term changes in the community structure (Sabater et al., 2007). The effects of acute (pulsed) and chronic (continuous) Cu exposure on photosynthesis, periphyton structure, metal content, community tolerance and Cu accumulation kinetics are discussed above.

Effects of Cu exposure on periphyton structure

Algal biomass was not clearly affected by Cu exposure since chlorophyll content was higher in the Cu-Pulsed than in the No-Cu and Cu-Continuous communities. The three periphyton communities reached relatively low algal biomass (1-3 μg/cm^2 chl-*a*), similar to the values found in oligotrophic streams, well below the threshold from oligotrophic to eutrophic conditions suggested by Dodds et al. (1998) which corresponds to 7 μg/cm^2 of benthic chlorophyll concentration.

The pulsed Cu exposure did not cause significant effects on the relative abundance of different algal classes (in comparison to the unexposed community). Neither the F(Bl), F(Gr), nor F(Br) presented differences from the ones obtained in the No-Cu community, indicating that the algal classes were similar. However, an increase in F(Bl), and F(Gr) linked with cyanobacteria and green algae respectively, and a high decrease in F(Br), linked with diatoms, were observed after the Cu-Continuous exposure. These results suggest that cyanobacteria and green algae were more tolerant to the continuous Cu exposure applied during the colonization period. On the other hand, the fact that the F(Br) decreased in the Cu-Continuous colonization experiment indicates that diatoms are the most sensitive class, and that a shift from diatoms to greens and cyanobacteria has occurred.

The composition of the periphytic community itself in terms of algal species is a vital element in determining tolerance to metals (Say and Whitton, 1981). The replacement of the most sensitive species for more tolerant ones has been described as a mechanism leading to an increase in community tolerance (Blanck et al., 1988). However, this change in the structure of the

community is not consistent between different studies reported in the literature. For instance, Soldo and Behra (2000) reported a decline of cyanobacteria and an increase in green algae after twelve weeks of periphyton Cu exposure. Genter et al., (1987) observed a shift of the community composition from diatoms to green algae and cyanobacteria, when exposed to zinc. Our findings are in agreement with the results from Barranguet et al., (2003), who also reported a shift from diatoms to cyanobacteria after long-term Cu exposure, as cyanobacteria are more resistant to Cu than diatoms (Barranguet et al., 2000).

Several studies reported effects of long-term metal exposure on periphyton diversity and richness (i.e. Leland and Carter, 1984; Soldo and Behra, 2000; Guasch et al., 2002; Barranguet et al., 2003; Morin et al., 2008). Some of these investigations reported a reduction in the number of species under metal polluted conditions (i.e. Kaufman, 1982; Leland and Carter, 1984; Morin et al., 2008). However, other studies showed an increase in species diversity in communities exposed to low metal concentrations (Gold et al., 2003). These late results are in agreement with our results, in which the Cu-Continuous exposure to 30 µg/L resulted in an increase in diatom diversity.

Our observations have been made on laboratory communities which, despite being multi-specific, are supposed to be less complex (in terms of species diversity) than natural communities developed in the field, due to the selection pressure derived from the channel conditions. We can explain this increase in diatom diversity by the fact that the diatom community grown in the experimental channels had low diversity with a dominant specie *A. minutissima*, which is a pioneer for colonization of new substrata (Sabater et al., 1989). Thus, continuous Cu exposure affected the dominant species causing an increase in the relative abundance of many other species. *A. minutissima* was described as a metal-sensitive species in previous mesoscosm (Blanck et al., 2003) and field studies (Sabater, 2000). The abundance of *A. minutissima* has been used by the US Environmental Protection Agency (EPA) as an index to express the disturbance produced after a toxic event (Barbour et al., 1999). In our study, continuous Cu exposure produced an increase in the diatom diversity index which accounts for the number of species and their equitability. It is important to point out that diatom diversity in the controls was very low compared to periphyton natural communities (Leira and Sabater, 2005), therefore caution

should be taken before extrapolating our results to field conditions. Overall, the similarity indices summarize the differences between the three diatom communities: No-Cu and Cu-Pulsed were very similar (similarity index of 68%) and differed slightly from the Cu-Continuous community (similarity index of 62%) indicating that a major part of diatom taxa present in the community was unaffected by the toxicant.

Influence of exposure history on Cu accumulation and toxicity

Our investigation demonstrates that the exposure history of periphyton is an important factor for modulating the response of these communities to heavy metals, since changes in the duration of exposure affect the community structure and the metal accumulation capacity of periphyton in addition to their sensitivity to heavy metals.

Metal uptake kinetics in algae generally involve an initial rapid surface adsorption, followed by a slower active metal transport into the intracellular pool also called absorption or bio-uptake (e.g. Xue and Sigg, 1990; Gonzalez-Davila et al., 1995; Knauer et al., 1997; Hudson, 1998; Sunda and Huntsman, 1998). Surface sorption may also include complexation with algal extracellular organic compounds and metal binding to negative charged functional groups of the cell surface (Zhou and Wangersky, 1989; Xue and Sigg 1990; Crist et al., 1994). On the other hand, intracellular concentration may also be influenced by Cu secretion (Soldo et al., 2005) affecting therefore the kinetics of metal accumulation.

The discrimination between extracellular and intracellular metal content in periphyton, has allowed us to evaluate differences in metal accumulation kinetics of these two compartments depending on the exposure treatment, thus, discriminating between adsorption and absorption processes.

In particular, total Cu accumulation (including both total and intracellular fractions) showed similar kinetics in the No-Cu and Cu-Pulsed communities, reaching a steady state within the first two hours of exposure. On the other hand, these two communities differed in the intracellular metal accumulation kinetics. Intracellular Cu concentration increased exponentially without reaching a steady state in the No-Cu community. In contrast, in the Cu-Pulsed community, the intracellular Cu concentration was higher at the beginning of the

experiment (due to the accumulation of the metal during the pulses received in the colonization), and increased slightly, reaching a steady state within the first two hours of Cu exposure. These results indicate that, although total Cu concentration increase was similar in both cases (around 30 µg Cu/gDW), the processes involved were different. Whereas the metal was being taken up by the cells in the unexposed community, Cu was not being internalized by the cells in the pulsed-exposed community. These results suggest that, although acute metal exposure did not produce any remarkable effect on periphyton structure or on total Cu accumulation kinetics, it clearly affected the intracellular Cu accumulation kinetics, by reducing the metal bio-uptake capacity of periphyton. This can be explained by the possible saturation of the metal binding sites in the cells (Di Toro et al., 2001; Rijstenbil and Gerringa, 2002; Morin et al., 2008) caused by the pulses that this community received limiting their future cell absorption capacity.

Meylan et al., (2003) reported that the time required to reach the steady state in metal accumulation by periphyton varies from a few hours to days depending upon the concentration of Cu, the presence of competing metals and the growth rate of the algae. Additionally to these findings, our study also revealed the importance of the Cu exposure history modulating the metal accumulation kinetics.

The chronic Cu exposure of periphyton during the colonization caused a large increase in total and intracellular metal concentrations reaching values of up to 300 µg Cu/gDW (total Cu concentration) at the end of the colonization period (which corresponds to the time 0 of the accumulation experiment). Cu accumulation in the chronically exposed community did not show any clear trend. This may be explained by the fact that the Cu concentration in the periphyton at the beginning of the accumulation experiment was higher than the concentration of the short-term exposure (100 µg/L, nominal concentration) thereby masking the adsorption and or desorption processes occurring in the biofilm. Our results are in agreement with the study of Soldo et al., (2005), where the tolerance of the green algae *Oocystis* was attributed to its high metal accumulation potential conferred by a high cellular metal binding capacity. Additionally, this suggests that the structural changes reported after chronic Cu exposure are associated with an increase in intracellular and extracellular metal

binding sites (Soldo and Behra, 2000; Soldo et al., 2005). This allows a metal-adapted community to immobilize high amounts of metals without causing toxicity (Mason and Jenkins, 1995).

Differences in the toxicological responses were also observed when comparing the three studied communities. Cu exposure during the accumulation experiments caused a reduction of photosynthetic efficiency in the unexposed and acutely exposed communities. In contrast, the chronically exposed community, despite having a high internal Cu content, had enhanced its tolerance and was able to cope with the potential effects of the metal. Pre-exposure of an organism to sub-lethal concentrations of metals confers a kind of acquired tolerance to pollutants. However, in order to enhance the tolerance to potentially toxic metals, pre-exposure should be sufficiently high to initiate cellular compensatory responses. According to this reasoning, the Cu-Continuous exposure tested in our study was probably long enough to produce changes in the community such as an increase in intracellular and extracellular metal binding sites, or changes in the community structure (the replacement of metal-sensitive species for more tolerant ones) that could lead to an increase in metal tolerance of the community. The fact that the large amount of intracellular Cu content found in the chronically exposed community had no effect on photosynthesis suggests the efficacy of intracellular ligands in protecting periphyton from excess metal accumulation.

Ecological implications

Cu concentration in biofilms is a very sensitive indicator of Cu exposure, even if this has been short without causing any apparent community damage. However, it is not possible to infer toxicity based on Cu content in biofilms since the sensitivity of the periphyton communities is affected by the temporal scale of the exposure episode, which may or may not lead to higher metal tolerance at the community level.

From our study, we can conclude that both acute and chronic periphyton Cu exposure has negative repercussions for the fluvial ecosystem. Acute exposure may lead to photosynthesis inhibition if the community has not been previously adapted. On the other hand, chronic exposure leads to community adaptation, which is often related to changes in species composition. However,

there is no clear evidence of the indicative value of periphyton taxonomy in terms of metal tolerance since community indices, like species diversity, commonly used to evaluate the ecological integrity of fluvial systems, are also ambiguous for metal pollution because they may either increase or decrease depending on the reference situation. Continuous Cu exposure may lead to an increase in the community tolerance but also to a higher metal content in periphyton. Metals will therefore be retained by periphyton and transferred to higher trophic levels in the fluvial food web. Our study highlights the influence of the exposure history to pollutants on modulating the toxicological response of organisms living in freshwater ecosystems. This investigation points out the need to combine tolerance measurements with metal accumulation kinetics studies in order to provide a better approach for assessing the fate and effects of contaminants in fluvial ecosystems.

Chapter V: Influence of phosphorus on copper sensitivity of fluvial periphyton: the role of chemical, physiological and community-related factors

Introduction

Among the many ecosystem stressors, eutrophication and metal pollution are the two major environmental problems in many developed and developing countries (Wang and Dei, 2006). Agricultural activity is one of the major sources of phosphorus and nitrogen to aquatic ecosystems (Carpenter et al., 1998; Eckholm et al., 2000). At watershed scale, excessive inputs of phosphorus derived from agricultural practices are closely linked to eutrophication of surface waters (Johnson et al., 1997). In addition to fertilizers, human activities have also contributed to a progressive increase in other substances, especially heavy metals in aquatic environments (Nriagu, 1979; Ma et al., 2003; Andrade et al., 2004) resulting in potentially toxic levels for aquatic organisms (Haughey et al., 2000). As metal pollution is often associated with eutrophic conditions in aquatic ecosystems (López-Flores et al., 2003), it is considered of great interest to elucidate the interaction between nutrients and metal toxicity, and this investigation specifically focuses on the interaction between copper and phosphorus which are commonly found together in fluvial systems draining industrial and urban watersheds (Twiss and Nalewajko, 1992). Metal bioavailability, and thus the toxic effects on the biota, can be modulated by the inorganic and organic chemistry of the water body (Genter, 1996). Water pH, conductivity, temperature, nutrient availability and inorganic and organic ligands have been described as factors strongly influencing metal toxicity (Stumm and Morgan, 1981; Luoma, 1983; Meador, 1991; Campbell, 1995; Sunda and Huntsman, 1998; Meylan et al., 2004).

Several investigations have focused on the interrelationships between trace metals and nutrients in phytoplankton and algal biofilms (Wang and Dei, 2001; Interlandi, 2002; Ivorra et al., 2002; Riedel and Sanders, 2003; Guasch et al., 2004). Many studies support the hypothesis that metal toxicity is reduced in response to increases in P concentrations (Meijer, 1972, Monahan, 1973, Harding and Whitton, 1977, Say and Whitton, 1977; Li, 1979; Chen C-Y, 1994). On the other hand, several studies indicate that metals can induce nutrient limitation which can result in reduced algal growth (e.g. Nalewajko and Olaveson, 1994; Paulsson et al., 2002). In these cases nutrient enrichment has been shown to compensate this effect of the metal as, for example, P-addition

may allow the formation of cellular polyphosphate bodies which can bind intracellularly heavy metals in a detoxified form (Jensen et al., 1982; Pettersson et al., 1985, Twiss and Nalewajko, 1992). For instance, a direct influence of phosphorus (P) supply on copper toxicity to algae has also been demonstrated (Hall et al., 1989; Twiss and Nalewajko, 1992; Hashemi et al., 1994; Nalewajko and Olaveson, 1994; Guasch et al., 2004). Although much information about the interaction between nutrients and heavy metal toxicity is already available, especially in phytoplankton or single species studies, field testing and studies at community level remain scarce (e.g. Guasch et al., 2004).

Periphytic communities are ubiquitous and ecologically important components of many rivers and streams, as they are in the basis of the fluvial food web they have been used for assessing the quality of these ecosystems (Boston et al., 1991) including metal pollution (Johnson et al. 1978; Ramelow et al., 1992; Ivorra et al., 1999). Based on the opportunity to evaluate the response of numerous species simultaneously, community ecotoxicology can provide a much broader context for the assessment of environmental contamination than the study of individual species (Clements and Newman, 2002).

In the present investigation, we hypothesize that eutrophication will lead to a reduction of copper sensitivity in natural periphyton communities due to variations in metal bioavailability as well as the phosphate regime during growth. Although both phosphorus and nitrogen supplies contribute to freshwater eutrophication (OECD, 1982), we have focused on the relationship between phosphorus (P) and copper (Cu) toxicity because phosphorus can often be the main limiting nutrient in freshwater environments (Horne and Goldman, 1994). This is also the case of our study area. In addition, P-Cu interaction was also of interest due to the contribution of phosphorus in metal detoxification mechanisms (e.g. Twiss and Nalewajko, 1992; Paulsson et al., 2002).

In order to test our hypothesis, the study design included three tiers: a field study including the characterization of land use and the ecological state of the corresponding river sections in the Fluvià River watershed, an experimental investigation performed with natural periphyton from the previously studied stream sites in indoor channels, and finally a toxicological study using algal cultures in the laboratory. Our multi-scale experimental design aimed to

elucidate the relative role of the different processes contributing to the interaction effects of P and Cu on fluvial periphyton communities. Specifically the contribution of phosphorus conditions during growth and phosphorus/copper ratio in media on Cu toxicity was evaluated in algal cultures as well as in natural periphyton communities. Whereas the use of algal cultures allows control of biological and environmental conditions, tests using natural communities incorporate the ecological variability of the natural system (Cairns and Niederkenher, 1987; Navarro et al., 2002; Sabater et al., 2007).

Materials and methods

Study design

This study was structured in three general sections: a field study, a microcosm study using experimental channels and natural periphyton communities and finally, a laboratory experiment with algal cultures. The field study was conducted in order to characterize the Fluvià watershed and was also used to choose the most suitable sites for the community ecotoxicology study. Natural periphyton communities collected from the chosen river sites were thereafter used to investigate the contribution of trophic conditions during growth on Cu toxicity. Finally, a benthic diatom was cultured under different P concentration and it was also exposed to Cu in the short term in order to elucidate the effect of cell nutrient status on copper toxicity. Moreover, the influence of P-supply in the media on Cu toxicity was also evaluated on both, periphyton and algal cultures.

Field study

The Fluvià River, located in northeast Spain, is a calcareous Mediterranean river which drains a volcanic zone, declared a Natural Park in 1982. The sampling included six sites located in the headwater of the River Fluvià and its main tributaries corresponding to first- and second-order streams (Fig.1).

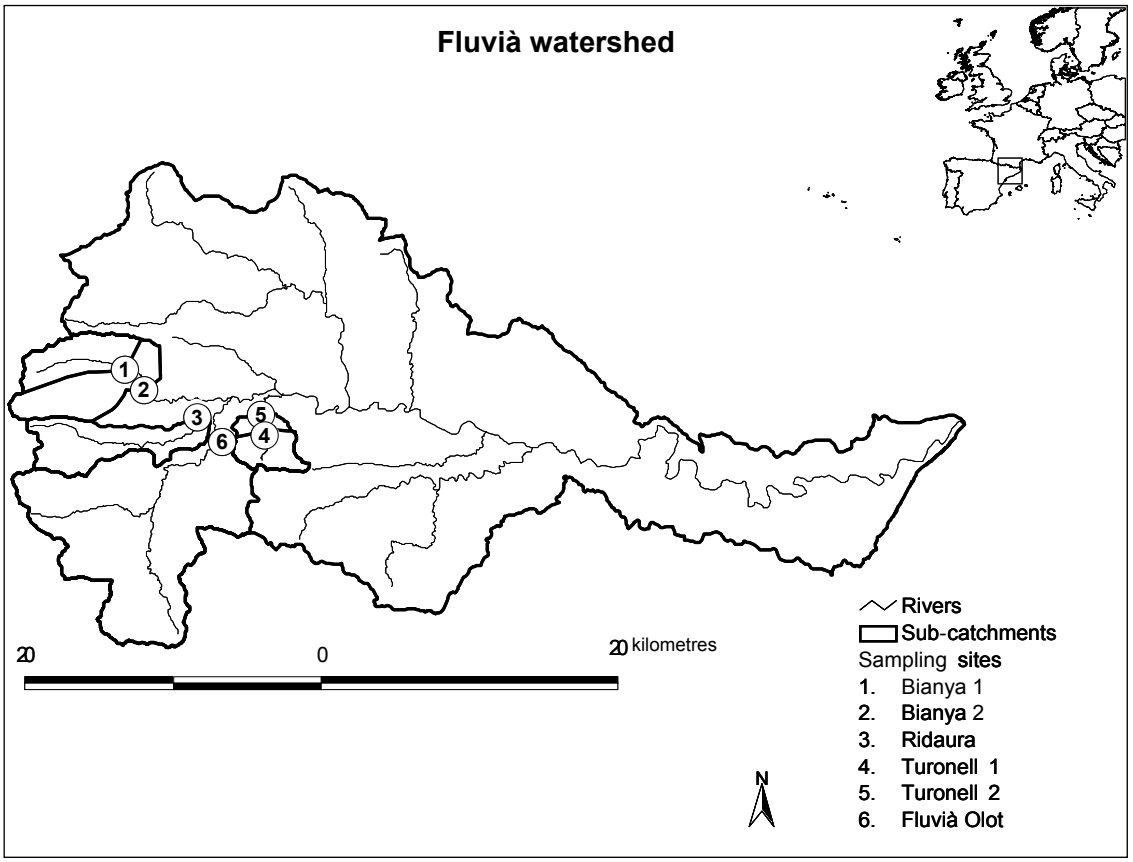


Figure 1. Map of the Fluvià watershed and location of the study sites. The sub-catchments are separated by a solid line.

The physicochemical parameters of each site were taken on five sampling dates from October 2002 to November 2003. On each sampling, water pH, temperature, conductivity, and dissolved oxygen were measured in the field. One liter of water samples per site were collected for their analysis in the laboratory. Samples were immediately filtered by GF/C Whatman glass microfibre filters, and refrigerated (4ºC). They were analysed between 24h for

ammonium and alkalinity measurements and frozen for soluble reactive phosphorus (SRP), nitrate and nitrite analyses following APHA (1989).

Periphyton samples for algal biomass measurements, were obtained from 1cm^2 of biofilm scraped off three stones from each sampling point in triplicate. Chlorophyll-*a* concentration was obtained after extraction in 90% acetone, and sonication for 5 min and determined spectrophotometrically, following Jeffrey and Humphrey (1975).

Land uses and land covers of each sub-catchment draining the six sampling sites were determined by using Geographic Information System (GIS). The entire watershed was subdivided into six sub-catchments corresponding to the six sites sampled. Land use and land cover maps were based on a 1:5000 orthophotomap provided by the Institut Cartogràfic de Catalunya (ICC), and digitized contours of sub-catchments from a 1:5000 topographic map using Arc-View GIS 3.2 software (California USA) in order to obtain the percentages of the different land uses.

Experimental channels

Once the gradient of eutrophication in the field was identified, three stream sites were selected for the toxicological study (B1, B2 and T1). Natural periphyton communities from these sites were used to assess Cu toxicity in indoor experimental channels. P-concentration in the media was experimentally manipulated in the Cu toxicity tests in order to evaluate the influence of both P-limitation and P-Cu chemical interaction on periphyton Cu toxicity.

Periphyton colonization and sampling. Mature periphyton communities were obtained after allowing the colonization of artificial sand-blasted glass substrata (1.4 cm^2) during two months (spring 2003) in each stream site. Colonized substrata were collected in the field and transported to the laboratory in cool boxes, filled with stream water (Guasch et al., 2003). Periphyton from three glass substrata from each sampling site were scraped and fixed with 4% of formaldehyde for taxonomical identification of the major algal groups under the light microscope. Three extra glass substrata from each sampling site were used for measuring algal biomass, estimated as the chlorophyll-a concentration (chl-*a*) as described above.

The physicochemical parameters of the stream water were measured in the field in each sampling site and date and 1L of stream water was collected and transported to the laboratory for nutrient analysis as described above.

Cu toxicity in periphyton. For each sampling site, the colonized substrata were incubated in twelve Perspex channels (87 cm long and 9 cm wide) using five replicates per channel (five artificial substrata). Ten liters of stream water (collected in the site of origin of each community) were recirculated in each channel from containers by centrifuge pumps. All the containers were placed in a water bath to maintain the field temperature. The pH was maintained within the range measured in the field by regular addition of diluted sulphuric acid during the incubation. The incubation was conducted inside a greenhouse allowing the penetration of natural light.

Periphyton from each site was exposed to two treatments, six channels were exposed to a gradient of copper concentration (referred to as controls in the text) and six channels to the same gradient of copper and with a supply of 20 µM phosphorus (referred to as +P in the text). Copper was added as copper chloride (copper titrisol, Merck, Darmstadt, Germany) to achieve nominal concentrations of 0, 20, 63.5, 201, 635, 2008 µg/L. Phosphorus was added as KH_2PO_4.

Analyses of copper concentration of the incubation water were performed periodically using ICP-MS (Agilent 7500c, Japan).

Based on the observations made by Barranguet et al. (2002), the commonly oberved negative relationship between Cu toxicity and periphyton thickness was explained as follows: the toxic effect of this metal is expected to increase slowly by indiscriminately moving from the biofilm surface to the deeper layers. Based on this argument, the toxicity tests lasted for 18h in order to allow the complete diffusion of Cu through the biofilm and minimize the influence of initial biomass on the measure of the Cu toxicity.

PAM fluorometry measurements. The effect of copper and phosphorus on periphyton was measured after 18h of exposure using a mini-pulse modulation chlorophyll fluorometer (PAM) (Walz Mess, und Regeltechnik,

Effeltrich, Germany) as described in Guasch et al. (2002). Before the PAM measurements, periphyton was previously dark adapted for 15 min. The effect of Cu on periphyton was evaluated directly on the colonized glass substrata by measuring the minimal fluorescence yield of dark-adapted cells (Fo), also called chlorophyll fluorescence. Although changes in pigment composition and photosynthesis inhibition due to toxicant exposure may also influence the Fo, this parameter is used in this study as an indirect indicator of biomass (Serôdio et al., 1997; Rysgaard et al., 2001).

Algal Cultures

Two independent sets of experiments were performed in order to obtain two replicates of the same experimental design. Each set of experiments started after 15 days of culture growth and included six short-term toxicity tests of Cu toxicity on n the diatom *Nitzschia perminuta*.

Before starting the toxicity tests, measurements of Alkaline Phosphatase Activity (APA) were performed in order to assess the degree of P limitation of the cultured diatom at P-repleted and depleted conditions. The procedure followed for the APA measurements is detailed below.

In each set of experiments, short-term toxicity tests were performed in duplicate using algal cultures differing in P regime during growth and/or P in the media during the toxicity test as follows:

i) P-depleted / -P = P-depleted culture without P in the media during the toxicity test
ii) P-repleted / -P = P-repleted culture without P in the media during the toxicity test
iii) P-repleted / +P = P-repleted culture with P in the media during the toxicity test

The treatment P-depleted / +P was not included in the experimental design in order to avoid interactions of a starved culture with the supply of phosphorus in the media during the experiment.

Culture preparation. Non-axenic precultures of the diatom *Nitzschia perminuta* were kept in 1000 mL Erlenmeyer flasks with 300–400 mL sterile WC medium (Guillard and Lorenzen, 1972) modified as follows: the concentration of

1.0 mg/L H_3BO_3 was lowered to 0.006 mg/L, and molybdenum was added as sodium salt (the same molybdenum concentration as in the original WC medium). HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid buffer was used to stabilise pH at 7.0. The medium was prepared following Van der Grinten et al 2004. 1 mL of the original pre-culture was transferred to 250 mL Erlenmeyer flasks containing 5 mL of glass beads (Ø 490-700 µm) that were used as substrate for the algal development, filled with 100 mL of new sterile WC medium modified as described above and containing different phosphate concentrations. The high phosphate regime used (100%) was that of the original WC medium, 50 µM representing a saturating concentration, corresponding to the P-repleted conditions and the low phosphate regime was 5 µM (10% of the original medium), corresponding to the P-depleted conditions (based on Van der Grinten et al., 2004).

The cultures were placed in an incubator, illuminated from above with fluorescent cool-white tubes at 100 µmol/m^2 s PAR (phostosynthetically active radiation) and the temperature was kept at 20°C using a cooling bath. The flasks were closed with cellulose plugs to allow CO_2 exchange with the air. Microalgae were grown in these conditions for 15 days and growth was monitored by using PAM fluorometry measurements daily, using the minimum fluorescence yield of dark adapted cells (Fo) as an indicator of algal biomass. Periodically and simultaneously to the PAM measurements, the number of algal cells was counted using a Burker-turk chamber, and these measurements were correlated with measurements of chlorophyll fluorescence of dark adapted cells (Fo) obtaining a good correlation ($r^2 = 0.83$; $p < 0.001$; $n = 80$) between these two variables.

Cu toxicity to Nitzschia perminuta. For each short-term toxicity test, cultures were shaked in order to detach the algae from the glass beads and obtain an homogeneus algal suspension. Afterwards, 10-15 mL of the algal suspension were centrifuged at 13000 rpm during 10 minutes and the pellet was resuspended in sterile media without EDTA in order to avoid chelation with copper to provide cell densities of approximately 1 x 10^5 cells/mL. 3 mL of this algal suspension were exposed during 3 hours to a growing gradient of copper

concentration; 0, 1.91, 6.35, 19.05, 63.5, 635, 1905 µg/L (nominal concentration) added as copper chloride.

PAM fluorometry measurements. PAM fluorometry was used for monitoring algal growth, using the Fo as the end point, as well as for measuring the toxic effects of copper on the photosynthetic activity of the algal cells by measuring the photochemical yield also called effective quantum yield (Y) (Drábková et al., 2007). All the PAM measurements in the algal cultures were performed in vials of 3 mL of algal suspension. The cells were dark adapted for 15 min before measurements. The parameters measured were: minimal fluorescence yield of dark-adapted cells (Fo) and the maximal signal of dark-adapted cells obtained with a saturating radiation pulse (Fm). These parameters allow the calculation of the maximal yield of PSII as (Fm – Fo)/Fm, also called photochemical yield (Y) which correspond to the capacity of dark-adapted cells to convert photon energy into chemical energy. Y is independent from the algal biomass. This nomenclature is according to Van Kooten and Snel (1990).

Alkaline phosphatase activity measurements. Alkaline phosphatase activity (APA) was determined spectrofluorometrically using 4-MUF-P (methylumbelliferyl phosphate) substrate from Sigma-Aldrich, following the methodology described in Romaní and Sabater (1999). 4 mL of algal suspension from the P-repleted and P-depleted cultures of *N. perminuta* were incubated with MUF-P at final concentration of 0.3 mM (saturation conditions). Incubations were performed in the dark with continuous gentle shaking for 1h at ambient stream temperature. Two blanks of sterilized medium were also incubated. After addition of 0.05 M glycine buffer pH 10.4 (1/1 v/v ratio buffer/sample), fluorescence was measured at 455 nm under 365 nm excitation (Kontron, SFM25).

Data treatment

The ordination of sampling points and sampling dates based on the water chemistry variables was carried out with Principal Components Analysis (PCA). The variables Ammonium, Phosphate and Nitrite were previously log-transformed to obtain the normality. Differences in phosphate concentration

between sampling sites and sampling dates were evaluated by means of two-way ANOVA.

Copper toxicity was estimated as Effective Concentration 50% (EC_{50}) by fitting the photosynthetic end points (Fo or Y) to the four-parameter logistic curve model as is described below.

$$y = \frac{(\max - \min)}{1 + (x / EC50)^{-hillslope}}$$

Where the variable *x* corresponds to the copper concentration of the dose-response test and the *y* is the value of the end-point measured (i.e. Yield or Fo).

Parameter *min* equals the baseline, and *max* is the plateau of the curve. Parameter EC_{50} gives the transition center and is the concentration that causes a reduction of the 50% of the end-point. The *hillslope* determines the slope of the curve at the transition center.

The effect of the phosphorus supply and copper concentrations on periphyton were tested by means of two-way ANOVA. The ANOVA included Cu concentration as Factor 1 (with 6 levels), and phosphorus during the dose-response test as Factor 2 (with 2 levels). For each Cu concentration and P treatment, Fo from 5 replicates were included (corresponding to 5 different colonized glass substrata). This statistical approach allows determining if the effect of Cu on the measured parameter differs between the two P treatments. The two approaches used, the estimation of EC_{50} using the four-parameter model, and the ANOVA are complementary since one provides a quantitative measure (an effective concentration) and the other provides information about the influence of P on Cu toxicity.

Results

Field study

The Fluvià watershed is mainly covered by forested land (68% of the total surface), it is also influenced by agricultural activities (25% of the total surface) with some areas covered by shrub lands (4%), built-up space (2%),

wetland vegetation (<1%) and denuded space (<1%). Land uses for each sub-catchment showed the following gradient: Bianya 1 has the highest percentage of forest and denuded space, Bianya2, Turonell 1, Ridaura, Turonell 2 and Fluvià Olot have greater surface occupied by agricultural and built-up space, and shrub lands (Fig. 2).

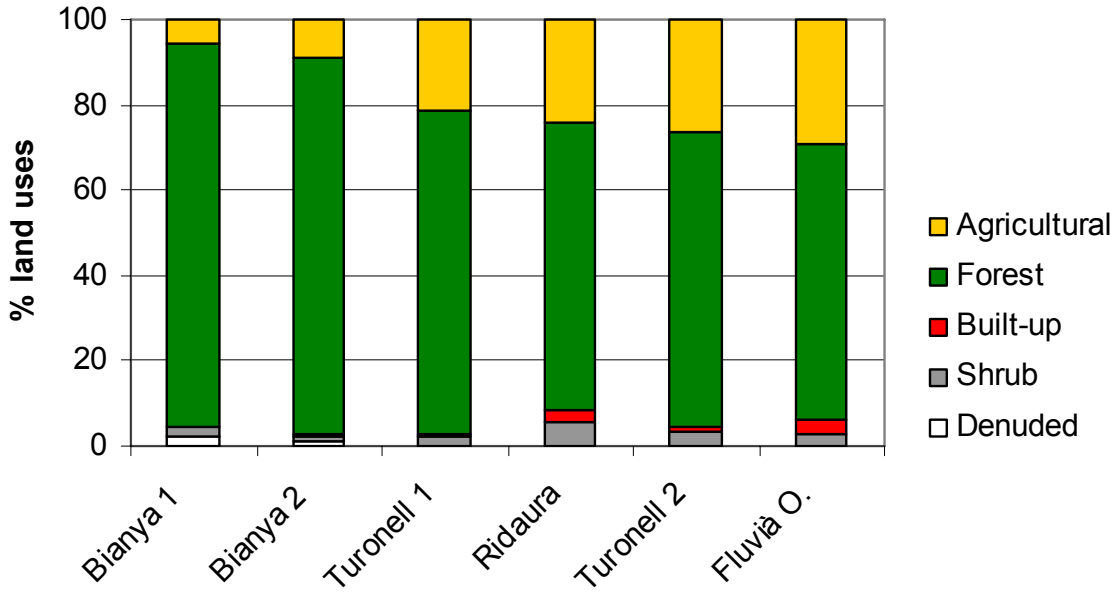


Figure 2. Distribution of percentage of land uses in the Fluvià watershed. Agricultural, Forest, Built-up, Shrub and Denuded correspond to agricultural space, forested space, built-up space, shrub lands and denuded space, respectively.

The PCA analysis based on water chemistry which is summarized in Table 1 (Fig. 3) shows the ordination of sampling points with respect to two principal axes. The first axis explained the 41% variance and separates two sites (Turonell 2 and Ridaura) with high phosphate concentration (higher than 20 µM), high conductivity, ammonium and nitrite concentrations, from the less polluted ones (Bianya 1, Bianya 2 and Turonell 1), where phosphate concentration does not exceed 2 µM. The second axis explained the 22% variance, placing the Fluvià Olot site on the positive side, with high pH, nitrate and dissolved oxygen concentrations. Algal biomass (estimated as chlorophyll-*a* concentration) of the periphyton colonizing the stream bed was lower in sites

with low nutrient concentration and higher in those with intermediate and high nutrient concentration (Table 1).

Table 1. Physical, chemical and biological parameters measured at the studied sites in five dates from October 2002 to November 2003, and percentage of the land uses and land covers of each sub-catchment.

Parameter	Bianya 1	Bianya 2	Turonell 1	Turonell 2	Ridaura	Fluvià O.
pH	8.07/8.16*	7.99/7.96*	8.18/8.14*	8.19	8.09	8.35
	(0.165)	(0.144)	(0.079)	(0.210)	(0.165)	(0.072)
temp (ºC)	9.15	10.7	10.0	11.3	14.0	10.8
	(3.61)	(2.51)	(3.46)	(3.09)	(2.65)	(3.97)
cond (µS/cm)	535/520*	630/588*	603/590*	1914	1590	639
	(98.4)	(118)	(103)	(708)	(307)	(106)
alk (meq/L)	6.6/4.56*	7.48/5.04*	7.81/5.64*	7.84	7.59	6.50
	(1.26)	(1.49)	(1.25)	(1.66)	(1.46)	(1.59)
oxy (% sat)	93.5	93.0	95.3	93.3	80.2	113
	(4.88)	(4.93)	(8.86)	(7.13)	(12.8)	(22.4)
NO_3 (µM)	31.9/34.3*	115/109*	155/172*	174	113	261
	(18.6)	(68.4)	(89.9)	(74.2)	(75.1)	(151)
NO_2 (µM)	0.016/0.06*	0.305/0.32*	0.128/0.22*	16.3	8.38	1.36
	(0.019)	(0.107)	(0.068)	(8.98)	(8.46)	(0.490)
NH_4 (µM)	1.71/0.46*	2.03/1.76*	1.01/1.18*	67.7	72.28	3.27
	(1.90)	(2.87)	(0.688)	(77.3)	(88.3)	(2.61)
PO_4 (µM)	0.085/0.095*	0.155/0.195*	1.05/1.228*	87.4	23.2	1.02
	(0.059)	(0.053)	(0.349)	(52.8)	(10.4)	(0.529)
N/P	424	1089	224	9.30	11.4	636
	(171)	(511)	(115)	(15.5)	(6.45)	(764)
chl-*a* (µg/cm^2)	7.06/20.41*	22.6/26.53*	21.9/33.74*	6.70	26.2	10.2
	(4.65)	(5.11)	(5.48)	(2.89)	(9.95)	(1.49)

The values are the average and the standard error (in parenthesis) for each site of water temperature (temp), conductivity (cond), alkalinity (alk), dissolved oxygen (oxy), chlorophyll-*a* content (chl-*a*).

* corresponds to the physicochemical parameters of stream water and algal biomass used in the channel experiments (June 2003).

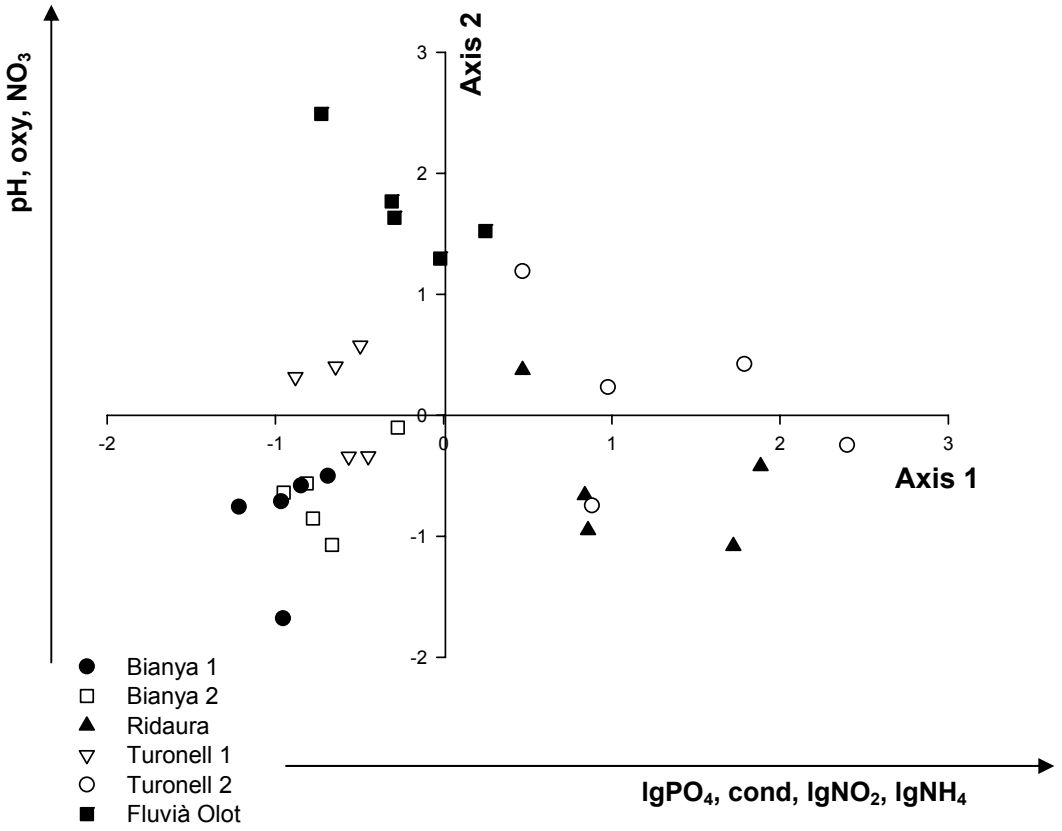


Figure 3. Principal Components Analysis based on the physicochemical parameters of stream water measured in each sampling date summarized in Table 1. Factor loadings correlated with a significance $p < 0.05$ for each factor are indicated under an arrow for clarity. Significance of each axis is $p < 0.05$.

The sites Bianya 1 (B1), Bianya 2 (B2) and Turonell 1 (T1) were selected for the toxicity study in the channels. These sites have been chosen because they have similar physical and chemical characteristics but they differ in their background nutrient concentrations (following a gradient in eutrophy derived from farming and agricultural activities developed in their sub-catchments) and this was persistent over the study period (Table 1). The selected sites showed significant differences in their phosphate background concentrations ($F_{2,8}$ = 25.74; $p < 0.001$) but no significant differences were found between the 5 sampling dates ($F_{4,8}$ = 1.24; $p = 0.368$). In addition, the three selected sites are the least polluted sites among the six sampled and their sub-catchments contain the highest percentage of forested surface (>75% of the total watershed

surface). In these sites, metal pollution, which could interfere with results from toxicity tests, was very low (Guasch et al. 2009). Cu was 0.65 ± 0.19 µg/L in B1,0.99 ± 0.35 µg/L in B2 and 1.44 ± 1.00 µg/L in T1; Zn was 16.58 µg/L in B1, 12.56 µg/L in B2 and 5.54 µg/L and 5.54 µg/L in T1; Cd was 0.055 ± 0.029 µg/L in B1, 0.057 ± 0.025 µg/L in B2 and 0.057 ± 0.033 µg/L in T1.

Experimental channels

The physicochemical parameters of the stream water, and algal biomass of the periphyton used in the channel experiments, are summarized in Table 1.

Periphyton communities were dominated by green algae in B1 and B2 (76% and 78% respectively), and were also abundant (56%) in T1. B2 and T1 communities had a high percentage of diatoms (19% and 44% respectively) compared to B1 (1.8%). Cyanobacteria were more abundant in B1 (22%) than in B2 (2.5%) and no cyanobacteria were found in T1. In addition, B1 presented filaments of *Rivularia spp.*, a colony-forming cyanobacterium which is characteristic of phosphorus-limited ecosystems (Guasch and Sabater, 1995).

The three studied communities showed differences in their sensitivity to copper. In the control treatments (without P-supply), differences in Cu tolerance followed the gradient of nutrient concentrations found in the field (Fig. 4). B1 was the most sensitive community, followed by B2 which was 1.6 times more tolerant than B1 and T1, being 3.6 more tolerant than B1. The increase of EC_{50} values caused by the P-amendment was of 3.5, 3.7 and 1.4 folds in B1, B2 and T1 communities, respectively (Fig. 4). Based on the ANOVA results, Cu sensitivity was significantly reduced after P-addition in B1 and B2 communities while no effect of P-supply was reported in the T1 community (Table 2).

Table 2. Two-way ANOVA results considering the effect of copper (Cu) on chlorophyll fluorescence (%Fo) of the three periphyton communities and the effect of phosphorus supply (P) on copper toxicity.

	B1		B2		T1	
ANOVA	**F**	**p-value**	**F**	**p-value**	**F**	**p-value**
Cu	41.56	<0.001	20.94	<0.001	22.00	<0.001
P	6.96	<0.05	4.84	<0.05	0.49	0.49

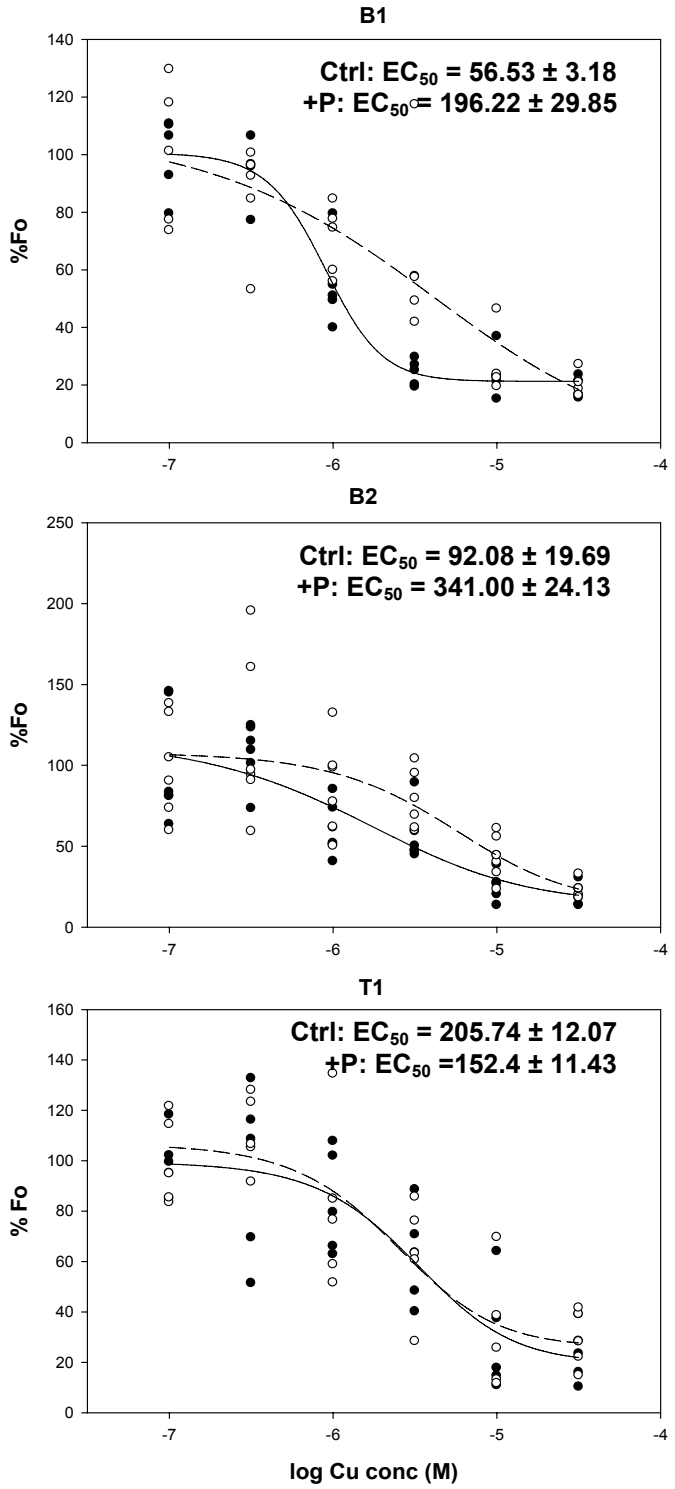


Figure 4. Dose-response curves of the three periphyton communities (B1, B2 and T1). Plots represents the inhibition of the algal biomass (expressed as the percentage of the control of the Fo parameter) measured without (black circle) and with (white circle) P-addition. Continuous lines represents the fitting of the four-parameter logistic curves for the control treatment (without P-amendment) and dashed lines for the +P treatment. Effective copper concentration (EC_{50}) for the three studied communities in the controls (Ctrl.) and after P-amended (+P) ± SE of the fitting are indicated in µg/L.

Algal Cultures

Phosphate concentration of the culture media was 4.75 ± 0.13 µM SRP and 46.33 ± 1.54 µM in P-depleted and P-repleted treatments SRP respectively. Clear differences in Alkaline Phosphatase Activity (APA) were found between P-depleted and P-repleted cultures in both sets of experiments (EXP 1 and EXP 2) as a result of differences in P availability in the media during growth.

APA activities measured in P-repleted cultures were $1.67\ 10^{-7} \pm 5.90\ 10^{-9}$ and $7.92\ 10^{-7} \pm 1.21\ 10^{-9}$ µM MUF/cell h (in EXP 1 and EXP 2, respectively). APA in the P-depleted cultures were $4.13\ 10^{-6} \pm 4.70\ 10^{-8}$ and $2.70\ 10^{-6} \pm 5.04\ 10^{-8}$ µM MUF/cell h (in EXP1 and EXP2, respectively); corresponding to 25 and 3.4 times higher than in P-repleted cultures.

EC_{50} values for Cu inhibited photosynthesis were highly similar comparing both sets of experiments (Fig. 5). The results showed differences in the toxicological responses of the algae depending on both the P status of the algae and the presence of P in the media. Comparing results from toxicity tests performed without P in the media, P-depleted cultures were 1.5 to 2 times more sensitive than P-repleted cultures. In conjunction, the toxicity tests performed with and without P in the media showed that Cu toxicity was reduced 1.6 times by adding P to the media.

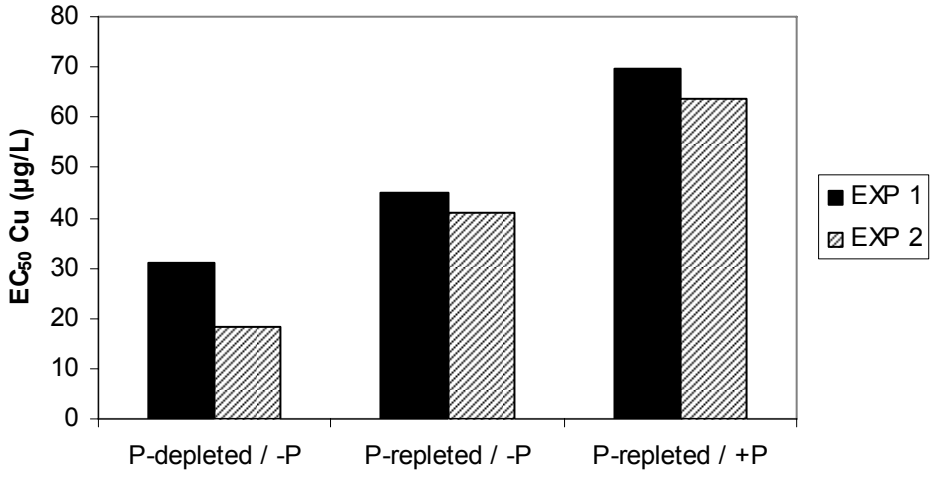


Figure 5. EC_{50} values calculated as the reduction of the photochemical yield (Y) in percentage of the control obtained after three hours of copper exposure in three different treatments. Bars represent the average of the EC_{50} values obtained in duplicate in the two experiments. Black and dashed bars correspond to the results obtained in the experiment 1 and experiment 2 respectively.

Discussion

The use of short-term physiological tests with natural water as the incubation medium and intact communities as key organisms (i.e. periphyton), has the great advantage of being simple methodologically and at the same time ecologically realistic. On the other hand, these tests introduce a high degree of complexity as the chemical behaviour of toxicants is affected by local water chemistry (Guasch et al., 2003). Some of these complexities were systematically analysed using cultures.

In this investigation we explored the influence of eutrophication, and more specifically P enrichment, on Cu toxicity to periphyton from a multi-scale perspective which allowed us to integrate different degrees of complexity. Observations from natural communities exposed to different nutrient conditions in the experimental channels supported our hypothesis that eutrophication leads to a reduction of copper sensitivity in natural periphyton. In addition, observations from algal cultures allowed us to clarify the relative contribution of phosphorus during algal growth and phosphorus in the media to copper toxicity.

Results from culture experiments, which allowed us to isolate the effect of phosphorus on Cu toxicity, confirmed that both the presence of phosphorus in the media and during algal growth leads to an increase in Cu tolerance of algae. Differences in Cu sensitivity between P-repleted and P-depleted cultures, both measured without phosphorus in the media showed that the nutritional state of the algal cells can itself explain differences in the Cu sensitivity of the algae. In agreement with our observations, Hall et al (1989) also found greater copper toxicity in P-limited cultures of *Chlorella vulgaris*. They concluded that P-limited cells were more sensitive to Cu, due to impaired metal exclusion/elimination mechanisms produced by P-limitation. Rijstenbil et al. (1998) also supported this idea. Verma et al. (1993) evidenced that Cu toxicity in cyanobacteria was due to Cu-induced phosphate starvation and that the exogenous addition of phosphate could antagonize the Cu-effect in *Nostoc calcicola.* It has been reported that Cu induces the deficiency of P directly by the inhibition of phosphate uptake, indirectly by reducing the permeability of cell membranes (Nalewajko and Olaveson, 1994). Other recent studies also supported this hypothesis; Wang and Dei (2006) found that P-deficient green algae responded much more dramatically to the increasing metal concentration than P-enriched cells. Metal toxicity in the P-deficient cells was observed within a very narrow range of ambient metal concentration or cellular concentration. One explanation was that the cells were more stressed under P-limited conditions, resulting in the greater toxicity of metals under such conditions.

In our culture experiments, when comparing results from the toxicity tests performed with and without P in the media, a reduction in Cu toxicity of 1.6 times was observed when P was added to the media (in both, EXP 1 and 2). Since the experiments were done with P-repleted cultures, it was not expected that the algae would suffer P-limitation during exposure, thus, the results support the alternative hypothesis that P-Cu interaction in the media leads to a reduction in Cu bioavailability. It has been shown that phosphate precipitates with other metals and that this leads to decreased metal availability (Schulze and Brand, 1978; Nalewajko and Paul, 1985). However, this argument is difficult to apply to natural systems since a copper phosphate precipitate is thermodynamically unlikely to occur in natural waters, because of the presence of competing ligands.

Concerning the experiments performed with natural periphyton, since they were performed with site water, it was expected that differences in Cu sensitivity would integrate the effect of differences in nutritional conditions during growth as well as differences in P content in the water. In fact, the increase in Cu tolerance of periphyton grown under more eutrophic conditions (T1) compared to periphyton from more oligotrophic conditions (B1) (with a 3.6-fold increase of EC_{50} values) was equivalent to the change in Cu toxicity found in the P-limited cultures exposed to Cu without P in the media and the P-repleted cultures exposed to Cu with P in the media (around 2-3 times). Since the increase of Cu tolerance observed in *N. perminuta* cultures may only be attributed to the influence of P, it indicates that differences in Cu tolerance observed in field communities from different sites could be attributed to *in situ* differences in P availability.

Higher metal tolerance found in both, algal cultures and communities, grown under more eutrophic conditions may be attributed to polyphosphate bodies (PPB) production in algal cells under non-limiting conditions. It has been well described that when the concentration of P in the medium is high, the levels of cellular P are elevated with P in excess of immediate cell requirements being stored as PPB (Rhee, 1972; Aitchison and Butt, 1973; Rhee, 1973). Several studies have reported the role of PPB in metal detoxification, (e. g. Jensen et al, 1982; Twiss and Nalewajko, 1992). These authors stated that intracellular polyphosphate is important in sequestering metals in a detoxified form and protecting the cell from the toxic effects of the metals. Thus, detoxification by PPB could also explain the high tolerance found in natural communities developed under more eutrophic conditions and P-repleted cultures in the present study, but this aspect was not directly addressed.

In the channel experiments, the exogenous addition of P during the toxicity tests enhanced Cu tolerance of the communities from the more oligotrophic sites (B1 and B2). These observations may be related to lowered nutrient limitation by the P-addition during the tests compensating the effects of Cu. These results are in agreement with a previous study (Guasch et al., 2004) where copper toxicity was slightly reduced when nutrients were simultaneously added in nutrient-limited communities. They suggested that the addition of metals produces a strong nutrient limitation, and that the addition of the toxicant

together with phosphorus may partially compensate the inhibitory effect on the physiological response of the community. Barranguet et al. (2002) found similar results.

Chemical factors other than phosphorus are known to modify the response of natural periphyton in fluvial ecosystems. The pH of the media has been considered an important factor influencing the toxicity of Cu on periphyton (Guasch et al., 2002) and on algal cultures (Starodub et al., 1987). These authors showed an increase in Cu toxicity when pH was reduced. This can be explained by copper speciation, because the amount of Cu^{2+} (which is considered the most available form of Cu for algae) increases at lower pH (Rai et al., 1993; Guasch et al., 2002). In our study, water pH in the experimental channels, which was done using natural periphyton and site water, ranged between 7.96 and 8.16 units of pH. These pH differences could influence the toxicity of Cu on periphyton. Taking into account these arguments, a higher sensitivity would be expected in B2, which had the lowest water pH during the channel experiments. This expectation contrasts with the results found: B1 and T1 showed marked differences in EC_{50}'s in spite of having similar water pH. We can therefore conclude that pH has not played a decisive role in explaining differences in the Cu sensitivity of natural periphyton in our experiments.

Differences in copper sensitivity between different periphyton communities have also been attributed to the protective role of biomass (Admiraal et al., 1999; Navarro et al., 2002; Guasch et al., 2003). Barranguet et al., (2002) suggested that the dependence between periphyton biomass and Cu toxicity was related to its unspecific mode of action. Cu toxicity was progressive by indiscriminately damaging the algae at or protruding progressively from the biofilm surface to deeper biofilm layers, thus, the magnitude of the effect depended on the initial biomass in the short term (Guasch et al., 2004). In our investigation, although the periphyton communities presented differences in algal biomass, a long duration of the toxicity tests (18h) was specifically selected to allow the metal to attain the deeper layers of the biofilm, minimizing the possible influence of algal biomass on the toxicological response of periphyton. Differences in the Cu sensitivity of natural communities could also be attributed to different algal taxa. However, taking into account that metal sensitivity is species-specific (Hutchinson and Stokes, 1975), although the three

studied communities showed differences between major algal groups, the taxonomic resolution obtained in our study did not allow us to derive this information.

Several ecological implications may be suggested if the experimental results obtained are extrapolated to the ecosystem scale. Based on the results of this study, it is expected that periphyton communities in fluvial systems draining forested areas will be more sensitive to copper since these sites will have low phosphate concentration. In addition, oligotrophic sites will also have a low probability of receiving metal inputs due to the dominating land use. On the other hand, periphyton communities located in more human-impacted areas will suffer from both eutrophication and potential metal inputs, as has been reported in previous studies (Guasch et al. 2009), and will be more tolerant to Cu.

In conclusion, the present study has demonstrated that elevated copper input into rivers has a measurable impact on natural periphyton communities and that such impact can be verified in cultured, single-species consortia. In both settings the interaction of phosphate availability and copper toxicity was found to be substantial, providing evidence for the presumed amelioration of copper toxicity by high phosphate concentrations. The observations implicate that potential ecological effects of metal input in rivers might be obscured by eutrophication.

Chapter VI: General discussion

Cu dynamics in rivers

Methodological remarks

In the present investigation, different approaches (linking traditional ecology and community toxicology concepts and methodologies) have been developed, used and/or applied to understand better the fate and effects of copper (Cu) in fluvial systems. The recent development made in the field of periphyton ecotoxicology has provided the tools for investigating the functional and structural responses of the community to pollutants entering stream ecosystems. On the other hand, in terms of experimentation, the studies performed so far have provided the basis for laboratory testing using complex communities in indoor experimental channels. In addition, the nutrient spiraling concept has linked the transport of solutes (nutrients) and their abiotic and biotic retention and provided the methodological and theoretical framework of this research.

A new method, based on the concepts and methodologies used for studying nutrient dynamics in rivers, has been developed and optimized in this thesis for studying metal dynamics in streams. In-stream Cu retention was studied at sub-toxic concentrations in a “pristine” system (in terms of chemical pollution) as well as in a chronically-exposed system. Additionally, the methodology used allowed the influence of different biotic and abiotic factors on metal retention to be evaluated, contributing thus to increase the ecological relevance in the field of experimental ecotoxicology.

Relevant findings and open questions

In agreement with our hypothesis, water discharge influenced Cu downstream transport and retention. Concretely in-stream Cu retention efficiency, which was estimated by the Cu uptake length (S_w), was reduced at high discharges. These results, which are in agreement with the findings from nutrient retention studies in the field (e.g. Butturini and Sabater, 1998; Martí et al., 2004), imply that metals will travel longer downstream distances at higher discharge conditions, thus this metrics gives an idea of the spatial scale of the Cu retention process in streams. On the other hand, and in contrast with what was expected, Cu retention was enhanced by the presence of biofilm but did

not increase further when algal biomass was higher. These results are probably related with the methodological approach used. The Cu retention measured by means of “constant-rate additions”, which are short in time, may only show the metal adsorption to the extracellular compartment of the biofilm (corresponding to the first phase of the metal retention mechanism). This may explain why a higher amount of Cu was removed from the water column with the presence of periphyton colonizing the channels compared to measurements done without periphyton, but retention efficiency did not increase with the thickness of the biofilm (higher biomass). Cu retention in the colonized system was much higher due to the presence of biotic metal binding sites. However, the temporal scale of the in-stream retention experiments did not allow the metal to diffuse through the biofilm and therefore, metal retention was not increased with biofilm thickness.

The comparison between uptake rates of Cu and phosphate provided interesting results. It was very clear that periphyton has a higher uptake and demand for phosphate than for Cu reflecting the behaviour of macronutrients vs. micronutrients of the two studied solutes (phosphate and Cu) through their dynamics.

It is well known that periphyton, and more specifically algae, are able to adapt to long metal exposures (e.g. Soldo and Behra, 2000). This adaptation may imply intracellular mechanisms which allow the accumulation of the metal without being toxic for the organism (e.g. polyphosphate bodies, phytochelatins, metal efflux pumps, etc.); extracellular mechanisms (EPS production) or taxonomical changes (shifts in species composition from sensitive to tolerant ones), but what had still not been explored was if this adaptation also affected metal retention in rivers. The results from our study showed that in-stream metal retention was reduced after chronic Cu exposure of the community. The possible saturation of metal binding sites produced by the Cu pre-exposure of the periphyton may explain the lower Cu retention efficiency of metal-polluted systems.

It might be concluded that the application of the nutrient spiraling concept and methodologies to the study of Cu dynamics allows a functional aspect of the stream ecosystem to be studied; the retention efficiency of the studied solute. However, this approach does not account for temporal responses, thus,

despite being a good tool for studying the efficiency of the system in terms of metal retention at a given time, it is not useful for studying processes occurring at longer time scales.

Cu accumulation and toxicity in periphyton

Methodological remarks

Accumulation of Cu in periphyton was studied along the time in a "static system". Metal accumulation kinetics has mainly been studied in phytoplankton or using algal monocultures (e.g. Knauer et al. 1997; Sunda and Huntsman, 1998; Hudson 2005). These studies describe a biphasic mechanism in which the metal is first adsorbed extracellularly and then is internalized by the cell. The discrimination between intra and extracellular fractions of the metal (Meylan et al., 2003) allows us to differentiate between these two processes of the metal accumulation by algae. In this thesis, the kinetics of Cu accumulation was studied in biofilms that differed in their metal exposure. This study complemented the previous findings of Cu dynamics in streams. While in the experiments of Cu dynamics, the Cu retention was studied at high spatial scale (reach scale), here the process of metal retention is studied in detail, using a smaller scale (biofilm) and including the time as a variable.

Relevant findings and open questions

Our study demonstrates that Cu accumulation in periphyton communities follows the biphasic mechanism described for planktonic species. The results also showed that previous metal exposure (pulsed exposure) of the biofilm leads to a decreased intracellular uptake. These results support our hypothesis that metal pre-exposure can lead to a decreased metal uptake capacity of periphyton due to the saturation of the metal binding sites, which could explain the reduced Cu retention efficiency of the system that was previously exposed to the metal as is discussed above. These results also confirm our hypothesis that Cu retention measured using constant-rate additions, which lasted for less than two hours, corresponds to the first phase of the accumulation mechanism,

the adsorption to extracellular compounds, since the internalization of the metal occurred later on.

The sampling frequency used in this study (every two hours) resulted appropriate to study the Cu accumulation kinetics and also to discriminate between the metal adsorption and uptake processes in the biofilm. Further studies aiming to provide a more detailed description of Cu accumulation in periphyton, should include a more frequent sampling (e.g. every 30 min.). In addition, if the kinetics of Cu accumulation is studied in communities that have received chronic Cu exposure (having high Cu content in the cells), caution should be taken in sufficiently increasing the concentration of the metal in the media to further detect an intracellular increase.

In this investigation we also found that long-term Cu exposures lead to structural changes which allow the community to survive under metal-polluted conditions. These changes consist of shifts in the taxonomic composition of the community (from sensitive to more tolerant species) and to increases in extra and/or intracellular binding sites, allowing the community to accumulate the metal without causing toxic effects. However, it is interesting to notice the lack of indicative value of taxonomic indices as species diversity for metal pollution monitoring purposes, since changes in community composition will depend on the reference conditions. In contrast with what was expected, chronic Cu exposure caused an increase in the diversity of diatoms. Conversely, short-term metal exposures (pulsed exposures) to the metal were not long enough to produce these structural changes in the community, therefore this community was not able to adapt to metal exposure being more sensitive than the adapted one.

Cu-phosphate interaction

Methodological remarks

Descriptive studies alone cannot be used to show causation, and so experimental studies are necessary to demonstrate cause-and-effect relationships. Microcosm and mesocosms approaches represent a link between standardized, single species toxicity tests and more expensive, logistically

difficult field experiments. An investigation which integrates experimental approaches at different scales is considered optimal for determining causation (Clements and Newman, 1992). Combining field observations with experimentation in the laboratory allows the application of the observations to field situations.

In our investigation, the influence of phosphorus on Cu toxicity was explored using a multi-scale approach (Serra et al., 2009*c*).

Relevant findings and open questions

This investigation demonstrates that phosphorus availability (during growth and in water during metal exposure) strongly influences the toxicological response of periphyton to Cu in the short-term. These results suggest that concurrent exposure to higher input of P and Cu leads to new and less intense ecological changes. This study highlights the relevance of investigating the relationship between land-uses and water chemistry to better understand causality. Based on the results obtained it might be concluded that Cu will have greater negative impacts on rivers draining forested areas, whereas nutrient export from the terrestrial to the aquatic systems will be higher in human impacted areas and metal sensitivity of the biota (periphyton) will be lower. The toxicological response of the biota will depend on the bioavailability of the metal, determined by water chemistry and the physiological status of the organisms. In the case of algae, the trophic status of the cells may contribute to the development of detoxification mechanisms such as the formation of poly-phosphate bodies.

General comments and applied issues

Fluvial conservation includes the conservation of structural traits, such as biodiversity as well as functional integrity, such as primary production or uptake capacity. The results of this investigation highlight the potential impact of an input of metals of short duration, as it may happen, for instance, after an accidental spillage, in fluvial ecosystems. This type of impact may be undetected in routine monitoring since metal concentration in water will

decrease very fast. This acute exposure may not have detectable effects on the structure of the community, since algal biomass or taxonomic composition may not be affected. However, it can produce marked effects on its function, as was shown by the inhibition of the photosynthesis and accumulation kinetics observed in this investigation.

This investigation points out very clearly the need for including measures of metal accumulation in the biota in monitoring studies. As stated before, metal can be very low in water (e.g. after a pulsed metal input) but be accumulated in the benthos (periphyton community). In this case, metal pollution may not be detected but, the ecological integrity may be at risk. Metal retained on the basis of the fluvial food chain will be available to higher trophic levels compromising the ecosystem's integrity. Therefore, analysing the metal content in the biota will provide a more reliable prediction of the ecosystem effects of metal pollution in rivers.

Land uses in the watershed will have strong repercussions on the fluvial ecosystem (Fig. 1). Rivers draining forested watersheds are expected to have low nutrient and metal concentrations. The periphyton communities will have low algal biomass due to nutrient limitation and the species composition will be characteristic of oligotrophic fluvial systems. It is also expected that these communities will show a high sensitivity to Cu due to the low protection capacity. The low biomass and low ability to immobilize intracellularly the metal, will determine the low protection capacity. In addition, the species sensitivity of these periphyton communities as well as the low presence of ligands characteristic of these oligotrophic waters may determine a high Cu sensitivity.

In agricultural watersheds, a high concentration of nutrients in water (phosphate) is expected. Eutrophication of the fluvial system is linked to high algal biomass of periphyton and a shift in species composition of the communities (Smith et al., 1999). These changes will reduce the Cu toxicity due to the protection of biomass and the lower availability of the metal due to the higher phosphorus content in water and in the algal cells.

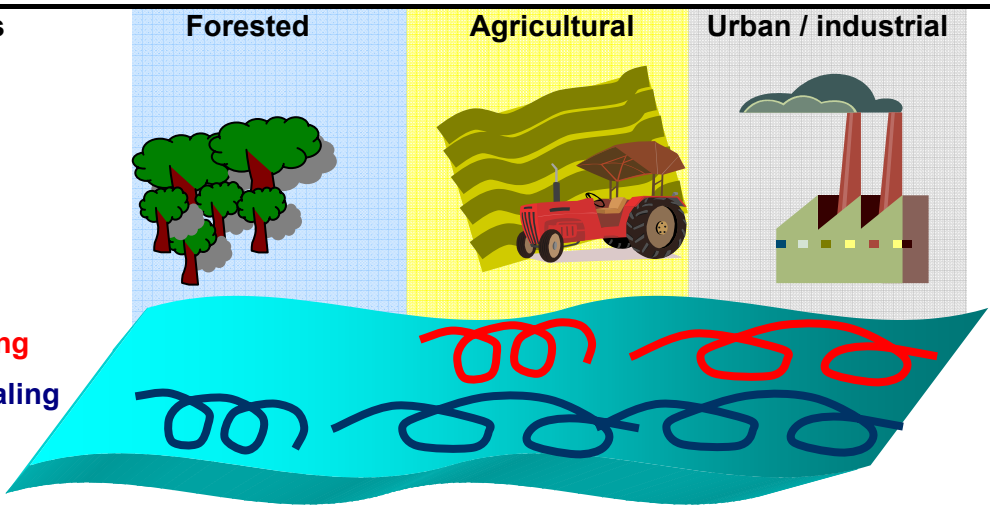
If metals are entering the fluvial systems together with nutrients (as is the case of urban and industrial watersheds) the stimulatory effect of nutrients on

algal biomass could be counterbalanced by Cu toxicity. Long-term Cu exposures will produce an adaptation of the communities by replacing the more sensitive species by more tolerant ones, and this adaptation will provide a higher community tolerance.

These different scenarios will also modulate the dynamics and retention of pollutants (Fig. 1). Previous nutrient dynamics studies showed that phosphate is retained more efficiently in oligotrophic than eutrophic systems (e.g. Martí et al., 2004). This investigation shows that Cu pollution will not directly affect phosphorus dynamics in nutrient-enriched systems. On the other hand, Cu retention efficiency will be reduced, being transported longer distances downstream.

In addition to the high Cu retention efficiency observed in the non-exposed community, a high Cu accumulation rate was also shown. This can be related to the availability of free metal binding sites in periphyton growing in non-metal polluted sites. Compared to this non pre-exposed periphyton, communities adapted to Cu after chronic exposure will have a higher Cu accumulation capacity due to the higher abundance of metal binding sites. However, it is not clear if the higher availability of metal binding sites will also provide a higher Cu accumulation rate or not since these binding sites might also be saturated.

Cu dynamics and accumulation have not been explored in oligotrophic fluvial systems. Future investigations in this direction would be very valuable for knowing the influence of eutrophication on the capacity of fluvial ecosystems to remove metals from the water column.



Water chemistry	↓ nutrients	↑ nutrients	↑ nutrients
	↓ Cu	↓ Cu	↑ Cu
Community	ref. Spp	shifts spp	shifts spp
structure	↓ algal biomass	↑ algal biomass	~ algal biomass
Cu toxicity	↑ toxicity	↓ toxicity	↓ toxicity
PO_4^{3-} dynamics	↓ S_w-$PO4_4^{3-}$	↑ S_w-PO_4^{3-}	↑ S_w-PO_4^{3-}
Cu dynamics	**?**	↓ S_w-Cu	↑ S_w-Cu
Cu accum. capacity	**?**	↓ Cu content	↑ Cu content
Cu accum. rate (kinetics)	**?**	↑ rate	**?**

Figure 1. Scheme summarizing the main findings of this investigation and remaining open questions.

Perspectives

Studies combining the theoretical bases and methodologies of both ecology and ecotoxicology disciplines are still scarce. This approach is necessary for understanding the causes of ecosystem damage and for its further restoration. Some specific aspects concerning the fate and effects of Cu in the fluvial system were explored in this investigation but many questions are still open.

The influence of nutrients on Cu toxicity was investigated. Since fluvial ecosystems are exposed to many stressors, it is also necessary to investigate

the influence of other environmental variables like water flow or light regime on toxicity.

The spiraling of toxicants developed in this study is a powerful tool. It was used to investigate Cu retention and to explore the influence of flow and biomass on it. The effects of a higher range of flow conditions or other environmental factors might also be explored as well as the retention of other toxic compounds.

Cu retention was studied in a simplified system. The use of larger systems including more complexity and their application, for instance, outdoors, might also increase the realism of the approach.

CONCLUSIONS

Measuring in-stream retention of copper by means of constant-rate additions

1. In-stream copper retention could be quantified by applying concepts and methodologies comprised in the nutrient spiraling theory in an artificial river system.

2. Copper was retained less efficiently than phosphate. This may reflect the different biological demand for both solutes.

3. Copper and phosphate retention efficiency were negatively influenced by water discharge. Periphyton accumulated greater copper amount at lower discharge conditions.

4. While phosphate uptake rate increased with algal biomass, no relationship was found between algal biomass and copper uptake. However, copper uptake was enhanced by the presence of biofilm in the channels.

5. The methods applied provide an ecological relevant approach for investigating the dynamics of toxicants in river ecosystrems.

Effects of chronic copper exposure on fluvial systems: linking structural and physiological changes of fluvial biofilms with the dynamics of toxicants

6. Chronic Cu-exposure caused clear effects on the structure of periphyton, affecting the Cu retention capacity of the system.

7. Structural effects of chronic Cu-exposure on periphyton included: a reduction of the community algal biomass; shifts in community composition from diatoms to green algae; marked increses in total and intracellular Cu content and slight increases in the EPS content.

8. The chronic Cu-exposure caused an induction of Cu tolerance of periphyton. This was shown by the maintainance of high photosynthetic efficiency, although having a high intracellular Cu concentration.

9. Cu retention efficiency was reduced after chronic Cu exposure which was indicated by longer Cu uptake lengths (S_w-Cu) and lower mass transfer coefficients (Vf-Cu). On the other hand, chronic Cu exposure did not influence the stream phosphate retention efficiency.

10. The exposure history of the community will influence downstream transport of copper in fluvial ecosystems.

Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history

11. Pulsed Cu exposure did not cause significant effects on the relative abundance of different algal classes. However, continuous Cu exposure caused a reduction in the proportion of diatoms and an increase of green algae and cyanobacteria.

12. The three studied communities (no-Cu, Cu-pulsed and Cu-continuous) had high similarity in the diatom assemblages (>62%). Continuous Cu exposure caused an increase in diatoms diversity which can be attributed to the effect of the toxicant by decreasing the abundance of the dominant specie of the relatively simple laboratory community and allowing the increase in the relative abundance of other species.

13. Pulsed Cu exposure affected the Cu uptake kinetics by decreasing the intracellular metal uptake. However, total Cu accumulation kinetics did not differed from the control community.

14. Continuous Cu exposure caused a large increase in both total and intracellular Cu content of the community but effects on Cu accumulation kinetics could not be evaluated from our experimental study.

15. Both pulsed and continuous metal exposure had negative effects on periphyton. While Cu pulsed exposure caused toxicity, continuous exposure leaded to community adaptation, which was linked to changes in species composition and higher metal contents.

Influence of phosphorus on copper sensitivity of fluvial periphyton: the role of chemical, physiological and community-related factors

16. Experiments done with natural communities exposed to different nutrient conditions demonstrate that eutrophication will lead to a reduction of copper sensitivity in natural periphyton.

17. Experiments performed with algal cultures allowed the relative contribution of phosphorus during algal growth and phosphorus in the media to copper toxicity to be clarified.

18. The investigation of the relationship between land-uses and water chemsitry allowed the experimental results to be upscaled. It can be stated that Cu will have greater negative impacts on rivers draining forested areas, whereas in more impacted areas, where the eutrophication is higher, the metal sensitivity of the biota (periphyton) will be lower.

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