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Variability in lotic periphyton community tolerance to zinc and atrazine, in relation to bioavailability

MARIA PAULSSON
Department of Plant Physiology
Göteborg University

Faculty of Science, Göteborg University

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Akademisk avhandling

För filosofie doktorsexamen i Miljövetenskap med inriktning mot fysiologisk botanik (examinator: professor Christer Sundqvist), som enligt beslut i Miljövetenskapliga sektionsstyrelsen vid Göteborgs Universitet, kommer att offentligen försvaras fredagen den 22 september 2000, kl. 10.15 i föreläsningssalen, Botaniska Institutionen, Carl Skottsbergs Gata 22B, 413 19 Göteborg.

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Maria Paulsson

Department of Plant Physiology, Göteborg University, Box 461, SE 405 30 Göteborg, Sweden

Abstract: The variability in community tolerance to zinc and atrazine for periphyton from different running waters were studied in relation to environmental factors, and how this affects the applicability of the Pollution Induced Community Tolerance (PICT) concept as well as the toxicity of zinc and atrazine.

Zinc had effects on periphyton biomass production at concentrations, which are presently exceeded in many European rivers. The low effect level on biomass production was due to an indirect effect of zinc on the phosphorus availability, which caused phosphorus deficiency in the periphyton. This indirect effect of zinc is assumed to be a problem particularly in phosphorus-poor environments.

Results on atrazine toxicity conformed well to earlier findings and suggest effects of atrazine only in the most polluted waters. However, a field study on atrazine tolerance suggested a positive correlation to atrazine pre-exposure and nutrients. This shows a slight indication that atrazine concentrations in some European rivers might cause a selection pressure on periphyton communities favouring tolerant life-forms.

Induced periphyton community tolerance to atrazine was difficult to detect with the short-term endpoints used. A very faint but consistent increase in tolerance that coincided with slight changes in algal community structure was found. This suggests a weak and slow selection pressure on natural communities for atrazine tolerance or that the short-term methods used to detect tolerance did not measure the tolerance in a meaningful way.

If bioavailability in a stream is low, tolerance measured in short-term tests may be overestimated. Atrazine was found to be highly bioavailable to periphyton, which should enable proper PICT measurements using short-term tests. A field study also showed low variability in atrazine tolerance. However, the detection difficulty, at least with the short-term endpoints used this far, suggests limited applicability of PICT as an ecotoxicological tool for atrazine, unless high precision measurements of community tolerance can be performed.

A field study revealed that many environmental factors correlate positively with zinc tolerance. One of the most important factors was zinc exposure, which suggests that several of the rivers studied had concentrations that had restructured the communities into more tolerant ones. Yet, there may be many confounding factors such as gradients in bioavailability, co-tolerance with other metals and inherent tolerance of the periphyton communities that can obscure the PICT response for zinc. Therefore, cautious analyses of the influence of these confounding factors are needed for PICT to be applicable as an ecotoxicological tool for zinc.

Keywords: running waters, algae, bacteria, heavy metal, *s*-triazine, toxicity, microcosm, PICT, photosynthesis, sulfolipid synthesis, thymidine incorporation, Redundancy analyses, PLS

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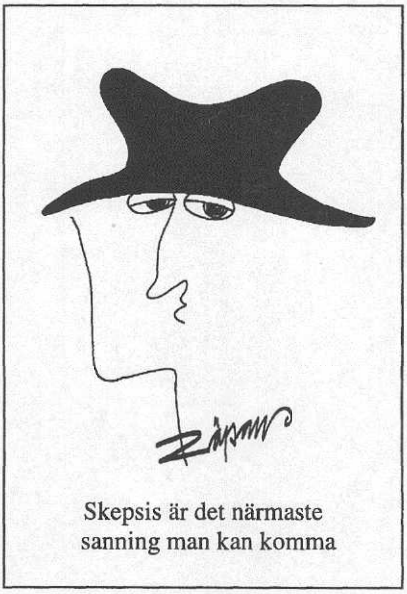

Skepsis är det närmaste
sanning man kan komma

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Maria Paulsson

Department of Plant Physiology, Göteborg University, Box 461, SE 405 30 Göteborg, Sweden

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. Paulsson M, Nyström B and Blanck H. 2000. Long-term toxicity to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosm study. Aquat. Toxicol. 47:243-257.

II. Nyström B, Paulsson M, Almgren K and Blanck H. 2000. Evaluation of the capacity for development of atrazine tolerance in periphyton from a Swedish freshwater site as determined by inhibition of photosynthesis and sulfolipid synthesis. Environ. Toxicol. Chem. 19:1324-1331.

III. Paulsson M, Månsson V and Blanck H. Effects of zinc on the phosphorus availability to periphyton from the river Göta Älv. (Manuscript).

IV. Nikkilä A, Paulsson M, Almgren K, Blanck H and Kukkonen JVK. Atrazine uptake, elimination and bioconcentration by periphyton communities and *Daphnia magna*: effects of dissolved organic carbon. (Submitted manuscript).

V. Blanck H, Admiraal W, Cleven RFMJ, Guasch H, van den Hoop MAGT, Ivorra N, Nyström B, Paulsson M, Petterson RP, Sabater S and Tubbing GMJ. Variability of zinc tolerance, measured as incorporation of radio-labelled carbon dioxide and thymidine in periphyton communities sampled from 15 European river stretches. (Manuscript).

VI. Guasch H, Ivorra N, Lehmann V, Paulsson M, Real M and Sabater S.1998. Community composition and sensitivity of periphyton to atrazine in flowing waters: the role of environmental factors. J. Appl. Phycol. 10:203-213.

CONTENTS

1. PREFACE

The current estimates of marketed chemicals vary widely from 20,000 to 70,000 and there is limited information about the toxicity of about 75% of these chemicals (EEA & UNEP 2000). In 1995 the worldwide chemical industry produced 400 million tonnes of chemicals (EEA & UNEP 2000). Of these 3 million tonnes were pesticides (Stanners & Bourdeau 1995). A lot of metals are also taken out of the earth's crust. The current mining rate of e. g. zinc is estimated to be 5 million tonnes per year (Levasseur 1993). In each step, from handling of the raw material, to production and to the use and waste there may be discharges of substances into the environment where they might pose a risk to living organisms. Substances that are spread into the environment and which are persistent (not easily broken down) or bioavailable are considered to be an environmental risk, if they produce adverse effects on the structure or function of a biological system. The characteristics of a biological system are influenced by the properties of its surrounding environment, and can therefore vary a lot between different systems. As a consequence, the sensitivity to a toxic substance may also vary. This has to be considered in environmental hazard assessment of the biological systems that is considered important to protect. To an ecotoxicologist, effects of toxic substances on any living organisms within an ecosystem are of concern. The focus of this thesis will be on the effects of zinc and atrazine on algae and bacteria living in periphyton communities in running waters.

2. BACKGROUND

2.1. Lotic ecosystems

Lotic ecosystems are the running waters like brooks, streams and rivers. These may have a special position in aquatic ecotoxicology since they collect and convey water and solutes that wash the biosphere, which is contaminated by toxicants through human activities. The worldwide rivers and streams are estimated to drain an area of land of approximately 150 million km^2 (Giller & Malmqvist 1998). Even though they only constitute about 1% of the world's freshwater resources (Keller 1984) they are important habitats for several organisms. They are also important as sources of drinking water and irrigation, for extracting energy and as conduit of effluents from different human activities (including toxicants).

The uniqueness of lotic systems compared to other aquatic habitats is that they are unidirectional, running from steep to flat, from shallow to deep. This means that they flow in one direction from one site to another that is hierarchically connected. Lotic systems can be classified into stream orders according to this

hierarchy. A 1st order stream is a single, unbranched headwater channel, and when two 1st order streams meet they form a 2nd order stream and so on. Low order streams or upstream systems will therefore always influence downstream reaches.

Lotic systems receive their water either directly from precipitation of rain or snow, but mainly indirectly through overland runoff, subsurface flow or ground water flow after percolation through the ground (Giller & Malmqvist 1998). For this reason the water reaching the lotic system has been in intimate contact with the surrounding land areas, including the polluted ones. The climate, which is influenced by geography, is responsible for the amount of water that precipitates over land (Fig. 1). The characteristics of the catchment area or drainage basin, which may differ in topography, geology, soil type and vegetation, is also decisive for the amount and quality of water and material that will reach a stream. Precipitation and the catchment characteristics will affect the water flow, which in turn affects the amount of material entering the stream. This affects the temperature, light, pH, salinity, water hardness, and concentrations of particles, dissolved organic matter (DOM), oxygen and nutrients as well as land-derived toxicants. The water chemistry and physics will affect the characteristics of the substrate and the biota, but also the other way around.

Toxic substances are typically introduced into lotic systems in pulses when precipitation of water is high or during snow melting. The concentration of a toxicant reaching a stream can be very high during these pulses but is often quickly diluted downstream a system, due to the continuous input of water from unpolluted lower order streams and through infiltration of groundwater. Lotic systems are therefore normally relatively resistant to change and can self-clean over time and space (Giller & Malmqvist 1998). However, regarding very extensive, concentrated or continuous inputs of toxicants, they may not be able to withstand damage.

How sensitive a lotic system is to a toxic input depends on many characteristics. Due to differences in geography, climate and catchment area, the variability both within and between different lotic systems is high, chemically as well as biologically. Thus, the bioavailability of toxicants introduced into the biosphere and the biotic response towards them are also expected to vary.

2.2. Periphyton communities

Microscopic algae living in periphyton communities are important primary producing biota in lotic environments (Weitzel 1979, Stevenson 1996). Algae, bacteria, fungi and different grazers such as e. g. ciliates and flagellates as well as detrital and silt particles trapped in the gelatinous polysaccharide matrix are all microscopic parts of a periphyton community. These communities are chemical modulators, in the sense that they transform many inorganic chemicals

into organic forms, they stabilise substrates and are an important food resource for many other organisms (Stevenson 1996).

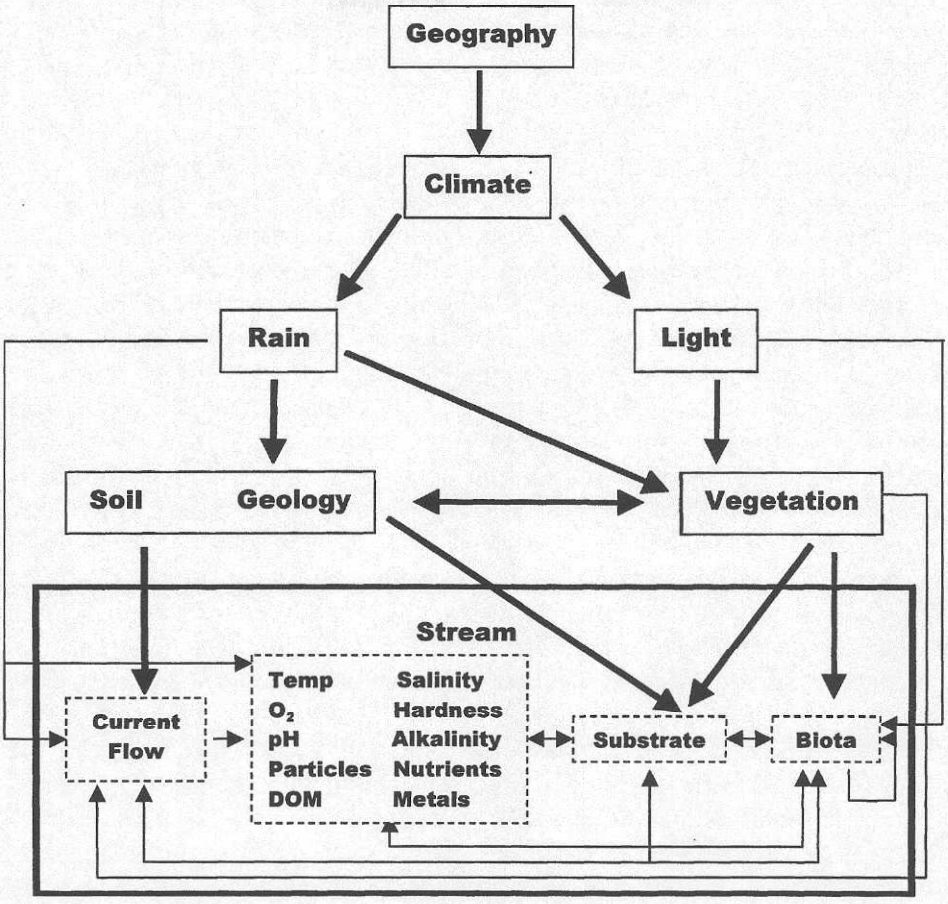


Figure 1. A schematic outline of the intricate network of some environmental factors that affect a lotic ecosystem.

The term community refers to an assemblage of populations of living organisms in a prescribed area or habitat that interact with each other (Krebs 1985, Chapman & Reiss 1992). The term periphyton was first used in 1924 as pointed out by Behning (1928) and literally means "around plants" (Weitzel 1979). Most often periphyton refers to micro-organism communities attached to or living on submerged substrates (Lewis 1995), sometimes specifically on artificial substrates (Sladeckova 1962 and references therein). Another term used

is Aufwuchs (German for "growth upon") although not so commonly used nowadays (Stevenson 1996). There are however many different terms referring more or less to the same assembly of organisms (Sladeckova 1962). Names referring to substrates can also be used. These are epilithon (on rocks), epiphyton (on plants or macroalgae), epizoon (on animals), epidendron (on wood), epipelon (on mud or sediments) and epipsammon (on sand) (Weitzel 1979). In this thesis the term periphyton is used since no better definition exists at present and the communities were established on artificial substrata (see Sladeckova 1962).

The immigration and colonisation of algal and bacterial periphyton species is a process dominated by passive settlement of cells (Biggs 1996). The settlement rate is dependent on the upstream species pool and the substrate characteristics. Toxicants that affect species composition in periphyton communities upstream of a system may therefore also have serious effects on downstream colonisation. The colonisation of a clean substrate starts with the development of an organic matrix, followed by bacteria. Then small adnate diatoms settle followed by apically attached colonial diatoms and finally filamentous green algae and cyanobacteria (Biggs1996). Periphyton communities consist mainly of non-moving organisms, but when the biofilm becomes thicker, moving organisms can be found (Round 1964). The periphyton biomass grows until it reaches its carrying capacity (Biggs 1996). That occurs when the biomass accrual equals the biomass loss. Sometimes parts of or the whole community can slough (Biggs 1996). This phenomenon may occur when nutrient and light conditions become unfavourable for the algae living closer to the substrate from which they detach. Other unfavourable conditions like toxic stress can also result in a spontaneous drift of species (Giller & Malmqvist 1998) enabling sensitive species to move to a cleaner site. Mechanic stress such as grazing and floods will also affect the amount of loss from the periphyton biofilm. Habitats with frequent floods and high grazing therefore tend to have low average biomass (Biggs 1996). Current velocity is hence one of the most important factors affecting the structure, biomass, export and colonisation rate of periphyton. It also affects the boundary layer and thereby diffusion of e. g. nutrients into the biofilm. The outer living organisms will receive more of their nutrients from the surrounding water than organisms deeper into the biofilm, which may have to depend more on internal cycling of nutrients or nutrients from the substrate. The internal cycling of nutrients will increase as the biofilm becomes thicker and the boundary layer increases. Due to this boundary layer, large gradients in e. g. pH can develop and the ability for substances, including toxicants, to reach the inner parts of the biofilm is decreased. It is therefore advisable to only use relatively thin periphyton communities in toxicity studies since realistic flow velocities are difficult to achieve.

2.3. Bioavailability

Bioavailability can be defined as the fraction of the total quantity or concentration of a substance in the environment that is available for action on e. g. the physiology of living organisms. However, the bioavailable fraction can be difficult to measure. Methods that have been used to measure bioavailable fractions for metals are e. g. dialysis resins, ion exchange techniques, chelating resins such as Chelex-100 and electrochemical techniques such as Anodic Stripping Voltammetry (ASV) (Florence & Batley 1977). Voltammetrically labile fractions reflect ionic and weakly complexed species of the substance, while more strongly bound complexes can be measured by using ion exchange or chelating techniques. Dialysis resins on the other hand attempt to resemble the biological membrane in bioaccumulation studies (see below). The resins are put into the water for a defined period of time during which substances in the water diffuse into the resin. The most common method to estimate the bioavailable fraction of an organic substance is its partitioning between octanol and water (K_{ow}). A high K_{ow} is an indication that the substance is lipophilic, and thus may flux through membranes of organisms. However, extremely hydrophobic organic substances may be less bioavailable than predicted by their high K_{ow} (Spacie et al. 1995). This may be due to steric hindrance of large molecules or poor diffusion through the aqueous boundary layer.

The direct way of studying bioavailability is to measure the amount of a substance that has been taken up by the organism. Bioaccumulation is the term for the general process by which a substance is taken up by an organisms directly from water, but also through other routes like via food ingestion. Bioconcentration is the measure of the net accumulation of a substance from water into an organism from simultaneous uptake and elimination.

Different environmental factors can modify the bioavailability of a substance. Bioavailability of a substance can consequently differ between different lotic systems. In Figure 2 some chemical and physical water parameters have been listed that can affect the bioavailability of a toxicant. The amount of a substance that is accumulated is also dependent on the organisms. The diffusive barrier of a thick periphyton biofilm can reduce the availability at least for those organisms that live deeper in the biofilm. Different species can have different uptake capacities and trace elements like manganese and calcium attached to the cell surface can reduce the uptake of e. g. metals.

2.4 Ecological realism in hazard assessment

To decide if a substance is or has the potential to be toxic to a biological system, a risk and hazard assessment is made. An ecological risk assessment can be defined as the process of identifying and quantifying risks and determine the

acceptability of those risks. A hazard assessment is the determination of the existence of hazard. If a substance is toxic to an organism a hazard arises. In hazard assessment toxicity is generally based on standardised single species tests. These tests have however low ecological realism since they are performed in artificial media and exclude many of the between-species interactions. To perform a test directly on a natural community in its natural medium should enable a better estimation of the sensitivity.

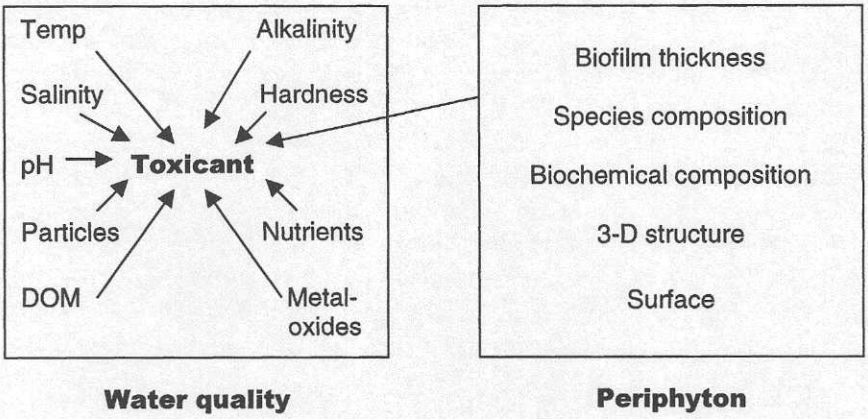


Figure 2. Some environmental factors that can modify bioavailability in a lotic system (cf. Fig. 2; Babich & Stotzky 1980, Rai et al. 1981, Genter 1996).

Growth is a common toxicological endpoint used in standardised single species. Yet, in a natural system a toxicant may exert a selection pressure where sensitive species are replaced by more tolerant ones. These species can take over niches from the excluded ones and may grow just as well. The function of the system may in this way be kept unaffected. However, a replaced species can be a key-species for other species or for a different function that is not measured. Species structure is therefore also an important endpoint.

The exclusion of sensitive species and individuals in a community and the replacement by more tolerant ones is the principle behind the Pollution Induced Community Tolerance (PICT) concept, first described by Blanck et al. (1988), but examined extensively in several theses since then (Wängberg 1989, Molander 1991, Dahl 1996, Nyström 1997). The restructuring of the community will result in an overall more tolerant community (Fig. 3). This tolerance increase can be quantified in a short-term metabolic toxicity test using e.g. the effective concentration inhibiting 50% (EC50) of the activity as a measure of tolerance. An increase in tolerance is direct evidence that the toxicant has

exerted a selection pressure and PICT is in this sense a particularly relevant endpoint to measure.

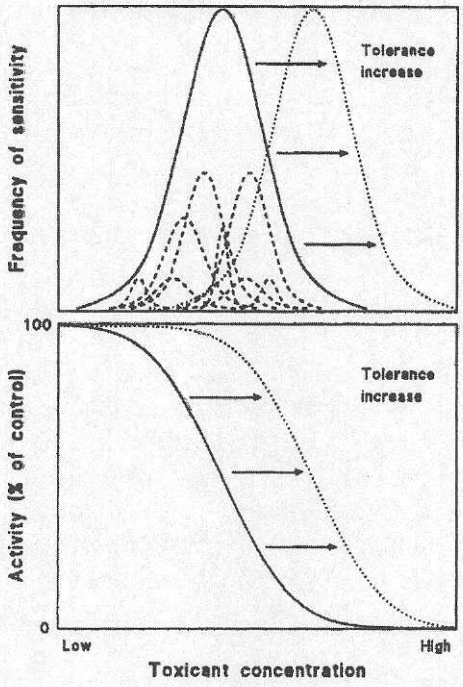


Figure 3. Schematic sensitivity distributions (upper panel) of populations (dashed lines) present in a community (solid line). When sensitive populations are excluded, the community tolerance will increase (dotted line). Community tolerance can be estimated from concentration-effect curves generated in short-term tests (lower panel) (from Dahl 1996 with permission).

Structural changes, like the ones reflected by PICT, will not be expressed in a standardised single species test. Long-term exposure on natural communities in model or experimental ecosystems like micro- and mesocosms should have a higher predictive capacity since the exposure is longer and the exposure regime is more similar to the natural ecosystem. However, the higher ecological realism of a model ecosystem is often accompanied by a higher complexity usually making these tests expensive, labour intensive and sometimes more difficult to estimate toxicity. Disadvantages of model ecosystems have been summarised by e. g. Gearing (1989) and Graney et al. (1995) and include among others poor statistical power due to low replicability and pseudoreplication. This is why people have argued against them being used as standardised procedure in hazard

assessment (Giesy 1985, Loewengart & Maki 1985, Mount 1985). Yet, the necessity of standardising and reproducing these types of studies can be questioned since the whole point is to resemble the ecosystem more closely, and an ecosystem can not be standardised.

2.5. Zinc

Zinc is one of the so-called heavy metals with a density ≥ 5 g·cm^{-3} (Zn = 7.133 g·cm^3 at 25 °C, 65.38 g·mol^{-1}). Zinc is a borderline metal and can bind to both soft (e. g. sulfur) and hard (e. g. oxygen and nitrogen) elements (Woolhouse 1983). It is a reasonably good conductor of heat and electricity. In air, a film of hydroxide is formed which protects against corrosion. Zinc is present in several minerals in the earth's crust.

In aquatic environments zinc is present in different forms (chemical species) dependent on water quality. The speciation influences the bioavailability of zinc to aquatic organisms. The free zinc ion (Zn^{2+}, actually $Zn(H_2O)_6^{2+}$) is generally considered to be the most bioavailable form. In Figure 4 some environmental factors are listed that modify the biovailability of zinc in lotic systems. An increase in the concentrations of particles, magnesium and calcium (water hardness), dissolved organic matter, phosphate, sodium, chloride and carbonates (alkalinity) generally decrease toxicity (Babich & Stotzky 1980, Rai et al. 1981, Lindeström et al. 1988, Genter 1996). Temperature and pH are more ambiguous in their modifying effects. The presence of other metal oxides normally decreases bioavailablity of zinc (Babich & Stotzky 1980). Organisms can regulate the concentration of zinc in the organisms by e. g. decreasing the uptake or storing it in vacuoles, excluding it or by complexing it in metal-binding proteins such as metallothioneins and phytochelatins or in polyphosphate bodies (Gadd 1990). Zinc is significantly bioconcentrated in algae but less so in fish (Cleven et al.1993).

Zinc is on the European Community's "Grey list" (Mason 1991) and the Swedish National Chemicals Inspectorate's "Sunset list" of candidates for risk reduction since it has a wide distribution and relatively high mobility, and is very toxic to several aquatic organisms (Landner & Lindeström 1998). Around 7.1 million tonnes of zinc are produced each year in the western world (Naturvårdsverket 1996). Zinc is used in many products such as flame retardants, rubber, paint pigments, biocides and pharmaceuticals (Landner & Lindeström 1998) and for galvanisation. Zinc may reach the environment through combustion, corrosion or leaching from smelter slags and waste deposits and around 1130 tonnes per year is estimated to be discharged into surface waters in Sweden of which the major source is corrosion (Landner & Lindeström 1998). Borg (1984) estimated a background concentration of zinc in Swedish freshwaters to be 0.5-5 µg·l^{-1} (8-80 nM). Concentrations of total zinc in European rivers can range from nmoles per litre to near a hundred µmoles per

litre in the most polluted ones (Whitton et al. 1982). The European Community water quality criteria of zinc is 30 $\mu g \cdot l^{-1}$ (0.46 µM) (hardness=10 $mg \cdot l^{-1}$) (Wiederholm 1999).

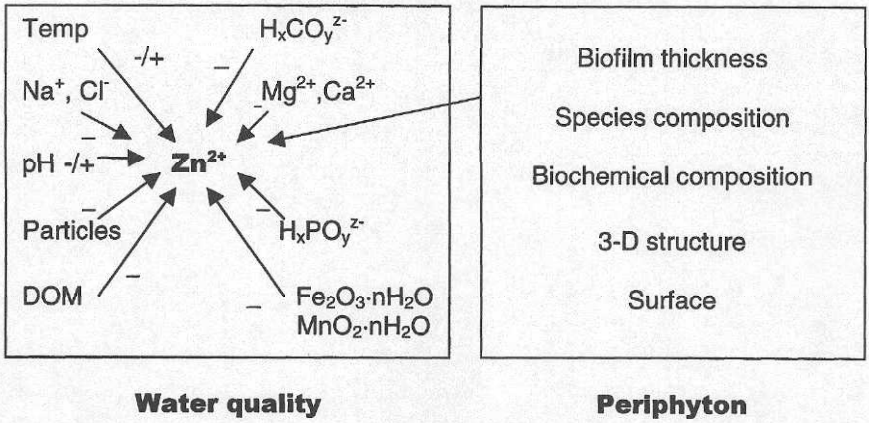


Figure 4. Some environmental factors that can modify bioavailability in a lotic system (cf. Fig. 2 and 3; Babich & Stotzky 1980, Rai et al. 1981, Genter 1996).

Zinc is also an essential micro-nutrient for living organisms including those living in periphyton communities. It is a component of at least 150 enzymes (Walker et al. 1996) like carbonic anhydrase, alkaline phosphatase, DNA and RNA polymerases, dehydrogenases and superoxide dismutase (Woolhouse 1983, Lindeström et al. 1988). Like many other essential elements zinc is toxic at higher concentrations. Zinc exerts its toxicity by binding to e. g. –SH groups, or replacing other metals in proteins like enzymes, and thereby altering or inhibiting their function (Lindeström et al. 1988). Zinc in this way has a rather unspecific mode of action and may interfere with many processes in an organism.

Zinc is considered to be among the least toxic of the heavy metals (Lindeström et al. 1988). Short-term toxicity to zinc measured for single freshwater species in standardised tests ranges from 0.46 µM for algae to more than 15300 µM for invertebrates (Table 1). The reported long-term toxicity on periphyton communities measured in model ecosystem tests range from 0.8 µM to 38 µM (Williams & Mount 1965, Genter et al. 1987, Colwell et al. 1989, Dean-Ross 1990, Niederlehner & Cairns 1992, 1993, Loez et al. 1995), which is in agreement with short-term toxicity on single algal species (Table 1). This suggests that zinc is toxic only in the most polluted rivers and streams. However, effects on biomass production in periphyton communities have also

been reported to occur at concentrations as low as 4.2 μg l^{-1} (0.06 μM) (Pratt et al. 1987) and 0.1-0.4 μM (**I, III**) indicating that relatively large differences between communities from different aquatic ecosystems can be expected.

Table 1. Short-term zinc toxicity ranges for freshwater single species

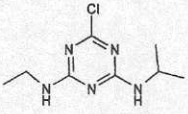
Species	Concentration range (μM)
Bacteria[1)]	4.6 - 3500
Algae[1)]	0.46 - 37
Invertebrates	0.61[2)] - >15300[1)]
Fish[2)]	2.1 - 612

[1)]Spear (1981)
[2)] VROM (1999)

2.6. Atrazine

Atrazine is a herbicide used to control annual broadleaf weed and grass mainly in cornfields but also for other crops. It is a polar, non-ionic compound with moderate water solubility and octanol-water coefficient (Table 2). This suggests that atrazine moves rather freely in water. Bioavailability of atrazine seems high (**IV**) and it is not likely to bioconcentrate (Verschueren 1996, Wang et al. 1996, Tang et al. 1998).

Table 2. Some physical and chemical characteristics of atrazine.

Name	Atrazine
Chemical name[1)]	2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine
Structure	
Molecular weight[2)]	215.68 g·mol^{-1}
Vapor pressure[1)]	40 μPa (20°C)
Water solubility[2)]	70 mg·l^{-1} at 25 °C
Log K_{ow}[3)]	2.68 at 25 °C

[1)] Verschueren (1996)
[2)] Windholz et al. (1983)
[3)] Solomon et al. (1996)

Atrazine has a low vapour pressure and thus volatilisation from the water is negligible. Half-lives of atrazine in aquatic systems may vary between 3 and 300 days (Huber 1993) depending on environmental conditions. Shorter half-lives

have been reported at low pH and exposure to short wavelengths (Solomon et al. 1996). Atrazine degradation occurs by hydrolysis, *N*-dealkylation and by splitting of the triazine ring (Esser et al. 1975).

Atrazine is highly selective in its pure state since the target plants do not have the same capacity as the resistant non-target crops to metabolize and detoxify the triazine herbicide (Tang et al. 1997, Huber 1993).

Atrazine was banned in Sweden in 1989 but is still commonly used in many other countries such as e. g. the USA, Spain and the Netherlands. About 35 million kg is applied annually in the USA (Hoagland et al. 1996), 192,000 kg in the Netherlands (1992) and 250,000 kg in Spain (1990) (**IV**). The heavy usage has led to the development of several resistant species such as e. g. *Amaranthus* spp. and *Chenopodium* spp. (Bandeen et al. 1982). According to the European Commission the emissions of atrazine is estimated to increase up to 2010 (EEA 2000). The main pathway for atrazine into the environment is through runoff from treated soils and further penetration into ground water (Huber 1993). Concentrations observed in lotic systems are generally below 20 $\mu g \cdot l^{-1}$ (0.09 µM) (Huber 1993, Solomon et al. 1996). In Europe however, a concentration of 10 $\mu g \cdot l^{-1}$ (0.05 µM) is seldom exceeded (Huber 1993).

Atrazine binds specifically to the D1 protein in the photosystem II (PSII) and thereby inhibits the photosynthetic electron transport (Moreland 1980, Bowyer et al. 1991). Due to its very specific mode of action, it is especially toxic for algae but much less so for other organisms (Table 3). Short-term atrazine toxicity for single species from different standardised tests ranges from 0.002 µM for algae to >464 µM for fish. Long-term toxicity in model ecosystem tests on periphyton communities ranges from 0.014 to 0.7 µM (Kosinski 1984, Krieger et al. 1988, Pratt et al. 1988, Pearson & Crossland 1996, Girling et al. 2000, **II**), which is in agreement with the range of short-term toxicity on single algal species (Table 3).

Table 3. Short-term atrazine toxicity ranges for freshwater single species.

Species	Concentration range (µM)
Bacteria[1)]	>46
Algae[2)]	0.002 – 4.6
Invertebrates[2)]	17 - >232
Fish[2)]	16 - >464

[1)] Verschueren (1996)
[2)] Solomon et al. (1996)

3. PROBLEMS, AIM AND APPROACH

3.1. Problems

Effects of zinc and atrazine toxicity have been studied extensively both in short-term single species tests (Table 1 and 3) and in long-term studies in model ecosystems (see references in Section 2.5 and 2.6). The results from these studies present a wide toxicity range. Either very few concentrations or large steps between the exposure concentrations were used in all the mentioned studies (except **I** and **III**). However, good estimations of no-effect levels are dependent on the range between each of the exposure concentrations and on the number of concentrations used. Hence, these studies might be poor estimates of the concentration level at which zinc and atrazine becomes a hazard.

PICT has the potential to be used as a relevant toxicological endpoint to predict long-term effects on communities. It has been used in many studies of algae (Blanck & Wängberg 1988b, Molander & Blanck 1988, Molander et al. 1990, Blanck & Molander 1991, Blanck & Wängberg 1991, Wängberg et al. 1991, Molander & Blanck 1992, Molander et al. 1992, Wängberg 1995, Gustavson & Wängberg 1995, Blanck & Dahl 1996, Dahl & Blanck 1996, Bérard et al. 1998, Admiraal et al. 1999, Gustavson et al. 1999, Arrhenius et al. 2000, Soldo & Behra 2000, **I-VI**), bacteria (Tubbing et al. 1993, Admiraal et al. 1999, Lehmann et al. 1999, Pennanen et al. 1996, Díaz-Raviña & Bååth 1996, Bååth 1992, **I-III**, Rutgers et al. 1998) and nematode communities (Millward & Grant 1995, 2000). To be used successfully as an ecotoxicological tool there are however certain proposed requirements that have to be fulfilled (Table 4) (Blanck et al. 1988).

Table 4. Requirements for a successful application of PICT as an ecotoxicological tool.

Requirement no.	
1	Tolerance can be measured in a meaningful way in short-term tests
2	PICT can be detected and distinguished from other causes of variation in community tolerance
3	PICT is associated with significant effects on the community as measured by conventional methodologies
4	The specificity of the selection pressure is high, or understandable patterns in co-tolerance can be found

Requirement no. 1 contends that the short-term tests used should be able to measure the tolerance in a meaningful way such as reflecting a toxicant's mode of action. However, if the tolerance mechanism is such that it reduces the toxic stress also for the measured metabolic endpoint, the short-term test may still

correctly estimate PICT like e. g. for tri-*n*-butyltin (TBT) (Dahl & Blanck 1996). To be able to *measure* a tolerance is a basic requirement for any PICT measurement.

Requirement no. 2 states that PICT should be distinguished from other causes of variation, which is of particular importance when used in field measurements. A positive correlation between tolerance and exposure concentrations in the field has been found for TBT (Blanck & Dahl 1996) and for metals (Wängberg 1995, Millward & Grant 1995, Pennanen et al. 1996, Lehmann et al. 1999, Millward & Grant 2000).

Requirement no. 3 says that PICT should be associated with significant effects on the community such as effects on structure or function. This is valid for arsenate (Blanck & Wängberg 1988b, Wängberg et al. 1991), 4,5,6-trichloroguaiacol (Molander et al. 1990), tri-*n*-butyltin (TBT) (Dahl & Blanck 1996), copper (Soldo & Behra 2000, Gustavson et al. 1999, Gustavsson & Wängberg 1995) and Seanine (Arrhenius et al. 2000).

Finally, requirement no. 4 means that the selection pressure of a toxicant must be specific or there are understandable patterns in co-tolerance. This has been studied thoroughly in PICT studies for arsenate (Blanck & Wängberg 1991) and diuron (Blanck & Molander 1991).

However, there are some confounding factors such as (a) difficulties in detecting PICT, (b) co-tolerance, (c) bioavailability and (d) natural variation in sensitivity, which will affect the applicability of PICT for predicting effects of some toxicants and detecting effects in the field.

It is recommendable that these possible confounding factors are examined before the specificity of PICT is challenged in field studies where gradients of confounding factors are allowed to covary with exposure and tolerance gradients.

3.1.1. Detection difficulties

No increase in tolerance was found for atrazine (Kosinski & Merkle 1984, Gustavsson & Wängberg 1995) and irgarol (Dahl 1996) despite changes in species composition. Also, for diuron, an increase in tolerance occurred only at very high exposure concentrations even though the assimilation ratio (incorporated $^{14}CO_2$ per chlorophyll *a*) increased at lower concentrations (Molander & Blanck 1992). This indicates that adaptation occurred at much lower concentration than PICT. Diuron, atrazine and irgarol are all inhibitors of PSII in the electron transport chain in photosynthesis. Despite that photosynthesis ($^{14}CO_2$-uptake) was the short-term endpoint used, an increase in tolerance was difficult to detect. There thus seems to be problems, concerning requirement no. 3 (Table 4) for PSII inhibitors such as atrazine since PICT was unable to reflect important structural community effects with the short-term endpoint used. There are however exceptions where increased tolerance to

atrazine has been found using photosynthesis as an endpoint (deNoyelles et al. 1982 and 1989).

3.1.2. Co-tolerance

Co-tolerance is when pre-exposure to one toxicant induces tolerance not just to this particular toxicant but also to other toxicants. Multiple tolerance can be defined as when e. g. a community possesses some tolerance mechanism that confers tolerance to several toxicants without pre-exposure to any of the toxicants. Co-tolerance can be expected to occur between toxicants that share some property related to chemical structure or mode of action (Blanck et al. 1988). This was evident for arsenate as shown by Blanck and Wängberg (1991) and for diuron (Blanck & Molander 1991). However, diuron also showed co-tolerance with TBT (Molander et al 1992) even though their mode of action differed. This implies that they share a common tolerance mechanism. There seem to be some aspects of generality in co-tolerance since there are slight increases in tolerance for toxicants even though they differ in chemistry and mode of action (Blanck & Wängberg 1991, Blanck & Molander 1991).

Co-tolerance is a common phenomenon for metals in microbial communities (Bååth 1992, Díaz-Raviña et al. 1994, Gustavsson & Wängberg 1995, Díaz-Raviña & Bååth1996, Soldo & Behra 2000) and multiple tolerance has also been reported (Foster 1982). Co-tolerance and multiple tolerance are therefore an obvious problem in field measurements, especially for metals such as zinc.

3.1.3 Bioavailability and natural variation in sensitivity

As discussed in Section 2.3, many environmental factors can affect the bioavailability of a toxicant. If the bioavailability is low a lotic ecosystem may actually be protected from toxicity. In this situation it is accurate to consider the influence of bioavailability on environmental hazard, because it modifies the *real* toxicity. However, bioavailability may also be a confounding factor that complicates the measurement of toxicity. Short-term tests may give erroneous results when bioavailability is low and the exposure insufficient during the test.

If there are gradients in environmental factors that affect the bioavailability of a toxicant or an inherent tolerance of the periphyton community, PICT may be difficult to distinguish from natural variation in sensitivity.

3.2. Aim

The aim of this thesis was to explore the applicability of the PICT method for zinc and atrazine and to study the potential confounding factors. The aim was also to study the environmental hazard that atrazine and zinc might pose to periphyton communities, which are important primary producers in lotic environments.

3.3. Approach

A model ecosystem was used to study long-term effects of zinc and atrazine on bacteria and algae in natural periphyton communities. The water and the communities originated from a relatively unpolluted site, thus avoiding any pre-exposure and selection. The communities were exposed to a gradient where short steps between the exposure concentrations were used. This enabled a better estimation of a no effect level and detection of a tolerance increase. PICT was compared to other relevant structural and functional endpoints. Different short-term endpoints were used to measure tolerance.

By performing the same short-term tests on different natural periphyton communities from a wide selection of lotic systems from different geographical areas, a measurement of the variability in sensitivity towards zinc and atrazine was accomplished. This variability was compared to the variability in exposure concentration and other environmental factors. In this way the magnitude of the PICT response contra the natural variability could be estimated.

Tests on natural communities in their natural medium enabled the simultaneous measurement of all the modifying factors that are important for the bioavailability of a toxicant.

4. PROCEDURES

4.1. Short-term tests

Short-term toxicity tests on different metabolic processes such as photosynthesis, sulfolipid synthesis and thymidine incorporation were used to estimate community tolerance. Periphyton communities were colonised for 2-6 weeks on glass discs (1.5 cm^2) to get reasonably mature communities but not too thick biofilms. The aim was to achieve a similar thickness between the different sites. The glass discs were mounted vertically on holders carrying 10 discs each, which were inserted in special racks. The racks were floating by means of floating bodies mounted on the racks parallel to the main current at a depth of about 0.5 m (Fig. 5) or mounted in aquaria (see Section 4.2). Photosynthesis was measured as the incorporation of $^{14}CO_2$ into acid-stable molecules (**I-III, V-VI**) while sulfolipid synthesis was measured as the incorporation of $^{35}SO_4^{2-}$ into sulfolipids (**II**). Sulfolipids are almost entirely synthesised by photosynthetic microorganisms (Cuhel & Lean 1987) and have been used to measure eukaryotic activity (Moriarty et al. 1985). The method was used as an alternative to photosynthesis to reflect algal growth to a somewhat higher extent. ^{3}H-thymidine incorporation is a common method used to measure bacterial production (Fuhrman & Azam 1982). Thymidine is mainly incorporated into bacterial DNA if low concentrations and short incubations (<1 h) are used (Moriarty 1986) and the method was used to reflect bacterial growth (**I, III, V-**

VI). The short-term tests were performed either in the field or in the laboratory in special incubators. The field experiments were conducted at ambient temperature and light conditions to simulate natural conditions as close as possible (**V**, **VI**), while more controlled conditions like fixed light exposure (80-190 µmol photons·m^{-2}·s^{-1}) were used in the laboratory (**I-IV**). The periphyton communities were exposed to a concentration series of the toxicant after which the radioactively labelled substrate was added. The relative amount of incorporation was plotted against the concentration of the toxicant. A lin-log interpolation was used to estimate the effective concentration inhibiting 50% of the metabolic process (EC50) (see Section 2.4). This EC50 was used as a measure of community tolerance.

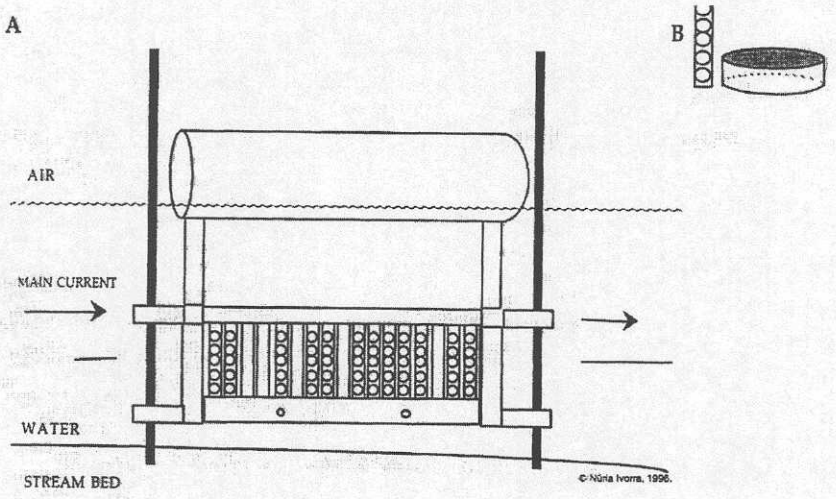


Figure 5. Sampling device for periphyton. A) Position of racks in the stream. B) Polyethylene holders with glass discs on both sides (n=10, 1.5 cm^2).

4.2. Long-term tests

To assess long-term toxicity on periphyton communities a flow-through microcosm system consisting of 12 22-l aquaria was used. Water and toxicants were pumped to a flow distributor, which had been modified to radial symmetry since the system was first described (Blanck & Wängberg 1988a), where they were mixed before entering the aquaria. Organisms coming in with the water settled on glass discs arranged vertically in the aquaria (see section 4.1). To avoid the influence of larger grazers, the water first passed a nylon filter with a mesh size of 1 mm which was placed before the flow distributor. The periphyton communities were exposed to the toxicants for approximately 4 weeks after

which structural and functional endpoints were measured. The endpoints were PICT (**I-III**), number of species, community structure, (**I, II**), biomass (**I-III**), assimilation ratio (**I**), phosphatase activity (**III**) and element content (**III**). Long-term No Effect Concentrations (NEC) of these different endpoints were estimated by using a regression analysis (Fig. 6). The NEC was determined as the intercept between the linear regression and the mean no-effect baseline.

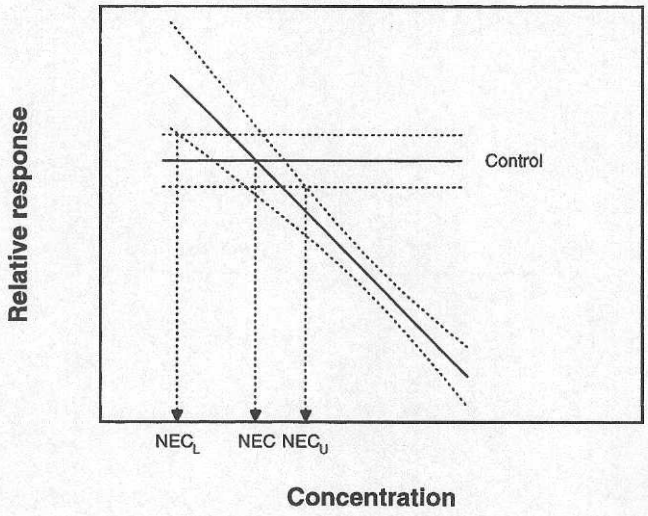


Figure 6. A schematic illustration of the calculation of the No Effect Concentration (NEC). The lower (NEC_L) and the upper (NEC_U) 95% confidence limits of the NEC are indicated (modified from Liber et al. 1992).

4.3. Bioaccumulation and depuration

Bioaccumulation and depuration of atrazine in periphyton communities were measured in Paper IV (Fig. 7). Bioaccumulation is the uptake of a toxicant from the medium as well as other routes such as via food. Bioaccumulation is thus the accurate term to use because the uptake is measured in a food web. However, in Paper IV, the term bioconcentration was used to avoid confusion, since the uptake in periphyton communities was compared with the uptake in *Daphnia magna* where only uptake from water was measured (see Section 2.3). Depuration is the loss of a toxicant from an organism after it has been placed in an environment devoid of the toxicant (Newman 1995). The kinetics is determined by modelling. Different models can be used depending on how many

compartments one considers is involved in the uptake or elimination of the toxicant, and how many sources of toxicant exposure there are. From these models, rate constants for uptake and depuration can be determined. In Paper IV the uptake data was fitted to a first-order two-compartment kinetic model (Newman 1995) even though the uptake appeared to be bi-phasic. However, the first uptake phase was so fast that it was impossible to measure. The depuration occurred also in a bi-phasic manner with a fast and a slow phase. Accordingly, a bi-exponential elimination model was used (Newman 1995). The modelling was performed using statistical software (SigmaPlot 4.00 for Windows, SPSS Inc.).

The bioaccumulation factor (BAF) was also determined and calculated as the concentration of the toxicant in the periphyton divided by the concentration in the water.

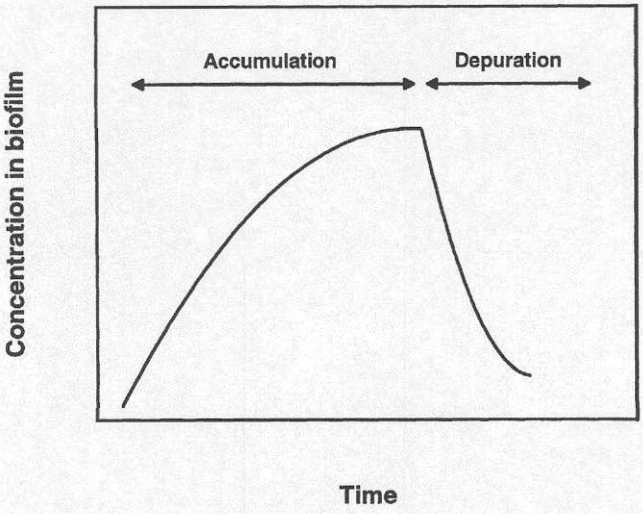


Figure 7. A schematic illustration of bioaccumulation and depuration measurements.

5. MAIN RESULTS

5.1. PICT

Development of tolerance to zinc and atrazine was studied in a flow-through microcosm system (see Section 4.2) on periphyton communities from the relatively unpolluted river Göta Älv ([Zn]= 0.08-0.09 µM, **I** and **III**, [atrazine]= <0.23-0.46 nM, unpublished results).

The communities were exposed to a nominal concentration series of zinc covering the range of long-term toxicity reported for zinc (0.09-32 µM). The highest concentration chosen equalled the zinc concentration found in the river Dommel (38 µM), in the Netherlands, downstream of the inlet of a zinc-polluted tributary, where a gradient in zinc tolerance was found (Admiraal et al. 1999). Zinc induced tolerance in Göta Älv periphyton at concentrations >9.7µM of total zinc for both bacteria and algae when using photosynthesis and thymidine incorporation as short-term measurement endpoints (Fig. 8). The increase in tolerance coincided with a marked change in algal species composition, implying that the restructuring of the community was the cause for this tolerance increase as depicted by the PICT concept (Fig. 9). However, effects on periphyton biomass were detected at much lower concentrations as determined by the NEC of 0.12-0.42 µM for dry weight (DW) (**I**).

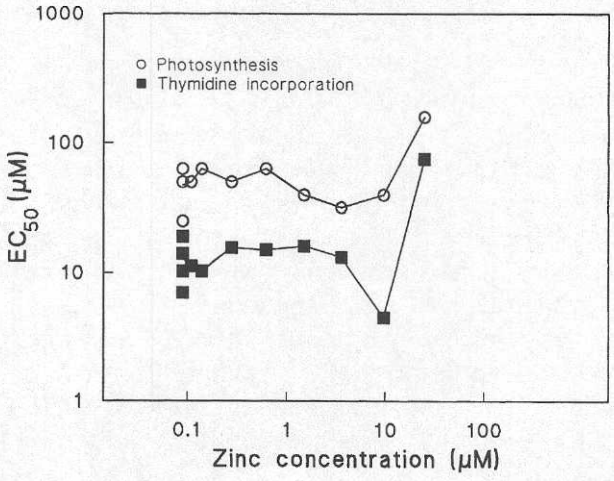


Figure 8. Periphyton community tolerance. The community tolerance was estimated as the EC50 from short-term tests (see **I**). Zinc concentrations are analysed zinc in the aquaria water.

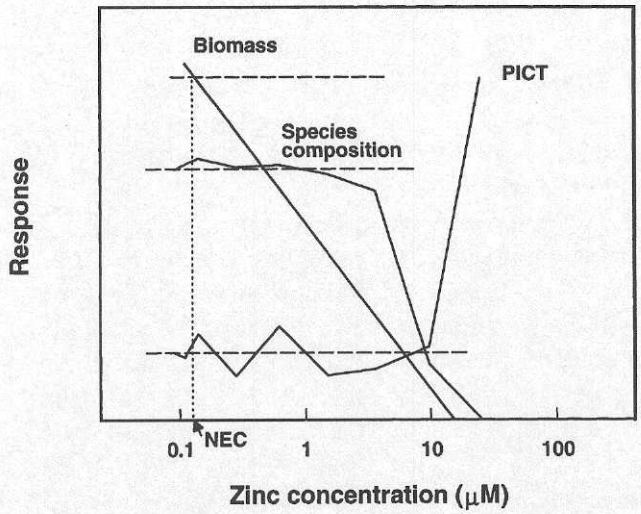


Figure 9. Long-term effects of zinc on periphyton communities after exposure in a microcosm experiment (**I**). Indicated are Pollution Induced Community Tolerance (PICT) for photosynthesis, species composition calculated as the Bray-Curtis similarity index based on relative abundance of species or groups of species, and the No-Effect Concentration (NEC) for biomass measured as dry weight.

The interval between atrazine concentrations where small (dilution factor = $\sqrt{\sqrt{10}}$, 8 concentrations between 0.056 and 3.2 μM) to be able to detect small induced tolerances as well as changes in structure and function. A minute but consistent increase in algal periphyton community tolerance occurred for both short-term endpoints used (photosynthesis and sulfolipid synthesis) (Fig. 10). This slight increase coincided with a slight change in species composition implying that this was a PICT response, even though no clear differences in tolerance were found compared to the controls (0 μM) (Fig.11). Biomass also decreased within the same concentration range. This suggests that PICT accurately detected long-term effects with the short-term measurement endpoints used. However, the concentration range for PICT was narrow (tolerance even decreased at higher concentrations) and difficult to detect in Göta Älv periphyton communities.

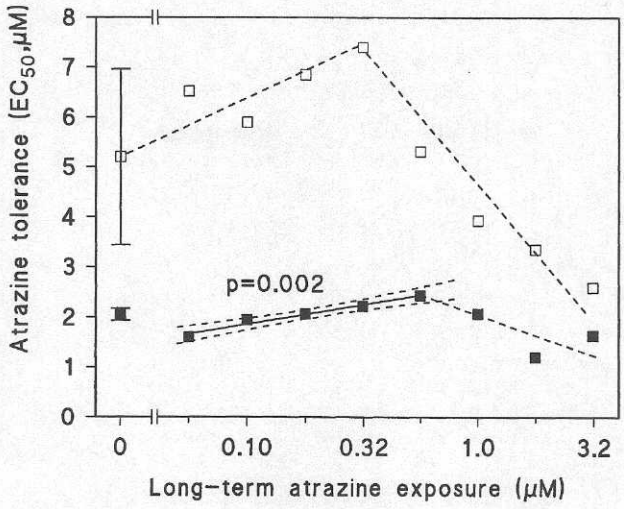


Figure 10. Atrazine periphyton community tolerance. EC50 values for short-term inhibition of photosynthesis (■) and sulfolipid synthesis (□) are plotted relative to the long-term exposure

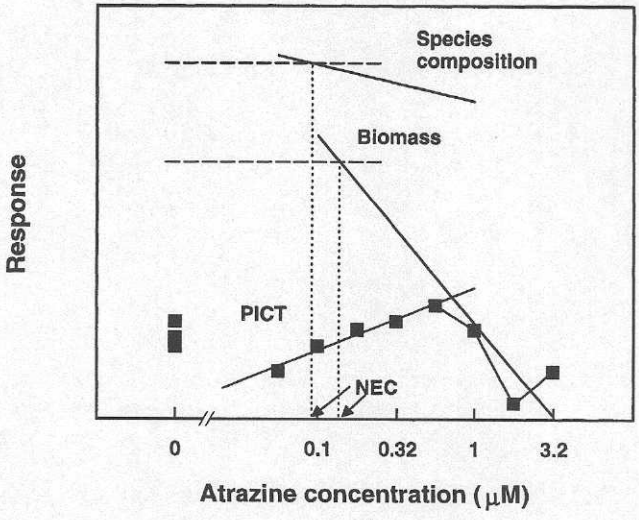


Figure 11. Long-term effects of atrazine on periphyton communities after exposure in a microcosm experiment (**II**). Indicated are the No-Effect Concentration (NEC) for algal biomass measured as chlorophyll *a*, Pollution Induced Community Tolerance (PICT) and species composition calculated as the Bray-Curtis similarity index based on relative abundance of species or groups of species.

5.2. Bioavailability

The discrepancy between long-term effects on biomass and PICT (**I**) was later shown (**III**) to be due to a zinc-dependent reduced bioavailability of phosphate. Alkaline phosphatase activity (APA), an indicator of phosphorus deficiency, increased as phosphorus reserves decreased with increasing exposure concentrations of zinc, indicating phosphorus depletion in the periphyton community (Fig. 12). This depletion coincided with a decrease in biomass production, measured as DW, which suggests that the phosphorus depletion was causing the production loss. It was thus shown that not only does water chemistry affect the bioavailability of zinc (see Section 2.7), but zinc might also change the bioavailability of phosphorus.

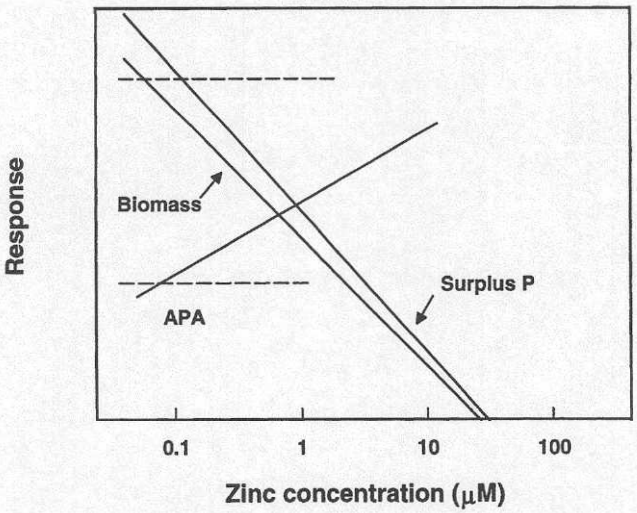


Figure 12. Long-term effects of zinc on periphyton communities after exposure in a microcosm experiment (**III**). Indicated are biomass measured as dry weight, alkaline phosphatase activity (APA) and surplus phosphorus.

Water chemical factors such as dissolved organic carbon (DOC), conductivity or pH did not affect the bioavailability of atrazine (**IV**). Atrazine uptake and depuration were studied in different periphyton communities in water with different water chemistry and compared with a synthetic fresh water with low concentrations of ions and organic matter (Fig. 13). No differences in uptake or depuration were detected that could be connected to differences in water chemistry. There were however differences in uptake rates and bioaccumulation

of atrazine between the different communities (Fig. 3, **IV**). This was assumed to be due to differences in other factors related to the biofilm such as e. g. protein content, biovolume or structure. However, differences in bioaccumulation of atrazine were rather small with a range in BAF values of 213-350 and no significant differences in depuration rates between the different communities were found (**IV**).

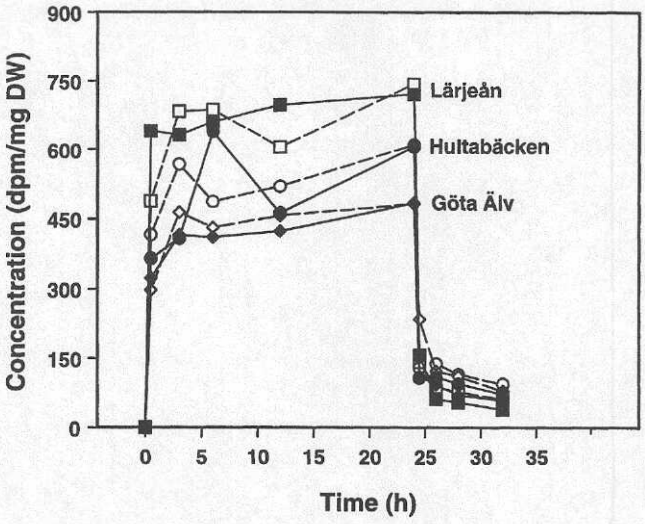


Figure 13. Atrazine uptake and depuration in periphyton communities from different running waters. Uptake and depuration were studied both in natural water (solid symbol and line) and in a synthetic freshwater with low concentrations of ions and organic matter (open symbols and dashed line) (**IV**). The first indicated measurement was done after 30 min of uptake or depuration.

5.3. Variability in tolerance due to natural variability and pre-exposure

Variability in tolerance to zinc and atrazine was studied in Paper V and VI using short-term toxicity tests on natural periphyton communities in their natural medium (see Section 4.1). The variability in tolerance for zinc, defined as the ratio between the highest and the lowest EC50 value, was 326 when measured as short-term inhibition of photosynthesis and 31 when thymidine incorporation was measured (Fig. 14, **V**). For atrazine this ratio was only 6.4 (Fig. 14, **VI**). According to multivariate statistical analysis, pre-exposure to either zinc (**V**) or atrazine (**VI**) was one factor responsible for the tolerance differences. However, many other factors also contributed to the variability in tolerance. The

$EC50_{max}/EC50_{min}$ ratio within one experiment (n=2, Göta Älv) was found to be 2.8 for zinc measured as short-term inhibition of photosynthesis. The variability between sites that were not previously exposed to zinc or atrazine should reflect the variability not induced by zinc or atrazine. This variability is an estimation of the natural or background variability, which is due to bioavailability, co-tolerance or inherent differences in sensitivity. The variability "not due to zinc pre-exposure" was defined as the ratio between the highest and the lowest tolerance values for sites with voltammetrically (ASV) labile zinc below detection limit, while for atrazine the ratio was defined for sites with total atrazine concentrations below detection limit. The $EC50_{max}/EC50_{min}$ ratio was found to be 22 (photosynthesis) and 17 (thymidine incorporation) respectively for zinc, and 6.1 for atrazine. The rest of the variability may then potentially be due to pre-exposure to zinc or atrazine. This part of the variability is rather large when using photosynthesis as the short-term measurement endpoint for zinc tolerance, less for thymidine and almost undetectable for atrazine.

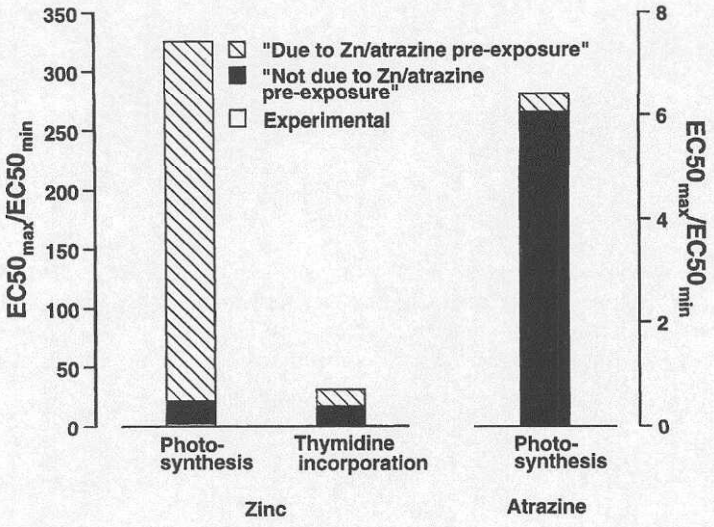


Figure 14. Variability in tolerance to zinc and atrazine for periphyton communities from different running waters in Sweden, Spain and the Netherlands (**V**, **VI**). Tolerance was measured as the concentration inhibiting 50% (EC50) of the photosynthesis or thymidine incorporation. Variability is indicated as the ratio between the highest EC50 and the lowest. Experimental variability is the $EC50_{max}/EC50_{min}$ measured within one experiment (n=2). The fraction denoted as "not due to Zn/atrazine pre-exposure" is defined as the ratio $EC50_{max}/EC50_{min}$ between zinc "unexposed" (voltammetrically labile zinc below detection limit) or atrazine unexposed sites. The "due to Zn/atrazine pre-exposure" fraction accounts for the rest of the variability between the different sites.

6. DISCUSSION

6.1. Zinc hazard to lotic periphyton communities

Long-term effects on biomass production in the Göta Älv periphyton occurred at concentrations presently exceeded in many European rivers (Fig. 15). These effects were, as shown in Paper III, most likely due to zinc-induced effects on phosphorus bioavailability. High alkaline phosphatase activity and low surplus phosphorus, both indicators of phosphorus deficiency (e. g. Torriani 1960, Kuenzler & Perras 1965, Healey 1973, Cembella et al. 1984, Siuda 1984, Chróst & Overbeck 1987, Jansson et al. 1988, Chróst 1991), indicated phosphorus depletion as biomass production decreased with increasing zinc exposure (Fig. 12). This suggests an interaction between zinc and phosphorus. Phosphate decreases zinc toxicity (Rana & Kumar 1974, Rai & Kumar 1980, Kuwabara 1985) possibly by making zinc less available due to precipitation of zinc phosphate (Rai & Kumar 1980, Hughes & Poole 1991). Other metals, such as lead and aluminium, can also can also form precipitates with phosphate (Schulze & Brand 1978, Nalewajko & Paul 1985). This precipitation was assumed to cause phosphorus depletion in algae. It seems thus likely that zinc can precipitate with phosphate, making phosphorus less available and consequently affecting biomass production. At equilibrium this is not likely to occur in natural waters with many competing ligands (Fig. 4) (Peter Croot personal communication). However, kinetically this might be feasible. Zinc was shown to bind strongly to titanium dioxide particles with little zinc in the dissolved phase (Kuwabara et al. 1986). Even so, when algae were introduced into the system, zinc concentrations in algae increased and zinc adsorbed by particles decreased. This suggests that algae were able to scavenge zinc from the particles, probably due to uptake of dissolved zinc, which drove zinc from the particles into solution. The same actually occurred for phosphorus even though the desorption seemed to be slower. Kinetics consequently is important.

Zinc may also interact with phosphorus inside the cells, as was shown by Kuwabara (1985). We have no evidence to distinguish between extracellular and intracellular Zn/P interactions although both seem likely. Nonetheless, phosphate can be expected to act as protection against zinc toxicity in phosphate rich environments like the river Congost in Spain ($[PO_4]$=18.6 µM, **V**). However, in nutrient-poor environments like the river Göta Älv ($[PO_4]$=0.2 µM, **V**) zinc toxicity may be higher. This is probably reflected to some extent in the EC_{50} values for the short-term inhibition of photosynthesis and thymidine incorporation which were 577 and 467 µM respectively in Congost, compared to 120 and 34 in the Göta Älv even though the total zinc concentrations were similar in both rivers (**V**). Waters with similar phosphate concentrations as the Göta Älv could therefore be at risk if exposed to concentrations higher than the No Effect Concentration (NEC) range measured in the Göta Älv. Actually, there

are some streams with similar phosphate concentrations as Göta Älv (**V**) and which have zinc concentrations within the NEC range (Fig.15). These are the Swedish Lillån, the Spanish Avencó and a tributary to Rió Major (**V**). The undisturbed Avencó actually lies below the NEC range and within the background concentration range. Lillån has been shown to occasionally have high zinc concentrations (Medin & Ericson 1993) and seems therefore particularly at risk.

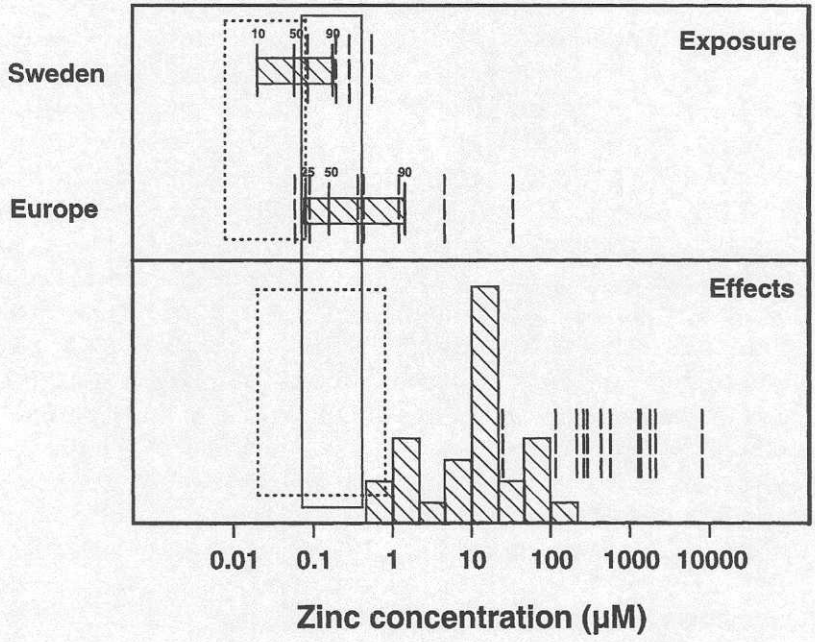


Figure 15. Zinc concentrations in lotic systems and effects on algae. The open bar that extends throughout the whole figure represents No Effect Concentration (NEC) levels on biomass dependent variables for Göta Älv periphyton communities after long-term exposure to zinc (**I**, **III**). *Exposure*: Horizontal bars represent zinc concentration percentiles in Sweden (n=71, Wiederholm 1999) and Europe (n=176, Stanners & Bordeau 1995). Dashed vertical lines are examples of zinc concentrations in European lotic systems (**I**, **III**, **V**). The dotted square is estimated background concentrations for zinc (Borg 1984). *Effects*: The histogram represents single species toxicity data (Spear 1981). Dashed vertical lines indicate short-term EC50 on photosynthesis in periphyton communities measured in different European lotic systems (**V**). The dotted square is the No Risk Area for zinc determined by van Assche et al. (1997).

Ecological hazard to periphyton communities at these sites could not be predicted by toxicity tests on single-algal species or short-term inhibition of periphyton community photosynthesis. This is probably due to the tests used and to bioavailability (see Section 3.1.3). If bioavailability is low in the test medium, and the exposure time too short, or if the test is not ecologically relevant or a less sensitive toxicity endpoint is used, the ecologically relevant toxicity can not be measured. Short-term single species tests can only show that zinc is toxic to lotic periphyton communities in zinc polluted Dutch rivers like the river Dommel (35 µM of zinc, **V**). More ecologically relevant tests like long-term exposure to natural communities in model ecosystems are therefore important aspects of hazard assessment.

It can be concluded that in phosphorus-poor lotic environments with similar water chemistry as Göta Älv, zinc can be hazardous at concentrations not much higher than the background concentrations in Swedish freshwater ecosystems (0.008-0.8 µM) (Fig.15). The so-called No Risk Area for zinc (0.02-0.8 µM) suggested by van Assche et al. (1997), which is based on effect concentrations in single-species tests, is clearly set too high since the NEC range is 0.07-0.42 µM (**I, III**). Also, the European Community water quality criteria for zinc of 30 $\mu g \cdot l^{-1}$ (0.46 µM) (water hardness=10 $mg \cdot l^{-1}$) (Wiederholm 1999) appears to be set too high for protection of periphyton in lotic ecosystems like Göta Älv.

6.2. Atrazine hazard to lotic periphyton communities

Atrazine appears to be a hazard to lotic periphyton communities only in very atrazine polluted sites according to the NEC range (0.04-0.28 µM) for atrazine measured in Paper II after four weeks exposure to atrazine in a model ecosystem (Fig.16). According to Huber (1993), concentrations of 0.05 µM are seldom exceeded in lotic systems in Europe. Neither would atrazine pose a risk for the running waters studied in this thesis (Fig. 16, **VI**). However, a multivariate analysis of the correlation between different environmental variables and tolerance to atrazine indicated a correlation with pre-exposure to atrazine and nutrients (**VI**). This implies that long exposure times to low concentrations of atrazine may act as a selection pressure on algae in periphyton communities. However, according to Figure 11 this PICT potential is very small and difficult to detect.

Single algal species toxicity tests and short-term periphyton communities seem to fairly well predict long-term community effects, since the measured short-term effects were in close vicinity of the NEC range determined after long-term exposure to atrazine (Fig. 16). Short-term toxicity on photosynthesis ($^{14}CO_2$ incorporation) and sulfolipid synthesis ($^{35}SO_4$ incorporation) and long-term effects measured in Paper II were also within this range. EC50 values for short-term inhibition of photosynthesis were 1.2-2.4 and for sulfolipid 2.6-7.4 µM.

This is in agreement with long-term inhibition of e. g. algal biomass production (chlorophyll *a*) for which EC50 was 0.7 µM of atrazine (**II**). Apparently photosynthesis is a sensitive endpoint for predicting hazard to algae in periphyton communities. It can thus be concluded that short-term inhibition tests on photosynthesis on periphyton communities and single species tests can predict concentrations at which long-term effects of atrazine to periphyton communities occur.

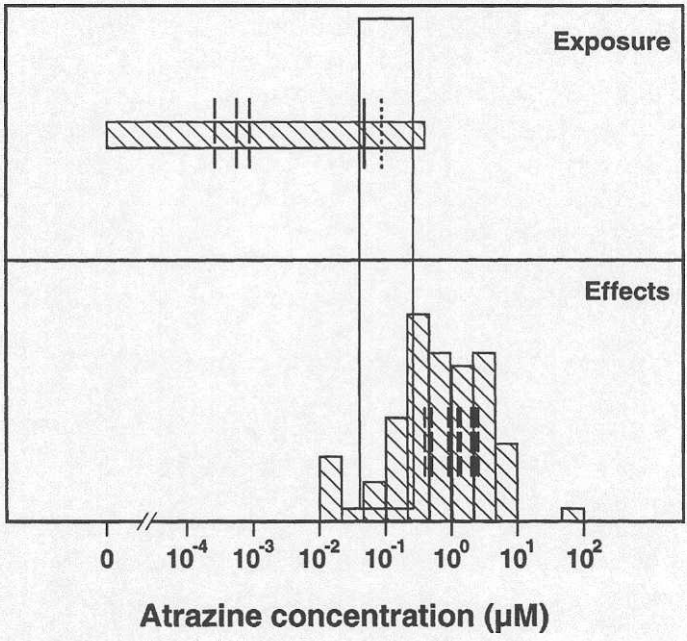


Figure 16. Atrazine concentrations in lotic systems and effects on algae. The open bar that extends throughout the whole figure represents No Effect Concentration (NEC) levels for Göta Älv periphyton communities after 4 weeks exposure to atrazine in a model ecosystem (**I, III**). *Exposure*: The horizontal bar covers unpolluted areas without atrazine to 0.4 µM (Huber 1993). The solid vertical line is the limit seldom exceeded in European waters (Huber 1993). The dashed vertical lines are examples of atrazine concentrations in European lotic systems (**VI**). The dotted vertical line is the concentration limit suggested by Huber (1993). *Effects*: The histogram represents single species toxicity data (Solomon et al. 1996). Dashed vertical lines indicate short-term EC50 on photosynthesis in periphyton communities measured in different European lotic systems (**VI**).

Recovery from long-term exposure to atrazine in model ecosystems has also been detected (Brockway et al. 1984, Hamala & Kollig 1985, Rocchio & Malanchuk 1986). However, only functional recovery was reported. Structure on the other hand seems to recover slowly after an exposure to 0.5 µM (Hamala & Kollig 1985, Hamilton et al.1988). This is however a concentration found only as short peak concentrations (Huber 1993). According to the lowest NEC level found in Paper II on thymidine to adenine incorporation ratio, 0.04 µM might change the community to a more bacteria dominated one. Long-term effects on community structure may thus occur in those waters having concentrations of atrazine higher than 0.05 µM. Therefore I do not agree with Huber (1993) who suggests that the limit can be set higher than 20 $\mu g \cdot l^{-1}$ (0.09 µM) since this limit is actually within the NEC area for atrazine.

6.3. The applicability of the Pollution Induced Community Tolerance (PICT) concept in hazard assessment of zinc and atrazine to periphyton communities

To be used successfully used as an ecotoxicological tool in predictive and retrospective assessment of toxicants, certain requirements have to be fulfilled (Table 4 and Blanck et al. 1988). This applies also for zinc and atrazine. I therefore intend to critically discuss each requirement separately to evaluate PICT's applicability in hazard assessment of these two toxicants for lotic periphyton communities.

6.3.1. Requirement no. 1

To fulfil requirement no. 1, tolerance should be measured in a meaningful way in short-term tests (Table 4). This refers to that the short-term tests used for measuring tolerance should reflect a toxicant's mode of action or that the tolerance mechanism is such that it reduces the toxic stress also for the measured metabolic endpoint.

A PICT response was detected for both zinc and atrazine with the short-term endpoints used as indicated by the simultaneous increase in tolerance with changes on species community structure (Fig. 9, 11, **I**, **II**).

Thymidine incorporation was a more sensitive short-term endpoint for measuring an induced zinc tolerance than photosynthesis was ($^{14}CO_2$ incorporation). The EC50 for photosynthesis was on average 50 µM for the controls and 13 µM for thymidine incorporation (Fig. 9). Thymidine incorporation thus corresponded better to the tolerance increase which occurred at >9.7 µM. Zinc inhibits enzymes and can therefore act on many metabolic processes. In this sense it has a rather unspecific mode of action and does not specifically inhibit photosynthesis. Thymidine incorporation, which is more closely related to growth, appears to be a more accurate short-term endpoint to use.

The faint increase in tolerance detected for atrazine was measured in a meaningful way when photosynthesis was used as the short-term endpoint. The EC50 for the inhibition of photosynthesis was on average 2 µM, which is in agreement with the slightly increased tolerance (Fig. 11). This is consistent with atrazine's mode of action, which is on PSII. Thus, it is not surprising that sulfolipid synthesis was a less accurate endpoint to use.

6.3.2. Requirement no. 2

Requirement no. 2 means that PICT should be detected and distinguished from other causes of variation in community tolerance (Table 4). This entails that the natural variability in community tolerance or the factors that are responsible for the variation are known.

One way to go is to look for the baseline tolerance in a controlled experiment with previously unexposed communities. Therefore periphyton communities from the relatively unexposed Göta Älv were exposed to zinc (**I**) and atrazine (**II**) in a model ecosystem for four weeks. Induced tolerance for zinc was found at 180-500 µM and 39-75 µM when using photosynthesis and thymidine incorporation as the short-term endpoints (**I** and unpublished results). Another way is to measure tolerance in different environments differing in exposure history and water chemistry and physics, and explore which factors contribute the most to the variability in tolerance. This was done in Papers V and VI where several running waters in Europe were sampled and multivariate analytical statistical methods were used to look for correlation patterns. If one hypothesises that sites where the ASV labile zinc is below the detection limit are relatively unexposed to zinc, this tolerance should be not-induced by
zinc". The highest EC50 for this "non-zinc induced" tolerance is actually higher than the zinc induced tolerance measured in Paper I (1995) and in 1997 (unpublished results) (Fig.17). This is most likely due to low bioavailability in the short-term test, co-tolerance or an inherent tolerance of the periphyton communities. In Section 3.1.3 it was discussed that bioavailability can be a confounding factor. When the bioavailability is low only an apparent toxicity can be measured, leading to an overestimation of tolerance. According to Paper V many factors co-varied with pre-exposure to zinc, among them phosphate which is known to decrease zinc availability (Rana & Kumar 1974, Rai & Kumar 1980, Kuwabara 1985). Co-tolerance can also be a confounding factor, but since no other metals except iron were measured this can neither be confirmed nor excluded. On the other hand heavy metals in the biofilms were measured and lead was positively correlated to zinc tolerance (Paper V). There are also strong indications of lead co-tolerance in the literature (Say et al. 1977, Díaz-Raviña et al. 1994). The baseline tolerance may also differ within the same site between different years (Fig.17). PICT may hence be over- or underestimated even within the same site. There is a possibility that the confounding effect of bioavailability in short-term tests can be overcome by

performing the tests in a medium with low ion and organic content as was used in Paper IV. This was actually done by Bååth (1992), probably for this particular reason. However, it has to be further explored whether this can be done without affecting the performance of the periphyton.

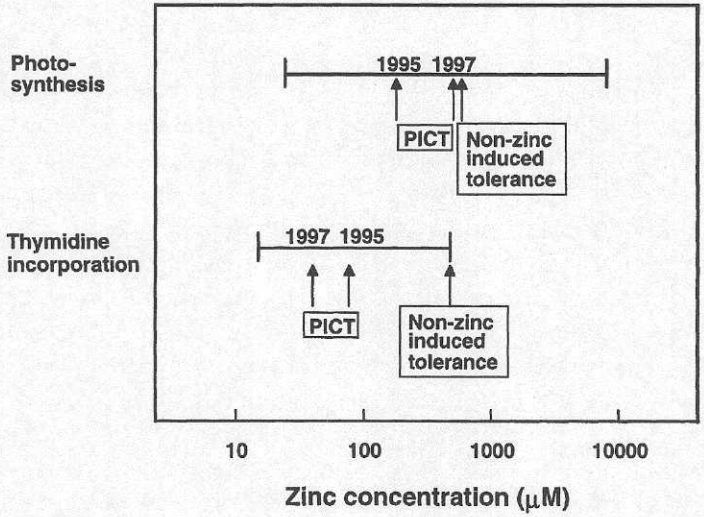


Figure 17. Ranges of tolerance either measured using photosynthesis as the short-term method or thymidine incorporation for different running waters in Sweden, Spain and the Netherlands. Indicated are the induced tolerance levels found for zinc (I and unpublished results) as well as tolerance not accounted for by zinc exposure (Non-zinc induced tolerance) (see Section 4.3).

For atrazine the tolerance induction measured in Paper II was too small to be used as a sign of induced tolerance in other environments. Tolerance measured as short-term inhibition on photosynthesis increased only 1.5-fold, from 1.6 to 2.4 μM (Fig.14). The tolerance measured in Paper VI varied from 0.4 μM in Swedish Hultabäcken to 2.56 μM in atrazine polluted Eijsden. Since periphyton communities from the normally atrazine unexposed Rió Major showed a tolerance of 2.42 μM, nearly all of this variation could be attributed to non-atrazine induced variability.

It can thus be concluded that it is difficult to distinguish PICT for atrazine and zinc from other causes of variation in community tolerance due to the large contribution to the variation from other environmental factors especially on a large regional scale, despite the fact that zinc and atrazine exposure were singled

out as one of the factors (**V**, **VI**). A baseline tolerance must probably be determined for each system individually and seasonally.

6.3.3 Requirement no. 3

Requirement no. 3 means that PICT is associated with significant effects of the community as measured by conventional methodologies. This refers to that PICT should be associated with significant effects on the community such as effects on structure or function.

PICT accurately indicated that a selection for tolerant species had occurred for both zinc and atrazine, assuming that the faint increase in tolerance for atrazine is a PICT response. The observed tolerance increases for both zinc and atrazine, measured as short-term inhibition of photosynthesis or thymidine incorporation, coincided with changes in algal community structure according to Bray-Curtis similarity indices (Fig. 9, 11, **I**, **II**). PICT was however unable to detect long-term effects of zinc on biomass production with the methods used ($^{14}CO_2$ and thymidine incorporation). This was suggested to be an indirect effect due to an interaction between zinc and phosphorus leading to phosphorus depletion and consequently biomass decrease (**I**). This idea was supported by the results in Paper III where indicators of phosphorus deficiency suggested phosphorus depletion in the area where effects on biomass production were found (**III**). Maybe a different short-term method that is related to phosphorus metabolism would be more suitable to use. There appears to be some species (one *Synedra* sp., *Tabellaria flocculosa* and one *Cosmarium* sp.) that increased their relative abundance where the biomass decreased (**I**), but since the community was so dominated by the two species *Achnanthes minutissima* and another *Synedra* species it did not influence the similarity index. *A. minutissima* is known to inhabit zinc polluted environments (Genter et al. 1987, Takamura et al. 1989, Ivorra et al. 1999) and it is thus not surprising to find that the communities dominated by this species did not increase their tolerance until exposed to high concentrations of zinc (>9.7 µM). One could speculate that the community is already tolerant to zinc, but the background concentration of zinc is low in the Göta Älv (0.08-0.09 µM, **I**, **III**) and has been for some time (Svärd 1990). Actually, it is known that there are both zinc tolerant and zinc sensitive strains of *A. minutissima* and that this tolerance can be a genetic trait (Takamura et al. 1989). Metal tolerant species have also been found in environments where no known pre-exposure has occurred before (Say et al. 1977), which suggests that metal tolerance may be a natural inherent trait (Foster 1982). Thus, even if a different short-term endpoint for measuring tolerance was used, it would probably still not induce any tolerance increases until high concentrations of zinc were reached, due to the naturally zinc tolerant composition. PICT most likely detected the direct effects of zinc on the Göta Älv periphyton community correctly. However, PICT can not be expected to detect any indirect effects that have a general negative effect on all species of a community.

For atrazine no potential indirect effects were foreseen by the methods (photosynthesis and sulfolipid synthesis) used to measure PICT for atrazine. The slight but consistent tolerance induction also conformed well, apart from structure, to other long-term effects (Fig. 11, **II**). Tolerance started to increase in the area where e. g. chlorophyll *a* started to decrease. However, this tolerance increase was so small that it was difficult to detect. Small or no tolerance increase has been seen for triazines on periphyton communities before (Kosinski 1984, Hamala & Kollig 1985, Gustavson & Wängberg 1995, Dahl 1996). It thus seems that natural periphyton communities have a low potential to increase their tolerance to atrazine. In those cases where resistance to atrazine in the D1 protein is reported, it has been in plants exposed for several years (Bandeen et al. 1982) and for cultured mutants (e. g. Galloway & Mets 1984, Trebst et al. 1993, Perewoska et al. 1994). The D1 protein is highly conservative between algae and plants investigated this far (Erickson et al. 1985) which does not suggest a spontaneous biochemical modification of this protein. Thus the tolerance mechanism may be elsewhere. Some resistant plants have an increased ability to metabolise atrazine (Jensen 1982). An ability to tolerate oxidative stress was suggested in Paper II to be a possible tolerance mechanism since that is one of the secondary effects caused by triazines (Moreland 1980). Membranes destroyed by oxidative stress need to be repaired. The sulfolipid synthesis actually appears to induce a stronger tolerance response than photosynthesis even though the variation in the controls were too high to conclude that this method detects tolerance better than photosynthesis. Another explanation might be the high mobility and bioavailability of atrazine that was shown in Paper IV. The very fast uptake (48-375 $ml \cdot g^{-1} \cdot dw\ h^{-1}$, **IV**) and depuration (11.7 to 28.6 h^{-1}, **IV**) rates suggest that bioavailability is high. As much as 20% was already taken up after 1 s and according to measurements in isolated chloroplasts, this fraction binds to the D1 protein and inhibits the electron transport (Arntzen et al. 1982). Maybe atrazine moves so quickly to the target site that there is no time for the tolerance mechanism to be expressed under severe exposure in a short-term test, unless it is a modification of the binding site. In all studies, where photosynthesis was used as the short-term endpoint, that failed or only managed to measure a small PICT response for triazines, the incubation times were not longer than 2 h (Gustavson & Wängberg 1995, Dahl 1996, **II**). There are however reports on induced tolerance for atrazine in phytoplankton communities (deNoyelles et al. 1982) but these communities were collected from a pond and were exposed for 2-3 months in a model ecosystem before tolerance was measured using much longer (24 h) toxicity measurements on photosynthesis. If there is a weak natural selection for atrazine tolerance, the chance of a mutation occurring should be higher with longer exposure times. Four weeks, as was used in the aquaria experiments, appears to be too short.

It can be concluded that PICT is associated with significant effects on the community for zinc and maybe atrazine as measured by conventional

methodologies, unless indirect effects occur that can not be measured as zinc tolerance. It can be noted that the tolerance increase for atrazine was rather small (Fig. 11). It was in fact so small that it was difficult to detect. The reason for this has to be further sorted out.

6.3.4 Requirement no. 4

Requirement no. 4 states that the specificity of the selection pressure is high or understandable patterns in co-tolerance can be found (Table 4). Co-tolerance has not been studied in this thesis so there are no direct evidence of co-tolerance for zinc or atrazine in the river periphyton. However, it seems quite clear from the literature (Foster 1982, Díaz-Raviña et al. 1994, Gustavson & Wängberg 1995, Díaz-Raviña & Bååth 1996, Pennanen et al. 1996, Soldo & Behra 2000,) that co-tolerance for metals can be expected. However, co-tolerance is not always found (Pennanen et al. 1996, Macnair 1993).

Co-tolerance in algae is also a common phenomenon among the PSII inhibiting herbicides, including atrazine resistant cultured mutants (e. g. Galloway & Mets 1984, Trebst et al. 1993, Perewoska et al. 1994). This confounds the specificity of atrazine selection pressure as well. However, the co-tolerance pattern differs quite a bit between different types of mutations, probably due to different binding capacities (Arntzen et al. 1982). Blanck and Molander (1991) reported co-tolerance patterns for different PSII herbicides. However, there was evidence of co-tolerance for triazines in diuron-treated marine periphyton, which implies that it is difficult to distinguish PICT responses especially for individual ureas and triazines.

It can be concluded that at present it seems that co-tolerance may be a confounding factor in PICT field studies of zinc as well as for atrazine if several metals or PSII inhibitors are present. More studies on mixtures may provide models that can result in understandable patterns.

7. FINAL REMARKS

PICT has been used successfully for TBT, arsenate, 4,5,6-trichloroguaiacol, copper and Seanine where the PICT response was connected in a meaningful way to long-term functional or structural changes (Dahl & Blanck 1996, Gustavson & Wängberg 1995, Molander et al. 1990, Wängberg et al. 1991, Blanck & Wängberg 1988b, Soldo & Behra 2000, Gustavson et al. 1999). However, results ón zinc and atrazine in this thesis have shown that there are several restrictions, such as detection difficulties, gradients in bioavailability and natural variability in sensitivity, to the applicability of PICT as an ecotoxicological tool: (a) An exposure towards a toxicant must induce a tolerance mechanism for at least some members of the community or favour those that are already tolerant (b) A short-term metabolic endpoint must be used

that is related to the toxic mechanism and the tolerance mechanisms, allowing the detection of the tolerance (c) If bioavailability of a toxicant is low, erroneously high tolerance levels can be measured in short-term tests, unless sufficient test duration is used to allow the uptake of toxicants to be completed. Thus, if a toxicant is expected to be modified to a large extent by different environmental factors, large variability in tolerance can be expected between different environments. For these toxicants, PICT studies in controlled long-term model ecosystem experiments are needed to explore whether environmental factors or pre-exposure accounts for the community tolerance response.

Even if there are limitations to PICT's applicability to assess effects of atrazine and zinc, unless the restrictions mentioned above are met, I would still like to emphasise PICT's value as an *eco*toxicological tool for basic research on tolerance development in natural communities.

Results in this thesis have shown that more ecologically relevant toxicity tests using natural communities are an important part in risk assessment of toxicants, otherwise the effects on sensitive ecosystems may be foreseen. Ecotoxicologists can measure effects on different types of organisms in different environments, in different ways. But, in whatever way an effect or a no effect level is measured, it still comes down to deciding what we want to protect. This is *the* most important issue for society to decide upon. However, it is most likely that no ecotoxicological tool will ever assess zero risk.

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