Helmholtz Centre
for Environmental Research – UFZ
Permoserstraße 15 I 04318 Leipzig I Germany
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Stefanie Rotter

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Pollution-Induced Community Tolerance of Periphyton as a Tool for Environmental Effect Assessment

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“It is good to have an end to journey toward; but it is the journey that matters, in the end.”

~ Ernest Hemingway

List of Contents

List of Abbreviations

ATP	Adenosine Triphosphate
CAS RN	Chemical Abstract Service Registry Number
BQE	Biological Quality Element
DBT	Dibutyltin
DMSO	Dimethyl Sulfoxide
EC_{50}	Median Effective Concentration
EDA	Effect-Directed Analysis
EPS	Extracellular Polymeric Substance
EQS	Environmental Quality Standards
ERA	Environmental Risk Assessment
F_0	Minimal Level of Fluorescence
F_m	Maximal Level of Fluorescence
GC-AED	Gas Chromatography-Atomic Emission Detector
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IBD	Indice Biologique Diatomées
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IPS	Specific Pollution Sensitivity index
ISO	International Organisation for Standardisation
MAC-EQS	Maximum Allowable Concentration Environmental Quality Standard
MBT	Monobutyltin
NOEC	No-Observed Effect Concenetration
OECD	Organisation of Economic Cooperation and Development
PAH	Polycyclic Aromatic Hydrocarbons
PAM	Pulse-Amplitude Modulation
PDMS	Polydimethylsiloxane
PEC	Predicted Environmental Concentrations
PICT	Pollution-Induced Community Tolerance
PNA	N-phenyl-2-naphthylamine
PNEC	Predicted No-Effect Concentration
POCIS	Polar Organic Chemical Integrative Sampler
PPCPs	Pharmaceuticals and Personal Care Products

PPFD	Photon Flux Density
P-site	Polluted Site
PSII	Photosystem II
PTFE	Polytetrafluoroethylene
REACH	Registration, Evaluation, Authorisation of Chemicals
R-site	Reference Site
SIM	Single Ion Modus
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SSD	Species Sensitivity Distribution
TBT	Tributyltin
TDI	Trophic Diatom Index
TIS	Toxicant-Induced Succession
TWA	Time-Weighted Average
UVR	Ultraviolet Radiation
WFD	Water Framework Directive

Summary

The pollution of surface water causes effects on structure and function of aquatic ecosystems and is of major concern worldwide (Schwarzenbach et al, 2006). To assess the risk of pollutants, environmental concentrations of pollutants are monitored and standardised single species tests are conducted to evaluate potential effects on model organisms. For an ecologically relevant effect assessment, species interactions, species diversity, chronic exposures as well as stressor interactions have to be considered.

Affective concentrations of chemicals exert a selection pressure in natural communities, which leads to the selection of more tolerant genotypes within the community and induces succession processes. The concept of pollution-induced community tolerance (PICT) is able to quantify the resulting tolerance increase of the community and therefore causally links chemical exposure to community level effects (Blanck, 2002). In order to propose PICT as a diagnostic tool for ecological effect assessment within environmental risk assessment, the objective of this thesis was to demonstrate the validity of PICT in complex field studies with various pollutants. The field studies of this thesis are based on an effect-directed analysis (EDA) conducted in a creek next to the industrial area of Bitterfeld (Germany), which revealed prometryn, N-phenyl-2-naphthylamine (PNA) and tributyltin (TBT) as phytotoxic, site-specific toxicants with a high potential to cause effects on phototrophic organisms (Brack et al., 1999).

In the first study, the PICT approach was used as a tool for hazard confirmation for the mentioned EDA study (Chapter 2). In order to assess the potential effect of prometryn, tributyltin and N-phenyl-2-naphthylamine, algal communities were cultivated at a reference and at the same sampling site where the EDA has been previously conducted by Brack et al. (1999). Exposure concentrations were monitored by using weekly spot sampling as well as passive sampling (POCIS and Chemcatcher®). The sensitivity of reference and “polluted” communities to each of the three compounds was quantified in short-term toxicity tests. The concentrations of prometryn and PNA were significantly higher at the polluted site. TBT concentrations were below the estimated no-effect concentration (0.3 to 0.5 nM) for algal communities (Blanck and Dahl, 1996). However, out of the three phytotoxic compounds only the effect of prometryn on the local algal community could be

validated by PICT. The PICT approach might be therefore an appropriate tool for an ecologically relevant hazard confirmation of site-specific compounds revealed by an EDA.

In a second field study, PICT has been used for active bio-monitoring in order to assess the potential of algal communities to recover from and adapt to chronic prometryn contamination in the field (Chapter 3). Therefore, algal communities were cultivated at the same reference and polluted site, but were subsequently transferred to the respective other site. The changes in sensitivity of communities to prometryn were determined in short-term toxicity tests and were related to structural changes in algal classes and diatom species composition. Time-weighted average concentrations of prometryn were determined by using polar organic integrative samplers (POCIS). Again, concentrations of prometryn were significantly higher at the polluted site compared to the reference site. Communities grown at the polluted site showed a seven times higher tolerance than communities grown at the reference site. However, communities from the reference site were more tolerant than the pre-exposed communities (from the polluted site) after 24 days adaptation at the polluted site. These functional changes were associated with structural changes of the community. The transfer of communities induces a shift of community structure towards the resident community. Thus, diatom species composition of the transferred pre-exposed community was similar to the local reference communities three weeks after the translocation. Based on the diatom composition, this study revealed that the recovery of formerly pollutant-exposed communities was faster, than the adaptation of reference communities to pollution. This study showed that *in situ* translocation studies based on PICT provide an ecologically relevant assessment of pesticide effects under field conditions. Therefore, PICT might be an appropriate approach to assess the success of the remediation, when PICT responses to certain stressors are measured prior and after remediation processes.

The two conducted field studies revealed beside the higher prometryn concentration also an enhanced conductivity of about 2000 µS cm^{-1} at the polluted stream. In a microcosm study (Chapter 4), the potential interaction between high ionic loads and prometryn was studied, in order to exclude high ionic load as confounding factor for the PICT found in the previous field studies. Therefore, two levels of high ionic loads (2000 µS cm^{-1} and 5000 µS cm^{-1}) were used as single stressor and in combination with prometryn. Structural parameters and the tolerance development towards

prometryn were determined over a growth period of six weeks. Furthermore, long-term community responses to the combined exposure were predicted using the mixture model of independent action. Based on diatom species data, a different community composition was observed for each stressor and combination of stressor. The combination of high ionic load and prometryn revealed concentration dependent joint effects. An exposure scenario similar to the conditions in the field (2000 µS cm^{-1} and prometryn) induced tolerance-levels towards prometryn, being in the same range as for algal communities, which were exposed solely to prometryn. Thus the microcosm approach validated the *in situ* PICT towards prometryn found in both field studies. However, the combined exposure of prometryn and a conductivity of 5000 µS cm^{-1} enhanced the tolerance to prometryn to levels higher than predicted by the model of independent action.

The studies of this thesis showed the validity of PICT in complex field studies and revealed that multiple stressors might change the magnitude of tolerance, but do not interfere with the causality of PICT. The strength of PICT to causally link exposure of single or various stressors to community-level effect in field situations makes it an appropriate tool for environmental effect assessment and distinguishes it against other community-level tools such as diatom indices. As multiple stressors might explain the failure to achieve a “good” ecological status for many European water bodies within the context of the EU-Water Framework Directive, I would like to propose PICT as a diagnostic tool for investigative monitoring. PICT might be able to clarify stressor conditions by testing the tolerances of local communities to “key” toxicants with different modes of action, in order to identify the type of stressor causing effects.

Chapter 1

General introduction

Background

Scope, Aims and Approaches

Background

Pollution – a global concern

The constantly growing human population is associated with the increase of water scarcity and pollution. Most ecosystems are directly or indirectly affected by human activities (Breitburg et al, 1998; Salbu et al., 2005). According to Schwarzenbach et al. (2006) more than one-third of the available freshwater is used for agricultural, industrial and domestic purposes. As a result, the impact of biological, chemical and physical stressors on aquatic ecosystems increased in the last decades (Folt et al., 1999). Especially, chemical pollution of surface and groundwater is due to unknown long-term effects on aquatic ecosystems and human health of major concern all over the world (Schwarzenbach et al., 2006). Sources of the contaminations with chemicals range from the wastewater of industries, over fertiliser and pesticides used for agriculture to pharmaceuticals and personal care products (PPCPs) released by humans.

A database study based on the observation of four European river basins revealed that 74% of the substances possessing a high or very high risk, are pesticides (von der Ohe et al., 2011). But many other compounds have been reported to trigger adverse effects on organisms and whole ecosystems e.g. estrogens causing intersexuality in fish (Jobling et al., 1998), nutrient pollution affecting ecosystem functions (Woodward et al., 2012) and elevated chloride concentrations (salinity) in freshwater influencing aquatic flora and fauna (Bäthe and Cöring 2011). Moreover, organisms are rarely exposed to one single pollutant, but rather to mixtures of chemicals or to multiple natural and anthropogenic stressors simultaneously. The possible interactions between different chemicals and/or other stressors can result in combined effects (Altenburger and Greco, 2009; Schindler et al. 1996). As an example, Brian et al. (2005) revealed that five estrogenic chemicals, mixed in concentration individually not causing effects, were acting additively in the mixture, inducing a significant response in male fathead minnows. The increasing number of pollutants makes the evaluation of effects on ecosystems challenging and requires targeted measures for the protection of ecosystems. The field studying the adverse effects of chemical and non-chemical stressors on organisms as well as on whole ecosystems is ecotoxicology. Environmental risk assessment (ERA) is the related management tool aiming at the assessment of possible risks as well as the development of prevention and safety measures to protect natural ecosystems.

How to assess the impact of pollutants on natural ecosystems?

Ecotoxicology and Environmental Risk Assessment

The term ecotoxicology was introduced in 1969 by René Truhaut, a toxicologist also interested in the impacts of contaminants on ecosystems. He defined ecotoxicology as a branch of toxicology studying toxic effects caused by natural or synthetic pollutants on organisms in the context of ecosystems (Truhaut, 1977). The bases for toxicology as well as ecotoxicology are dose-response models, estimating effect concentrations by using for example the number of individuals in a population responding to different concentrations of a chemical. A frequently estimated effect concentration is the EC_{50}, the concentration which induces lethal or sublethal responses to 50% of the population. Most of the ecotoxicological tests focus on survival or reproduction of fish or daphnids, on population growth of unicellular algae as well as on sublethal effects of individuals (Calow and Forbes, 2003). These simplified, single species tests are conducted according to internationally harmonised guidelines provided for example by the Organisation of Economic Cooperation and Development (OECD) or the International Organisation for Standardisation (ISO) and are comparable, relatively quick, easy and inexpensive (van Straalen, 2003). The data retrieved by these laboratory tests provide important information on intrinsic toxicities and mechanisms of toxicants, enable a comparison of toxicity between different contaminants and are most frequently used for ERA. However, natural ecosystems are characterised by different organisms living in a community and interacting with their physical and biological environment. The high environmental complexity of an ecosystem including species interactions, high species diversity and trophic complexity can be hardly reflected by these single-species approaches. Furthermore, the majority of the ecotoxicological data are acute tests (short-term exposure using high concentrations), which are in contrast to the usual long-term exposure to sublethal, chronic concentrations in ecosystems. Thus, these approaches generally oversimplify real ecological conditions (Calow and Forbes, 2003) and therefore lack ecological relevance (Newman and Clements, 2008). Furthermore, the extrapolation from responses at the individual level to effects on complex ecological systems is hardly predictable due to the high complexity and interactions in ecosystems (van Straalen, 2003) and involves a lot of uncertainties (Calow and Forbes, 2003). In this context, van Straalen (2003) encouraged to integrate the way of thinking as well as the concepts of ecology and merge ecotoxicology with “stress ecology”. This becomes even more relevant as

ecotoxicology is the science supporting the ERA. For this reason, Eggen et al. (2004) considered the implementation of ecosystem complexity in ecological risk assessment as one of the major challenges in ecotoxicology.

Ecotoxicology has to provide more ecologically relevant data!

ERA aims to analyse and evaluate the probability of adverse effects exerted by pollutants and other substances to ecological systems. It is designed to provide information to risk managers and forms the basis for a sustainable management of chemicals and international regulations as REACH (Registration, Evaluation, Authorisation of Chemicals) or the European Water Framework Directive (WFD). A distinction is made between prospective risk assessment, assessing the likelihood that predicted exposure levels cause adverse effects (e.g. used for authorisation of compounds) and retrospective risk assessment, which aims to identify causes of adverse effects that have already occurred at specific sites (Calow and Forbes, 2003). In both cases, the process of ERA consists of three sections: the formulation of the problem, the assessment of the exposure as well as the effect assessment and finally the risk characterisation (Hill et al., 2000). In general, all schemes of risk assessment are tiered and consider mode of actions, substance properties, produced mass of compounds (REACH) and results of lower tiers (Hommen et al., 2010). The earlier tiers are designed to be protective and are based on the conservative data of single-species tests provided by ecotoxicology (Solomon and Sibley, 2002). This clearly conflicts with the aims of various ERA schemes, focussing on the protection of populations, communities or ecosystems (Hommen et al., 2010).

So far, two methods are used for the extrapolation from single species tests to complex systems. First, by applying fixed extrapolation factors or so called safety factors to an endpoint of the most sensitive test species (e.g. EC_{50}) and secondly by using available effect data of different species in order to generate the species sensitivity distribution (SSD). The latter enables the estimation of a safe concentration, affecting less than 5% of the species (Posthuma et al., 2001). If the relation of the predicted environmental concentrations (PEC) and predicted no-effect concentration (PNEC) reveals a risk, the risk analysis continues with a refined and detailed assessment. This means that depending on the objective of the assessment more accurate exposure data are produced (e.g. site-specific exposure information) and more sophisticated tests may be used (e.g. higher tier tests). According to Boxall

et al. (2001) higher tier tests can be used to reduce the magnitude of uncertainty/safety factors in ERA. They provide a more realistic assessment of effects as well as input data for ecological models; however, these results might be more difficult to interpret and to compare between different studies and toxicants. The guidance document on aquatic ecotoxicology (Sanco/3268/2001) describes different options for higher tier testing as microcosm and mesocosm studies for the refinement of risk assessment. Nevertheless, at this level of ERA ecological effect assessment has to move closer to the ecosystem taking environmental complexity and chronic exposure scenarios into account.

In order to achieve a full understanding of the impacts of pollutants at higher levels of biological organisation, the available ecological knowledge has to be applied and more ecological data has to be integrated in toxicological studies (Galic et al., 2010). Most of the community metrics available in ecology are useful to characterise differences between different sites, but lack a quantification of responses to contaminants and do not allow a prediction (Solomon and Sibley, 2002). As an example, biological metrics as diatom indices rather classify freshwaters in categories than causally link exposure of contaminants to effects on diatom communities. These classifications might be relevant for environment-oriented regulatory tools, such as the WFD, but are not suitable for prospective ERA. Furthermore, ecological models can potentially increase the ecological relevance of predictions, but are rarely mentioned in directives and related guidance documents so far (Hommen et al., 2010). Available ecological models, such as ecosystem and population models predicting for example the impact of recovery processes, indirect effects in food webs and bioaccumulation to ecosystems, might be relevant for ERA (Galic et al., 2010). However, further development and improvement of these models is depending on the needs identified by industry and regulators. Furthermore, ecological models cannot be based solely on effect data of single-species tests but require additional ecological data itself (Hommen et al., 2010). Thus, appropriate higher tier tests for the detailed risk assessment and ecologically relevant data for ecological modelling are required. This emphasises the need for higher tier approaches which enable the quantification of multispecies responses to chronic exposures of contaminants and causally link exposure to effects.

The concept of pollution-induced community tolerance (PICT) is presently one of few approaches, which have the qualities to fulfil these requirements. PICT might not be

suitable for a first screening, but PICT is predestined for hazard confirmation in site-specific risk assessment as well as for prospective microcosm approaches e.g. assessing concentrations of pollutants that will not affect natural communities containing species with different sensitivities.

ERA needs higher tier tests!

Pollution-induced community tolerance (PICT)

The PICT concept has been introduced by Blanck et al. (1988) and uses the ability of organisms and communities to survive in polluted environments. In order to withstand the exposure to toxicants, communities can increase their tolerance through three mechanisms: (1) physiological acclimatisation of individuals (e.g. modification of the target site), (2) selection of tolerant genotypes within a population, and (3) due to the inhibition or elimination of sensitive species, which results in the replacement of sensitive species with more tolerant ones (Blanck et al., 1988). Therefore, the presence of toxicants in natural environments can play a fundamental role for the natural selection of genotypes. Dependent on the concentration and exposure time, toxicants exert a selection pressure which leads to succession processes, called toxicant-induced succession (TIS) (Blanck, 2002). As a result, the community will consist of more tolerant species, being able to tolerate the prevailing concentrations of the toxicants. This results in an increased tolerance of the entire community, the so-called PICT response. In order to relate the long-term toxicant exposure to the impact on communities, tolerance increase is quantified in the detection phase (Blanck, 2002). For this purpose, communities from polluted sites are sampled, exposed a second time to the respective toxicant and effects on specific metabolic processes, such as photosynthesis, are measured in short-term toxicity tests. Because the detection of PICT highly depends on the short-term test, the measured endpoint has to be appropriate for the mode of action of the studied compound. A long metabolic distance between the process affected by the investigated toxicant and the measured endpoint (indirect effect) might obscure the detection of PICT (Eriksson, 2008). Furthermore, only biologically relevant concentrations of a toxicant induce an increase of the community tolerance, which can be detected as soon as the most sensitive species are eliminated (Blanck, 2002). Although other environmental factors might influence the structure of communities as well, the increase of the overall community tolerance is limited to the mode of action of the

toxicant itself (Blanck and Dahl, 1996). This makes PICT a sensitive and ecologically relevant approach for ecological effect assessment, which causally links toxicant exposure to community level effects.

The PICT response is defined as the deviation from the baseline tolerance, for this reason a complete PICT approach requires the determination of the community tolerance of an unaffected community (Blanck, 2002). This means the tolerance of exposed communities has to be related to the tolerance of unaffected control or reference communities, which were not exposed to the respective toxicant or a toxicant with a similar mode of action. Due to the increasing pollution of surface water, it is often challenging to find uncontaminated reference sites in field studies. Nevertheless, it is inevitable to determine a proper baseline tolerance and to make sure that the investigated compound is not or in non-effective concentrations present at the reference site.

The first evidence of an increased tolerance level due to a chronic exposure to a toxicant was found for 0.1 to 0.3 µM arsenate (Blanck et al., 1988). Since that time, the PICT response of aquatic communities has been shown for several pesticides and heavy metals: atrazine (Nyström et al., 2000), copper (Gustavson et al., 1999), diuron (Dorigo et al., 2007; McClellan et al., 2008), irgarol (Blanck et al., 2009), isoproturon (Schmitt-Jansen and Altenburger, 2005), lead (Fechner et al., 2012a), prometryn (Schmitt-Jansen et al., 2004; Rotter et al., 2011), sea-nine (Larsen et al., 2003), 4,5,6-trichloroguaiacol (Molander et al., 1990), TBT (Blanck and Dahl, 1996; Dahl and Blanck, 1996a) and zinc (Blanck et al., 2003; Tlili et al., 2011a).

Furthermore, several microcosm studies using algal communities determined no-effect concentrations (NOEC) for atrazine (Nyström et al., 2000), irgarol (Dahl and Blanck, 1996b), sea-nine (Larsen et al., 2003) and TBT (Dahl and Blanck, 1996a). Consequently, PICT approaches conducted under controlled conditions (e.g. microcosms or indoor channels) may be used for predictive effect estimations, whereas field studies may be more retrospective, proving that certain toxicants lead to changes in the community structure (Blanck et al., 1988).

PICT integrates concepts of ecology into ecotoxicology.

PICT communities

The majority of the PICT studies are based on the effect assessment of phototrophic algal communities such as periphyton (e.g. Dorigo et al., 2010a, b; Blanck et al., 2009) or phytoplankton (e.g. Larsen et al., 2003; Berard and Benninghoff, 2001), and soil bacteria (e.g. Demoling and Baath, 2008; Schmitt et al., 2005). In addition, the PICT concept has been applied to the heterotrophic fraction of periphyton (e.g. Fechner et al., 2012a, b), nematodes (Millward and Grant, 2002), the food web of soil invertebrates, microbes (Salminen et al., 2001) and sediment microbial communities (Ogilvie and Grant 2008).

The exposure history within a PICT study is crucial for the selection of species and for the detection of PICT. However, the exposure in field studies is often not stable and fluctuates, especially in flowing waters. As a result, the exposure history of aquatic communities is generally hard to characterise. Passive sampling techniques such as POCIS or Chemcatcher® allow the measurement of average bioavailable concentrations and may simplify the exposure assessment of sessile communities. However, for floating and drifting phytoplankton the exposure assessment remains difficult. Thus, plankton is rather suitable for effect assessment in homogenous pollutions of small lakes. Peripyton has the advantage to combine a sessile life-form with a short generation time, which allows a rapid response to changing environmental conditions. Furthermore, the sessile life-form enables the translocation of established periphyton communities to different sites in the field, from the field to the laboratory and vice versa. This makes periphyton an appropriate model community to study the effect of contaminations especially of herbicides in controlled microcosms as well as in field studies.

Periphyton is a suitable model community for PICT studies.

Periphyton

Periphyton, also known as phytobenthos, biofilm or “Aufwuchs”, is a benthic community, which has been widely used as bio-monitoring tool for the indication of water quality. These communities are living attached to substrate and consist mainly of microalgae such as chlorophytes, cyanobacteria and diatoms but also of protozoa and bacteria, embedded in an extracellular polymeric substance (EPS). The EPS improves the initial attachment of cells in early stages of development, stabilises and protects the biofilm against environmental stress (Vu et al., 2009). During the

development of the biofilm, biomass measures such as biofilm thickness, algal density, biomass and chlorophyll a concentration are increasing until a climax is reached (Sekar et al., 2004). Furthermore, the communities are undergoing a succession process from small, fast reproducing, initial colonisers to larger late colonisers and good competitors (Sekar et al., 2004). This process is similar to higher plant succession, developing from low to high physical statures (Hoagland et al., 1982). Furthermore, periphyton integrates ecological interactions as predation or competition for nutrients and space.

In aquatic ecosystems, periphyton communities are primary producers and the first to interact with dissolved substances such as nutrients, organic matter and toxicants (Sabater et al., 2007). Thus, they are among the first to be affected by physical, chemical and biological disturbances, which might lead to effects at higher trophic levels as well. Their short life-cycles enable a rapid growth and a fast response to environmental changes, which makes periphyton a good “early warning system” for chemical pollution in aquatic ecosystems (Sabater et al., 2007).

Diatoms often represent the major autotrophic part of periphyton (McClellan et al., 2008; Battin et al., 2003) and respond to environmental changes with changes in species composition and relative contribution to the community (Sabater and Admiraal, 2005). As a result, especially diatom indices have been widely used as indicators for water quality (Kelly and Whitton, 1995; Kelly, 1998; Rott et al., 1998). Furthermore, periphyton is implemented as one of the Biological Quality Elements (BQE) in the monitoring strategy of the European WFD. In Germany, the taxonomic composition and abundance of diatoms is used to calculate the trophic and the saprobic index. For the remaining phytobenthos Schaumburg et al. (2004) developed a first method considering 13 classes of benthic algae (excluding diatoms) for the assessment of the ecological status of running waters.

Periphyton is a suitable and fast indicator for environmental pollution.

Tolerance mechanisms and co-tolerance

One of the major concerns of PICT is the occurrence of co-tolerance. The chronic exposure of organisms to one toxicant exerting a selection pressure may also induce tolerance to other compounds with the same mode of action not being present. Blanck and Wängberg (1991) investigated the specificity of PICT by exposing arsenate tolerant communities to a set of uncouplers and photosynthetic inhibitors.

Only one out of five tested chemicals showed a significant co-tolerance, therefore the authors concluded that co-tolerance is not very common in algal communities. However, the study of Knauert et al. (2010) revealed that PICT cannot discriminate between the photosynthesis II inhibitors atrazine, isproturon and diuron, targeting the D1 protein at slightly different domains. Furthermore, Tlili et al. (2011b) investigated the effects of copper, zinc and arsenic on phototrophic and heterotrophic biofilm communities, showing that co-tolerance may vary for different communities, is dependent on the compound the communities were chronically exposed to and that, beside the mode of action, the mechanisms of detoxification is most important for the occurrence of co-tolerance. Interestingly, the exposure of periphyton communities to an increased level of ultraviolet radiation (UVR) also increased their tolerance to cadmium (Navarro et al., 2008). The authors assumed that the induction of anti-oxidative enzymes might be the common defence mechanism against the oxidative stress exerted by both stressors. For these reasons, co-tolerance can be expected for compounds inducing similar detoxification mechanisms (Navarro et al., 2008; Tlili et al., 2011b) or for compounds with similar modes of action, especially when they are transported by the same carriers or affect the same enzymatic site (Blanck, 2002). According to this, PICT might not be able to distinguish between compounds inducing similar tolerance mechanisms but enables the discrimination between different modes of action and reveals the impact of a type or class of similarly acting compounds. The occurrence of co-tolerances might lead to misinterpretations especially in field studies dealing with unclear contaminations. In order to establish causality between the exposure of a toxicant and the increase of tolerance, co-tolerance has to be excluded (Blanck, 2002). Therefore, PICT studies conducted in the field should be supported by chemical target analysis.

The specificity of PICT is not absolute.

Multiple stressors and the role of confounding factors

The manifold anthropogenic usage of natural resources increased the impact of chemicals, biological and physical stressors on ecosystems (Breitburg et al., 1998; Folt et al., 1999). Therefore, organisms or biological networks are often exposed to combinations of diverse anthropogenic and natural stressors simultaneously (Breitburg et al., 1998). Possible interactions among the stressors may lead to combined effects (Altenburger and Greco, 2009). The occurrence of multiple

stressors and their possible interactions might also influence the development of community tolerance and PICT. Only a few PICT studies investigated the influence of environmental factors on the tolerance development of communities to toxicants in controlled laboratory studies so far. Boivin et al. (2005) found that heterotroph biofilms enhanced their tolerance to copper with an increase of temperature and assumed a higher internal concentration of copper due to an enhanced active transport in the cells. Guasch et al. (2007) showed in a long-term study that phosphate enrichment has no influence on atrazine toxicity, whereas Tlili et al. (2010) revealed that an increased level of phosphate enhanced the tolerance of phototrophic biofilms to copper. However, most PICT field studies have to deal with various contaminations, unknown exposure backgrounds and possible interactions between abiotic and biotic stressors. Therefore, Pesce et al. (2010) conducted a field survey with an intensive chemical and physical monitoring over eight months, investigating the variations in diuron tolerance of periphyton communities. Statistical analysis revealed that the mean exposure level of diuron was the main factor inducing diuron tolerance, however, conductivity and the concentration of nitrate were significantly related to diuron sensitivity as well. Also, Fechner et al. (2012b) investigated *in situ* the impact of a complex mixture of copper, cadmium, nickel, lead and zinc on metal tolerance, concluding that community tolerance is a sensitive indicator of an urban disturbance by mixtures of contaminants, but cannot identify the impact of a specific toxicant. Furthermore, the exposure to several selecting stressors simultaneously might lead to the development of multiple tolerances for a set of stressors (Blanck et al., 1988). However, this should not interfere with the interpretation of PICT responses, as similar-acting compound might lead to a common PICT response and dissimilar-acting compound might increase the tolerance to the different modes of action, however, in both cases the PICT response should still be detectable.

Nevertheless, the reliability of PICT has been shown in many complex field studies. Rutgers et al. (1998) even linked the increasing community tolerance towards zinc to the increasing exposure level of zinc concentrations in the field. According to Blanck (2002), a field PICT approach requires a stronger validation approach: First of all, one has to examine if the single toxicant is able to induce a PICT response, which is usually validated in controlled microcosm studies. Secondly, PICT should appear even though other contaminants and confounding factors occur. Finally, the presence

of the toxicant is compulsory, thus, it needs to be present to induce PICT, conversely, PICT should disappear when the toxicant has vanished. The validation of the last point can be achieved by conducting recovery studies.

Do interactions of multiple stressors lead to combined effects?

Recovery studies

The EU WFD aims to achieve a "good chemical and ecological status" of all European water bodies by 2015 (Directive 2000/60/EC). In order to achieve this ambitious goal, pesticides frequently detected in fresh and groundwater monitoring may be restricted in their application or withdrawn from the market and highly contaminated sites have to be remediated. The potential of a disturbed ecosystem to return to a similar state as before the contaminations is called recovery (Dorigo et al., 2010b).

Blanck and Dahl (1998) investigated the recovery of marine periphyton after the TBT ban in Sweden in 1989 and found a successive decrease of the community tolerance from 1988 to 1991. Since the concentration level of TBT decreased and the effects on periphyton disappeared, the authors were able to conclude that the TBT ban was efficient. Another, promising approach to study the course and the timeframe of recovery is the transfer of communities from a contaminated site (e.g. downstream) to a reference site (e.g. further upstream). An *in situ* study, investigating the effects of copper and diuron on periphyton, revealed that nine weeks after the transfer to the reference site, the communities were still more similar to the structure of the contaminated site and community tolerance towards copper and diuron were intermediate compared to the local communities (Dorigo et al. 2010a). On the contrary, copper tolerant heterotrophic communities reached the tolerance level of controls only 15 days after the transfer to the reference site (Fechner et al., 2012c). Lambert et al. (2012) showed that the presence of pristine biofilm influences the process of recovery greatly. In the presence of pristine biofilm the structure of transferred communities recovered and tolerance to copper disappeared within six weeks. In contrast, communities without pristine biofilms kept their enhanced tolerance towards copper. Therefore, immigrations processes play a major role in the recovery of phototrophic biofilm communities (Lambert et al., 2012).

Recovery studies are often based on structural changes within communities. However, the identification of species (e.g. diatoms) is time consuming. The above

mentioned studies showed that PICT enables the estimation of functional recovery of phototrophic and heterotrophic communities. PICT is able to reflect structural and functional changes within the communities in complex field studies and might be a promising tool to study the success of remediation processes.

PICT enables the detection of functional recovery in communities.

Scope, Aims and Approaches

Environmental risk assessment (ERA) aims the assessment of risks exerted by chemical and non-chemical stressors on ecosystems. For an ecologically relevant estimation of environmental risks environmental complexity, including possible interactions of stressors as well as interactions of species living in natural communities, has to be considered. For this reason, ERA needs appropriate higher tier tests, which causally link exposure of toxicants to community-level effects. The concept of pollution-induced community tolerance (PICT) enables the quantification of multispecies community responses to chronic exposures. Diverse studies showed the reliability of PICT in controlled microcosm studies as well as in complex field conditions with various contaminations. So far, most of the available higher-tier tests are purely descriptive and do not allow a quantification of community-level responses and a linkage of community effects to toxicant exposure. For that reason, this thesis deals with PICT as a potential tool for ecologically relevant effect assessment. The general aim of this thesis was to demonstrate the validity of PICT in complex field studies with various contaminations, in order to propose PICT as a diagnostic tool for ecological effect assessment within environmental risk assessment. Therefore, the objectives of the thesis were to:

- evaluate the suitability of PICT for hazard confirmation in effect-directed analysis studies as well as for retrospective environmental risk assessment,
- strengthen the causal link of the PICT approach between exposure of site-specific toxicants and community-level effects, by combining PICT with passive sampling techniques and relating sensitivity changes to shifts in the species composition of periphyton communities,
- describe the timeframe of recovery processes of algal communities from chronic contaminations, in order to explore the opportunity of using PICT as active bio-monitoring tool for the measure of remediation success,
- investigate individual and combined effects of ionic and toxic stress on the structure and function of periphyton and predict joint effects by using the mixture toxicity model of independent action,
- validate an *in situ* PICT according to the requirements defined by Blanck (2002).

In order to achieve these goals two *in situ* studies and one microcosm approach were conducted. The studies are based on an effect-directed analysis (EDA) conducted in a creek next to the industrial area of Bitterfeld (Germany), which revealed prometryn, N-phenyl-2-naphthylamine (PNA) and tributyltin (TBT) as phytotoxic, site-specific toxicants (Brack et al., 1999).

In **Chapter 2**, the PICT approach was used as tool for hazard confirmation for an EDA study. The potential effects of prometryn, PNA and TBT on local freshwater periphyton were evaluated in the field in order to validate the findings of Brack et al. (1999), that these phytotoxic compounds have a significant ecotoxicological potential to affect indigenous organisms. On account of the different modes of action of the toxicants, different exposure times and three different measures of photosynthesis were tested for their suitability in the short-term toxicity test. This approach also enabled the examination of potential co-tolerances between the investigated compounds. Furthermore, the exposure levels of prometryn, PNA and TBT were determined in the field by using passive sampling techniques, supporting the results of the effect assessment. Out of the three phytotoxic compounds only the hazard of prometryn could be validated.

For this reason, PICT has been used for active bio-monitoring in order to assess the potential of periphyton communities to recover from and adapt to chronic prometryn contamination in the field (**Chapter 3**). Therefore, local periphyton communities grown at a reference site and the prometryn contaminated sampling site were transferred to the respective other site and structural and functional changes were examined over 44 days. In order to develop a mechanistic understanding, the tolerance shifts towards prometryn were related to structural changes in algal classes and diatom species composition of the communities. This approach enabled the systematic analysis of the timeframe of recovery and adaptation processes of periphyton communities based on succession processes after toxicant removal and toxicant-induced succession. The decrease in tolerance after the removal of environmental stressors is essential for the validation of PICT in the field (Blanck, 2002) and might be a relevant measure of restoration success for retrospective risk assessment. However, the field studies of **Chapter 2 and 3** revealed beside the higher prometryn concentration also an enhanced conductivity at the polluted stream.

In **Chapter 4**, the potential interaction between high ionic loads and prometryn was studied, in order to exclude high ionic load as confounding factor for the PICT found in the field. Therefore, the individual and combined effect of high ionic loads and prometryn on structure and function of freshwater periphyton were investigated in a controlled microcosm approach. The specific succession of diatom species was monitored for each stressor solely and the combination of stressors. Tolerance to high ionic loads was measured by using the index of salinity, while tolerance to prometryn was tested in short-term toxicity tests. The applicability of the mixture toxicity model “independent action” to ecologically relevant long-term community responses was assessed for the first time. **Chapter 4** describes the development of community tolerances under toxic, ionic and multiple stressors. The hazard confirmation of **Chapter 2** was supported by the results from **Chapter 3** and **4**.

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Chapter 2

Pollution-induced community tolerance as a diagnostic tool for hazard assessment

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Rotter, S., Gunold, R., Mothes, S., Altenburger, R., Schmitt-Jansen, M., 2013. Manuskript.

Abstract

The impact of chemical pollution in aquatic ecosystems is globally of concern. Most of the sites are contaminated with various different compounds, which makes chemical target analysis susceptible for ignoring compounds potentially affecting ecosystems. Effect-directed analysis (EDA) enables a non-target analysis of complex contaminated environmental samples, but requires several toxicity and effect confirmation steps. In this study, the community approach of pollution-induced community tolerance (PICT) was used instead of single species tests in order to increase ecological relevance of effect confirmation. An EDA study conducted in a small creek next to an industrial area, revealed three phytotoxic compounds with a high potential to cause effects on phototrophic organisms (Brack et al., 1999). In this study, the effects of these compounds on local algal communities have been assessed for validation of EDA results. For this reason, periphyton was cultivated at a reference (R-site) and at the same sampling site where the EDA has been conducted (P-site). The sensitivity of R- and P-site communities to prometryn, tributyltin and N-phenyl-2-naphthylamine was quantified in short-term tests by using three measures of photosynthesis (effective- and maximum quantum yield, ^{14}C-incorporation). Exposure concentrations were determined by using weekly spot sampling as well as passive sampling (POCIS and Chemcatcher®), providing time weighted average concentrations.

Prometryn as well as PNA concentration were significantly higher at the P-site, whereas TBT concentrations were in the same range at both sites. Out of three site-specific phytotoxic toxicants, only effects of prometryn on local periphyton could be confirmed. The impact of TBT could not be proven as no comparison to the baseline toxicity of not exposed communities was detected. For PNA no differences in sensitivities between R- and P-communities were detected as PNA concentration were very low with 0.026 nmol L^{-1} at the P-site.

In conclusion, the PICT approach can be recommended for the confirmation of site-specific compounds revealed by an EDA, even if restricted to phytotoxic compounds so far. A chemical characterisation of the site is essential for an *in situ* PICT-approach, because the development of possible co-tolerances as well as combined effects have to be considered. Furthermore, the selection of an appropriate endpoint and exposure time for the short-term tests is crucial for the success of the confirmation by PICT.

Introduction

Manifold industrial and agricultural activities have increased chemical pollution of surface waters with unknown impacts on aquatic ecosystems throughout the world (Schwarzenbach et al., 2006). In order to protect environmental health and identify hazardous chemicals in the environment, environmental risk assessment (ERA) is based on three pillars: firstly, hazard identification, secondly, exposure assessment and thirdly, effect assessment. In Europe, the Water Framework Directive (WFD; European Parliament and European Council) has defined Environmental Quality Standards (EQS) for 33 priority substances (Directive 2008/105/EC), in order to achieve a "good chemical and ecological status" of European water bodies. The general approach of exposure assessment integrates data from chemical target analysis e.g. of priority pollutants and predictions of exposure pathways from mathematical models (Escher and Leusch, 2012). However, target analysis is based on prior structure knowledge and precludes the determination of unknown, site-specific compounds or key toxicants, causing specific effects on the ecosystem (Weiss et al., 2011). A promising but time consuming tool for non-target analysis of environmental samples, containing a high number of compounds, is the effect-directed analysis (EDA). This method combines several fractionation steps in order to reduce the chemical complexity of, for example, water or sediment samples, tests the toxic potency of these subsamples on different bioassays, and identifies potentially hazardous compounds with chemical analysis (Brack, 2003). A full EDA study requires a tiered toxicity confirmation based on analytical, effect and hazard confirmation steps (Brack et al., 2008). The analytical confirmation focuses on the match of the suggested chemical structure with present compounds in the sample. Whether the identified compounds are causing the measured effects is proved in the effect confirmation, based on acute single species tests or on sublethal *in vitro* effects. Both steps are crucial for the evaluation of an EDA study but do not consider chronic exposure scenarios, sensitivity differences of species as well as species interactions within communities and therefore have limited ecological relevance. An *in situ* test using communities living in the investigated sampling site would enable an ecologically relevant hazard confirmation, usually not conducted in EDA studies.

An EDA conducted on sediments from a highly contaminated, industrial area in Bitterfeld (Germany) revealed, among other toxic substances, three phytotoxic site-specific compounds in concentrations probably causing effects: N-phenyl-2-naphthylamine (PNA), tributyltin (TBT) and prometryn (Brack et al., 1999). The concept of pollution-induced community tolerance (PICT) could successfully confirm

effects of the photosystem II (PSII) inhibitor prometryn on the local communities of the same site, measured as tolerance increase to prometryn in comparison to reference communities (Schmitt-Jansen et al., 2004). However, possible effects of PNA and TBT have not been analysed, up to now.

The PICT concept was introduced by Blanck et al. (1988) and is based on succession processes triggered by the chronic exposure to toxicants, the so called toxicant-induced succession (TIS). This means that sensitive species of the community are replaced by more tolerant ones, resulting in a measurable increase of community tolerance compared to reference communities. For the quantification of the PICT response, communities are sampled, exposed a second time to the studied toxicant and effects on specific metabolic processes, such as photosynthesis, are measured in short-term tests. Therefore, PICT causally links exposure of toxicants to integrated community responses. The reliability of PICT has been shown in several field studies e.g. for heavy metals (Lehmann et al., 1999), the PSII inhibitor irgarol (Blanck et al., 2009), TBT (Blanck and Dahl, 1996) and metabolites of diuron (Pesce et al., 2010). Nevertheless, co-tolerance for chemicals with similar mode of action or chemical structures may occur as a byproduct of the tolerance to the selecting compound (Blanck, 2002) and has to be considered for establishing causality.

PICT has been discussed already as a tool to confirm EDA results, but limits as the restriction to organisms with simple and short life cycles, the possibility of the occurrence of co-tolerances and the uncertainty if the concept is valid in multiple contaminated sites, demonstrate some lacks in the approaches of the concept so far (Brack et al., 2008).

In order to overcome the uncertainties regarding the validity of the PICT concept in multiple contaminated sites and the occurrence of possible co-tolerance, we used the knowledge from a previous study (Brack et al., 1999) by analysing the effects of three phytotoxic, site-specific compounds on local periphyton communities.

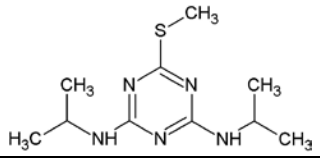
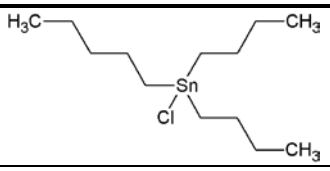
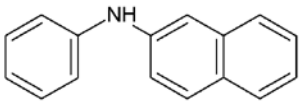
The selected compounds have different fields of application (see Table 1) and differ in their mode of actions. Prometryn is a specifically phytotoxic compound, interfering with the D1 protein of the PSII (Huppatz, 1996). In contrast, TBT affects the energy metabolism of chloroplasts and mitochondria and additionally interacts with proteins and membranes (Fent, 1996). At higher concentrations between 10 to 100 µM, TBT also inhibits the proton translocation at the cytochrome *bf* complex within the photosynthetic electron transport (Klughammer et al., 1998). Therefore, the possibility of a co-tolerance between prometryn and TBT must be taken into account

in this study. Furthermore, PNA affects organisms due to a reactive toxicity (Altenburger et al., 2006).

The objective of the study was to reveal the impact of the phytotoxic, site-specific toxicants TBT, PNA and prometryn on local algal communities in order to validate the findings of Brack et al. (1999) with respect to an *in situ* hazard confirmation. Furthermore, we aim to draw general conclusions about the applicability of PICT for the hazard confirmation in EDA as well as for ERA.

According to Eriksson (2008), the method for PICT detection is crucial, because a long metabolic cascade between the processes affected by the compound and measured by the endpoint (target unspecific measures) results in a poor or slow connection between exposure and detected effect and may limit the ability to detect PICT. Therefore, the evaluation of appropriate exposure durations and a set of different short-term tests are necessary. For this reason, the time dependency of the effects were analysed and three different measures of photosynthesis were chosen to enable the detection of the effects for all selected compounds.

Table 1 Identity, biological, physical-chemical properties and environmental concentrations of the studied compounds.

Compound	**Prometryn**	**TBT-Cl**	**PNA**
Molecular structure			
Full name	2-methylthio-4,6-bisisopropyl-amino-1,3,5-triazine	Tri-n-butyltin chloride	N-phenyl-2-naphthylamine
CAS RN	7287–19–6	1461-22-9	135-88-6
Chemical class	s-triazine	trialkyl organotin compound	aromatic amine
Mode of action	Photosystem II inhibitor (blocks D1 protein) [1]	inhibits oxidative phosphorylation, alters mitochondrial structure and function [4]	reactive toxicity [5]
Use	herbicide	antifouling agent	rubber antioxidant
Log K_{OW}	3.34 [2]	4.76 [5]	4.2 [6]
water solubility	140 µmol L^{-1} [2]	>30.7 µmol L^{-1} [5]	28.8 µmol L^{-1} [6]
Environ. conc.	6.34 mg/kg sediment [3]	2.44 mg/kg sediment [3]	15.8 mg/kg sediment [3]

[1] Huppatz, 1996; [2] Gaggi et al. 1995; [3] environmental concentration detected by Brack et al. (1999) in the sediment of the polluted creek Spittelwasser (Germany); [4] Fent, 1996 ; [5] Ciucani et al., 2004; [6] Altenburger et al., 2006.

Methods

Sampling site and design for periphyton cultivation

This study was carried out in two streams of the river Elbe basin in the area of Bitterfeld (Germany) from May to June 2011. The region has been a major industrial site for chlorine and chemical industry for more than 100 years (Chemie AG Bitterfeld-Wolfen, 1993). As a result of poor waste-water treatment, sediments of surrounding rivers are highly contaminated with organic chemicals as well as heavy metals (Bunge et al., 2001). With regard to the findings of Brack et al. (1999) the creek Spittelwasser was chosen as polluted sampling site (P-site), expecting contaminations of prometryn, TBT and PNA. The reference site (R-site) was located in the vicinity of the P-site (linear distance 3 km) further upstream the river Mulde, assuming a low chemical background of these compounds.

Periphyton was colonised on circular glass discs (1.77 cm²) mounted in plastic racks according to Blanck (1985). Seven racks, each supporting 100 glass discs were placed at the R-site and the P-site. Disks were exposed vertically to the water surface and parallel to the current at a water depth of 10-15 cm. Periphyton samples were collected after 7, 14, 21 and 28 days for characterisation of colonisation. After 20, 21, 22 and 28 days about 120 glass discs were removed from the racks at the R- and P-site for short-term tests, respectively. In order to minimise abrasion and detachment of periphyton during the transport to the laboratory, racks were placed in boxes completely filled with water from the corresponding site. For acclimatisation of periphyton, racks were incubated at 20°C and a photosynthetic photon flux density (PPFD) of about 130 µmol photons m^{-2} s^{-1} for 24 h before testing.

Physico-chemical analyses

Water parameters such as conductivity, light attenuation, oxygen concentration, pH and current velocity were measured directly on site. For further analyses, water samples were taken about 20 cm below the water surface using amber glass flasks. Samples were kept cool during transport to the laboratory and were stored at 4°C until analysis. The chemical analyses of dissolved ions (Cl^-, F^-, NO_2^-, NO_3^-, SO_4^{2-}: ion chromatography; NH_4^+, PO_4^{3-}: photometry; Ca^{2+}, K^+, Mg^{2+}, Na^+, Si: ICP-OES) and trace elements (As, Cd, Cu, Fe, Mn, Zn: ICP-OES) were conducted following standard techniques. Furthermore, subsamples were used for quantification of

prometryn, N-phenyl-2-naphthylamine (PNA) and Tri-n-butyltin (TBT), to enable the linkage of toxicant exposure and effects on local communities.

Prometryn and PNA analyses

In order to analyse prometryn and PNA concentrations of freshwater samples, a solid phase extraction (SPE) was performed using two replicates per sampling date and site. Therefore, each water sample of 250 ml was filtered through a glass fibre filter type GF/F (0.7 µm, Whatman Inc., Buckinghamshire, UK) and was passed through 500 mg conditioned polymeric SPE sorbents (Strata X 33 µm; Phenomenex, Torrance, CA, USA). Absorbed compounds were eluted with methanol (LiChrosolv®, Merck, Darmstadt, Germany) and elutes were concentrated by rotary vacuum evaporation at 40°C. Each residue was re-dissolved in 50 µl toluene (LiChrosolv®, Merck, Darmstadt, Germany). Prometryn and PNA were quantified by gas chromatography (GC; model 6890, Hewlett Packard, Germany) with a mass spectrometer (MS; HP 5973, Hewlett Packard, Germany) on a 5% diphenyl dimethyl polysiloxane capillary column (model HP-5MS, Agilent, 30 m * 250 µm * 0.25 µm film thickness + 5 m precolumn) using a constant flow of helium (1.3 ml min^{-1}). Aliquots of 1 µl per samples were injected splitless at a temperature of 250°C. The column temperature was kept at 60°C for 1 min, increased with a rate of 30°C min^{-1} up to 150°C, then 6°C min^{-1} to 186°C and with 4°C min^{-1} up to 280°C, where it was set for 16.5 min. The measurements were done in single ion modus (SIM). Concentrations were corrected using deuterated benzo[a]pyrene-d12 as injection standard.

TBT analyses

For the quantification of TBT, water samples were filtered through GF/F filters and were extracted using solid-phase microextraction (SPME) within 48 h after sampling. For processing of the samples 9.5 ml acetate buffer (pH 4.75) and 20 µl sodium propyl borate ($NaBPr_4$, Merseburger Spezialchemikalien, Schkopau, Germany) in tetrahydrofuran (2%) (Merck, Darmstadt, Germany) was added to 1 ml water sample. The solution was stirred for 10 min. For an automatic processing, the samples were transferred to the tray of the MultiPurpose Sampler (MPS 2) under a stable temperature of 25°C. Analytes were concentrated using a 100 µm polydimethylsiloxane (PDMS)-coated fused silica fibre (Supelco, Bellefonte, PA, USA) at 40°C for 30 min. Subsequently, the fibre was automatically transferred to the

GC injector and compounds were thermally desorbed at 250°C for 1 min. Analyses of TBT were carried out on a 6890 gas chromatograph (Hewlett Packard, Germany) with an atomic emission detector (G2350A, Joint Analytical Systems, Moers, Germany), using a HP-5MS capillary column (30 m x 250 µm x 0.25 µm, Agilent, Santa Clara, CA, USA) under constant helium flow (2.5 mL min^{-1}).

The PDMS-fiber was calibrated using different concentrations of TBT standards dissolved in acetone.

Passive sampling

Passive sampling methods involve a SPE technique for the accumulation of environmental pollutants *in situ*. The target compounds were accumulated into the receiving phase of the passive sampling device during exposure in the water phase. Hence the use of passive sampling techniques enables a continuous monitoring of bioavailable compounds during the growth period of periphyton and represents a similar exposure history when exposed simultaneously. Two different passive sampling configurations were used in order to detect time-weighted average (TWA) concentrations of prometryn, PNA and TBT. The samplers were fixed in a stainless steel cages in order to protect them from floating material and were exposed next to the biofilm racks.

Polar organic chemical integrative sampler (POCIS)

POCIS were used for analyses of PNA and prometryn. Sampler design and operating principles have been described by Alvarez et al. (2004). POCIS contained 300 mg Strata-X 33 µm (Phenomenex, Torrance, CA, USA) enclosed between two polyethersulfone membranes (Supor®-100, pore size 0.1 µm; Pall Life Science, Washington, NY, USA) with a total exchanging area of 41 cm^2. A total of four samplers were exposed at each sampling site for 21 days. After sampling, POCIS were covered with aluminium foil, kept cool during the transport in the lab and stored at -20°C until analysis.

The extraction of POCIS was modified after Alvarez et al. (2004). POCIS were lyophilised and compounds were eluted from sorbents with 50 ml methanol. Extracts were evaporated to 5 ml via rotary evaporation. After a solvent change (dichloromethane; LiChrosolv®, Merck, Darmstadt, Germany) they were evaporated

further under nitrogen to dryness. Samples were re-dissolved into 200 µl toluene. For quantification of prometryn and PNA, the GC-MS method was used as described for water samples (see Prometryn and PNA analyses).

For the calibration experiment, ten POCIS were exposed in a flow through system similar to that described by Vrana et al. (2006). The calibration system consisted of a 20 L calibration tank and two peristaltic pumps, one for a constant water flow of 10 ml min^{-1} and one delivering the stock solution prepared in methanol (flow 0.26 ml min^{-1}). Thus, the water in the tank was completely renewed every 33 h. A nominal concentration of 0.39 µg L^{-1} prometryn and 0.38 µg L^{-1} PNA was kept during the experiment and methanol concentrations in the water did not exceed 2.6%. POCIS were removed in duplicates after 10 sec, 74 h, 165 h, 338 h and 504 h (0 to 21 days) and replaced by POCIS-holders with an inert glass fibre filter only so that the flow regime in the calibration tank was not altered. The calibration was conducted at 20°C and a simulated flow velocity of 0.15 m s^{-1}. The extraction and analyses of POCIS was performed as described above, extracts were re-dissolved in 1 ml toluene.

The sampling rate (R_s) of prometryn (R_s= 0.59 L d^{-1}) and PNA (R_s= 0.57 L d^{-1}) was determined by measuring the mass of both compounds in the retrieved POCIS. During the 21 days of calibration the compounds were still in the linear uptake phase which is in accordance with Alvarez et al. (2004). POCIS removed immediately after the beginning of the calibration served as blanks, and all subsequent measurements were blank corrected. TWA concentrations in the water were calculated according to Mazzella et al. (2008).

Chemcatcher

The Chemcatcher® passive sampler was used for analyses of TBT according to Aguilar-Martínez et al. (2008). The design of the polytetrafluoroethylene (PTFE) sampler body used in this study has been described in detail by Kingston et al. (2000). C_{18} Empore Extraction discs (47 mm diameter; 3M, St. Louis, USA) were used as receiving phase. For preconditioning, discs were soaked and stored in methanol until required and rinsed with HPLC-grade water before usage. One disc was fixed between the two parts of the PTFE sampler body with a diffusion-limiting cellulose acetate membrane (0.45 µm pore size; Sartorius Stedim Biotech GmbH, Göttingen, Germany) being placed on top of the disc. Finally, the cavity of the Chemcatcher® body was filled with tap water and closed with the PTFE cap. The

prepared samplers were stored at 4°C until exposure at the sampling site (max. 92 h). At each sampling site, three Chemcatcher® bodies were exposed in two periods of 14 days each. Directly after sampling, each sampler was filled with water from the sampling site. The samplers were closed with the cap again and stored at 4°C until extraction.

Tributyltin was extracted from the Empore discs as described in Aguilar-Martínez et al. (2008). GC-grade solvents (methanol, hexane) and reagents (acetic acid, sodium acetate, tripropyltin) were purchased from Merck (Darmstadt, Germany) and were of analytical grade with a purity of at least 97%. The derivatisation agent sodium tetraethylborate ($NaBEt_4$, CAS RN: 15523-24-7) was obtained from Sigma-Aldrich (Steinheim, Germany) with a purity of at least 97%.

For quantification of TBT, GC-AED device was used as described for TBT analyses of water samples (see TBT analyses). However, here aliquots of 1 µl per sample were injected splitless at a temperature of 250°C. The column temperature was kept at 60°C for 1 min, increased with a rate of 20°C min^{-1} up to 180°C, then with 30°C min^{-1} to 300°C, where the temperature was kept constant for 3 min. All TBT concentrations were corrected using tripropyltin as internal standard. The TWA concentrations were calculated based on the calibration data of Aguilar-Martínez et al. (2008).

Biological analyses

In order to detect PICT, short-time inhibition tests were performed and the sensitivity of communities exposed to pollutants was related to a reference community. Three different measures of photosynthesis were used to compare tolerances of communities to prometryn, PNA and TBT. Preliminary tests were conducted for each chemical for range finding of the concentration-response relationships. Short-term tests of prometryn, PNA and TBT were conducted at three consecutive days after three and four weeks of colonisation, respectively. Due to technical problems ^{14}C-incorporation tests were not measured after four weeks. The tests for one compound were conducted within 10 h. Periphyton communities were characterised weekly by using structural parameters such as biomass, algal class composition and diatom composition.

PAM fluorometry

Pulse-amplitude modulation (PAM) - fluorescence is an non-invasive method based on the principle that light energy absorbed by PSII pigments can be used for the photochemical conversion at the PSII reaction centre or is dissipated by heat or emitted as chlorophyll a fluorescence (Schmitt-Jansen and Altenburger, 2008). Due to the complementarities of the pathways, fluorescence measurements serve as a good measure of efficiency of the electron transport from the PSII. Periphyton of each site was exposed to different concentrations of prometryn ($1.26 \cdot 10^{-4}$ to 126 µmol L^{-1}, CAS RN: 7287-19-6, Riedel-de Haën, Seelze, Germany), tributyltin chloride ($1.11 \cdot 10^{-4}$ to 11 µmol L^{-1}, CAS RN: 1461-22-9, Merck, Darmstadt, Germany) and PNA ($5.66 \cdot 10^{-2}$ to 28 µmol L^{-1}, CAS RN: 135-88-6, Sigma-Aldrich, Germany), respectively. Stock solutions of compounds had been prepared in dimethyl sulfoxide (DMSO; CAS RN: 67-68-5, Merck, Darmstadt, Germany). Short-term tests were conducted in small glass vials, to keep losses due to adsorption low. Each test consisted of six concentrations, a solvent control containing DMSO and an untreated control with three replicates, respectively. DMSO concentration within the test was kept at 0.1% and did not show effects in the solvent controls. Preliminary tests using periphyton cultivated in microcosms were conducted to decide on appropriate exposure duration for the compounds. As PAM tests are non-destructive the same sample was measured after 1, 2, 4, 6, 8 and 24 h of exposure. For prometryn and TBT best results were obtained after one hour and for PNA after 24 h of exposure, which were chosen for following tests. Periphyton was incubated on a rotary shaker and under a PPFD of 110 µmol m^{-2} s^{-1} at 20°C.

Two different measures of photosynthetic quantum yield were determined for each sample. First, in order to calculate maximum quantum yield (Φ_{PSII}), the minimal (F_0) and maximum level of fluorescence (F_m) was measured by exposing dark adapted periphyton to measuring light, which displays only marginal effects on the photosynthesis (Genty et al., 1989). Second, the effective quantum yield (Φ'_{PSII}) was estimated under actinic light, driving the photosynthetic electron transport (Schreiber et al., 2007), by measuring the steady state fluorescence (F_0') and the maximum fluorescence in the light (F_m').

$$\phi_{PSII} = \left(\frac{F_m - F_0}{F_m} \right) \quad \text{and} \quad \phi'_{PSII} = \left(\frac{F'_m - F'_0}{F'_m} \right)$$

Samples were measured in time series with four observations respectively, using the MAXI-Imaging-PAM (Walz, Effeltrich, Germany). The average of these measurements was used for calculation of photosynthesis inhibition.

^{14}C-incorporation

The incorporation of radiolabelled carbon into macromolecules was applied as a more integrated measurement of photosynthetic rate (Eriksson, 2008). Sample preparation, concentration ranges and total incubation times were the same as for PAM fluorometry. Due to a higher biological variation of the ^{14}C-incorporation, five replicates per concentrations and controls were used. In addition, one set of samples remained unlabelled and the rate of dark fixation was estimated by keeping further controls in the dark. Further, five samples were inactivated with formol (0.25% final concentration) to detect the abiotic CO_2 fixation by the biofilm. After pre-incubation of 30 min to TBT and prometryn and 23 h 30 min to PNA, respectively, 25 µl $NaH^{14}CO_3$ (PerkinElmer LAS, Rodgau, Germany) was added to glass vials giving a final activity of 0.0625 µCi mL^{-1} (2.31 kBq mL^{-1}). Carbon fixation was terminated by adding 50 µl formol (10%) to each sample after 30 min. Glass discs were carefully removed from the vials and were alternately washed in 1 N HCl and water for ten times each, to remove $^{14}CO_3$ not incorporated in the biofilm. Discs were transferred to scintillation vials containing 1 ml DMSO and were shaken on a rotary shaker for 15 min for lysis of cells. Finally, 7 ml of scintillation cocktail (Ultima gold XR, Perkin Elmer, Waltham, MA, USA) were added and samples were shaken until the solution became clear. Radioactivity was measured in a Wallac 1414 liquid scintillation counter (Perkin Elmer Wallac GmbH, Freiburg, Germany).

Calculation of effect parameters

For each short-term test mean values of controls were set to 100% of activity and relative inhibition of each exposure concentration was calculated to model concentration-response relationships, using log-logistic analysis. Maximum response (A_{max}) was set to 100%, minimal response (A_{min}) to zero, y represents the effect, x the concentration, x_0 the concentration at median efficacy (EC_{50}) and p the slope of the curve.

$$\gamma = A_{\max} + \frac{A_{\min} + A_{\max}}{1 + (x / x_0)^p}$$

However, there are exceptions for some ^{14}C-incorporation tests, showing considerable (-25%) relative effects below 0%. In this case, the mean of the relative effect values of lowest tested concentration was used as lower asymptote and EC_{50} was recalculated by using parameters of the respective concentration-response curve. Analyses were performed using nonlinear regression least-squares curve fitting with the software OriginPro 8G (Microcol Software Inc., Northhampton, MA, USA).

Algal class composition (Phyto PAM)

Algal class composition was analysed based on fluorescence measurements of a four-wavelength-excitation PHYTO-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Diatoms, chlorophytes and cyanobacteria are characterised by different signature pigments with typical absorption spectra. These allow discrimination on the level of algal classes by multiwavelength excitation. Three spots per glass disc with five replicates per site and sampling date were measured according Schmitt-Jansen and Altenburger (2008).

Biomass

Chlorophyll a concentration was used as measure for biomass. In order to extract lipophilic pigments, colonised glass discs were placed in amber glass vessels containing 1 ml acetone (90%) and were sonicated at 56°C for 4 min. After storage of 24 h at -80°C, samples were sonicated at room temperature until biofilms were completely removed from glass discs. The extracts were filtered through at 2 µm syringe filter (Spartan 13/ 0.2 RC, Whatman GmbH, Freiburg, Germany) and were stored at -80°C until analyses. A total of five samples per site and sampling date were analysed according to Woitke et al. (1994) using reverse-phase high performance liquid chromatography (HPLC). Chlorophyll a was calibrated using standards supplied by Sigma Aldrich (Steinheim, Germany).

Taxonomic analyses of diatoms

After three and four weeks of colonisation, ten glass discs per sampling site were preserved in 5% formaldehyde for identification of diatom species. Preparation and identification of the samples was conducted according to Krammer and Lange-Bertalot (1986-1997). Organic matter was removed by boiling the scraped algal material in hydrogen peroxide (30%) until the solution was colourless. Clean diatom samples were used to identify and to count at least 530 valves per sample using light microscopy.

For each sample the Shannon Diversity Index, Evenness, Specific pollution sensitivity index (IPS), Trophic index and Saprobic index were calculated using Omnidia software v8.1 (Lecointe et al., 1993). Furthermore, the Index of Salinity was calculated according to Ziemann et al. (1999).

Results

Physico-chemical characterisation of the sampling sites

The effect of pollution at the P site is mirrored in its four times higher conductivity than at the R-site, which is due to higher concentrations of Cl^-, NO_2^-, SO_4^{2-}, NH_4^+, Ca^{2+}, Fe, K^+, Na^+, Mg^{2+}, Mn and Si (Table 2). Flow velocity, pH, light attenuation but also the concentration of O_2 and F^- were similar at both sites, whereas temperature, NO_3^- and PO_4^{3-} were higher at the reference site. The concentrations of As, Cd, Cu and Zn were below detection limits at both sites.

Table 2 Mean values of physical and chemical parameters (concentration in mg L^{-1}) of the stream water at the reference (R) and polluted site (P), determined weekly during the experimental period of 28 days (5n).

Physico-chemical parameters [mg L^{-1}]	R-site		P-site	
	Mean value	SD	Mean value	SD
Temperature [°C]	21.1	0.6	17.4	1.5
pH	7.47	0.53	7.27	0.09
Conductivity [µS cm^{-1}]	560	6	1934	99
Flow velocity [m s^{-1}]	0.02	0.01	0.03	0.01
Relative light ratio 30 cm under water surface [%]	38	6	39	3
O_2	9.22	0.80	8.01	0.68
Cl^-	50	1	221	25
F^-	0.18	0.06	0.19	0.04
NO_2^-	0.17	0.04	0.37	0.07
NO_3^-	11	1	6	1
PO_4^{3-}	0.21	0.05	<0.1[a]	0.00
SO_4^{2-}	118	3	578	42
Ca^{2+}	63.3	2.3	274.3	15.2
K^+	8.03	0.48	30.66	2.46
Mg^{2+}	14.52	0.47	38.37	2.68
Na^+	39.43	1.28	127.74	13.54
NH_4^+	0.25	0.07	1.90	0.28
As	<0.1[a]	0.00	<0.1[a]	0.00
Cd	<0.05[a]	0.00	<0.05[a]	0.00
Cu	<0.05[a]	0.00	<0.05[a]	0.00
Fe	0.10	0.01	0.18	0.06
Mn	0.04	0.01	0.81	0.14
Si	2.36	0.20	7.13	0.79
Zn	<0.05[a]	0.00	<0.05[a]	0.00

(SD = standard deviation, [a] < detection limit)

Prometryn and PNA had higher water concentrations at the polluted site than at the reference site throughout the duration of the experiment (Fig. 1). Prometryn concentrations found for spot sampling showed a mean of 1.67 nmol L^{-1} at the P-site and were significantly (t-test, $p<0.05$) higher than at the R-site (0.006 nmol L^{-1}). In contrast, mean concentrations of TBT were with 0.31 nmol L^{-1} at the P-site and 0.24 nmol L^{-1} at the R-site similar at both sites. The average PNA concentrations of 0.026 nmol L^{-1} at the P-site were significantly higher than the average of 0.008 nmol L^{-1} at the R-site (t-test, $p<0.05$). Time-weighted average concentrations derived by passive sampling confirmed the trend of concentration differences between the compounds for each site, but were two to three times smaller than concentrations found for spot sampling. The standard deviation of mean values of POCIS (4n) used for determination of TWA concentrations of prometryn and PNA were low, but Chemcatcher® replicates (two times 3n) varied for the R-site. Based on the Grubbs outlier test one value of the Chemcatcher® replicates was excluded for data evaluation. Nevertheless, four out of the five remaining Chemcatcher® replicates showed TBT concentrations below 0.01 nmol L^{-1}, whereas the extract of another showed a concentration of 0.56 nmol L^{-1} for the R-site.

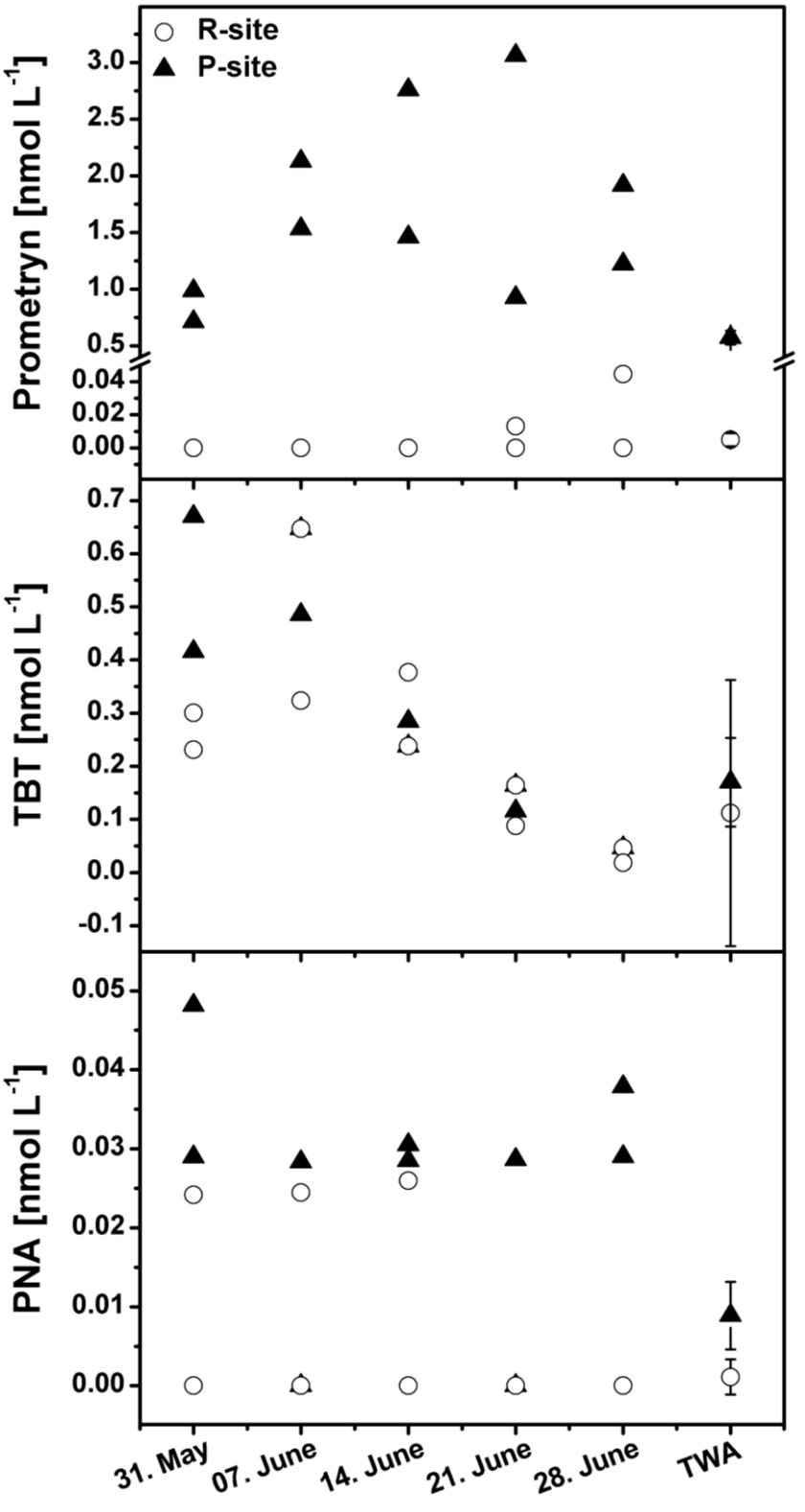


Fig. 1. Water concentrations of prometryn, TBT and PNA in nmol L^{-1} determined by weekly spot sampling and by passive sampling at the reference (R) and the polluted (P) site. For spot sampling of compounds two replicate water samples were used per sampling date. Passive sampling of prometryn and PNA was conducted with four replicates and for TBT two times three replicate passive samplers were analysed. Error bars of time-weighted average (TWA) concentrations show standard errors associated with each mean.

Characterisation of local periphyton communities

Periphyton communities of R- and P-site differed in biomass, taxa richness and IPS classification, while other biological parameters were similar (Table 3). The chlorophyll a concentration indicated that communities from R-site had twice as much biomass as communities grown at the P-site (Table 3). However, relative abundances of algal classes were comparable with about 7% cyanobacteria, 35% chlorophytes and 60% diatoms. A higher taxa richness of diatoms was found for communities of the P-site, but Shannon diversity and Evenness were in the same range. According to the IPS values of diatom communities, the R-site showed a good and the P-site a high ecological status (based on Eloranta and Soininen (2002)). Furthermore, the R-site was in the eutrophic and the P-site in the mesotrophic class. Both sites showed a moderate pollution level and were characterised as β-oligohalob. Except for cyanobacteria, differences between the third and the fourth week were low for all biological measures, therefore a climax state of the periphyton communities can be assumed.

Table 3 Biological parameters for characterisation of periphyton communities from R- and P-site after three and four weeks of colonisation. The taxa richness and species indices such as Shannon diversity index, evenness, Specific Pollution Sensitivity index (IPS), Trophic index, Saprobic index and the Salinity index are based on diatom abundances.

Biological parameters	R-site		P-site	
	3rd week	4th week	3rd week	4th week
Chlorophyll a [µg cm^{-2}]	4.08	4.87	2.29	2.27
Cyanobacteria [%]	0.2	7.7	0.6	6.4
Chlorophytes [%]	36.4	34.7	39.6	34.6
Diatoms [%]	63.5	57.6	59.8	59.0
Taxa richness	49	54	79	70
Shannon diversity	2.24	2.20	2.23	2.30
Evenness	0.40	0.38	0.35	0.38
IPS	15.2	15.1	17.4	17.3
Trophic index	2.5	2.5	1.8	1.9
Saprobic index	1.9	1.9	1.8	1.9
Salinity index	1.3	1.6	1.0	1.4

Toxicity estimation

The short-term tests revealed that the effect of each compound was increasing with higher concentrations (Fig. S1 of the Supporting Information) and was dependent on the exposure time (Table 4). Prometryn provoked highest effects on maximum and effective quantum yield closely followed by TBT. Both were acting rapidly within the first hours of exposure and effects on the effective quantum yield were still in the same range for 24 h. However, median efficacies of maximum quantum yield were slightly decreasing for prometryn within the first 8 h, whereas the photosynthesis rate of TBT treated communities recovered already after an exposure of 4 h. For prometryn and TBT, EC_{50}s were low for both PAM measures after 24 h, but variation of replicates increased. The effects of PNA developed during a longer exposure time, with lowest observed EC_{50} after 24 h. Moreover, highest EC_{50} values were observed for this compound. Measures of the effective quantum yield were, depending on the compound, one to three orders of magnitude more sensitive than the maximum quantum yield, the highest differences were observed for TBT.

As a result of these experiments, exposure durations were adapted to the more sensitive effective quantum yield. Thus, for the following periphyton experiments (PAM and ^{14}C-incorporation) with prometryn and TBT measurements were performed after 1 h of exposure, whereas PNA samples were exposed for 24 h.

Table 4 Effective concentrations (EC_{50}) in µmol L^{-1} determined as photosynthesis inhibition, using the two methods of PAM fluorometry as measure of maximum (Φ_{PSII}) and effective quantum yield (Φ'_{PSII}) after different exposure times to prometryn, TBT and PNA. EC_{50}s were modelled from concentration-response relationships using log-logistic data analyses, italic values represent their standard errors.

EC_{50} [µmol L^{-1}]	Φ_{PSII} - maximum quantum yield			Φ'_{PSII} - effective quantum yield		
	Prometryn	**TBT**	**PNA**	**Prometryn**	**TBT**	**PNA**
1 h	0.435	23.809	239.211	0.016	0.037	17.213
	±0.084	*±12.666*	*±146.364*	*±0.004*	*±0.013*	*±5.703*
2 h	0.272	13.456	190.910	0.018	0.050	18.482
	±0.049	*±6.921*	*±95.180*	*±0.005*	*±0.015*	*±6.017*
4 h	0.135	22.714	126.210	0.017	0.030	15.154
	±0.049	*±17.076*	*±38.767*	*±0.006*	*±0.007*	*±4.296*
6 h	0.153	72.437	107.695	0.017	0.030	11.754
	±0.026	*±88.999*	*±32.142*	*±0.004*	*±0.009*	*±3.623*
8 h	0.126	233.836	87.847	0.016	0.026	9.077
	±0.021	*±402.630*	*±20.615*	*±0.004*	*±0.010*	*±2.842*
24 h	0.180	29.048	25.052	0.006	0.013	4.775
	±0.026	*±33.181*	*±3.554*	*±0.002*	*±0.006*	*±0.751*

Effect evaluation at community-level

The sensitivity of periphyton from the R- and the P-site to prometryn, PNA and TBT detected with three different measures of photosynthesis is shown in Table 5. The median efficiencies (EC_{50}s) were calculated from the concentration-response relationships, model fits of log-logistic data analysis are shown in Fig. S1 and model parameters in Table S1.

The EC_{50} values for prometryn calculated from the different measures of photosynthesis varied in their range, showing the lowest values for the effective quantum yield, closely followed by ^{14}C-incorporation, whereas the maximum quantum yield gave 50 to 95 times higher EC_{50} values. Nevertheless, periphyton from the polluted site was in all experiments about two (Φ'_{PSII} and ^{14}C-incorporation) to 3.8 (Φ_{PSII}) times more tolerant than the reference community. Considering the standard errors of modelled EC_{50}, no overlap between median efficiencies has been observed. The data retrieved after four weeks of colonisation showed a 3.6 to 5.4 times higher tolerance of the P-site community, confirming the results after three weeks of colonisation.

Table 5 Effective concentrations (EC_{50}) in µmol L^{-1} of prometryn, TBT and PNA for periphyton communities colonised at the reference (R-site) and the polluted site (P-site), determined with three measures of photosynthesis after three and four weeks of colonisation. Median efficiencies were modelled from concentration-response relationships using log-logistic data analyses, italic values represent their standard errors.

EC_{50} [µmol L^{-1}]	**Prometryn**		**TBT**		**PNA**	
	R-site	**P-site**	**R-site**	**P-site**	**R-site**	**P-site**
3rd week						
Φ_{PSII} PAM	1.797	6.896	0.323	0.148	198.72	175.90
	±0.489	*±2.366*	*±0.028*	*±0.022*	*±78.46*	*±93.00*
Φ'_{PSII} PAM	0.035	0.072	0.139	0.006	173.35	69.84
	±0.004	*±0.011*	*±0.009*	*±0.002*	*±104.77*	*±69.84*
^{14}C-incorporation	0.054	0.096	0.011	0.101	6.034	4.197
	±0.004	*±0.01*	*±0.003*	*±0.014*	*±3.926*	*±0.658*
4th week						
Φ_{PSII} PAM	4.146	22.218	0.445	0.336	167.96	-
	±1.113	*±5.606*	*±0.055*	*±0.033*	*±43.39*	
Φ'_{PSII} PAM	0.036	0.129	0.083	0.084	31.184	-
	±0.001	*±0.011*	*±0.016*	*±0.014*	*±7.786*	

Φ_{PSII} = maximum quantum yield; Φ'_{PSII} = effective quantum yield; ± represent standard errors of EC_{50}s.

With regard to TBT, sensitivity of effective quantum yield (Φ'_{PSII}) and ^{14}C-incorporation were in the same range, whereas maximum quantum yield gives again highest EC_{50} values (two to 26 times higher). For the measures of maximum and effective quantum yield, reference communities are two to 24 times more tolerant than P-communities. However, the quantification of ^{14}C-incorporation reveals communities of the P-site being nine times more tolerant than R-communities. After four weeks of colonisation, measurements of maximum and effective quantum yield showed similar tolerances of both communities. Thus, the difference between the tolerance of R-communities and P-communities to TBT varied, showing different trends of TBT tolerance for different measures of photosynthesis and time points.

For PNA ^{14}C-incorporation is the most sensitive measure of photosynthesis inhibition. For both maximum and effective quantum yield maximum inhibition of photosynthesis did not exceed 40% and replicates showed high variations. Therefore, the calculation of EC_{50} values is not reliable. However, the highest test concentration used, caused similar relative photosynthesis inhibition of R- and P-communities, indicating similar sensitivities. With regard to ^{14}C-incorporation, reference communities are slightly (1.4 times) more tolerant than P-communities. Since the standard errors of modelled EC_{50} are overlapping the difference in tolerance is small and not significant. The short-term test for the P-site after four weeks revealed high variations of replicates and maximum inhibitions below 40% and could therefore not be evaluated. For this reason, a comparison of data between the third and the fourth week is not possible.

Discussion

Relevance of choosing an appropriate endpoint for PICT detections

For a reliable PICT detection the endpoint as well as the exposure duration of the short-term test is crucial. In the following section, the suitability of the chosen endpoints is discussed, which is the basis for the evaluation of PICT responses in the field approach.

Depending on the mode action of compounds measurable effects might occur within different time ranges (Arrhenius et al., 2006). In general, one can assume that a long metabolic cascade between the endpoint and the target of the compound leads to a delayed detection of the effect (Eriksson, 2008). Furthermore, the level of the effect value is highly depending on the sensitivity of the endpoint, e.g. an endpoint being just indirectly affected by the compound might underestimate the toxicity of the compound if the exposure duration is not sufficient. Thus, a mismatch between endpoint and mechanism of action may limit the detection of PICT (Eriksson, 2008). The mode of action of PNA has not been fully explained, but PNA affects organisms most probable due to a reactive toxicity (Altenburger et al., 2006). Thus, measures of photosynthesis might not be the most sensitive effect parameter for PNA, which is also indicated by the extended exposure time required for effect assessment in our study. The aim of the short-term test is to detect changes that have been developed in the selection phase (Blanck, 2002). Thus, the induction of selection processes, such as significant growth of tolerant species, within the short-term test would influence the PICT detection and must be avoided (Blanck, 2002). On this account, the exposure time of 24 h should not be further extended. The effective and the maximum quantum yield measure the efficiency of the electron transport in the light reaction, whereas ^{14}C-incorporation gives an estimation of the light independent (dark) reaction within the Calvin cycle. In comparison to PAM measures, ^{14}C-incorporation represents a more integrated measure of photosynthesis with more and closer links to other parts of the metabolism (Eriksson, 2008) and was therefore more sensitive for effect detection of PNA. For prometryn, all measures of photosynthesis determined effects on photosynthesis within the first hour. This finding is consistent with the specific mode of action of prometryn, inhibiting the D1 protein of the PSII which leads to a decrease of the electron transport capacity through the PSII (Huppatz, 1996) and suppresses carbon uptake (Brown and Lean, 1995). Thus the

chosen methods seem to be suitable endpoints for tolerance detection towards prometryn.

TBT alters mitochondrial structure and function and inhibits oxidative phosphorylation as well as the synthesis of ATP (Fent, 1996). Although, TBT only affects the photosynthesis directly in concentrations exceeding concentrations applied in this study (Klughammer et al., 1998), it led to fast effects on all measures of photosynthesis.

To conclude, for prometryn and TBT the used measures of photosynthesis are suitable for the detection of tolerance, but effective quantum yield was more sensitive than maximum quantum yield. For PNA, PAM measures might be not suitable, as the mode of action may not specifically affect photosynthesis. According to Eriksson (2008), ^{14}C-incorporation might be more qualified to detect effects of compounds with mode of actions different from inhibition of photochemistry and was the most sensitive measure for PNA in our field study. For this reason, endpoints have to be chosen according to the mode of actions of the compound to give accurate tolerance values (Blanck, 2002). For a reactive mode of action an integrated endpoint might be more reliable.

Exposure and effects of studied compounds

The first objective of the study was to reveal effects of the phytotoxic, site-specific compounds on local algal communities. In order to link the exposure of prometryn, TBT and PNA to possible effects on local periphyton, R- and P-site were chemically and their respective communities biologically characterised.

Analysis of physico-chemical parameters showed differences in temperature, nutrient concentrations and conductivity, which might influence species composition of periphyton. For instance, the higher concentration of phosphate and nitrate at the R-site might be one reason for the categorisation in the eutrophic class according to the trophic index for diatoms and a higher biomass of communities from the R-site. The difference in biomass has to be considered for comparison of community sensitivities, as in biofilms with high biomass the transfer of substances and contaminants is limited (Stevenson and Glover, 1993), resulting in decreasing sensitivities (Ivorra et al., 2000). Nevertheless, differences in chloride concentrations and conductivity were not reflected in the salinity index, and also diversity, evenness and saprobic status of

sites were in the same range. As phytobenthos is one of the biological quality elements of the WFD, diatom indices such as IPS are frequently used indicators for the assessment of the ecological status within the WFD. According to the IPS values in this study, P-site was surprisingly categorised as site with high ecological status, whereas the R-site showed a good ecological status (based on limits proposed by Eloranta and Soininen (2002)). However, for the classification of the entire quality element abundances, indices and species compositions of macrophytes, benthic diatoms and remaining phytobenthos have to be combined (Schaumburg et al., 2004).

Prometryn concentration was significantly higher (1.66 nmol L^{-1}) at the P-site than at the R-site and was about two times lower than the no-observed effect concentration found for *Scenedesmus vacuolatus* (NOEC = 3.4 nmol L^{-1}, Faust et al., 2001). Nevertheless, periphyton communities colonising the P-site were two to 5.4 times more tolerant than R-communities. This is in accordance to the result of a previous field study at the same sampling site, reporting a five times higher tolerance than compared to the reference site (Schmitt-Jansen et al., 2004). Furthermore, a microcosm study conducted by Schmitt-Jansen and Altenburger (2005), showed an increase of tolerance by a factor of three to six for communities exposed to prometryn concentrations equal or higher than 41 nmol L^{-1}, but not for concentrations below. This study shows that a low but chronic exposure of 1.66 nmol L^{-1} is still affecting periphyton communities in the field and triggers community changes as TIS, resulting in a PICT response. Therefore, *in situ* PICT studies seem to be more sensitive than studies conducted in microcosms. The low effect thresholds in the field might be due to a more restricted species input in microcosm studies and a possible underestimation of field exposure or confounding factors (Schäfer et al., 2012). For an additional confirmation of the detected PICT for prometryn, periphyton from the P-site have been translocated to the R-site and vice versa in order to study recovery and adaptation processes (Rotter et al., 2011). Also considering the aim of the WFD to achieve a “good ecological status” the study of recovery trajectories and community resilience in aquatic ecosystems is of major interest (Morin et al., 2010).

With regard to TBT, exposure at the R- and P-site was in the same range (0.24-0.31 nmol L^{-1}) and was about hundred times higher than the maximum allowable concentration environmental quality standard (MAC-EQS) of 0.0025 nmol L^{-1} defined by the WFD due to endocrine disruption in fish (CIS Guidance No. 19, 2009). Due to

the equal exposure of both sites, communities of the R-site were most probably affected to the same extent than P-communities. As a result, no baseline tolerance of unaffected communities was detected, which does not allow PICT detection (Blanck, 2002) and causes unclear relations between the sensitivity of R- and P-communities for the different measures of photosynthesis after three weeks. Furthermore, the mode of actions of TBT might lead to different results between the specific measure of PAM and the more holistic ^{14}C-incorporation tests. The uncoupling of energy transduction and the inhibition of the ATP synthetase deprives the cells from energy (ATP) needed for the incorporation of CO_2 within the Calvin cycle. Thus, ^{14}C-incorporation might be more directly affected by TBT than the effective and maximum quantum yield providing a measure of the light reaction of photosynthesis. However, R- and P-communities showed similar sensitivities after four weeks of exposure, but ^{14}C-incorporation could not be measured. With regard to the findings of Blanck and Dahl (1996), exposure in this study was in the range of the no-effect concentration (0.3 to 0.5 nM) estimated for marine periphyton. Furthermore, a similar study (Molander et al., 1992) using ^{14}C-incorporation for the quantification of periphyton sensitivity to TBT, detected an EC_{50} value of 0.012 µmol L^{-1} for reference communities, which is in accordance to our study, and an EC_{50} of 0.53 µmol L^{-1} for communities exposed to 5 nmol L^{-1} TBT, being five times higher than found in our study. To conclude, due to the exposure below the estimated no-effect concentration for periphyton communities and effect values being in the same range of reference communities of other studies, no effect of TBT on periphyton communities is assumed. Nevertheless, this can not be proved as no comparison to the baseline toxicity of unexposed communities was detected.

The PNA exposure at the P-site (0.026 nmol L^{-1}) was by factor of three higher than at the R-site. However, concentrations at both sites were low, being three to four orders of magnitudes lower than an EC_{50} (0.153 µmol L^{-1}) detected for *Scenedesmus vacuolatus* (Altenburger et al., 2006). Furthermore, a metabolomics approach studying the effect of PNA on *Scenedesmus vacuolatus* reported on first pharmacological effects for a concentration of 0.007 to 0.228 µmol L^{-1} and toxic effects from 0.456 µmol L^{-1} on (Sans-Piché et al., 2010). Thus, the chronic exposure found in this study is still two to three orders of magnitudes lower than the concentrations triggering first effects on metabolome level. In comparison to single species tests, median efficacies detected for periphyton were with 4-6 µmol L^{-1} one

magnitude higher than for single species tests. As PNA might interfere with photosynthesis only indirectly, maximum and effective quantum were less sensitive than the more integrated ^{14}C-incorporation, and EC_{50} values could not be estimated for PAM measures. No differences in sensitivity between R- and P-communities were detected for ^{14}C-incorporation, which led us to the conclusion that PNA did not affect local communities in our study.

As a result, out of three site-specific phytotoxic toxicants, only effects of prometryn could be confirmed. As the EDA was conducted in sediment and not in the water, bioavailability, water solubility and stability of each compound have to be considered for risk assessment. Thus, compounds found in the sediment do not necessarily be present in adverse concentrations in the water phase. In comparison to PNA and TBT, prometryn has the lowest log K_{OW}, is readily soluble (Table 1) and stable in water (Erickson and Turner, 2002). In contrast, TBT has a high lipophilicity and is accumulating in sediments, but slowly degrades to less toxic dibutyltin (DBT) and monobutyltin (MBT) in water (Aguilar-Martínez et al., 2008). Both degradation products have been found in our study as well (data not presented). PNA is hardly soluble in water and due to its covalent bonding to humic materials in the water column it may partition to the sediment phase (TCI America, 2011). Thus, the physico-chemical parameters of the studied compounds are supporting the low water concentrations of TBT and PNA found in this study. Furthermore, the EDA study has been conducted more than ten years ago, therefore a reduction of the concentrations in the sediment due to degradation, desorption or release to the water phase can be assumed.

PICT as confirmation for EDA?

A further objective of this study was to clarify the issue whether EDA studies can be confirmed by PICT approaches. Brack et al. (2008) proposed PICT already as a potential tool, but had three major concerns which we address in the following section.

The first concern relates the inability of PICT to confirm effects of long-lived organisms with complex life-cycles. In fact, PICT is restricted to microorganisms, periphyton and phytoplankton so far. Nevertheless, these algal communities are due to their short life cycle suitable early warning systems for toxic stress (Sabater et al., 2007) and constitute the basis of the food chain in aquatic ecosystems. Thus,

monitoring of algae might prevent effects on higher organisms. It is obvious that for compounds mainly affecting higher organisms as PAHs or estrogens, periphyton might not be the most sensitive target organism, but this is also the reason why risk assessment is always based on effect values of different groups of organism (algae, daphnids, fish). Thus, PICT is not supposed to be an all-rounder, it is rather a sensitive, environmentally relevant, effect based tool which should be taken into consideration for detecting effects of phytotoxic compounds on the primary trophic level.

Second, the occurrence of co-tolerances for chemicals with similar modes of actions might limit the PICT approach. However, it is important to distinguish between multiple tolerances due to simultaneous exposure to several compounds and co-tolerance, which means that the exposure to one compound also induces the tolerance to other compounds (Blanck and Wängberg, 1991). EDA is conducted in order to provide integrative parameters for the presence of various compounds affecting the ecosystem, by using different biotests and aims the identification of site-specific toxicants (Brack, 2003). In the best case scenario, EDA provides a list of compounds being present in environmental relevant concentrations. Therefore, candidates for possible co-tolerances can be figured out before and are anyway present in considerable concentrations at the studied site. Thus, compounds with similar mode of actions present at the site would rather lead to a combined effect, as previously shown in a study conducted by Knauert et al. (2008). As a result, the increase of tolerance measured by PICT can not be attributed to one single compound, but to the chemical class of compounds (Knauert et al., 2010). Furthermore, it was shown for arsenate exposed communities that co-tolerance occurred only for one of five compounds with similar mode of actions, demonstrating that co-tolerance is not common and indicates highly comparable toxicity and tolerance mechanisms (Blanck and Wängberg, 1991). In this study, modes of actions of compounds differed, thus tolerance patterns between compounds were different and no co-tolerance was found.

The last concern refers to the uncertainty if PICT is valid in a multiple contaminated site. As described above, PICT has been successfully applied in many field studies already. Aquatic ecosystems are rarely contaminated with one single compound and rather contain various compounds simultaneously (Schwarzenbach et al., 2006). For this reason, the conducted PICT field studies were most probably performed in multiple contaminated sites and could nevertheless link the exposure of toxicants to

sensitivity changes in the community. However, the issue of multiple contaminations has been rarely addressed in these field PICT studies. To our knowledge, only multiple stress of atrazine exposure and grazing pressure (Muñoz et al., 2001) and Cu as well as diuron exposure to phosphate gradients were studied so far (Tlili et al., 2010). Pesce et al. (2011) exposed passive sampling extracts, containing pesticide mixtures of the sampling site to natural phototrophic biofilms retrieved from the same sampling site and found an increasing tolerance of local biofilm communities to the pesticide mixture from upstream to downstream. Thus, a tolerance increase was detected to a whole mixture and not just to one single compound. However, the authors claimed for a wider analytical screening, because important information might be missed, as the sampling efficiency depends on the hydrophobicity of the compound and calibrated data is missing for a large range of chemicals. For this reason, PICT studies should be underpinned with detailed chemical analysis in order to characterise sampling sites chemically and to enable addressing possible mixture effects on community-level.

Beside the conditions for PICT studies mentioned in Blanck (2002), for a reliable hazard confirmation the following requirements have to be fulfilled: First, in order to consider the possible occurrence of multiple- or co-tolerance for compounds with similar mode of actions, a chemical characterisation of the site is essential as most of the ecosystems are contaminated with various compounds simultaneously. An EDA might provide the required information for site-specific toxicants in advance. Second, the design of the PICT study in particular the selection of an appropriate endpoint and exposure time for the short-term tests depends on the mode of action and is crucial for the effect evaluation.

In conclusion, the PICT approach enables the confirmation as well as the denial of effects of suspected, phytotoxic compounds on local communities and can be recommended for hazard confirmation. The hazard confirmation is not only implemented in the tiered confirmation of an EDA study, it is also an important part of ERA in general and in particular in site-specific risk assessment. To our knowledge, there are no tools available so far which causally link exposure of compounds to effects on communities and therefore allow an *in situ* hazard confirmation on higher biological levels. Thus, PICT might be a diagnostic tool for contaminated sites where suspected compounds are known already, but is, as shown in Pesce et al. (2011), also able to determine multiple tolerances from mixtures of putative toxicants.

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Supporting information

Figure S1. Concentration-response relationships

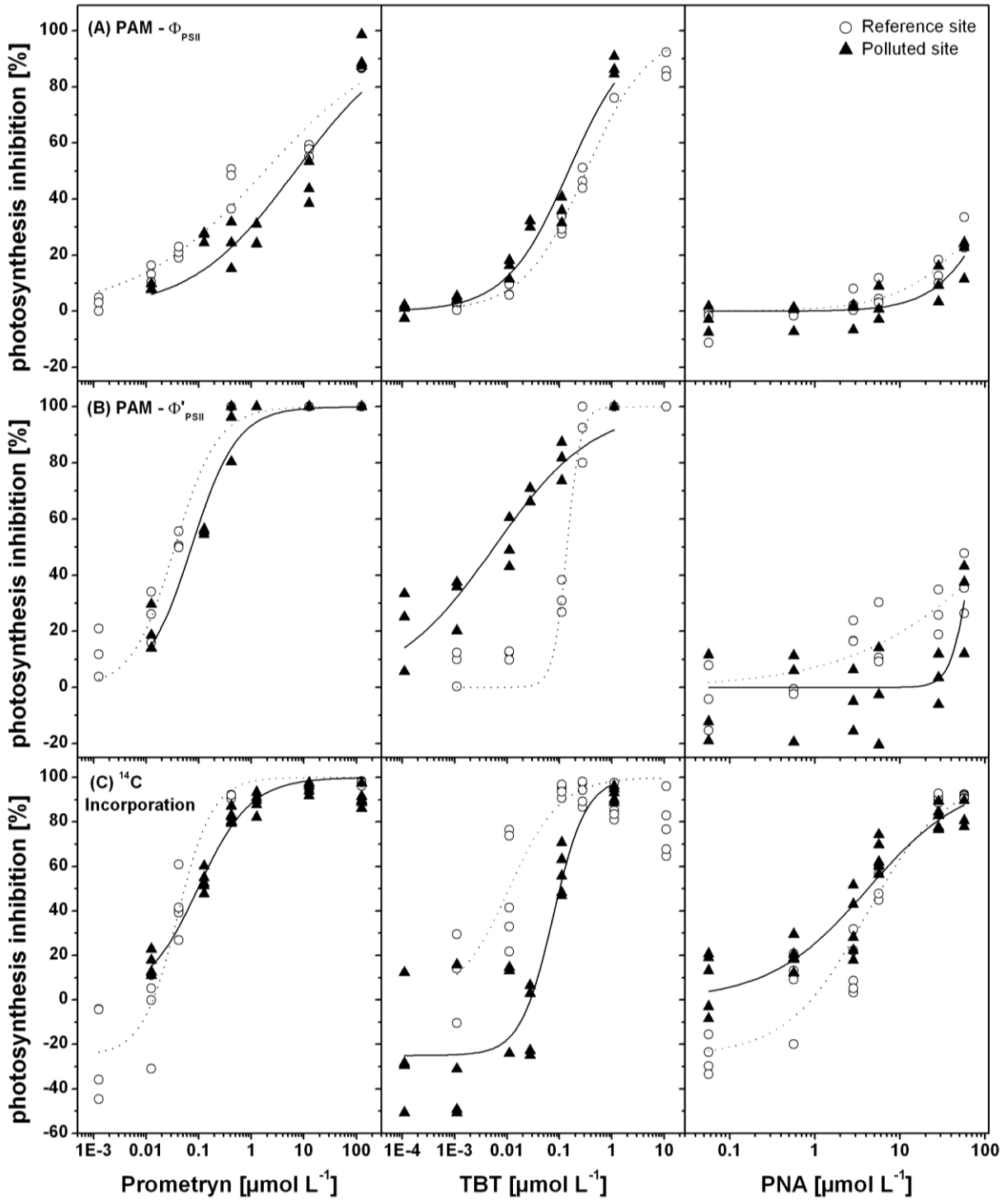

Fig. S1. Concentration-response relationships of prometryn, TBT and PNA for periphyton communities colonised at the reference (○, dotted line) and the polluted site (▲, closed line), determined with three measures of photosynthesis: (A) maximum quantum yield (Φ_{PSII}), (B) effective quantum yield (Φ'_{PSII}) and (C) ^{14}C-incorporation. Tests were conducted after 21 days of periphyton colonisation at the sampling sites. Lines are model fits to the data (log-logistic data analysis).

Table S1. Parameters of concentration-response relationships

Table S1 Slope (p) and coefficient of determination (R^2) of the log-logistic concentration-response relationships for photosynthesis inhibition of periphyton communities from the reference (R-site) and the polluted site (P-site) induced by prometryn, TBT and PNA. Effects of short-term tests were determined with three measures of photosynthesis: (1) maximum quantum yield (Φ_{PSII}), (2) effective quantum yield (Φ'_{PSII}) and (3) ^{14}C-incorporation. Maximum response (A_{max}) was set to 100% and minimal response (A_{min}) to zero, *indicates tests were A_{min} was set to -25. Tests were conducted after three and four weeks of periphyton colonisation at the respective sampling site. Tests were parameter could not be fitted are indicated with n.a. (data not available).

EC_{50} [µmol L^{-1}]	R-site				P-site			
	3rd week		4th week		3rd week		4th week	
	p	R^2	p	R^2	p	R^2	p	R^2
Prometryn								
Φ_{PSII} PAM	0.352	0.948	0.397	0.946	0.435	0.851	0.432	0.909
Φ'_{PSII} PAM	1.066	0.968	1.404	0.996	0.999	0.944	1.741	0.964
^{14}C-incorporation	1.291*	0.949	-	-	0.858	0.952	-	-
TBT								
Φ_{PSII} PAM	0.727	0.984	0.492	0.972	0.728	0.958	1.032	0.987
Φ'_{PSII} PAM	3.314	0.965	0.700	0.934	0.458	0.899	1.223	0.946
^{14}C-incorporation	0.890	0.677	-	-	1.387*	0.853	-	-
PNA								
Φ_{PSII} PAM	0.858	0.820	0.603	0.899	1.242	0.699	n.a.	n.a.
Φ'_{PSII} PAM	0.497	0.687	0.568	0.829	3.847	0.457	n.a.	n.a.
^{14}C-incorporation	0.943*	0.918	-	-	0.752	0.873	-	-

Chapter 3

Active bio-monitoring of contamination in aquatic systems - An *in situ* translocation experiment applying the PICT concept

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Rotter, S., Sans-Piché, F., Streck, G., Altenburger, R., Schmitt-Jansen, M., 2011. Aquatic Toxicology 101, 228-236.

Abstract

The environmental risk assessment of toxicants is often derived from chemical monitoring, based on single species tests performed in the laboratory. However, to provide ecologically relevant information, community approaches are required. The aim of this study was to causally link prometryn exposure to community-level-effects in complex field situations and to identify response times of adaptation to pollution and recovery from pollution. For this reason sensitivity shifts in communities were detected and related to structural changes within the periphyton community. Furthermore, it was intended to illustrate the possibility of a combined approach of community translocation and sensitivity assessment for active monitoring of polluted sites.

Periphyton was grown at a reference (R) and at a polluted (P) site of the river Elbe basin for 26 days, was subsequently transferred from the polluted site to the reference site and vice versa. Sensitivity of communities to prometryn was determined according to the PICT-concept in short-term tests by measuring photosynthesis inhibition and was related to structural changes in algal class and diatom species composition. Exposure to prometryn was determined by using polar organic integrative samplers (POCIS), giving time-weighted average concentrations. Environmental concentrations of prometryn were significantly higher at the polluted site compared to the reference site.Communities grown at the polluted site showed a higher tolerance to prometryn in comparison to the reference site. 17 days after the translocation to the reference site, EC_{50} decreased 2-fold compared to the non-translocated P-community of the same age. By contrast, EC_{50} of the community grown at the reference site was 5 times higher after 17 days exposure at the polluted site. Furthermore, R-P communities were less sensitive to prometryn (higher EC_{50}) than P-R communities, 24 days after translocation. These changes in sensitivity to prometryn were consistent with changes in species composition and clearly indicate that the exposure history of communities is defining the time-response of recovery and adaptation.

In conclusion, the PICT-concept is proofed to be a suitable tool for analysis of recovery and adaptation processes of communities under natural conditions. Therefore, it improves the link between cause and effect in field situations. *In situ* translocation studies provide an ecologically relevant assessment of pesticide effects under field conditions and could be used as a diagnostic tool in active monitoring for decision-making frameworks as used in the implementation of the European Water Framework Directive (WFD).

Introduction

Chemical pollution of aquatic ecosystems is a major environmental problem (Schwarzenbach et al., 2006), which leads to changes in all trophic levels of the ecosystem (Geiszinger et al., 2009). The assessment of ecotoxicological effects is often based on chemical monitoring to evaluate environmental concentrations and subsequently linked to biological effects. This effect assessment is mainly based on simplified approaches performed at suborganism or organism level under standardised laboratory conditions. Communities are taxonomically diverse and consist of interacting species differing in physiology, sensitivity and autecology. McClellan et al. (2008) demonstrated that community approaches, considering species interactions, revealed lower effect thresholds for toxicants than estimated by current risk assessment procedures. Thus, there is a lack of data for realistic exposure situations at higher biological levels in the ecosystem, which is difficult to compensate by extrapolations from simplified single species tests (Seitz and Ratte, 1991).

Therefore, community approaches, like Pollution-Induced Community Tolerance (PICT) seems to be appropriate tools to provide ecologically relevant information on chemical effects in the environment (Blanck, 2002). The PICT-approach is based on the assumption that toxicant-exposed communities increase their tolerance due to the replacement of sensitive species by more tolerant species (Blanck et al., 1988), known as toxicant-induced succession or TIS (Blanck, 2002). Several PICT-studies have been conducted in complex field situations to determine effects on community-level caused by organic pollution and heavy metals (e.g. Blanck et al., 2009; Blanck and Dahl, 1996; Lehmann et al., 1999).

Algal assemblages (e.g. periphyton) are frequently used for bio-monitoring in aquatic ecosystems. Their rapid response to environmental changes, due to their short life cycle, makes them suitable early warning systems for toxic effects (Sabater et al., 2007; Geiszinger et al., 2009). Furthermore, their community composition can dramatically affect the upper trophic levels (Lewis, 1992) and play an important role in the energy and nutrient cycles of running waters (Allan, 1995). Diatoms often represent the major autotrophic proportion of periphyton (McClellan et al. 2008; Navarro et al., 2002). They are widely used to assess water quality (Kelly and Whitton, 1995; Kelly et al., 1995) and microphytobenthos is one of the biological quality elements surveyed in the Water Framework Directive (WFD). The species-

specific sensitivities to environmental variables such as pH, salinity and nutrients are well characterised (Lange-Bertalot, 1979; Kim et al., 2008), which makes them suitable indicators for many types of pollution. Furthermore, diatom indices can be used to directly observe pollution-related succession processes over time like recovery (Lavoie et al., 2008).

Active bio-monitoring is based on the translocation of organisms from one place to another and quantifying their biochemical, physiological and/or organismal responses for the purpose of water quality monitoring (De Kock and Kramer, 1994). Translocation studies were conducted for active monitoring to assess recovery and stress responses of algal communities in complex aquatic field situations (Dorigo et al., 2010a; Dorigo et al., 2010b; Hirst et al., 2004; Iserentant and Blancke, 1986; Ivorra et al., 1999; Gold et al., 2002; Morin et al., 2010; Rimet et al., 2005). These studies set up a field transfer of microalgae communities between polluted and uncontaminated sites. Changes in the diatom species composition and derived diatom indices are mainly determined and have been recommended to regulatories to assess eutrophication in rivers for monitoring (Kelly, 1998).

In general, the interactions between anthropogenic stressors and natural perturbations are poorly understood and effects on organisms in the field have been rarely investigated (Clements and Newman, 2002). *In situ* translocation experiments combined with chemical and biological monitoring (PICT- and diatom analyses) might be a way forward to directly link community changes to toxic effects in the field. However, a systematic analysis of the time-responses of recovery and adaptation processes and related processes e.g. species replacement is missing at present. The understanding of these time-responses may be essential to support decision-making frameworks like the European WFD.

In the present study, time-responses in respect of sensitivity changes and succession processes of periphyton were investigated at two sites from the Elbe river basin (Germany) after translocation. The pollution of the creek Spittelwasser results from the discharge of an untreated effluent from a former chemical plant. A previous effect-directed analysis (EDA) detected, among others, prometryn as a phytotoxic, site-specific toxicant (Brack et al., 1999). Prometryn was produced in Bitterfeld from 1959 to 1990 as a herbicide (Chemie AG Bitterfeld-Wolfen, 1993) and was quantified in the sediment of the polluted stream Spittelwasser (6.34 mg kg^{-1}) by Brack et al. (1999). However, effects from a constant drain of prometryn on local communities

were unproven, yet, but may be relevant to consider before starting restoration efforts, e.g. removal of the sediments of this contaminated site. Additionally, understanding of the time-responses of local communities after removal of the stressor may be essential for monitoring restoration success.

The aim of this study was (1) to causally link the exposure of prometryn to effects in natural periphyton communities, by using a PICT-approach connected with integrated passive sampling, (2) to relate sensitivity changes to an observed toxicant-induced succession (TIS) after translocation and (3) to study the time-responses of recovery from chronically contaminated periphyton and for adaptation of reference communities to chronic contamination. Therefore, this translocation study focuses on the dynamics of adaptation and recovery processes of periphyton, supported by integrated passive sampling giving a long-term overview for chemical levels in the aquatic environment.

Methods

Sampling site

The study was conducted in the river Elbe basin located in Eastern Germany, next to Bitterfeld, which has been a major industrial site of intensive chlorine industry, production of solvents, pesticides, dyes, disinfectants and plastics (Chemie AG Bitterfeld-Wolfen, 1993). Due to the lack of effective wastewater treatment, surrounding areas are highly contaminated with many organic chemicals as well as heavy metals (Bunge et al., 2001). Therefore, a high amount of inorganic and organic pollutants has remained in the sediment of the small creek Spittelwasser (in the further text called P-site). The reference site at the river Mulde (R-site) is located 8 km upstream of the junction with the Spittelwasser and has a low chemical background in water and sediments.

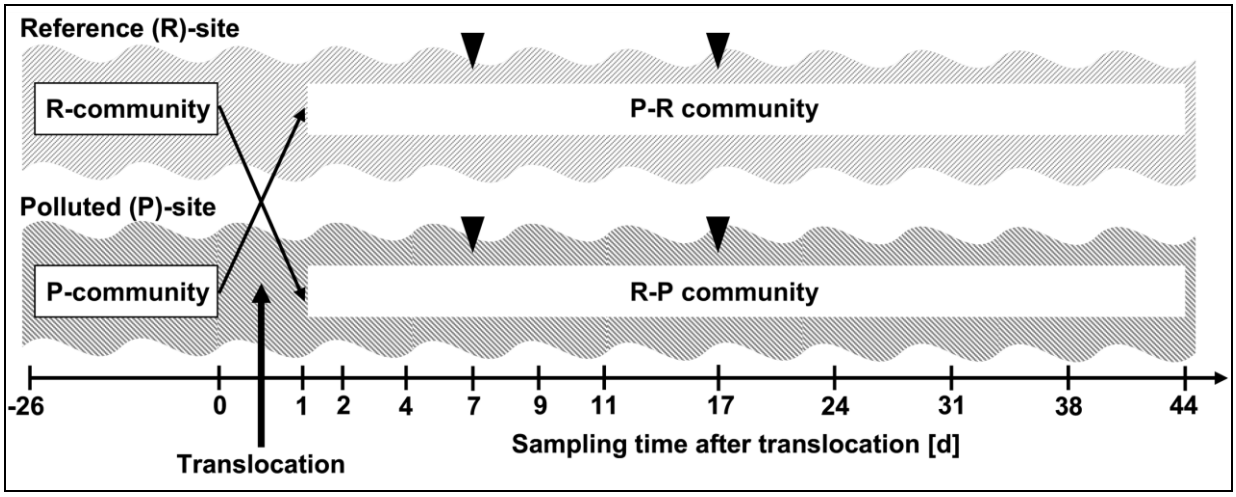

Fig. 1. Schematic overview of sampling design. Time points on the scale represent sampling dates before and after the translocation of the R- and P-community. (▼) indicates sampling of non-translocated communities.

Experimental design for biofilm growth

To enable comparable growth conditions of biofilms, periphyton was grown on glass discs (1.5 cm diameter) inserted in plastic racks according to Blanck (1985). Eight racks per site, each containing 100 glass discs were placed vertically to the water surface and parallel to the current at a water depth of 10-15 cm. After the colonisation of 26 days, 6 racks of periphyton from the polluted site were transferred to the reference site and vice versa (Fig. 1). The holders were placed in boxes, containing surface water from the corresponding site. Transfer between the sites was conducted

within 30 min. To allow to the development of control communities 2 racks were kept at the R- and P-site. Two different sampling strategies were carried out: (1) remaining samples of the original sites were analysed to follow recovery and adaptation processes of translocated communities over longer time-intervals (7, 17 days after translocation) and (2) translocated communities were surveyed in shorter time intervals of 1-7 days directly after the translocation to follow the processes of adaptation in terms of changes in sensitivity and toxicant-induced succession (TIS).

The translocation experiment was performed from May until August 2008. Periphyton samples were collected just before the translocation and on days 1, 2, 4, 7, 9, 11, 17, 24, 31, 38 and 44 after the translocation. Non-translocated control communities were sampled after 7 and 17 days. Each time, 40 glass discs were removed from the racks at the R- and P-site, respectively.

In the laboratory, boxes were incubated in a climate chamber at 20°C and a photosynthetic photon flux density (PPFD) of about 130 µmol photons m^{-2} s^{-1} until analysis (maximal storage 18 h).

Physico-chemical analyses

Conductivity, pH, temperature, oxygen concentration, light attenuation and current velocity were measured *in situ* at each sampling site and date. Water samples were taken weekly using amber glass flasks. Samples were transported cooled to the laboratory and stored at 4°C until analysis. An aliquot was used to analyse the prometryn concentration (Method see Appendix S1). Remaining water samples were analysed for total and dissolved inorganic and organic carbon (TOC-Analysator Dimatoc), dissolved ions (Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, F^-: ion chromatography; NH_4^+, PO_4^{3-}: photometry; Ca^{2+}, K^+, Na^+, Mg^{2+}, Si: ICP-OES) and trace elements (As, Cd, Cu, Fe, Mn, Zn: ICP-OES) following standard techniques.

Polar organic chemical integrative sampler (POCIS)

The POCIS approach is a suitable tool for detecting time-weighted average concentrations of bioavailable contaminants in water (Alvarez et al., 2004). POCIS used in this study had a surface area of approximately 41 cm^2 and contained 300 mg Strata-X 33 µm (Phenomenex, Torrance, CA, USA) enclosed between two polyethersulfone membranes (Supor®-100, pore size 0.1 µm; Pall Life Science, Port

Washington, NY, USA). The sampler design, the preparation of the membranes and the operating principles has been described previously (Alvarez et al., 2004). POCIS were exposed in stainless steel canisters at a water depth of 30-60 cm, close to the biofilm racks. A total of four POCIS were deployed at each site in four exposure periods (21 May-12 June, 12 June-3 July, 3 July-24 July and 24 July-21 August 2008). After 21-28 days, POCIS were transported cooled into the lab and stored at -20 °C until analysis.

Calibration was conducted according to Vrana et al. (2006); see Appendix S2. The extraction of POCIS was modified after Alvarez et al. (2004). POCIS were lyophilised and compounds were eluted from sorbents with 50 ml methanol. Extracts were evaporated to 5 ml via rotary evaporation and after a solvent change (dichloromethane) under nitrogen to dryness. Samples were re-dissolved into 200 µl toluene. Concentrations of prometryn were quantified using GC-MS (model 6890 N, Hewlett Packard, Germany) on a 5% diphenyl dimethyl polysiloxane capillary column (model HP-5MS, Agilent, 30 m x 250 µm x 0.25 µm film thickness + 5 m precolumn) using a constant flow of helium (1.3 ml min^{-1}). Aliquots of 1 µl per sample were injected at a temperature of 250°C. The column temperature was kept at 60°C for 1 min, increased with a rate of 30°C/min up to 150 °C, then 6 °C/min to 186°C and with 4°C/min up to 280°C where it was set for 16.5 min. A mass spectrometer HP 5973 (Hewlett Packard, Germany) was used for identification and measurements were done in single ion modus (SIM). Concentrations were corrected using deuterated benzo[a]pyrene-d12 as injection standard.

Biological analyses

Short-term toxicity test

Short-term inhibition tests of periphyton to prometryn were performed and effects on photosynthesis were derived from pulse-amplitude modulation (PAM) - fluorescence measurements. Periphyton of each sampling date and site were exposed to prometryn (CAS RN: 7287-19-6, Riedel-de Häen, Seelze; Germany) in geometrical concentration steps from 3 x 10^{-7} to 30 mg L^{-1} dissolved in DMSO (CAS RN: 67-68-5, Merck, Darmstadt, Germany). Three replicates were used per concentration and control. Samples without contamination and samples containing DMSO served as controls. DMSO concentration within the test was kept at 0.1% and did not show effects in the solvent controls. Periphyton was exposed to prometryn for 1 h on a

rotary shaker and under a photosynthetic photon flux density of 110 µmol m^{-2} s^{-1} at 20°C. After incubation fluorescence was measured with the MAXI-Imaging-PAM fluorometer (Walz GmbH, Effeltrich, Germany).

The relative inhibition of variable fluorescence in relation to controls was calculated to model concentration-response relationships. The community tolerance to prometryn (expressed as EC_{50}) was calculated using the log-logistic analysis

$$\gamma = A_{\max} + \frac{A_{\min} + A_{\max}}{1 + (x / x_0)^p}$$

where A_{max} and A_{min} denote the minimal and maximal response, x the concentration, x_0 the concentration at median efficacy (EC_{50}) and p the slope. After data inspection A_{max} was fixed to 100% and A_{min} to zero. Analyses were performed using nonlinear regression least square fitting of the software Origin (Microcol, Software Inc., Northhampton, MA, USA).

Algal class composition

The PHYTO-PAM fluorometer (Walz GmbH, Effeltrich, Germany) was used to distinguish between cyanobacteria, green algae and diatoms according to Schmitt-Jansen and Altenburger (2008). The signature pigments for the different algal classes allow discrimination by multiwavelength-excitation. Fluorescence was measured for three spots per glass disc with three replicates per site and sampling date.

Taxonomic analyses of diatoms

Five colonised discs per sampling and site (R, P) were fixed with formaldehyde (5%) until further processing. Preparation of the samples as well as taxonomic analysis of diatom species was conducted according to Krammer and Lange-Bertalot (1986-1997). Organic matter was removed by boiling the scraped algal material in peroxide (30%) until the solution was colourless. Determination of the cleaned diatoms was based on their microscopic exoskeleton using a light microscope. In terms of the relative distribution of species, 400 diatoms were counted per sample. Further diatoms were analysed for occurrence, but were not counted anymore. The most dominant species were ranked according to their proportion on the total number of

counted species. According to this data the Trophic Diatom Index (Rott et al., 1999), Saprobic Index (Rott et al., 1997), Index of Salinity (Ziemann, 1999), Shannon Diversity Index and Evenness (Mühlenberg, 1989) were calculated. The Bray-Curtis Index was computed using EstimateS Version 8.2 (Colwell, 2009).

Non-metric multi-dimensional scaling (NMDS) using Bray-Curtis Dissimilarity and species abundances were performed to identify specific differences of the reference and the polluted site, as well as temporal changes in species structure of translocated periphyton communities. Diatom species occurring in less than 10% of all samples were removed for statistical analyses, giving a total of 30 diatom species. Library vegan of the R statistical environment (http://www.R-project.org) was used.

Results

Physico-chemical parameters of the sites

The physical and chemical parameters are shown in Table 1. At both sites the pH was around 7.5, light attenuation, NO_2^-, F^-, NH_4^+ and PO_4^{3-} values were comparable. Higher temperature, oxygen content and NO_3^- concentrations were detected at R-site. The P-site offered a high conductivity (1919 µS cm^{-1}), a higher flow velocity and higher concentrations of Cl^-, SO_4^{2-}, Ca^{2+}, K^+, Na^+, Mg^{2+}, Si, DIC and DOC.

Table 1 Mean values of physical and chemical parameters (concentration in mg L^{-1}) of the stream water at the reference (R) and polluted site (P), determined during the experimental period. (DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, SD = standard deviation, [a] < detection limit).

Physico-chemical parameters [mg L^{-1}]	R-site		P-site	
	Mean value	SD	Mean value	SD
Temperature [°C]	20.9	1.78	17.1	1.89
pH	7.83	0.28	7.16	0.25
Conductivity [µS cm^{-1}]	527	29	1919	298
Flow velocity [m s^{-1}]	0.03	0.01	0.15	0.04
Relative light ratio 20 cm under water surface [%]	33.81	13.55	39.22	7.31
O_2	7.82	0.85	4.95	0.58
DIC	17.23	1.33	44.32	3.88
DOC	5.00	0.31	9.09	2.39
Cl^-	44.42	4.23	247.51	66.93
F^-	0.29	0.02	0.34	0.08
NO_2^-	0.12	0.04	0.39	0.29
NO_3^-	13.59	2.85	6.28	0.83
PO_4^{3-}	0.19	0.07	0.13	0.03
SO_4^{2-}	102.72	5.30	535.18	101.84
Ca^{2+}	58.07	3.11	235.65	39.37
K^+	7.22	0.72	22.25	5.18
Mg^{2+}	14.18	0.82	35.29	5.42
Na^+	35.34	4.78	139.73	27.61
NH_4^+	0.35	0.52	1.29	0.66
As	<0.10[a]	-	<0.10[a]	-
Cd	<0.05[a]	-	<0.05[a]	-
Cu	<0.04[a]	-	<0.04[a]	-
Fe	0.06	0.04	0.26	0.19
Mn	0.04	0.02	0.74	0.40
Si	1.27	0.66	6.98	1.12
Zn	<0.04[a]	-	<0.04[a]	-
Prometryn [µg L^{-1}]	0.01	0.00	0.70	0.18

Concentrations of Cd, Cu, Zn were below the detection limit of 0.05 mg L^{-1} and As concentrations were also below the limit of analytical determination (< 0.10 mg L^{-1}), showing a similar concentration range at the two sites. However, mean concentration of Mn and Fe were higher at the P-site compared to the R-site.

The quantification of prometryn measured by POCIS showed a mean concentration of 0.01 µg L^{-1} at the R-site and determined concentrations at the P-site ranged between 0.54 and 0.92 µg L^{-1} (Fig. 2). The prometryn concentrations found for spot sampling confirmed this range of concentration (0.23 - 0.97 µg L^{-1}), see Table S1 in the Supporting Information. Therefore, prometryn showed during the whole sampling period on average 85 times higher concentrations at the P-site than at the R-site.

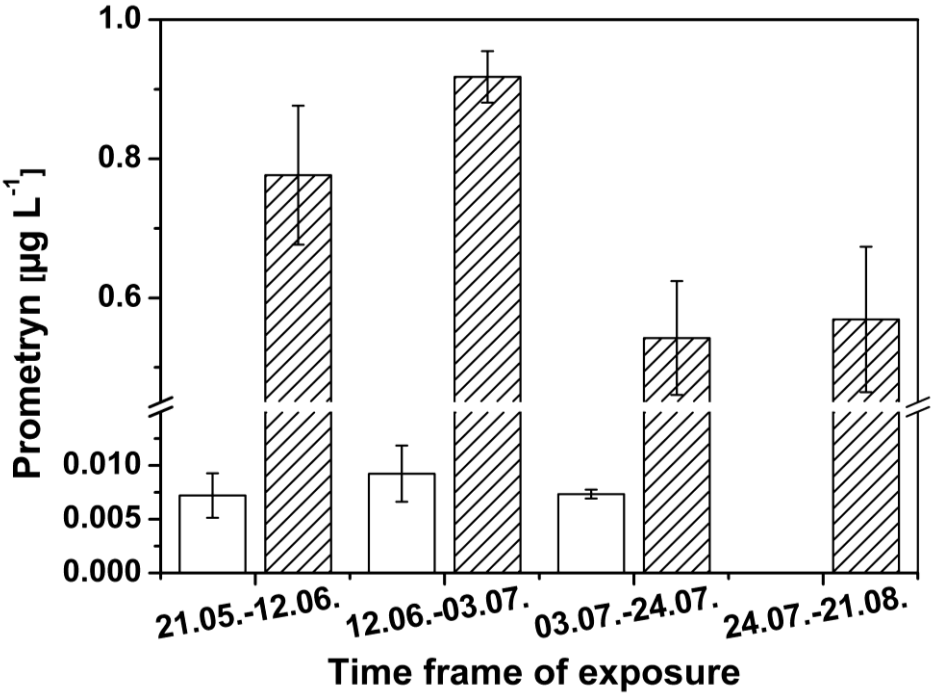

Fig. 2. Prometryn concentrations in µg L^{-1} recovered in POCIS after their deployment at a reference (□ R-site) and a polluted site (▨ P-site). Results shown are obtained by taking the mean of four samplers deployed at the same time for periods of 21-28 days. Error bars show standard errors associated with each mean. POCIS of R-site were lost during the last exposure time.

Pollution-induced community tolerance – sensitivity changes

Short-term inhibition tests of photosynthesis were performed using local P- and R-communities as well as translocated P-R and R-P-communities on different time points of the experiment.

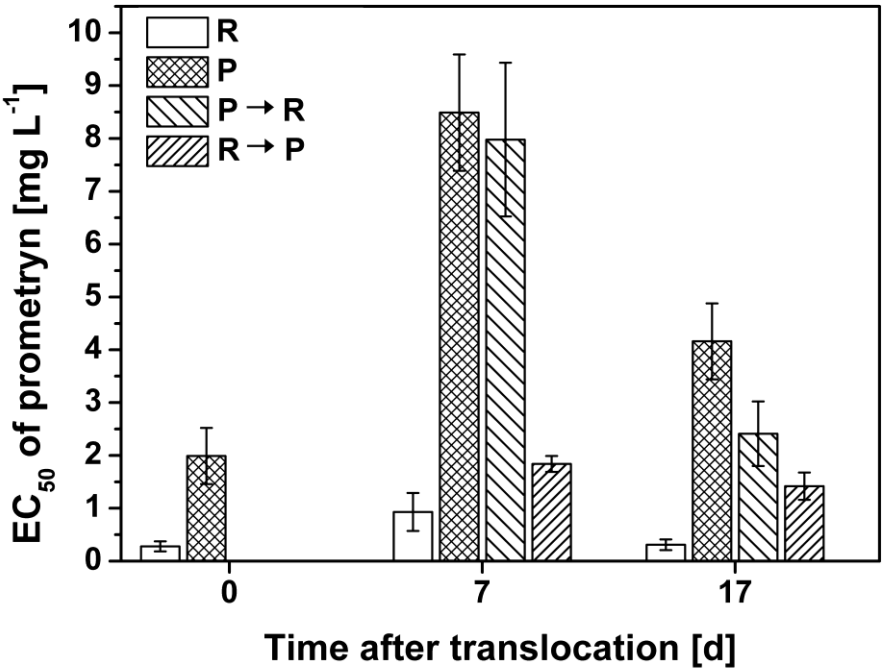

Fig. 3. Changes in tolerance to prometryn of resident communities in comparison to translocated communities after 7 and 17 days of translocation, shown as EC_{50} of photosynthesis inhibition. P- and R-communities were translocated to the respective other site (R→P, P→R), after an initial growth of 26 days at the prometryn polluted (P) and the reference site (R). Non-translocated controls communities were kept at the sites of initial growth. Median efficiencies (EC_{50}) were calculated from concentration-response relationships using log-logistic data analysis.

Local communities grown for 26 days at the polluted site showed a higher EC_{50} (1.99 mg L^{-1}) in comparison to the community grown at the R-site (0.28 mg^{-1}). Non-translocated R-communities did not show remarkable changes in sensitivity over a period of 17 additional days of growth (Fig. 3). In contrast P-communities had a peak value in tolerance (EC_{50} = 8.49 mg L^{-1}) after 7 more days of growth and decreased to an EC_{50} of 4.16 mg L^{-1} after 17 days. Despite translocation to reference conditions, P-R communities increased their tolerance in the same range as the resident P-community after 7 days, but decreased in tolerance in comparison to resident P-community after 17 days. However, they were still less sensitive than non-translocated R-communities. The same trend was observable for R-P communities, which increased in tolerance after 7 days already, but did not reach the same tolerance as the resident P-communities. EC_{50} of R-P community was about 5 times higher compared to the non-translocated R-community of the same age, 17 days after translocation to the P-site. These trends were confirmed from the analysis of sensitivity using a shorter sampling frequency: Higher EC_{50} values of communities

originally grown at the P-site persisted over 9 days after the translocation to the reference site (Fig. 4) and increased significantly on day 4, 7 and 9 up to a maximum of 8 mg L^{-1}. After 17 days exposure at the R-site, EC_{50} of P-R community decreased to 2.4 mg L^{-1}, which is twofold less than non-translocated P-community of the same age. During the 17th and 38th day after the translocation the sensitivity became more stable with EC_{50} between 1.5 and 2.7 mg L^{-1}.

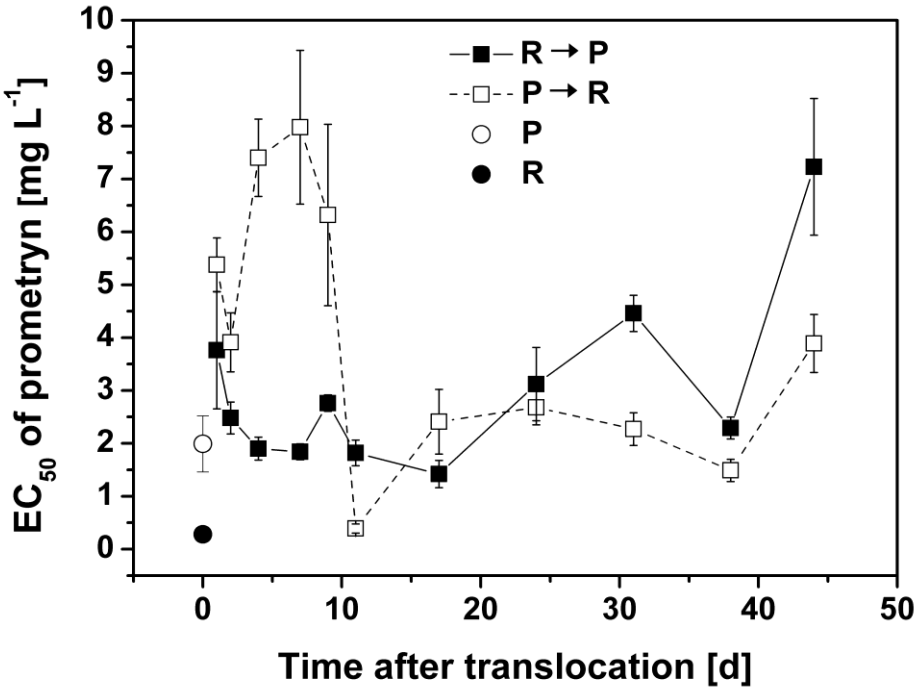

Fig. 4. EC_{50} development over time of translocated periphyton communities, measured as photosynthesis inhibition after translocation of resident P- and R-communities to the respective other site (R→P, P→R). Translocation was conducted 26 days after growing at the prometryn polluted (P) and the reference site (R). Median efficacies (EC_{50}) were calculated from concentration-response relationships using log-logistic data analysis.

The median effect concentrations characterising original R-communities increased after one day exposure at the P-site to 3.8 mg L^{-1}, decreased again to 1.9 mg L^{-1} on day 4 and remained almost stable (+/- 0.5 mg L^{-1}) until the 17th day after translocation. After 24 days a slight increase up to 3.1 mg L^{-1} occurred and therefore P-R communities were less sensitive to prometryn (higher EC_{50}) than R-P communities, a phenomenon which remained unchanged until the end of the experiment. Thus after 44 days exposure of primary R-communities on P-site, EC_{50} values increased 25-folds to 7.2 mg L^{-1}. By contrast P-R-communities showed 44 days after the translocation an EC_{50} of 3.9 mg L^{-1}, which is only twice as high as the primary P-community without translocation.

Algal class composition

Algal class composition of local communities differed from each other, R-periphyton consisted of 82.4% diatoms, 9.1% cyanobacteria and 8.5% chlorophytes, and P-periphyton of 60.5% diatoms, 27.3% cyanobacteria and 12.2% chlorophytes (Fig. 5).

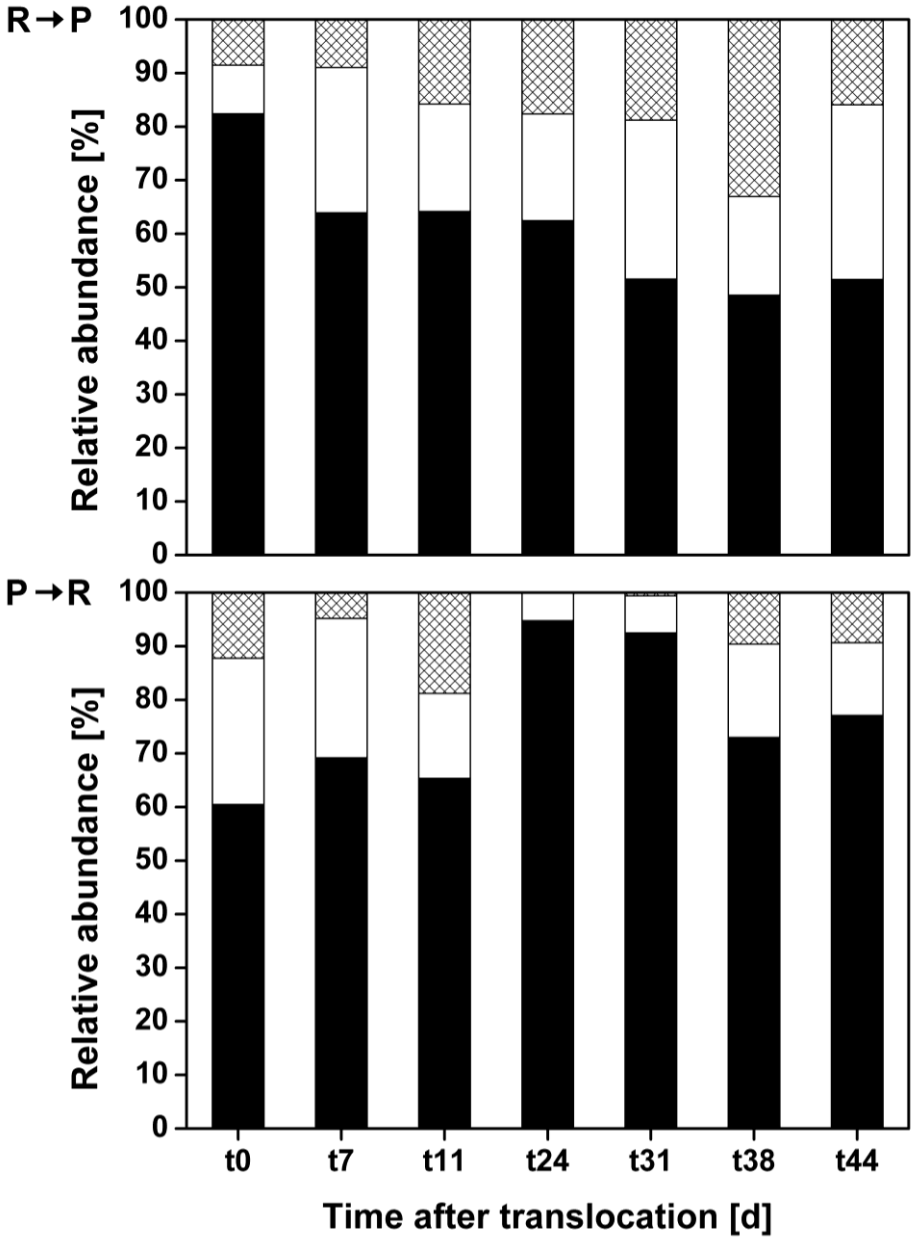

Fig. 5. Relative distribution of algal classes in periphyton after R→P and P→R translocation measured by multiwavelength-excitation PHYTO-PAM fluorometry (n per day = 3) (■ diatoms; □ cyanobacteria; ⊠ chlorophytes).

After translocation the relative abundance of algal classes changed towards the composition of the local communities. Six weeks after the translocation the relative abundance of diatoms in R-P-periphyton decreased to 51.5%, chlorophytes increased to 15.9% and cyanobacteria increased to 32.6%. Within the P-R-

periphyton the relative abundance of diatoms increased up to 77.1%, chlorophytes stayed nearly constant with 9.3% and cyanobacteria decreased to 13.6%. Therefore, algal class composition of transferred communities was more similar to the local community of the sampling site they were transferred to (mean variation of algal classes 4.8% (± 2.7)) than to their original local community (mean variation 15.8% (± 10.3)).

Diatom communities

To follow toxicant-induced succession during translocation, changes in the diatom community were analysed during short sampling intervals directly after translocation and weekly until the end of the experiment. In total, 79 diatom species were determined during this experiment. Relative abundance of local diatom communities differed between the sites and changed dramatically after translocation (Fig. 6), but no significant difference between taxa richness appeared. NMDS ordination (see Fig. S1) showed a clear differentiation between R- and P-communities, translocated communities seem to be similar 24 days after translocation. Only the most abundant diatoms are leading to the variation between the samples. *Cocconeis placentula* is separating the reference community from the whole dataset and *A. minutisima* and *Gomphonema parvulum* the P-community from the rest. The stress value of the NMDS is 0.051 and represents for this reason good quality without major distortion. R-communities were dominated by *C. placentula* (95%) and P-communities by *Achnanthes minutissima* (87.9%) and *G. parvulum* (7.3%). The rest of the community was formed by species with relative abundances below 5%. After translocation, relative abundances of diatoms changed towards the local communities but showed characteristic time-responses in dependence of their exposure history: *A. minutissima* and *G. parvulum* were abundant at the P-site, but decreased during their exposure for 44 days at the R-site in their relative abundance to 7% and 1%. In contrast, *C. placentula* increased its abundance from 0.2% to 76.4% and *A. lanceolata* occurred with 5.7%. The community of the R-site was originally dominated by *C. placentula* (95%), which decreased 44 days after the translocation to the polluted site to 48%. Simultaneously, *A. minutissima* increased from 0.2% to 40%. After 31 days, abundance of *C. placentula* and *A. minutissima* of P-R-community was similar to the local R-community. In contrast, the R-P-community still differed in their relative abundances compared to the local P-communities after 44 days exposure at

the P-site. Calculated indices are shown in Table S2. Both sites had the same richness of taxa, were β-mesosaprobous and Index of Salinity was in the range of freshwater. The reference site was in the mesotrophic and the polluted site in the eutrophic stage. Already 31 days after translocation Trophic Diatom Index, Saprobic Index and Bray-Curtis Dissimilarity of P-R communities were in the range of the local R-community. Even after 44 days R-P community did not reach the range of the local community.

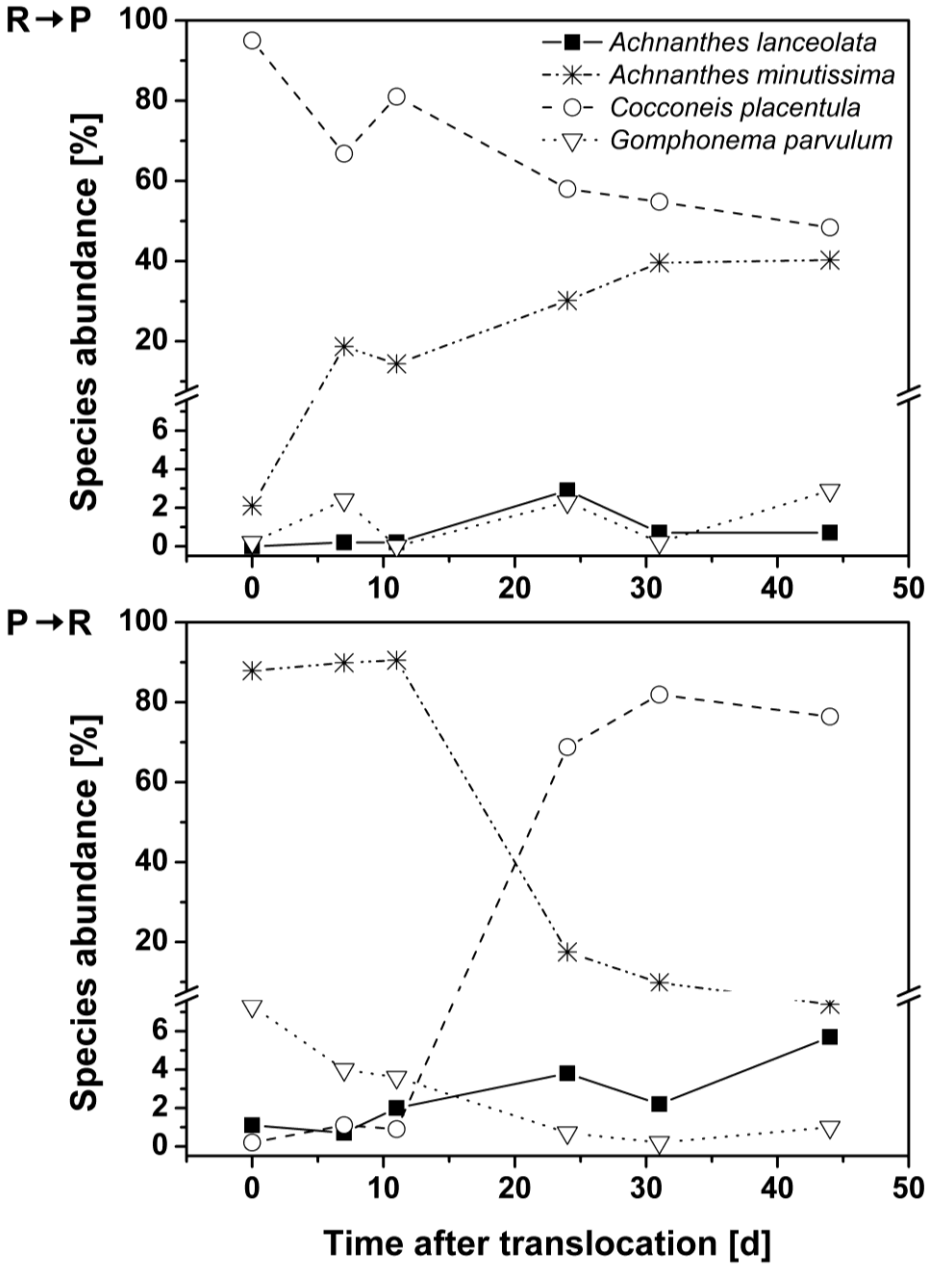

Fig. 6. Relative abundances of periphyton diatom species after translocation of resident P- and R-communities to the respective other site (R→P, P→R). Diatom species with a relative abundance below 5% are not shown. Translocation was conducted 26 days after growing at the polluted (P) and the reference site (R).

Discussion

Causality of exposure and effects in communities

The first objective of the study was to link exposure of environmental toxicants to effects in periphyton. The advantage of benthic communities in effect screening of environmental toxicants is that they integrate the exposure history during growth. Thus, periodic or peak concentrations of toxicants are integrated in the overall exposure. However, most studies analysing effects of environmental toxicants on communities use spot sampling for chemicals, resulting in uncertainty of the exposure estimation. Therefore, in this study pollution of prometryn was continuously monitored by integrated passive sampling and PICT was determined as a measure of community changes.

Results of biological and chemical analyses show that both sites are within the β-mesosaprobe category but differed in prometryn exposure. Prometryn recovered from POCIS indicates time-weighted average (TWA) concentrations over the exposure duration in the aquatic ecosystem and represent the concentrations aquatic organisms were exposed to. Concentrations of prometryn at the P-site ranged from 0.54 to 0.92 µg L^{-1} and were at the reference site at a clearly lower pollution level, less than 0.01 µg L^{-1} for passive sampling during the whole sampling campaign. Taking the known co-tolerances to compounds with similar chemical structure and mode of action (Blanck, 2002; Knauer et al., 2010) into account, we can conclude that a PS II inhibitor must be the main driver of the observed tolerance development in periphyton. However, due to the former effect-directed analysis (Brack et al., 1999), there is a high probability that prometryn is the main PS II inhibitor occurring in effective concentrations in this creek, because no other PS II inhibitors and s-triazines were detected in the river sediment. Furthermore, Brack et al. (1999) stated a significant toxic potential of the compound in aquatic ecosystems and confirmed prometryn as the chemical causing toxicity to algae.

Communities of the polluted site were more tolerant to prometryn than the local community of the R-site. Furthermore, 24 days after translocation sensitivity shifted and R-P communities became more tolerant to prometryn than P-R communities. Changes in the sensitivity to prometryn can be caused by long-term exposure of a community to a toxicant. This exerts, depending on concentration and exposure time, a selection pressure resulting in succession processes (TIS). Therefore, the

community will consist of species more tolerant to the particular toxicant, inducing an increased tolerance of the entire community, the so-called PICT response. The crucial step in the PICT-approach is the metabolic short-term test, in which the community is exposed a second time to the toxicant, to quantify tolerance adaptation. The so called detection phase brings causality to PICT, because long-term exposure to a chemical is forcing the community to a succession sequence in the direction of an increased tolerance and should lead to PICT for that chemical only (Blanck, 2002). Hence, a couple of field PICT studies could link toxicant exposure like TBT and metals to effects in the community (e.g. Blanck and Dahl, 1996; Lehmann et al., 1999; Ivorra et al., 2000). Therefore, the effect of single toxicants on the sensitivity of communities in multiple stress field situations was clearly demonstrated already. Additionally, long-term effects of prometryn on periphyton were already analysed in a controlled microcosm study in a concentration range from 0.0025 to 0.32 mg L^{-1} by Schmitt-Jansen and Altenburger (2005a). They reported an increase of the community tolerance to prometryn by factor 3 to 6, which is in the same range as found in this study. This shows that prometryn itself is able to induce a PICT response, because the experiment was conducted under controlled lab conditions and prometryn has been the only varying parameter. The detected field concentrations of prometryn were at least 10 times lower than in the microcosm study, showing the high sensitivity of the PICT method and its suitability for field application. These differences between field and microcosm studies were demonstrated in other studies, too (Morin et al., 2008). Higher total numbers of species (79 species in the field study, described here; 56 species found in a comparable microcosm study (Schmitt-Jansen and Altenburger, 2005a)) and constant upstream-input of species in field studies could explain the difference in effective concentrations between field and microcosm studies.

In conclusion, the analysed concentrations of prometryn and the detected PICT response clearly indicate the selective pressure induced by prometryn at the P-site and causally link exposure to the site-specific toxicant.

Toxicant-induced succession (TIS)

The second aim of the study was to observe TIS as a feasible causal explanation for PICT. Therefore, analyses on the level of algal classes and on the level of diatom species were conducted. Diatoms were the dominant algal class over the whole experiment, which is in accordance with other studies (e.g. Navarro et al., 2002; McClellan et al., 2008; Morin et al., 2010) and the overall finding that diatoms are usually dominant in rivers. The translocation of periphyton communities caused significant changes at the algal class level within 6 weeks, e.g. relative proportion of diatoms decreased after R-P translocation by 30.9% and increased after P-R translocation by 16.6%. Therefore, the changes in sensitivity (PICT) after the translocation were accompanied by succession processes detected as changes in the algal class composition. This is according to the results of the microcosm studies of McClellan et al. (2008) and Schmitt-Jansen and Altenburger (2005b), which detected changes in the algal class composition for concentrations that induced an increase of the community tolerance. In this study the detection of algal class composition was even sensitive enough to detect changes within field periphyton communities, whose environmental conditions changed after the initial growth phase. However, concerning the PICT-concept, further changes are supposed to take place at the species level within the algal class of diatoms.

Analyses of diatoms showed significant structural differences within the local communities of the P- and R-site and related indices. *C. placentula* dominated the R-communities and *A. minutissima* and *G. parvulum* were abundant at the P-site, which was confirmed by the NMDS ordination. *A. minutissima* is known to be a small, early coloniser (Ivorra et al., 1999), possessing a strong tolerance to metals (Kim et al., 2008). Also *G. parvulum* has been found at metal contaminated sites in several studies (Kim et al., 2008). It is highly abundant in disturbed environments (Ivorra et al., 2002a) and tolerates polysaprobic conditions (Lange-Bertalot, 1979). Furthermore, it is assumed that *G. parvulum* is able to adapt to wide ranges of chemical stress (Ivorra et al., 2002b). *A. minutissma* as well as *C. placentula* are colonizers of the early phase, whereas *G. parvulum* is following afterwards (Korte and Blinn, 1983). Therefore *A. minutissma* and *C. placentula* seem to be direct competitors, which may be also the case in our study. The increase of the relative abundance of one of these two species results in the decrease in the relative abundance of the other one (Fig. 6).

After the R-P translocation, tolerant and robust species like *A. minutissima* and *G. parvulum* increased in their relative abundance, which was also detectable as increase in the tolerance of the whole community. Therefore, these two species might be favoured by prometryn exposure, because other species could not resist the exposure. The high tolerance of the local P-community may be due to the high abundance of these two species, also causing the increasing tolerance of the R-P community after the translocation. On the other hand, the decrease of community tolerance of the P-R community was correlated with the decrease of the relative abundance of *A. minutissima* and *G. parvulum.* Moreover, the sensitivity shift after the transfer of the communities resulted from succession processes at the level of algal classes and at the species level.

We conclude that shifts in the sensitivity of the whole community were correlated with changes in the abundance of indigenous diatom species and algal classes, caused by prometryn.

Response time of recovery and adaptation to pollution

The last objective of the study was to analyse the response time required for recovery of chronically contaminated periphyton and the adaptation of reference communities to chronic contamination, respectively.

Our study revealed different time responses in terms of functional and structural processes of recovery and adaptation. So far, there are few translocation studies analysing recovery within periphyton, applying the PICT-concept (Dorigo et al, 2010a; Dorigo et al, 2010b). But none of them are studying the adaptation processes in terms of sensitivity changes simultaneously. In many cases structural changes in the diatom composition and changes in diatom indices (e.g. Iserentant and Blancke, 1986; Morin et al., 2010; Rimet et al., 2005) are focus of the investigations. All translocation studies had a change in the community structure towards the local community in common but differed in response time required for recovery and adaptation, respectively. First changes within the community composition were detected already after 6 days by Hirst et al. (2004), which is in accordance to our study (7 days). Translocation studies conducted by Ivorra et al. (1999), Gold et al. (2002) and Hirst et al. (2004) analysed effects over short periods of 12-28 days. Morin et al. (2010) found a rapid recovery of the quantitative parameters (dry weight, chlorophyll a) within 1 month, but no recovery for the BDI (biological diatom index).

Different time patterns for functional and structural recovery after translocation were also found by Dorigo et al. (2010a and 2010b). With regard to the diatom composition we found a slow adaptation of reference communities to pollution but a rapid recovery of communities originating from the polluted site. However, the response time in the processes of structural adaptation (diatom composition) to pollution and recovery, respectively differ in our study in comparison to literature (Iserentant and Blancke, 1986; Rimet et al., 2005; Lavoie et al., 2008). They reported a rapid adaptation of communities originating from clean streams to organic pollution, but a clear hysteresis effect in the process of recovery. For example the process of recovery from high organic and trophic loads was not finished after 45 days (Iserentant and Blancke, 1986), respectively after 60 days (Rimet et al., 2005). But these studies focused on monitoring adaptation of diatom communities to clear changes in organic and trophic loads and not on toxic stress, which may be considered as a true perturbation for a community (Lavoie et al., 2008). In our study functional recovery was not achieved after 17 days, as tolerance of translocated communities was still intermediate. But the survey of translocated communities showed an inverse tolerance pattern (R-P higher than P-R) after 24 days of the translocation. In contrast, structural recovery was almost stabilised after 24 days and was clearly faster than the response to perturbation, which was not finished after 44 days. These results were confirmed by Bray-Curtis Dissimilarities, Saprobic Indices and Trophic Diatom Indices. Communities from low-polluted sites showed a higher stability in composition and therefore a higher resistance to environmental changes, but removal of the perturbation resulted in a rapid change of species composition and sensitivity.

These findings may be attributed to different mechanisms: There are three possibilities to adapt to or recover from the exposure to toxicants, (1) through physiological acclimation (2) genetic adaptations (Eriksson et al., 2009) or (3) by undergoing a successional change. We hypothesise that physiological acclimations as well as successional changes occur in a consecutive reassembly of the community. This is in accordance with the conclusion of Dorigo et al. (2010a) that the detection of sensitivity and community structure indicates different kind of reaction to stress (physiological adaptation and selection). Furthermore, adaptations to toxicants may have specific metabolic costs, e.g. the induction of metal-binding proteins increases tolerance of the organism, but utilises also energy normally available for

metabolic processes (Clements and Newman, 2002). The debasement of water quality due to the presence of toxicants may enforce the community towards physiological acclimations, which are required to survive. Communities from the reference site may still have the adaptive capacity for detoxification before succession processes start. On the other hand, in communities of polluted sites, the capacity for acclimation may be exceeded already, but removal of the perturbation may decrease metabolic costs and succession processes may start quickly.

Conclusion

Active bio-monitoring of periphyton applying the PICT-concept has been showed to be able to discriminate between polluted and unpolluted sites and improved the stressor - effect diagnosis at the contaminated site. Within this study the exposure to a site-specific toxicant was causally linked to community-level effects. Prometryn exposure appears to be the driving force for the change of the community composition at the species-level and the sensitivity shifts within the community. Furthermore, the relevance of exposure history for response times for adaptation to chronic contamination and recovery processes from contaminations became achievable by active bio-monitoring of periphyton, which makes them suitable biomonitors for site-specific contamination.

It is obvious that *in situ* studies provide a lot of uncertainties and uncontrolled variables, like fluctuating exposures and stressor interactions. Nevertheless, they are environmentally more relevant than laboratory toxicity experiments conducted with single-species tests under controlled conditions. *In situ* surveys of indigenous biota provide the most realistic assessment of field conditions (Crane et al., 2007) and have a high potential use for assessment of hazards and risks. The adaptive response of natural communities such as periphyton can lead to a better understanding of pesticide effects in aquatic ecosystems. Therefore, active bio-monitoring applying translocation of communities and PICT for causal analysis could contribute to investigative monitoring, when the causes of failing ecological integrity are unknown. It has the potential to improve accuracy and relevance in weight-of-evidence based decision-making frameworks as used in the implementation of the European Water Framework Directive (WFD). The implementation of active monitoring of the sensitivity of periphyton communities may contribute an additional line-of-evidence in environmental risk assessment and be a reliable linkage between

the field surveys of biological quality elements and the assessment of the Environmental Quality Standard (EQS) for toxic substances in aquatic systems.

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Supporting information

Appendix S1. Pesticide concentration in water samples

To analyse the prometryn (CAS RN: 7287-19-6, Riedel-de Häen, Seelze, Germany) concentration of freshwater samples, SPE (solid phase extraction) was performed with 2 replicates per sample, by passing 250 ml filtered water through 500 mg conditioned sorbents (Strata X 33 µm; Phenomenex, Torrance, CA, USA). Adsorbed prometryn was eluted with methanol, elutes were concentrated by rotary vacuum evaporation. Each residue was redissolved in 50 µl toluene. Prometryn was quantified by gas chromatography (model 6890, Hewlett Packard, Germany) on a 5% diphenyl dimethyl polysiloxane capillary column (model HP-5MS, Agilent, 30 m * 250 µm * 0.25 µm film thickness + 5 m precolumn) using a constant flow of helium (1.3 ml min^{-1}). Aliquots of 1 µl per samples were injected at a temperature of 250°C. The column temperature was kept at 60°C for 1 min, increased with a rate of 30°C/min up to 150 °C, then 6°C/min to 186°C and with 4°C/min up to 280°C where it was set for 16.5 min. A mass spectrometer HP 5973 (Hewlett Packard, Germany) was used for identification and measurements were done in single ion modus (SIM).

Table S1. Prometryn concentrations

Table S1. Mean concentrations of prometryn (µg L^{-1}) at the reference (R) and the polluted site (P), determined during the experimental period on different sampling days.

Sampling dates	R-site		P-site	
	Mean value	SD	Mean value	SD
21.05.2008	0.03	0.00	0.23	0.00
12.06.2008	0.02	0.01	0.85	0.05
18.06.2008	0.01	0.00	0.77	0.25
25.06.2008	0.01	0.00	0.47	0.17
03.07.2008	0.01	0.00	0.97	0.04
10.07.2008	0.02	0.01	0.78	0.25
17.07.2008	0.02	0.00	0.60	0.55
24.07.2008	0.02	0,00	0.90	0.18
30.07.2008	0.06	0.01	0.55	0.29
21.08.2008	0.09	0.08	0.27	0.09

Appendix S2. Calibration polar organic chemical integrative sampler (POCIS)

In the calibration experiment ten POCIS were exposed in a flow through system similar to that described by to Vrana et al. (2006). The calibration system consisted of a 20 L calibration tank and two peristaltic pumps, one for a constant water flow of 10 ml min^{-1} and one delivering the stock solution prepared in methanol (flow 0.26 ml min^{-1}). Therefore the water in the tank was completely renewed every 33 h. A nominal concentration of 385.7 ng L^{-1} prometryn was kept during the experiment and methanol concentrations in the water did not exceed 2.6%. POCIS were removed in duplicates after 10 sec, 74 h, 165 h, 338 h and 504 h (0 to 21 days) and replaced by POCIS-holders with an inert glass fibre filter only so that the flow regime in the calibration tank was not altered. The calibration was conducted at 20°C and a simulated flow velocity of 0.15 m s^{-1}. The extraction and analyses of POCIS was performed as described above (2.4), extracts were re-dissolved in 1 ml toluene.

The sampling rate of prometryn (R_s= 0.59 L d^{-1}) was determined by measuring the mass of prometryn in the retrieved POCIS. During the 21 days of calibration prometryn was still in the linear uptake phase which is in accordance with Alvarez et al. (2004). POCIS removed immediately after the beginning of the calibration served as blanks, and all subsequent measurement were blank corrected. Time-weighted average (TWA) concentrations in the water were calculated according to Mazzella et al. (2008).

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Figure S1. Non-metric multi-dimensional scaling on a Bray-Curtis dissimilarity index

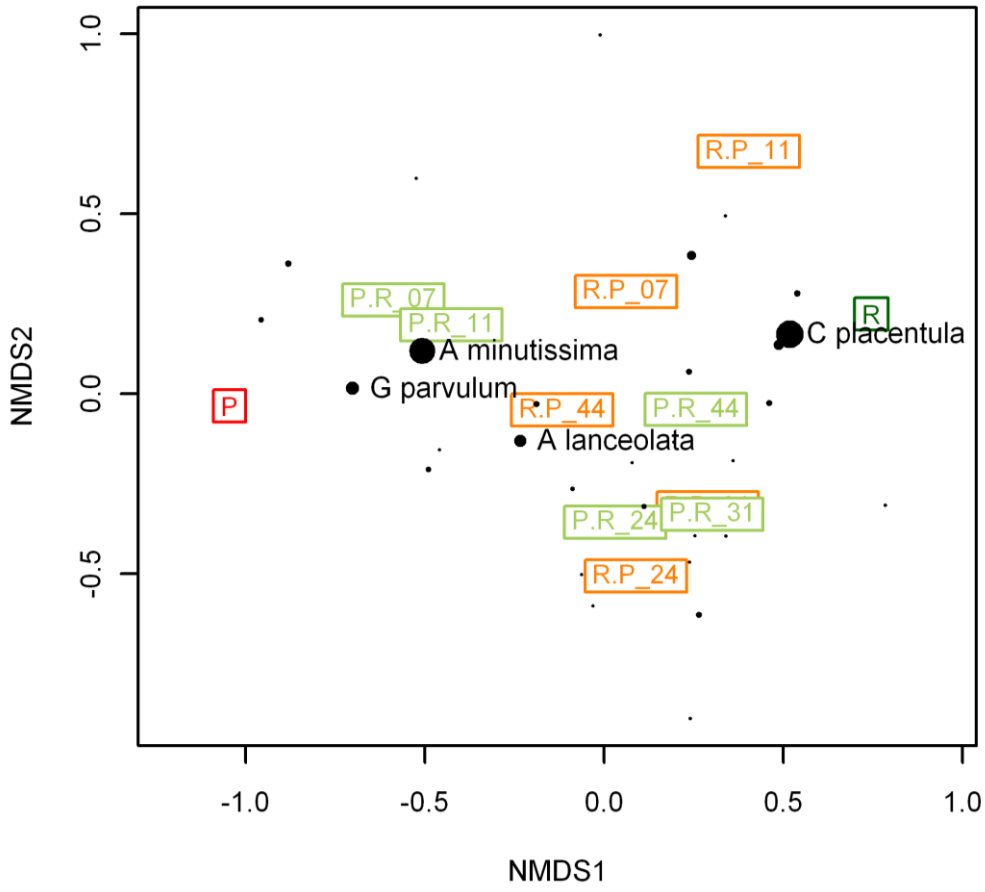

Fig. S1. NMDS ordination of local periphyton (R = reference site, P = polluted site) translocated to the respectively other site (R-P, P-R). The distance matrix of each sample was compiled using the Bray-Curtis dissimilarity index on species composition of samples. Boxes represent periphyton communities, labels refer to direction and time after translocation in days. Each data point indicates a diatom species, dimension of points are proportional to absolute species abundance in all samples. Stress value of NMDS is 0.051.

Table S2. Diatom richness and species indices

Table S2. Diatom richness and species indices based on diatom abundances per site (R = reference, P = polluted site) and sampling date after the translocation (tx).

Biofilm sample	Sampling date	Trophic Diatom Index	Saprobic Index	Index of Salinity	Richness (diatoms)	Shannon Diversity Index	Evenness	Bray-Curtis Dissimilarity (R-tx)	Bray-Curtis Dissimilarity (P-tx)
R	**t0**	2.60	1.82	0.0	11	0.30	0.12	0.00	0.98
R-P	**t7**	2.47	1.84	7.8	21	1.20	0.40	0.08	0.73
	t11	2.49	1.81	5.0	11	0.70	0.28	0.02	0.82
	t24	2.44	1.82	9.2	21	1.20	0.40	0.16	0.56
	t31	2.28	1.81	8.5	15	1.00	0.36	0.22	0.44
	t44	2.33	1.81	4.3	19	1.20	0.42	0.28	0.39
P	**t0**	1.60	1.68	0.0	11	0.50	0.22	0.98	0.00
P-R	**t7**	1.57	1.75	5.3	14	0.50	0.20	0.97	0.00
	t11	1.56	1.73	13.0	13	0.50	0.19	0.97	0.00
	t24	2.53	1.84	2.7	22	1.20	0.38	0.07	0.76
	t31	2.57	1.83	8.2	19	0.80	0.27	0.02	0.88
	t44	2.60	1.88	5.2	16	1.00	0.37	0.03	0.90

Chapter 4

Multiple stressors in periphyton - comparison of observed and predicted responses to high ionic loads and herbicide exposure

Rotter, S., Heilmeier, H., Altenburger, R., Schmitt-Jansen, M., 2013. Journal of Applied Ecology 50, 1459-1468.

Abstract

As a result of the increasing human impact on aquatic ecosystems, freshwater organisms are often exposed to multiple stressors simultaneously. The joint actions between stressors can result in combined effects and unexpected ecological effects. Therefore, a better understanding of the interactive effects on ecosystems is required.

This study aimed to identify potential interactions between high ionic loads and herbicides. A microcosm study, using periphyton as model community, was conducted with a factorial design. Two levels of ionic loads were used as single stressor and in combination with prometryn. Structural (biomass, algal class and diatom composition) and functional parameters (tolerance development) were determined over a growth period of six weeks. The concept of pollution-induced community tolerance (PICT) was applied to quantify integrated community responses. Long-term community responses to the combined exposure were predicted using the model of independent action.

No co-tolerance of high ionic loads and prometryn or vice versa was found. Stress-induced succession resulted in a distinct community structure for each stressor and combination of stressors. Multiple stressors led to the selection of opportunistic species and higher tolerances to prometryn than predicted by the model of independent action. However, joint effects for high ionic loads and prometryn were concentration and time dependent. The PICT concept enabled the quantification of community level effects in systems receiving multiple stresses.

Synthesis and applications. Multiple stressors might explain the failure to achieve good ecological status for many European water bodies within the context of the EU-Water Framework Directive (WFD). We propose PICT as a diagnostic tool for investigative monitoring to clarify stressor conditions by testing the tolerances of local communities to pre-selected site-specific compounds.

Introduction

Aquatic organisms and biological networks are often exposed to diverse natural and anthropogenic stressors simultaneously. Interactions among multiple stressors might be more commonly non-additive than simple additive, which might cause ´ecological surprises´ (Darling and Côté, 2008). The type of interactive effect is dependent on stressor pairs, trophic level and biological response levels, i.e. species interactions within communities might dampen or exacerbate the impacts of multiple stressors (Crain et al., 2008). Thus, there is a clear need for concepts and appropriate approaches to analyse, quantify and predict community-level responses to multiple stressors (Breitburg et al., 1998).

For chemical mixtures, two basic concepts have been developed for calculating the expected combined effect based on known responses of the individual toxicants: (1) concentration addition for compounds with a similar mode of action and (2) independent action for dissimilar-acting compounds (Faust et al., 2003). While concentration addition formalises the dilution principle, independent action is based on statistically independent effects. Several studies successfully applied these concepts to chemical mixtures (Altenburger and Greco, 2009) and combinations of different stressors (Coors and De Meester, 2008) but were mainly applied in single species tests.

Backhaus et al. (2004) have taken a first step by applying the principles of mixture toxicity at a community level, using a short-term community assay. However, there is a lack of long-term studies considering integrated community parameters, such as community tolerance based on species shifts, for prediction. One reason for this might be the absence of suitable community approaches that allow for a quantification and prediction of long-term community level responses.

The concept of pollution-induced community tolerance (PICT) was introduced by Blanck et al. (1988) and enables quantitative measures of community-level responses by causally linking exposure to community level effects. Tolerance is the result of adaptation processes which maintain overall functions within organisms (physiological acclimatisation) and communities (species shifts). The PICT approach is based on the assumption that chronic exposure to toxicants entails changes in species composition, known as toxicant-induced succession (Blanck, 2002). The reliability of PICT has been demonstrated for several compounds in microcosm and

field studies using periphyton (e.g. McClellan et al., 2008; Rotter et al., 2011), but few studies address the effect of simultaneous stressors on periphyton (e.g. Muñoz et al., 2001; Tlili et al., 2010). However, under multiple stressor conditions one stressor might change the vulnerability of a biological system to a second stressor (De Lange et al., 2010). One single stressor might induce tolerance mechanisms triggering co-tolerances to similar-acting stressors or the exposure to several selecting stressors simultaneously might lead to the development of multiple tolerance mechanisms for the entire set of stressors (Blanck et al., 1988).

Apart from pesticide contamination, the increasing secondary salinisation of freshwaters has been recently described as one of the most important stressors for freshwater ecosystems, causing environmental risks worldwide (Cañedo-Argüelles et al., 2013). Furthermore, high ionic loads and pesticides often occur simultaneously in freshwater (Hart et al., 1991; Bäthe and Cöring, 2011, von der Ohe et al., 2011) and revealed joint effects on macroinvertebrates (Schäfer et al., 2011). However, approaches quantifying community responses to multiple stressors, in order to enable the prediction of joint effects, are still lacking.

In this study, we address the question of individual and combined effects of salt and toxic stress on long-term community level responses. Stressors were chosen according to a former field study of the Elbe River basin with the simultaneous presence of prometryn and high conductivity (Rotter et al., 2011). The herbicide prometryn inhibits the photosynthetic electron transport by competing with the plastoquinone Q_B for its binding sites at the D1 protein (Huppatz, 1996). Therefore, algae exposed to prometryn have a diminished photosynthetic rate (Brown and Lean, 1995), which reduces the growth rate. In contrast, environments with increased ion concentrations lead to disturbed ionic steady states of Na^+, Cl^-, K^+ and Ca^{2+} in organisms (Hasegawa et al., 2000). The loss of water activates several secondary acclimation processes, which increase the demand for energy supplying reactions (Fodorpataki and Bartha, 2004). Based on these considerations, we assume dissimilar modes of action of prometryn and high ionic loads.

We hypothesise that (1) exposure to high ionic loads, herbicides and the combination of both triggers a stressor-specific replacement of species resulting in (2) enhanced tolerance to prometryn in single and combined prometryn exposures. However, due to dissimilar modes of action we (3) do not expect co-tolerance between high ionic loads and prometryn. The objective of this study was to quantify the single and joint

effects of both stressors on changes in community tolerance in order to enable the prediction of joint effects using the mixture toxicity model of independent action. For a mechanistic understanding of tolerance development in multiple stressor environments, a microcosm study in a factorial design was conducted and structural changes of periphyton (biomass, algal class and diatom composition) were related to community tolerance development under control conditions as well as under single and combined stressors. Periphyton pre-exposed to one single stressor was used to investigate co-tolerance. While the tolerance to prometryn was tested in short-term toxicity tests, the index of salinity was used as measure of tolerance to high ionic loads.

Material and methods

Periphyton cultivation

Microcosm experiments were conducted over a period of six weeks. Periphyton was used as model community and was grown on glass discs (1.5 cm diameter) according to Blanck (1985). Unfiltered freshwater from the River Parthe (Germany) was used as inoculum to obtain a naturally sourced community of algae. River monitoring showed no prometryn contamination, conductivity values around 800 µS cm^{-1} and low pesticide levels within the nanogram range (Sächsisches Landesamt für Umwelt, Landwirtschaft und Geologie). The light regime was set to a 14:10 h light:dark cycle using neon lamps (36 W; photosynthetic photon flux density above water surface = 200 µmol m^{-2} s^{-1}), cultivation temperature was approximately 22°C and water was stirred continuously. In total, 14 microcosms each containing 14 L of water and 200 vertically exposed glass discs were used. Water was replaced weekly and pH, oxygen and conductivity were measured in the fresh river water and in aquaria directly before water exchange.

Two aquaria without additives served as controls (Co) and two further containing 0.01% (v/v) of dimethyl sulfoxide (DMSO, CAS RN: 67-68-5, Merck, Darmstadt, Germany) were solvent controls (DMSO-Co). Two aquaria were exposed to a nominal concentration of 15 µg L^{-1} prometryn (Prom) (CAS RN: 7287-19-6, Riedel-de Haën, Seelze, Germany), which is known to cause an increase in tolerance (Schmitt-Jansen and Altenburger, 2005). The stock solution of prometryn was dissolved in DMSO and was freshly spiked each week subsequent to the changing of water. Two levels of ionic loads were selected: the first corresponding to the former field study with a mean conductivity of 1919 µS cm^{-1} (Rotter et al., 2011) and the second was taken to mimic mean conductivities of the lower River Werra, which has been heavily polluted by direct discharges of salt wastewater from the potash industry since 1950 (Bäthe and Cöring, 2011). In order to increase ionic loads, sodium sulphate (CAS RN: 7757-82-6, Merck, Germany) and calcium chloride (CAS RN: 10043-52-4, Merck, Germany) were added in equal weight ratios. The salt concentrations were elevated to a final conductivity of about 2000 µS cm^{-1} (C2000) and 5000 µS cm^{-1} (C5000) in two aquaria each. Finally, four aquaria were exposed to mixtures of 15 µg L^{-1} prometryn and elevated conductivity, two microcosms to 2000 µS cm^{-1} (P2000) and two to 5000 µS cm^{-1} (P5000), providing a factorial design. The sodium

and chloride concentrations in C5000 treatments were in the range of 24.6–31.8 mM NaCl (see Table S1 in Supporting Information).

In the following, we refer to increased conductivity as high ionic load, because salinity is based on chloride concentrations and no limiting nutrients such as ammonium, nitrate or phosphate were introduced to the system.

Chemical analyses

A preliminary experiment was conducted to analyse the stability of prometryn over the exposure period of one week (see Appendix S1 for details). Due to minimal deviations from applied concentrations, we refer to nominal concentrations.

Furthermore, the physico-chemical water parameters (temperature, pH, oxygen concentration, conductivity) of one control microcosm were measured directly after water renewal and after seven days. Analyses for dissolved ions (Cl^-, SO_4^{2-}: ion chromatography; Ca^{2+}, Na^+: ICP-OES) were conducted following standard protocols.

Biological analyses

Short-term toxicity tests for quantifying PICT

Community tolerance was determined by short-term inhibition tests of photosynthesis measured by a pulse-amplitude modulation (PAM) - fluorescence based method. After three, four, five and six weeks of growth, 24 colonised glass discs of each microcosm were sampled. Tests were performed in 24 well plates, each containing 2 ml of freshwater. Periphyton was exposed to six concentrations of prometryn in triplicate, ranging from 3×10^{-5} to 30 mg L^{-1} dissolved in DMSO. Samples without contamination and samples containing 0.1% DMSO served as controls. Short-term tests were incubated on a rotary shaker under a photosynthetic photon flux density of 110 µmol m^{-2} s^{-1} at 20°C. After 1 h of exposure to prometryn, chlorophyll a fluorescence was measured using a MAXI-Imaging-PAM (Walz, Effeltrich, Germany).

Prometryn tolerance was quantified as EC_{50} (effect concentration at median efficacy) for inhibition of photosynthesis. Relative inhibition of fluorescence in relation to controls was calculated to model concentration–response relationships, according to Seefeldt et al. (1995).

$$\gamma = A_{\max} + \frac{A_{\min} + A_{\max}}{1 + (x / EC_{50})^{p}}$$

where A_{min} and A_{max} denote the minimal and maximum responses, *x* the concentration and *p* stands for the slope. After data inspection A_{max} was fixed at 100% and A_{min} at zero. Analyses were performed using nonlinear regression least-squares curve fitting with the software OriginPro 8G. EC_{50} data were tested for outliers using Grubbs` test (Grubbs, 1969).

For prediction of joint effects the concept of independent action was used. The equation was formulated following Backhaus et al. (2004)

$$E_{Mix} = 1 - \prod_{i=1}^{n} [1 - E(c_i)]$$

where E_{Mix} represents the expected effect (scaled between 0–1) of an *n*-compound mixture and $E(c_i)$ the individual effect of each stressor i. The transformation of the observed effects to proportional data and the rescaling of predicted E_{Mix} to absolute values were conducted according to Coors and De Meester (2008)

$$E(c_i) = \frac{(e_i - e_{control})}{(e_{\max} - e_{control})}$$

whereas e_i is the observed effect in absolute units, $e_{control}$ is the mean of all controls (DMSO-controls and controls) per week. The maximum possible effect e_{max} was defined based on the maximal observed EC_{50} of this experiment (41.13 mg L^{-1}). More details and an example calculation of the prediction are shown in Appendix S2. We consider interactions between stressors present where the predicted EC_{50} lies outside of the standard error for observed effects of combined stressors.

Algal class composition and biomass

Fluorescence-based measurements of photosynthesis pigments were conducted to analyse algal class composition and biomass of periphyton. The PHYTO-pulse-amplitude-modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany) allows discrimination by multiwavelength-excitation and was used according to Schmitt-Jansen and Altenburger (2008). Minimal fluorescence (F_0) was measured after three,

four, five and six weeks for three spots per glass disc with three replicates taken from each microcosm.

Taxonomic analyses of diatom

For identification of diatom species seven discs per microcosm were preserved in 5% formaldehyde. Samples were taken after three, four, five and six weeks of cultivation. Preparation of the samples was conducted according to Krammer and Lange-Bertalot (1986–1997). Clean diatom samples were used to count and to identify at least 400 valves per sample using light microscopy and taxonomic literature of Krammer and Lange-Bertalot (1986–1997).

The index of pollution sensitivity (IPS) of each sample was calculated using Omnidia software v8.1 (Lecointe et al., 1993). Furthermore, Index of Salinity (Ziemann, 1999), Shannon Diversity Index and Evenness (Mühlenberg, 1989) were calculated.

Statistical analyses

A repeated measures ANOVA was conducted to analyse the effects of high ionic loads, prometryn (between-subject factors) and time (within-subject factor) and their interaction on community tolerance, biomass and algal class composition (von Ende, 2001). Data of controls and DMSO-controls were pooled into one control group after statistical testing indicated that there were no significant differences between the two control groups. (Table S2). Normal distribution had to be assumed due to few replicates. Mauchly`s test of sphericity was used to test for equal variances, Greenhouse Geisser correction was applied if assumption of sphericity was violated. For significant ANOVA results ($P < 0.05$), the source of the differences was located using the Tukey post-hoc test, comparing all possible pairs of groups. The Bonferroni-adjusted *P*-value for multiple testing was applied for responses of cyanobacteria, green algae and diatoms. Statistical analyses were conducted using the software SPSS v19 (IBM, Chicago, IL, USA) and SigmaPlot v11 (Systat Software Inc., San Jose, CA).

Canonical ordination analyses were conducted to relate the effects of prometryn, high ionic load and time to the species composition. Diatom species with a relative abundance below 1% were excluded from analyses. Species abundances were transformed using Hellinger transformation (Legendre and Gallagher, 2001).

Furthermore, *Cocconeis pediculus* had to be excluded, because it was either present in high numbers or absent, which led to an imbalanced analysis. A Detrended Correspondence Analysis on the diatom data revealed a linear gradient, which requires a Redundancy Analysis (RDA). RDA was carried out on the reduced diatom dataset of 31 species against the variables conductivity, prometryn and time. Statistical significance for RDA axes and environmental parameters were assessed using permutation test with 999 random permutations. Multivariate analyses were carried out using vegan 1.17-9 of the R statistical environment (R Development Core team, 2010).

Results

Physico-chemical parameters

The actual prometryn concentration in the microcosm was constant during one week (mean = 14.0 ± 1.3 µg L^{-1}) and matched well with the nominal concentration of 15 µg L^{-1}. Water temperature in microcosms was stable (22.1 ± 0.9°C), pH increased from 8.03 (± 0.43) to 8.38 (± 0.16), oxygen concentration (8.76 ± 0.49 mg L^{-1}) decreased by 9% and mean conductivity of 650 (± 191) µS cm^{-1} decreased by 7.5% to 601 (± 120) µS cm^{-1} within one week in control microcosms.

Community tolerance

According to the Grubbs` test, nine out of 56 EC_{50} values were identified as outliers. A total of five of these values were excluded from statistical analysis, because maximum inhibition of the corresponding concentration–response curve was less than 60%, which prevents the accurate modelling of EC_{50} values.

All factors (ionic load, prometryn and time) significantly increased community tolerance and showed significant interactions among each other (Table S3).The controls and DMSO-controls showed similar sensitivities to prometryn (mean EC_{50} = 2.2 ± 1.5 mg L^{-1}), which were stable for the duration of the experiment (Fig. 1; exact values are shown in Table S4). Sensitivities of treatments with high ionic loads (C2000 & C5000) were in the same range as controls (mean EC_{50} = 2.6 mg L^{-1}). In contrast, all prometryn exposed communities showed an increase of EC_{50} over time. Thus, Prom treatments were significantly more tolerant than controls from the fifth week (Tukey`s Test, $P < 0.001$). The community tolerance of the combined treatments P2000 were in the range of single Prom treatments from the fourth week and were significantly higher than controls in week five (Tukey`s Test, $P = 0.01$). Apart from the sixth week, the predicted EC_{50} for P2000 were in the range of the observed values.

The combined treatments with high ionic loads (P5000) continuously increased EC_{50} values over time and developed tolerances beyond all other treatments (13 times higher EC_{50} than controls after six weeks). EC_{50}s of P5000 were significantly higher than controls, C2000, C5000 and Prom from the fourth week and were significantly higher than all other treatments after five weeks (Tukey`s Test, $P < 0.001$). The

tolerances of P5000 treatments were clearly higher than predicted values (independent action model) from the fourth week, also reflected in a significant interaction of high ionic loads, prometryn and time ($P = 0.009$).

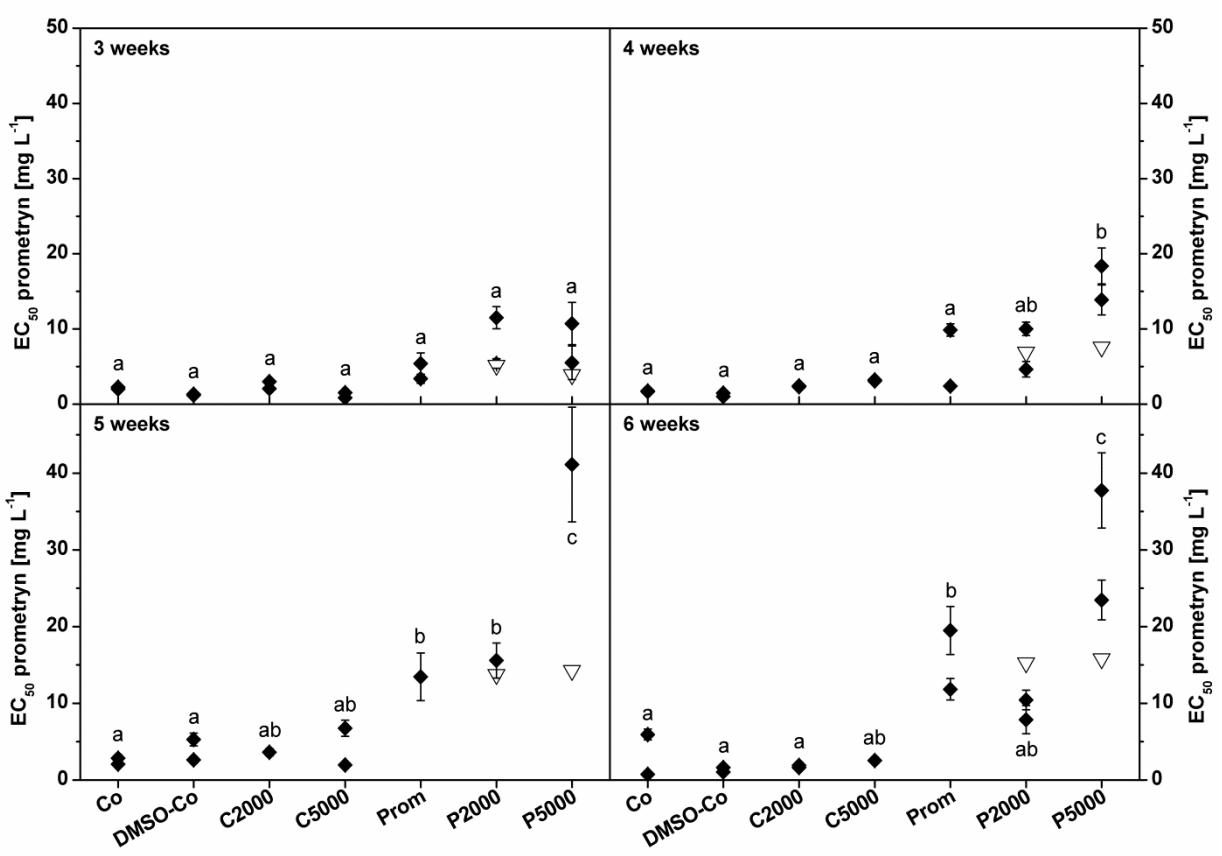

Fig. 1. Effective concentrations (EC_{50}) as a measure of community tolerance to prometryn after three to six weeks of exposure. Two replicate microcosms were conducted per treatment (Co = control; DMSO-Co = solvent control; C2000 = 2000 µS cm^{-1}, C5000 = 5000 µS cm^{-1}, Prom = 15 µg L^{-1} prometryn; P2000 = prometryn + 2000 µS cm^{-1}; P5000 = prometryn + 5000 µS cm^{-1}). EC_{50}s were calculated from concentration–response relationships (log-logistic data analysis) based on photosynthesis inhibition. Error bars represent standard errors of modelled EC_{50}. Different lowercase letters indicate significant differences between treatments. Triangles are predicted EC_{50}, based on the concept of independent action.

Structural parameters

Biomass

The minimal fluorescence (F_0) was measured as indicator for algal biomass. The biomass of each treatment is shown in Fig. 2 for the third and sixth weeks. The whole data set was used for statistical analyses and is shown in Table S5, whereas P5000 and C5000 significantly increased in biomass from the third to the sixth week

(significant interaction between high ionic loads and time, $P < 0.001$), neither the effect of prometryn nor the combined effect of high ionic load and prometryn changed significantly with time (Table S3). After six weeks of exposure, biomass of C2000 was in the range of controls, P2000 and Prom showed lowest level of biomass and C5000 and P5000 doubled in biomass compared with controls.

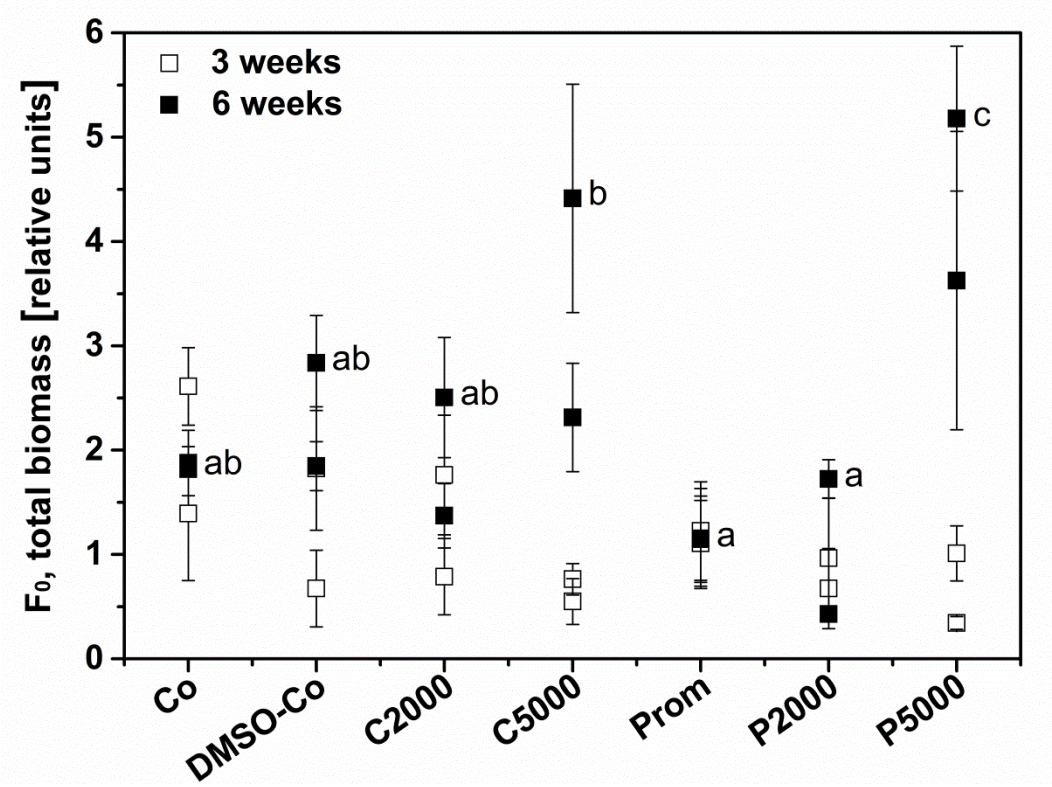

Fig. 2. Biomass development of periphyton communities per treatment from the third to the sixth week. Data were derived from minimal fluorescence (F_0 in relative units) measured by multiwavelength-excitation PAM fluorometry. Error bars represent standard deviations of three measurements of each three glass discs per microcosm. Different lowercase letters indicate significant differences between treatments after six weeks, no significant differences were found after three weeks. Abbreviations are as in Fig. 1.

Algal classes

A detailed description of algal class composition is presented in Appendix S3. All communities significantly changed in their algal class composition over time, (Bonferroni-adjusted *P*-value < 0.017, see Table S3). All controls and C2000 had similar compositions with equal relative proportions of diatoms, chlorophytes and cyanobacteria. After three weeks of growth C5000, Prom, P2000 and P5000 had slightly more diatoms and less chlorophytes than the control group. However, these

treatments clearly developed in different directions within six weeks of colonisation. After six weeks, P2000 and C2000 were similar to the control group and had about 70% diatoms and less than 5% cyanobacteria, whereas the high ionic load treatments (C5000 & P5000) were highly dominated by diatoms (> 85%). However, Prom communities were dominated by diatoms (55%), chlorophytes were in the range of controls, but the relative abundance of cyanobacteria was significantly higher (23%) than in all other treatments (Tukey`s test, $P < 0.01$).

Diatom community

A total of 104 diatom species were classified from 14 different microcosms covering four weeks. Values for community metrics (Shannon Weaver Index, species richness, evenness, salinity index and IPS) are shown in Table S6. The Shannon Weaver diversity, species richness and evenness tended to decrease from the third to the sixth week but showed no consistent differences between treatments. According to the salinity index, periphyton exposed to high ionic loads (C5000, P5000) was α-oligohalobic and β-mesohalobic indicating low and moderate salt effects, respectively, whereas all other treatments were classified as typical freshwater (β-oligohalobic) after six weeks of colonisation. Control communities had on average significantly higher IPS values than treated communities (unpaired t-test, d.f. = 54, t = 5.29, $P < 0.001$) indicating good ecological quality of controls. In contrast, C2000 and P2000 belong to the moderate and C5000, Prom and P5000 to the poor ecological quality class.

The RDA was carried out on three variables (conductivity, prometryn, and time) and 31 common diatom species (species are listed in Table S8). The first three axes of the RDA explained 45.1% of the variance in the diatom data. The result of the RDA is illustrated in Fig. 3 as a biplot of species data and treatment factors. RDA1 explains 23.6% of the variance and leads to a separation of prometryn exposed communities (Prom, P2000, P5000) and non-exposed communities (Co, DMSO-Co, C2000 and C5000). The second axis of the RDA contributed an additional 14.9% to the explained variance and is characterised by the conductivity gradient, discriminating treatments with elevated ionic loads from other treatments. Six of the 31 analysed diatom species were clearly related to treatment conditions in terms of control conditions, prometryn exposure and increased conductivity. The species *Cocconeis*

placentula (Ehrenberg), *Cymbella silesiaca* (Bleisch) and *Achnanthes minutissima var. minutissima* (Kützing) are clustered in the “control-area”. *Fragilaria capucina var. gracilis* (Hustedt) is related to prometryn as single stressor, whereas *Nitzschia palea* (Kützing) appears more with high ionic load/prometryn mixtures. *Navicula halophila* (Grunow) is related to RDA2 and correlates to high conductivity. According to the permutation test, RDA axes and treatment factors (conductivity, prometryn and time) were significant at $P = 0.001$ (Table S7).

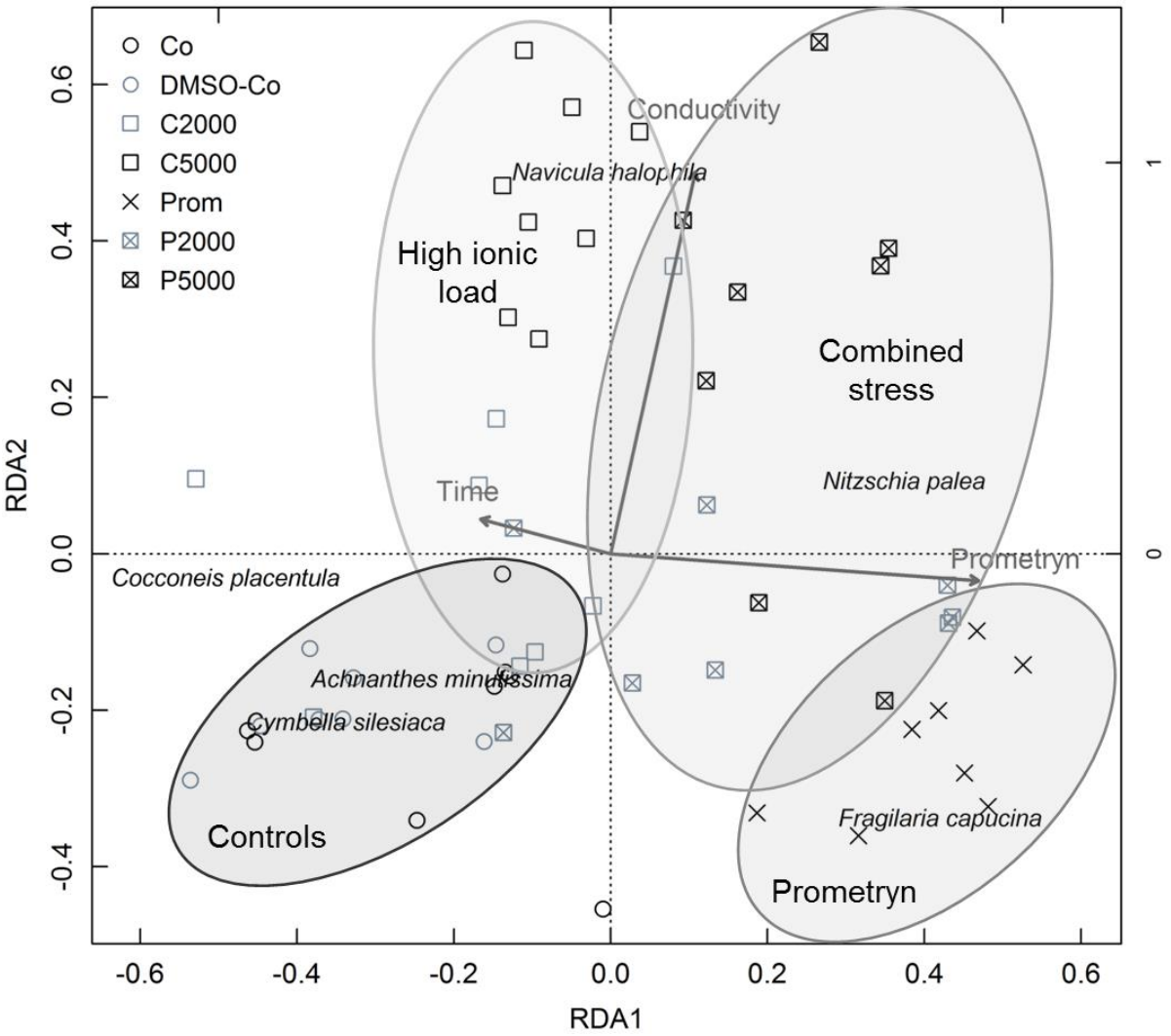

Fig. 3. Redundancy analyses (RDA) based on most abundant (> 1%) diatom species of periphyton communities colonised in microcosms with different treatments and sampled after three, four, five and six weeks. For a better visualisation, species in the centre are suppressed and species supporting the separation of the treatments are highlighted. For illustration, treatment groups are framed. Abbreviations are as in Fig. 1.

Discussion

Response to toxicant induced stress

Our results support the first and the second hypothesis that prometryn exposure triggers a replacement of species resulting in an increased tolerance to prometryn.

Prometryn exposed communities showed 50% less biomass than controls, reflecting the diminished photosynthetic rate. To survive in a polluted environment species must develop tolerance mechanisms such as increased detoxification of compounds or alterations of the target site, for example the D1 protein (Eriksson et al., 2009). We found shifts not only in the species composition, but also in an enhanced abundance of cyanobacteria on the level of algal classes, which might indicate sensitivity differences in both structural levels. Previous studies on other PSII inhibitors found comparable results, for example Bérard et al. (1999) reported that the cyanobacterium *Oscillatoria limnetica* was stimulated by atrazine exposure in the summer months. Also, Schmitt-Jansen and Altenburger (2005) found a 20% increase in relative abundance of cyanobacteria for isoproturon (PSII inhibitor) concentrations of 20–40 µg L^{-1}.

On the species level, *Fragilaria capucina var. gracilis* showed the highest relative abundances in Prom treatments (43% after six weeks). Therefore, we assume *Fragilaria capucina var. gracilis* is a prometryn tolerating species. Also Guasch et al. (1998) found *Fragilaria capucina var. vaucheriae* as one of the dominating species in an atrazine polluted stream. According to our second hypothesis, prometryn treated communities had significantly higher tolerance (four to seven times) compared with controls from the fifth week on. This indicates a time dependency in the development of community tolerance, which goes along with the succession process. The detected tolerance increase was in the range of other studies, which found an increase by a factor of three to six in prometryn exposed communities in microcosms (Schmitt-Jansen and Altenburger, 2005), and a seven times higher tolerance in pre-exposed field communities (Rotter et al., 2011). By impacting the physiology of sensitive species, toxicants such as prometryn clearly exert selective pressure in communities, resulting in toxicant-induced succession and an increase in the overall community tolerance.

Response to ionic stress

The responses to high ionic loads were concentration dependent. The increase of conductivity to 2000 µS cm^{-1} did not affect the community at the level of algal classes (Fig. S1), also the species composition of diatoms differed just slightly from controls (Fig. 3). Furthermore, community biomass was in the range of controls. These findings are in accordance with the gradient study from 200 to 3000 µS cm^{-1} of Leung et al. (2003), who found effects on the community composition of phytoplankton, but not on total biomass, indicating functional redundancy. C5000 communities were clearly affected by the high ionic load. After six weeks of colonisation, the overall biomass of the C5000 community was almost twice that of controls. The increase of ions in our study stimulated periphyton, whereas the toxic input of prometryn reduced the photosynthetic rate and thus also biomass. This finding supports the perturbation theory of Odum et al. (1979), considering a system as subsidised if ecosystem functions (e.g. biomass) are enhanced due to an input into a system, even if certain species may be stressed. Several studies analysing salt effects reported decreased biomass with increasing levels of NaCl (100–800 mM) (e.g. Fodorpataki and Bartha, 2004). However, lower concentrations of NaCl (17–85 mM), which are comparable to C5000 in our study, increased the biomass of the green algae *Botryococcus braunii* (Rao et al., 2007). Due to equal or higher biomass than that of controls, we assume that C2000 and C5000 communities were not limited by osmotic or ionic stress.

Given the decrease of chlorophytes in C5000 treatments, we assume that chlorophytes of the community were less tolerant to high ionic loads than diatoms. The high relative abundance of *Navicula halophila* in C5000 communities and the associated correlation to C5000 treatments in the RDA indicate an elevated tolerance to high ionic loads. This is in accordance with Lange-Bertalot (2001) characterising *Navicula halophila* as salt tolerant species, preferring waters with high electrolyte contents of various ion concentrations. According to our first hypothesis, enhanced ionic loads inhibit sensitive species (e.g. *Cymbella silesiaca*) or algal classes, giving salt tolerant species the opportunity to increase their growth extensively, resulting in a specific community composition.

Apart from the measurement of tolerance to prometryn by using short-term tests, the index of salinity can be used as a metrics-based measure of tolerance to high ionic loads. Both approaches, based on species shifts due to the exposure to either toxicants (PICT) or salt (index of salinity), enable a “translation” of structural changes

into a nominal scale, which simplifies comparisons to other treatments or time points. The index of salinity indicated a clear development of salt tolerance in C5000 communities. Yang et al. (2003) found for a salinity gradient of lakes the highest changes in diatom assemblages between 1.5 and 1.9‰, which is the transition point from slightly oligosaline to eusaline lakes. For our study, 2000 µS cm^{-1} equates to 1‰ and 5000 µS cm^{-1} to about 2.8‰, which supports the findings of Yang et al. (2003) and might explain the clear difference between C2000 and C5000 diatom compositions. There might be a threshold of resistance for high ionic loads from which changes within communities become obvious at all structural levels.

High ionic loads as a single stressor did not induce an increase of tolerance to prometryn, and thus, no co-tolerance was found, supporting our third hypothesis. Also, prometryn exposure did not trigger an increase in salt tolerance, as measured by the index of salinity. As no co-tolerance was found for high ionic loads and prometryn, we confirm the expectation of dissimilar tolerance mechanisms for both stressors. This further indicates different qualities of selection pressure for ionic and toxic stress that might shift communities to different alternative stable states (Clements and Rohr, 2009).

Combined effects of toxic and ionic stress

Our most important finding is that enhanced ionic loads in P5000 seem to stimulate productivity of tolerant species, compensating for the inhibitory effect of prometryn on biomass and inducing tolerances higher than predicted by the model of independent action. Interestingly, this was not found for P2000 indicating a concentration dependent effect. Similar results were found for the simultaneous presence of 150 mM NaCl and copper, leading to higher biomass production of *Scenedesmus opoliensis* as compared to single treatments of copper (Fodorpataki and Bartha, 2008). Crain et al. (2008) stated that nutrients and toxicants as well as salinity and toxicants have opposing effects and interact antagonistically, pointing out that the positive effect of nutrients or high ionic load can mitigate or overcompensate for the negative effect of toxins.

Compositional changes in communities revealed different mechanisms of species selection of single and combined stressors. Whereas single stressors led to a higher occurrence of specialists, combined stressors caused increasing numbers of

omnipresent and opportunistic species, such as *Nitzschia palea* and *Gomphonema parvulum*. Furthermore, *Fragilaria capucina var. gracilis* was less abundant than in Prom treatments and *Fragilaria ulna acus* almost disappeared in comparison to C2000 and C5000. Dissimilar-acting stressors may target a wider range of species than single stressors with similar mode of actions. Therefore, communities developed distinct species compositions for each specific stressor or stressor combination. Furthermore, the strong influence of elevated ionic loads was indicated by the diatom index IPS and the index of salinity categorising C2000 and P2000 communities (moderate ecological class; β-oligohalobic) as well as C5000 and P5000 (poor ecological quality class; α-oligohalobic to β-mesohalobic) in the same functional classifications. According to the index of salinity, the high ionic load as single stressor and in combination with toxic stress led to an increase in salt tolerance.

Whereas the single stress of prometryn increased the tolerance to prometryn up to a factor of eight, tolerance levels of combined stress (P5000) were up to 16 times higher than that of controls and were clearly higher than predicted by the independent action model. This finding was supported by the statistical significant interaction of high ionic load and prometryn ($P < 0.002$). These elevated tolerances might be due to a higher biomass (limits the transfer into the biofilm), the species composition itself or physiological adaptations of species such as modifications at the receptor site of the stressor. Since both stressors were acting in parallel, one stressor might influence the tolerance of the other simply by selecting specific species. Functional redundancy within communities might also have led to the selection of opportunistic species with a high growth rate. This is in accordance with Howarth (1991), who postulated that ecosystems dominated by opportunistic species are more resistant to stress than those dominated by specialists. In comparison to controls, we did not find a clear loss in biodiversity, rather species characterising control communities were no longer abundant in other treatments. This means that sensitive species were replaced by pollution tolerant opportunistic species (Howarth, 1991).

An increase of tolerance clearly reflects serious stress responses and might indicate the loss of ecological resistance accompanied by the shift of communities to alternative stable states. Our results confirm the hypotheses that single and combined stressors trigger stressor-specific succession processes resulting in distinct species compositions and changes in community tolerance.

The determination of community tolerance causally links structural and functional changes to toxicant exposure and enables quantification of an integrated community response. This study revealed that the combined stresses of a toxicant and salt foster clear changes in the community composition accompanied by an increase of community tolerance, which exceeded tolerance levels of single stressors and model predictions. Using the PICT approach, this study conducted a first step in assessing the applicability of the independent action concept to long-term community responses in a multiple stressor environment. Our findings on combined effects of high ionic loads and pesticides also provide a first insight into effects of secondary salinisation in multiple stress environments.

Management implications

Multiple stressors might be one of the factors explaining the critical ecological status of many European water bodies. Therefore, a better understanding of stressor interactions is crucial for water quality assessment and the success of remediation activities. In unspecific stressor conditions, investigative approaches are required, enabling diagnosis and prioritisation of impacts of stressors by causally linking exposure to effects. Although, several taxonomy based metrics, for example for eutrophication (trophic diatom index) or high salinity (index of salinity) exist, no suitable metrics exists for toxicants. Thus, diagnostic tools based on other criteria such as toxicant-induced community tolerance may support decision making within the WFD. If biological quality elements (e.g. diatom metrics) implemented in EU-WFD refer to a poor or bad ecological status (Directive 2000/60/EC, Annex V) and chemical analyses point to various contaminations and stressors, PICT might be a tool to identify the worst triggering compounds. The tolerance of local communities towards site-specific stressors could be tested for a selection of candidates, in order to find the most adverse compounds. This might enable an adaptation of remediation processes and an increase in remediation success. We propose PICT due to its specificity, causality and the ability to quantify community level responses as an advanced tool for ecological effect assessment in multiple stressor environments.

Acknowledgements

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Supporting information

Appendix S1. Chemical analyses of prometryn

To analyse the stability of prometryn over one week one microcosm was prepared and performed as described in the section “periphyton cultivation”, using freshwater of the river Parthe and a prometryn concentration of 15 µg L^{-1}. Water samples (about 20 ml) were taken just before dosing of prometryn stock solution and after 0.5, 1, 2, 7, 23, 29, 50, 80, 121, 168 h exposure in the microcosm. Aqueous samples were measured with pre-equilibrium solid-phase microextraction (SPME) in combination with GC-MS according to Bandow et al. (2010). DVB/CAR/PDMS fibre was loaded for 13 min at 20.9°C and stirred by a glass covered stirrer bar at 250 rpm. The fibre was subsequently injected on a Varian CP-3800 gas chromatograph equipped with a CP-Sil-8 column (Varian, Palo Alto, CA, USA) and compounds were thermally desorbed into the injector of the GC-MS using a splitless mode at 270°C. Fibre was calibrated in the range of 0.16 to 20 µg L^{-1} before conducting measurements. For details of calibration and oven program see Bandow et al. (2010). Analysed water samples were returned to the microcosm.

Bandow, N., Altenburger, R., Brack. W., 2010. Application of nd-SPME to determine freely dissolved concentrations in the presence of green algae and algae-water partition coefficients. Chemosphere 79. 1070-1076.

Appendix S2. Assumptions for prediction of tolerance

For the prediction of community tolerance (EC_{50}) under multiple stress conditions, parallel concentration-response curves for the different treatments were assumed. Therefore, data and curves of concentration-response relationships of different treatments were inspected week by week. The concentration-response curves showed similar shapes and slopes (average *P*-value = 0.53) and met the criteria of parallelism. Thus, it suffices to use only one single effect parameter for calculation of the prediction. For this purpose, the EC_{50} has been chosen as robust parameter of the concentration-response curve. Furthermore, a continuous scaling for the tolerance between the tolerance level of controls and highest observed tolerances of treatments (41.13 mg L^{-1}) was assumed. On this account, tolerance (EC_{50} values of concentration response curves) was rescaled to values between 0 and 1 in order to calculate the prediction of community tolerance using the model of independent action. In the following, we show exemplarily the scaling of EC_{50} values, the

calculation of the prediction using the model of independent action and the rescaling of predicted values to effect values.

Example (week4, C2000 & Prom, prediction of P2000)

Scaling of effect values:

$$E(c_i) = \frac{(e_i - e_{control})}{(e_{\max} - e_{control})}$$

$$E(c_i) = \frac{(EC_{50}\ (treatment) - EC_{50}\ (mean\ controls\ per\ week)}{(EC_{50}\ (\max.\ observed\ tolerance) - EC_{50}\ (mean\ controls\ per\ week)}$$

$$E(C2000\,a) = \frac{(2.44 - 1.47)}{(41.13 - 1.47)} = 0.024 \qquad E(\mathrm{Pr}om\ a) = \frac{(2.39 - 1.47)}{(41.13 - 1.47)} = 0.023$$

$$E(C2000\,b) = \frac{(2.32 - 1.47)}{(41.13 - 1.47)} = 0.021 \qquad E(\mathrm{Pr}om\ b) = \frac{(29.86 - 1.47)}{(41.13 - 1.47)} = 0.212$$

$$E(C2000\,) = \frac{(0.024 + 0.021)}{2} = 0.023 \qquad E(\mathrm{Pr}\,om) = \frac{(0.023 + 0.212\,)}{2} = 0.117$$

Prediction using the model of independent action:

$$E_{P2000} = 1 - \prod_{i=1}^{n} [1 - E(c_i)]$$

$$E_{P2000} = 1 - \left[(1 - 0.023) \times (1 - 0.117)\right] = 0.138$$

Rescaling of prediction:

$$E_{P2000} = 0.138 = \frac{(x - 1.47)}{(41.13 - 1.47)}$$

$$x = pred.\ E_{P2000} = 6.93\ mg\ L^{-1}$$

Appendix S3. Figure and description of algal class distribution

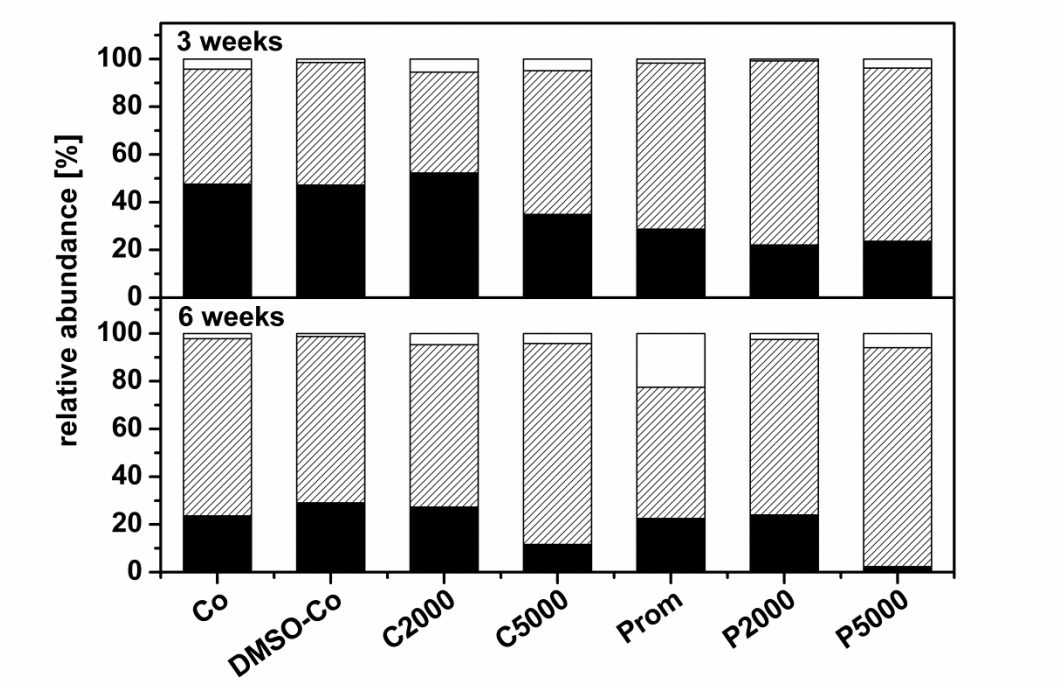

Fig. S1. Relative distribution of algal classes within periphyton communities of different treatments (mean of two microcosms per treatment and three glass discs per microcosm) derived by multiwavelength-excitation PHYTO-PAM fluorometry. (▨ diatoms; ■ chlorophytes; □ cyanobacteria)

Composition of algal classes within periphyton of different treatments after three and six weeks is shown in Fig. S1 (mean values of the two replicate microcosms). Data of week four and five are demonstrating the trend from week three to week six and are not presented because no additional knowledge is gained. The effect of the factor time was significant for all algae classes (chlorophytes: $P < 0.001$, cyanobacteria: $P = 0.003$, diatoms: $P = 0.003$). Referring to the algal class composition control, DMSO-control and C2000 were comparable. After three weeks of colonisation about 50% of periphyton community were represented by chlorophytes, almost 50% by diatoms whereas cyanobacteria were only present with 1% in DMSO-Co and with 5% in Co and C2000. This similarity was consistent, after six weeks diatoms increased their abundance by 20%, simultaneously green algae were decreasing by this proportion, while cyanobacteria did not change in relative abundance. In contrast, C5000, Prom, P2000 and P5000 communities had 22 to 35% less chlorophytes, which is reflected in a significant ionic load ($P = 0.007$) and prometryn factor ($P = 0.006$) for chlorophytes. Simultaneously, relative proportions of diatoms were with 60 to 72% higher than for the control-group, also represented by the significance of the ionic load factor for diatoms ($P = 0.016$). Cyanobacteria were contributing 0.8 to 4.9%

to the overall biomass of these treatments. These treatments developed in different directions and were clearly different after six weeks. The high ionic load treatments were increasing in relative abundance of diatoms, C5000 had 85% and P5000 92% diatoms, simultaneously chlorophytes were decreasing to 12% (C5000) and to 2% (P5000) and relative abundance of cyanobacteria remained in the same range as after three weeks. P2000 did not change from the third to the sixth week. In contrast, Prometryn clearly increased relative abundance of cyanobacteria to 23%, at the same time diatoms decreased their abundance to 55% and relative proportion of chlorophytes remained stable. After six weeks prometryn treatment was significantly different in relative abundance of cyanobacteria to each of the other treatments (Tukey`s test, $P < 0.05$).

Table S1. Molar concentrations of different treatments

Table S1. Molar concentrations of calcium, chloride, sodium and sulphate for control microcosms (natural freshwater) and for microcosms where sodium sulfate and calcium chloride were added to increase the conductivity to 2000 and 5000 µS cm^{-1} respectively.

	Ca^{2+}	Cl^{-}	Na^{+}	SO_4^{2-}
Control	3.9 mM	2.0 mM	1.6 mM	3.3 mM
2000 µS cm^{-1}	7.7 mM	9.9 mM	7.5 mM	6.3 mM
5000 µS cm^{-1}	18.7 mM	31.8 mM	24.6 mM	11.5 mM

Table S2. Statistical differences between controls and DMSO-controls

Table S2. Results of the statistical tests for differences between controls and DMSO-controls.

Effect parameter	Statistical test	n	d.f.	t-value	*P*-value	z-value
Community tolerance	Mann-Whitney test	8	-	-	0.195	-1.365
Biomass	t-test	8	14	0.452	0.658	-
Chlorophytes	t-test	8	14	-0.176	0.863	-
Cyanobacteria	t-test	8	14	2.049	0.060	-
Diatoms	t-test	8	14	-0.207	0.839	-

n =number; d.f. = degrees of freedom

Table S3. Results repeated-measure ANOVA

Table S3. Results of repeated-measure ANOVAs comparing the effect of ionic loads and prometryn as single stressors and in combination on community tolerance, biomass and algal class composition after three, four, five and after six weeks of exposure. The ANOVA model was intercept + ionic loads + prometryn + ionic loads x prometryn + error. The within-subject factor was time (three, four, five and six weeks). The Greenhouse Geisser correction was applied to the within-subject effects of community tolerance.

Effect parameter	d.f.	MS	F-ratio	*P*-value
Community tolerance				
(A) Within-subject effects				
Time	1.684	264.835	72.74	**< 0.001**
Time x ionic load	3.369	50.598	13.897	**0.007**
Time x prometryn	1.684	225.036	61.809	**< 0.001**
Time x ionic load x prometryn	3.369	45.450	12.483	**0.009**
Error	5.053	3.641	-	-
(B) Between-subject effects				
Ionic load	2	214.843	100.884	**0.002**
Prometryn	1	956.981	449.370	**< 0.001**
Ionic load x prometryn	2	227.305	106.736	**0.002**
Error	3	2.130	-	-
Biomass				
(A) Within-subject effects				
Time	3	4.273	12.432	**< 0.001**
Time x ionic load	6	2.377	6.915	**< 0.001**
Time x prometryn	3	0.086	0.252	0.859
Time x ionic load x prometryn	6	0.524	1.525	0.213
Error	24	0.344	-	-
(B) Between-subject effects				
Ionic load	2	2.473	2.653	0.131
Prometryn	1	2.077	2.228	0.174
Ionic load x prometryn	2	0.217	0.233	0.798
Error	8	0.932	-	-
Chlorophytes				
(A) Within-subject effects				
Time	3	531.149	12.902	**< 0.001**
Time x ionic load	6	61.063	1.483	0.226
Time x prometryn	3	182.637	4.436	**0.013**
Time x ionic load x prometryn	6	55.019	1.336	0.280
Error	24	41.170	-	-
(B) Between-subject effects				
Ionic load	2	784.351	9.872	**0.007**
Prometryn	1	1097.352	13.811	**0.006**
Ionic load x prometryn	2	139.488	1.756	0.233
Error	8	79.455	-	-

Table S3. (continued) See previous page for heading of the table.

Effect parameter	d.f.	MS	F-ratio	P-value
Cyanobacteria				
(A) Within-subject effects				
Time	3	86.807	6.007	**0.003**
Time x ionic load	6	24.644	1.705	0.163
Time x prometryn	3	48.305	3.343	0.036
Time x ionic load x prometryn	6	31.238	2.162	0.083
Error	24	14.451	-	-
(B) Between-subject effects				
Ionic load	2	28.705	0.726	0.513
Prometryn	1	99.782	2.525	0.151
Ionic load x prometryn	2	98.036	2.481	0.145
Error	8	39.522	-	-
Diatoms				
(A) Within-subject effects				
Time	3	303.736	6.169	**0.003**
Time x ionic load	6	104.362	2.120	0.088
Time x prometryn	3	379.718	7.712	**0.001**
Time x ionic load x prometryn	6	112.769	2.290	0.069
Error	24	49.236	-	-
(B) Between-subject effects				
Ionic load	2	904.626	7.219	**0.016**
Prometryn	1	535.157	4.271	0.073
Ionic load x prometryn	2	138.427	1.105	0.377
Error	8	125.312	-	-

d.f.= degrees of freedom; MS = mean square; bold value denotes a significant result.

Table S4. Community tolerance data

Table S4. Effective concentrations (EC_{50}) and standard error (SE) of each microcosm per week, measured in short-term tests of prometryn as photosynthesis inhibition. Median efficiencies were calculated from concentration-response relationships using log logistic data analysis. Values in parentheses were identified as outlier (based on Grubbs outlier test and a maximum inhibition of less than 60% in the corresponding concentration-response curves) and have been excluded from further data analyses.

	week 3		week 4		week 5		week 6	
[mg L^{-1}]	EC_{50}	SE	EC_{50}	SE	EC_{50}	SE	EC_{50}	SE
Co-1	2.27	*0.34*	1.74	*0.31*	2.07	0.26	0.76	*0.17*
Co-2	2.01	*0.44*	1.66	*0.33*	2.86	0.47	5.94	*0.71*
DMSO-Co-1	1.32	*0.19*	1.03	*0.10*	2.63	0.47	1.05	*0.21*
DMSO-Co-2	1.20	*0.26*	1.46	*0.19*	5.28	0.83	1.63	*0.41*
C2000-1	3.00	*0.41*	2.44	*0.29*	3.63	0.50	1.92	*0.42*
C2000-2	2.07	*0.29*	2.32	*0.34*	(12.91)	(3.46)	1.62	*0.23*
C5000-1	0.85	*0.22*	3.09	*0.42*	1.98	0.28	2.55	*0.46*
C5000-2	1.53	*0.23*	3.23	*0.41*	6.75	1.06	(20.95)	*(2.82)*
Prom-1	3.38	*0.61*	2.39	*0.30*	13.46	3.11	11.84	*1.41*
Prom-2	5.40	*1.42*	9.86	*0.81*	(88.65)	(19.76)	19.50	*3.12*
P2000-1	11.50	*1.46*	10.01	*0.89*	29.86	6.57	7.87	*1.84*
P2000-2	5.40	*0.64*	4.65	*1.03*	15.59	2.29	10.45	*1.28*
P5000-1	5.52	*2.23*	18.39	*2.38*	41.13	7.47	37.75	*4.89*
P5000-2	10.72	*2.81*	13.86	*2.00*	(98.59)	(41.26)	23.47	*2.59*

The incorporation of the outliers in the analysis would partly change statistical significances, but would not influence the main finding of the manuscript, that joint effects of high ionic loads and prometryn (P5000) significantly increased community tolerance towards prometryn.

Table S5. Biomass data

Table S5. Biomass and standard error (SE) of each microcosm per week with two replicates per treatment. Data was derived from minimum fluorescence (*f0* in relative units) measured by multiwavelength-excitation PAM fluorometry. Standard error represents deviations of three measurements of three glass discs per microcosm.

	week 3		week 4		week 5		week 6	
[relative units]	***f0***	**SE**	***f0***	**SE**	***f0***	**SE**	***f0***	**SE**
Co-1	2.6	*0.4*	1.2	*0.3*	1.4	*0.6*	1.9	*0.3*
Co-2	1.4	*0.6*	3.1	*0.5*	1.7	*0.6*	1.8	*0.0*
DMSO-Co-1	1.8	*0.6*	1.7	*0.4*	1.9	*0.2*	2.8	*0.5*
DMSO-Co-2	0.7	*0.4*	1.9	*0.4*	1.3	*0.1*	1.8	*0.2*
C2000-1	0.8	*0.4*	2.2	*1.1*	2.6	*0.9*	2.5	*0.6*
C2000-2	1.8	*0.6*	1.6	*0.2*	2.4	*0.4*	1.4	*0.3*
C5000-1	0.8	*0.2*	2.0	*0.2*	2.2	*0.2*	2.3	*0.5*
C5000-2	0.5	*0.2*	2.2	*0.5*	4.2	*1.0*	4.4	*1.1*
Prom-1	1.1	*0.4*	1.8	*0.2*	1.2	*0.4*	1.2	*0.5*
Prom-2	1.2	*0.5*	1.3	*0.5*	1.9	*0.3*	1.1	*0.4*
P2000-1	0.7	*0.4*	1.3	*1.1*	1.7	*0.2*	0.4	*0.0*
P2000-2	1.0	*0.6*	2.0	*0.7*	1.6	*0.5*	1.7	*0.2*
P5000-1	1.0	*0.3*	2.3	*1.1*	2.9	*1.2*	5.2	*0.7*
P5000-2	0.3	*0.1*	0.5	*0.2*	1.6	*0.3*	3.6	*1.4*

Table S6. Values of diatom metrics

Table S6. Values of the Shannon-Weaver biodiversity (SW) index, species richness, Evenness, salinity index and the specific pollution sensitivity index (IPS) of each microcosm after three and six weeks of colonisation.

	SW index		Richness		Evenness		Salinity index		IPS	
week	**3**	**6**	**3**	**6**	**3**	**6**	**3**	**6**	**3**	**6**
Co-1	2.55	1.97	32	26	0.73	0.60	2	1	12.6	14.4
Co-2	2.94	2.40	48	38	0.76	0.66	4	2	8.3	14.8
DMSO-Co-1	2.67	2.01	37	39	0.74	0.55	2	1	12.1	15.3
DMSO-Co-2	2.36	1.55	31	23	0.69	0.50	1	1	12	14.9
C2000-1	2.74	1.74	43	34	0.73	0.49	9	8	10	12.8
C2000-2	3.03	2.35	52	34	0.77	0.66	7	2	9.6	14.6
C5000-1	2.63	2.50	31	33	0.77	0.72	14	28	7.5	7.7
C5000-2	2.73	2.24	38	27	0.75	0.67	13	30	5.5	5.9
Prom-1	2.03	2.11	38	35	0.56	0.59	4	4	5.6	13.8
Prom-2	1.51	2.02	30	37	0.44	0.56	1	1	4.5	11.2
P2000-1	2.26	2.44	46	31	0.59	0.71	6	8	7.2	8.3
P2000-2	1.88	1.22	34	23	0.53	0.39	1	2	12.7	14.3
P5000-1	1.92	1.62	30	27	0.56	0.49	15	55	4.2	5.4
P5000-2	2.13	2.30	38	33	0.59	0.66	2	17	10.3	8.7

Table S7. Permutation test - RDA

Table S7. Result of the permutation test for environmental parameters and axes using 999 random permutations.

	d.f.	Var.	F-ratio	*P*-value
Environmental parameter				
Conductivity	1	0.0319	14.149	**0.001**
Prometryn	1	0.0454	20.132	**0.001**
Time	1	0.0181	8.016	**0.001**
Residual	52	0.1172		
Axes				
RDA1	1	0.0504	22.355	**0.001**
RDA2	1	0.0318	14.103	**0.001**
RDA3	1	0.0139	6.176	**0.001**
Residual	52	0.01172		

d.f. = degrees of freedom; Var. = variance; bold values denote a significant result.

Table S8. Abundances of diatom species used for RDA

Table 8. List of common diatom species, of periphyton communities cultivated under different exposure conditions, used for RDA. (*relative abundance of species * ≥ 1%, ** ≥ 10%, *** ≥ 30%).

	Control-1				Control-2				DMSO-Co-1				DMSO-Co-2				C2000-1				C2000-2				C5000-1				C5000-2			
weeks	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6
Achnanthes lanceolata													*									*										
Achnanthes lanceolata ssp. frequentissima					*					*							*	*				*	*	*							
Achnanthes minutissima var. minutissima	*	*	**	*	*	**	**	**	*	*	**	**			*	*		*		*	*	*	**	**		*				*	*	*
Cocconeis placentula	**	*	*	**	**	*	*	***	**	**	**	***	**	**	**	***	**	**	**	***	*	*	**	**	**	**	**	**	*	*	*	*
Cyclotella meneghiniana	*				*	*	*			*							*	*	*		*				*	*	*	*	*	*	*	
Cymbella minuta	*	*									*		*																			
Cymbella silesiaca	*	*	**	**	*	**	**	**	**	**	**	**	*	**	**	*	*	*	*	*	*	*	*	*						*	*	*
Fragilaria capucina var. gracilis	**	***	*	*	*	*	*	*	**	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*
Fragilaria capucina var. mesolepta	*	*	*	*		*	*	*	*	*	*	*	*				*	*	*	*	*	*	*	*	*				*			*
Fragilaria capucina var. rumpens	*	*			*																				*						*	*
Fragilaria capucina var. vaucheriae	*		*		*	*	*	*	*	*	*		*	*			*		*		*	*	*	*	*	*	*	*	**	*	*	*
Fragilaria construens f. venter					*												*	*				*			*			*				
Fragilaria fasciculata																			*						*			*	*	*		
Fragilaria nanana						*																										
Fragilaraia ulna	*	*	*											*			*	*			*			*				*	*	*		*
Fragilaria ulna acus-Sippen	**	*	*	*	*	*	*	*	*	*	*		*	*			**	**	*	*	**	**	*	*	**	*	*	*	*	**	*	**
Gomphonemacf. amoenum																																
Gomphonema gracile													*																			
Gomphonema parvulum	**	**	**	**	**	*	*	*	*	**	**	**	**	**	*	*	**	*	*	**	*	**	**	**	**	**	*	**	**	**	*	*
Melosira varians	*	*	*		*	*	*	*	*	*							*	*			*				*			*	*	*	*	*
Navicula capitata																	*															
Navicula cryptocephala					*				*																							
Navicula halophila		*				*	*	*		*	*					*	*	*	**	*			*		*	**	***	**	*	*	**	**
Navicula veneta																			*		*							*			*	
Nitzschia archibaldii																					*	*	*						*	*	*	
Nitzschia capitellata																					*											
Nitzschia fonticola	*	*			*	*			*		*	*					*	*					*	*								*
Nitzschia fruticosa						*																										
Nitzschia palea	*	*	*		**	**	**	*	**	*	*		*	*	*		*	**	***		**	**	**	*	*	**	**	*	**	*	**	**
Nitzschia paleacea					*				*				*				*	*			*	*				*			**	*	**	*
Surirella brebissonii	*																*												*			

Table S8. (continued) See previous page for heading of the table.

	Prom-1				Prom-2				P2000-1				P2000-2				P5000-1				P5000-2			
weeks	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6
Achnanthes lanceolata																								
Achnanthes lanceolata ssp. frequentissima								*							*							*		
Achnanthes minutissima var. minutissima		*	*	**		*		*		*		*		*	*	*						*		*
Cocconeis placentula	*		*	*	*	*		*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*
Cyclotella meneghiniana	*								*								*	*	*					
Cymbella minuta			*				*				*		*											
Cymbella silesiaca										*	*	*		*	*				*					
Fragilaria capucina var. gracilis	**	***	***	***	**	***	***	***	***	***	***	**	**	*	*		*	*	*	*	**	**	*	*
Fragilaria capucina var. mesolepta							*			*		*	*						*					
Fragilaria capucina var. rumpens																					*			
Fragilaria capucina var. vaucheriae													*									*		*
Fragilaria construens f. venter	*																	*						
Fragilaria fasciculata												*						*		*	*	*	*	*
Fragilaria nanana																								
Fragilaraia ulna	*	*		*				*	*	*		*	*				*	*		*				
Fragilaria ulna acus-Sippen	*	*	*	*	*	*	*	*	*	*		*					*	*	*			*		
Gomphonemacf. amoenum												*												
Gomphonema gracile																								
Gomphonema parvulum	*	**	**	**	*	*	*	**	*	*	**	**	**	**	**	*	**	*	*	*	**	**	**	**
Melosira varians																					*			
Navicula capitata																								
Navicula cryptocephala				*																				
Navicula halophila	*	*	*	*					*	*	**	*			*	*	**	**	**	***			*	**
Navicula veneta																								
Nitzschia archibaldii									*															
Nitzschia capitellata	*																							
Nitzschia fonticola																								
Nitzschia fruticosa																								
Nitzschia palea	***	**	**	*	***	***	**	**	**	***	**	**	*	*	*	*	***	***	***	**	**	*	**	*
Nitzschia paleacea	*	*							*														*	*
Surirella brebissonii																					*			

Chapter 5

General discussion

and Outlook

General discussion

The general aim of this thesis was to demonstrate the validity of PICT in complex field studies with multiple contaminations, in order to propose PICT as a diagnostic tool for ecological effect assessment within environmental risk assessment (ERA).

According to Calow and Forbes (2003) a better understanding of species sensitivities and their effects on communities and ecosystems is required to improve ecological effect assessment procedures. Thus, there is a need to develop a better understanding of the complex interactions and mechanisms of biological organisation in ecosystems (Eggen et al., 2004). Beside the potential role in ERA, PICT studies also give an insight into species interactions and tolerance mechanisms of communities, which might help to establish a causal understanding of community-level effects. Furthermore, PICT studies provide ecologically relevant data that might be used for ecological modelling. **Chapter 3**, for example, revealed that the recovery from toxic stress is faster than the adaptation to toxic stress and associated these processes to changes in abundances of two diatom species. On the basis of this result, a process-based computer simulation model was conducted, in order to find key parameters and processes influencing the timeframe of adaptation and recovery processes (Metzger, 2010). This study revealed that adaptation and recovery times depend on the way a community is affected. If the toxicant reduces the carrying capacity (maximum number of individuals that can be supported sustainably by a given environment) of population growth the adaptation is faster than the recovery, if it reduces the growth rate the adaptation takes longer than the recovery (Metzger, 2010). The latter is likely for the toxicant prometryn, therefore the modelling approach is in accordance to the results of **Chapter 3**.

Moreover, also ERA needs to integrate ecologically relevant data and has to consider environmental complexity, in order to assess the impact of pollutants on natural ecosystems (Eggen et al., 2004). The concept of PICT enables the quantification of community-level effects to toxicants and was applied in many microcosm and field studies (e.g. McClellan et al., 2008; Blanck et al., 2009). Even if the PICT response has been shown in many field studies, which were very unlikely only contaminated with one single compound and rather contained multiple stressors (e.g. Pesce et al., 2010; Rotter et al., 2011; Fechner et al., 2012), the conclusiveness of PICT in an environment containing multiple stressors is still uncertain (e.g. Brack et al., 2008). However, the strength of PICT is to causally link exposure to community-level effects

in the field. Therefore, especially the *in situ* application of PICT seems to be useful for the use within retrospective ERA. In the studies described in **Chapter 2**, **3** and **4** PICT has been applied as tool for hazard confirmation, for the assessment of recovery-processes and to proof the validity of PICT under exposure to multiple stressors. On the basis of these studies the *in situ* PICT was validated according to Blanck (2002). Furthermore, the significance of PICT responses to multiple stressors and mixtures was discussed in order to propose PICT for ecologically relevant effect assessment in the framework of ERA:

Validation of *in situ* PICT

First of all, the tolerance measure of the short-term test needs to be meaningful for both *in situ* and microcosm PICT studies (Blanck et al., 1988). According to Eriksson (2008) a long metabolic distance between the measured endpoint and the process influenced by the toxicant may limit or even hinder the detection of PICT. Thus, inappropriate, target unspecific measures may detect measurable effects with a time delay. An extension of the exposure time might compensate for a weak connection between the affected target and measured endpoint. However, the exposure time in the detection-phase of PICT (short-term test) should not allow further succession processes of the community and has to be therefore shorter than the generation time of the organisms (Blanck, 2002). For this reason in **Chapter 2**, three different measures of photosynthesis were tested at different exposure times in order to find appropriate test conditions for the phytotoxic compounds prometryn, TBT and PNA. For prometryn and TBT, effects on photosynthesis developed fast within the first hour of exposure, thus all measures of photosynthesis were appropriate. However, for the reactive compound PNA (Altenburger et al., 2006), which might not affect photosynthesis directly, a longer exposure duration of 24 h was required to establish measurable effects on photosynthesis. Only, ^{14}C-incorporation was appropriate to measure effects of PNA on photosynthesis. This is in accordance with Eriksson (2008), who stated that ^{14}C-incorporation represents a more integrated measure of photosynthesis with more and closer links to other parts of the metabolism than PAM-fluorescence measures. Thus, for each compound an appropriate measure and exposure time was found in order to enable the detection of PICT.

Secondly, the single stressor has to be able to induce a PICT response. In the controlled microcosm study of **Chapter 4**, periphyton communities exposed solely to

prometryn were four to seven times more tolerant than unexposed control communities and showed therefore a clear PICT response. This is in accordance with Schmitt-Jansen and Altenburger (2005), who found a tolerance increase by a factor of seven for periphyton communities exposed to 0.01 mg L^{-1}. Furthermore, *Cymbella silesiaca*, a diatom which was abundant in the controls, was absent in prometryn exposed communities, simultaneously *Fragilaria capucina* was highly abundant. Also Guasch et al. (1998) found *Fragilaria capucina* as a dominating species in an atrazine polluted stream, therefore a high tolerance of *Fragilaria capucina* towards triazines can be assumed. This study showed that the exposure of prometryn to periphyton communities induces a toxicant-induced succession, replacing sensitive species by more tolerant ones. These structural changes within the community result in significantly higher tolerances towards prometryn.

Thirdly, the presence of the toxicant is compulsory for the induction of PICT. For this purpose, natural periphyton communities were cultivated at a prometryn contaminated and a reference site without prometryn contamination for 26 days (**Chapter 3)**. Both communities were subsequently transferred to a respective other site. Pre-exposed communities were seven times more tolerant than communities grown at the reference site. However, four weeks after the translocation to the reference site, pre-exposed communities became more sensitive towards prometryn and were less tolerant than communities from the reference site. Also, Dorigo et al. (2010) found that pre-exposed, diuron tolerating eukaryotic periphyton communities decreased their tolerance at the unpolluted site and showed a similar tolerance as control communities after five weeks. Conversely, reference communities transferred to the contaminated site increased their tolerance towards prometryn from 0.28 mg L^{-1} at the reference site to 7.2 mg L^{-1} after six weeks at the contaminated site. This clearly shows that the exposure to prometryn was required to induce PICT and that PICT disappeared when prometryn was absent. These functional changes of the community were induced by structural changes within the community. The increase of community tolerance was related to the increasing abundance of *Achnanthes minutissima*, which is associated with continuous physical and chemical stress (Sabater, 2000) and might be able to tolerate the exposure to prometryn, and the decreasing abundance of *Cocconeis placentula,* which was dominating the reference communities and might be more sensitive. On the other hand, relative abundance of *Cocconeis placentula* enormously increased from 0.2% to 76% after

pre-exposed communities were transferred to the reference site, whereas abundance of *Achnanthes minutissima* decreased from 88% to 7%. The diatom species composition of the transferred pre-exposed community was similar to reference communities after three weeks. Also, other studies found that the transfer of communities induces a shift of community structure towards the resident community (Ivorra et al, 1999; Gold et al., 2002; Hirst et al., 2004). Therefore, the presence of the toxicant induces a toxicant-induced succession, which results in an increase of community tolerance. As soon as the toxicant vanishes, the selection pressure of the toxicant relieves and species which are, without the toxicant, more competitive enter the community. Lambert et al. (2012) showed that these immigration processes play a major role for the timeframe of recovery. This means that contaminated river sites with a high immigration of species from a not or only slightly contaminated upstream section potentially recover faster than rivers with continuous contaminations.

Finally, PICT should appear even though other contaminants and confounding factors occur. As most of the aquatic environments are disturbed by several natural and anthropogenic stressors (Breitburg et al., 1998, Schwarzenbach et al., 2006, Tockner et al., 2010), every *in situ* PICT linking exposure of contaminants to effects on communities might show that PICT is induced in multiple contaminated environments as well. An *in situ* study at a polluted site with a complex mixture of copper, cadmium, nickel, lead and zinc showed that community tolerance is a sensitive indicator of urban disturbance by mixtures of contaminants (Fechner et al., 2012). Pesce et al. (2010) found in a field study that diuron tolerance is mainly driven by the exposure level of diuron itself, but that conductivity may be related to diuron sensitivity as well. Beside the difference in the prometryn concentration in the *in situ* studies described in **Chapter 2** and **3**, also the conductivity of the contaminated site (1934/1919 µS cm^{-1}) was about 3.5 times higher than at the reference site with mean values of 560 µS cm^{-1} or rather 527 µS cm^{-1}. Nevertheless, significant difference in the tolerance towards prometryn between pre-exposed and reference communities were detected in both field studies. In order to assess the effect of high ionic loads on the development of PICT towards prometryn and to proof the validity of PICT in an environment with multiple stressors, a more controlled microcosm study was conducted. **Chapter 4** shows that the simultaneous exposure of periphyton communities to prometryn and enhanced levels of high ionic loads did not prevent the development of a PICT towards prometryn. An exposure scenario similar to the

conditions in the field (prometryn exposure and ionic load equivalent to 2000 µS cm^{-1}) induced tolerance-levels towards prometryn which were in the same range as for periphyton which was exposed solely to prometryn. Both periphyton communities were about five times more tolerant than unexposed control communities. Therefore, the *in situ* PICT response found in **Chapter 2** and **3** was validated by the microcosm study of **Chapter 4**. In contrast, the combined exposure of prometryn and an enhanced conductivity of 5000 µS cm^{-1} even enhanced the tolerance to prometryn. These communities were about ten times more tolerant than unexposed communities. Thus, communities receiving ionic and toxic stress simultaneously revealed concentration dependent joint effects. Nevertheless, they clearly showed the validity of PICT responses towards the toxicant also in the presence of another confounding factor.

Therefore, the *in situ* PICT responses detected in **Chapter 2** and **3** were confirmed according to the requirements of Blanck et al. (2002).

Significance of PICT responses to multiple stressors and mixtures

One of the challenges within ecotoxicology is the simultaneous exposure of organisms to chemical mixtures or multiple stressors (Eggen et al., 2004), because possible interactions might lead to combined effects (Altenburger and Greco, 2009) and unexpected ecological surprises (Darling and Côté, 2008). **Chapter 4** studied the single and combined effect of ionic and toxic stress and examined concentration dependent interactions between both stressors. This might be an important finding, because secondary salinisation of freshwater bodies is increasing worldwide (Cañedo-Argüelles et al., 2013). For this reason, the simultaneous occurrence of pesticides and high ionic loads becomes more probable, which might lead to extensive effects in aquatic environments. Furthermore, **Chapter 4** revealed that the type of interactions might depend, beside the stressor pairs, trophic level and biological response levels (Crain et al., 2008), also on the concentration level of stressors. It was shown that ecotoxicological mixture models, so far mainly applied in single species tests or rarely in short-term community assays (Backhaus et al., 2004; Arrhenius et al., 2004; Arrhenius et al., 2006), might be used for the prediction of long-term effects in communities as well. For stressors with the same mode of action the mixture model “concentration addition” might allow the prediction of combined effects (Faust et al., 2001) and for dissimilar acting stressors the model “independent

action" (Faust et al., 2003). However, for ionic and toxic stress different modes of action were assumed. **Chapter 4** showed the applicability of the "independent action" model for long-term community responses to stressors with dissimilar modes of action. The mixture models "independent action" and "concentration addition" assume no interactions between the mixture components (Backhaus and Faust, 2012). Therefore, interactions of stressors or chemical mixtures are revealed as deviation of the detected effect values from the predicted effect. So far, **Chapter 4** is the only PICT study predicting community tolerance, in order to study potential interactions of stressors.

Pesce et al. (2011) conducted another approach to study the effects of mixtures on local communities. They exposed passive samplers at a site with various contaminations and simultaneously cultivated periphyton communities at the same site as well as a bit more upstream, where lower contamination levels were assumed. In a short-term test, both communities were exposed to extracts of the passive samplers, containing mixtures of pollutants. The study revealed that downstream communities were more tolerant to the extracts of passive samplers than the upstream communities. This may indicate that communities exposed to complex mixtures develop multiple tolerances against the whole set of pollutants. To confirm the effect of single contaminants, further testing with identified compounds of the extract would have been required. However, this does not seem practical as chemical analyses are time-consuming and periphyton cannot be stored over longer periods without inducing structural changes. Thus, this approach enables a first screening for effects from mixtures and confirmed their potential combined effect but does not relate the effect to single compounds or interactions of compounds.

Also Fechner et al (2012) related low exposure levels of a complex mixture of copper, cadmium, nickel, lead and zinc to metal tolerance in heterotrophic biofilms. The tolerance towards zinc was increasing even though the concentration in the river was lower than 20 µg L^{-1}, which did not induce PICT in a microcosm approach (Fechner et al., 2011). Furthermore, the mean concentrations of dissolved metals were below the Environmental Quality Standard (EQS) defined by European WFD (Fechner et al., 2012). Metals are known to induce co-tolerances, because they commonly induce oxidative stress and therefore defense mechanisms are effective also against other metals (Soldo and Behra, 2000). As the exposure levels of the single metals were most probably too low to induce toxicant-induced succession in

communities, the tolerance increase found in heterotrophic biofilm might be based on the sum of the concentrations levels of the entire set of metals. Therefore, not one single metal, but the entire metal mixture had a significant effect on periphyton communities, which could have not been detected if the specificity of PICT would be absolute.

The above mentioned studies of **Chapter 4**, Pesce et al. (2011) and Fechner et al. (2012) clearly showed that multiple stressors or mixtures might change the magnitude of tolerance, but still show the causality of PICT. The advantage of PICT is that the detection phase of PICT is able to confirm the effects of stressors on the one hand and establishes causality of stressor exposure and community-level effects on the other hand. Thus, PICT links exposure to effects also in complex field studies with various contaminations. However, tolerance developments have to be interpreted more carefully. In the presence of various contaminants communities might develop multiple tolerances against the whole group of contaminants. Toxicants with similar mode of actions induce similar tolerance patterns, which do not allow the separation of the single effects afterwards. The compound chosen in the short-term test represents an entire group of contaminants with the same mode of action. Therefore, PICT responses in multiple contaminated sites should be seen as response to a certain mode of action (e.g. PSII inhibitors, oxidative stress) rather than to one compound itself, unless one can exclude the occurrence of toxicants with the same mode of action. The strength of PICT to causally link exposure of single or various toxicants to community-level effect in field situations makes it an appropriate tool for environmental effect assessment and distinguishes it against other community-level tools such as diatom indices.

PICT - a suitable tool for environmental effect assessment?

As already mentioned in the introduction ERA can be divided in prospective risk assessment and retrospective risk assessment. The European Water Framework Directive (WFD) covers the retrospective hazard assessment of chemicals and is meant to protect aquatic ecosystems across borders (von der Ohe et al., 2009). The main objective of the WFD is to achieve “a good chemical and ecological status” for surface waters by 2015 (Directive 2000/60/EC). Therefore, EQS have been set up for 33 priority substances (Directive 2008/105/EC). These compound specific thresholds aim to prevent effects on aquatic communities. A water body with exceeding

concentrations of these thresholds fails to achieve a “good chemical status”. The assessment of the ecological status is type specific (e.g. lowland rivers or alpine regions) and is determined by the biological quality elements phytoplankton, macrophytes, phytobenthos, benthic invertebrate and fish fauna as well as physical and chemical properties of water bodies (Schaumburg et al., 2004). This approach provides a full picture of flora and fauna, but does not allow to link exposure to effects. A good ecological status is achieved, if all biological quality elements are characterised as “good”, EQS are complied and functionality of ecosystem is guaranteed (BMU und UBA, 2010). In Germany, the majority of rivers (88%), lakes (92%) and coastal areas (98%) achieve “a good chemical status” (BMU und UBA, 2010). In contrast, only 10% of the surface waters achieve a ”good” or “high” ecological status, which mirrors basically the ecological status of the rivers, having the largest share of water bodies (BMU und UBA 2010). Thus, often aquatic communities are affected, but the chemical status (based on 33 priority substances) does not seem to be the reason for it.

One cause for this might be the occurrence of many unknown chemicals in water bodies, not being monitored or chemicals which are not in the list of priority substances yet. Hein et al. (2010) recommended a tiered approach to assess the impact of chemicals using, beside the recently applied PEC/PNEC ratios and modelled exposure data, a third approach based on field relevance. They proposed an evidence approach based of effect-directed analysis (EDA) to derive candidates for monitoring and prioritisation. Thus, potential chemicals causing effects in the investigated ecosystems can be revealed. However, the hazard of the chemicals revealed by EDA has to be confirmed. An ecologically relevant confirmation can be achieved by using the endangered biological community at the contaminated site. **Chapter 2** showed that only one out of three suspected phytotoxic compounds revealed by an EDA had significant effects on algal communities. Thus, PICT enables an ecologically relevant hazard confirmation of phytotoxic compounds, using local communities from the investigated site. In water bodies with a “good” chemical status and a “poor” or “bad” ecological status the combined approach of EDA and PICT might reveal the chemicals causing effects at the aquatic organisms and communities. Another advantage of the hazard confirmation at the contaminated site is that the average concentration of contaminants can be measured by passive sampling during the entire cultivation time of local communities. Therefore, the

effects on the community and a potential PICT response could be directly related to concentrations the community was exposed to.

With regard to the aim of the WFD to reach a “good” ecological status of surface waters, risk managers have to decide if a remediation of e.g. the sediment of the contaminated site is necessary to improve the ecological status. As shown in **Chapter 3**, the presence of the contaminant is required to induce a PICT response, as soon as the contaminant vanishes the selective pressure is gone and communities recover. When PICT responses to certain stressors are measured prior and after remediation processes, PICT might be an appropriate approach to assess the success of the remediation. Although, this thesis dealt primarily with effects on the phototrophic part of periphyton and is therefore restricted to phytotoxic compounds, the scope of application might be broadened by measuring the effect on the heterotrophic part of periphyton communities e.g. as shown by several PICT studies of Fechner et al. (e.g. 2011, 2012) investigating effects of metals. However, so far, the detection methods of PICT are restricted to PAM-fluorescence measures and ^{14}C-incorporation for the phototrophic part of periphyton as well as to thymidine incorporation (e.g. Blanck et al., 2003) and β-glucosidase activity (e.g. Fechner et al. 2010) representing effects on heterotrophic communities. As the measured endpoint has to match with the mode of action of the compound, PICT is currently limited to compounds affecting processes of the photosynthesis or to metals exerting oxidative stress. In order to increase the field of application of PICT, there is a need to develop new endpoints for short-term tests.

Another reason for the failing to achieve a “good” ecological status might be the simultaneous occurrence of multiple stressors or several chemicals in mixtures. Darling and Côté (2008) revealed in their literature study that interactions among multiple stressors are more commonly non-additive than simple additive, which might cause unexpected ´ecological surprises´. Also the joint toxic effect of various chemicals in a mixture used to be higher than the individual effect of its components (Kortenkamp et al., 2009). Even if components of mixtures occur at concentration levels which would individually not affect organisms, the mixture might have significant toxicity (e.g. Fechner et al., 2012; Altenburger and Greco, 2009; Faust et al., 2001). As discussed in the previous part, PICT might be able to reveal effects from single contaminants in complex mixtures or from a group of similarly acting contaminants. The ability of PICT to causally link exposure to community effects

might enable a limitation of potential candidates causing effects in the water body to a group of contaminants with similar mode of actions.

In Germany, for the Biological Quality Element (BQE) phytobenthos the metrics species composition and abundance as well as the saprobic and trophic index are combined for the assessment of the ecological status (Schaumburg et al., 2004). Furthermore, the remaining phytobenthos was classified in sensitive species, less sensitive species, tolerant species and species preferring strong eutrophication. This classification of species allows a comparison of comparable sites (e.g. alpine regions or lowlands) within Germany. However, other countries within the EU defined their own criteria for the BQE phytobenthos and are using different diatom indices such as the Indice Biologique Diatomées (IBD) in France, the Trophic Diatom Index (TDI) in Ireland or the Specific Pollution Sensitivity Index (IPS) in Belgium and Luxembourg (Commission Decision 2008/915/EC). This makes a comparison between different countries difficult. Furthermore, these metrics are purely descriptive and do not allow to relate community characteristics to chemical exposure. So far, no approach implemented in the WFD establishes causality between chemical exposure and community effects in the environment and allows a comparison between effects found in different countries.

The strength of PICT is to causally link exposure of site-specific toxicants to community-level effects. The increase of tolerance related to the respective reference community allows a comparison of studies conducted in different eco-regions and countries. This thesis showed the validity of PICT in complex field studies and the significance of PICT responses to multiple stressors and mixtures. For those reasons I would like to propose PICT as a diagnostic tool for environmental effect assessment within retrospective risk assessment.

Outlook

So far, PICT studies are limited to phytotoxic compounds and metals exerting oxidative stress. In order to broaden the application of PICT to compounds or stressors with other modes of action, new endpoints for the short-term toxicity tests have to be developed.

The study in **Chapter 4** showed that high ionic loads affect the structure and function of periphyton communities. On the basis of these results, a short-term test for ionic stress was established. Krumbiegel (2013) adapted the spectrometric method for the quantification of the proline content in fresh leaf material of plants (Bates et al., 1973) to periphyton communities. The amino acid proline serves as compatible osmolyte in organisms exposed to salinity stress, for this reason the proline content in plants or algae increases with increasing levels of salinity (Szabados and Savouré, 2009). The first PICT response to salt stress was detected for periphyton communities exposed to a concentration of 120 mM NaCl for a period of five weeks (Krumbiegel, 2013). Even though, secondary salinisation of freshwaters is a major problem worldwide (Cañedo-Argüelles et al., 2013), the sensitivity of the proline short-term test does not match the range of salt pollutions in the environment so far (e.g. of the lower River Werra; Bäthe and Cöring, 2011). Therefore, the sensitivity of the method has to be improved for an application in environmental relevant effect assessment. However, the proline short-term test might extend the application of PICT to the detection of salt stress.

Furthermore, “omics” techniques such as genomics or metabolomics might be used to detect mode of action or even compound specific stress responses of organisms. Eriksson et al. (2009) combined a meta-genomic approach with the PICT concept to study tolerance mechanism of periphyton communities to the PSII inhibitor irgarol. This study revealed that the exposure to irgarol leads to an increase of tolerance, based on the selection of high-turnover D1 proteins (PSII) in periphyton communities. Furthermore, Sans-Piché (2011) investigated the impact of short-term and long-term exposures to prometryn on the meta-metabolome of periphyton communities. Therefore, a GC-MS based metabolomics approach was established, which was sensitive and robust enough to detect toxicant-induced metabolic changes. Sans-Piché (2011) showed that metabolomics approaches might be useful to detect short-term effects of toxicants. Additionally, it was shown that toxicant-induced successions, induced by long-term exposures to prometryn, go along with global

changes of the metabolic profile of the community. Furthermore, the metabolic differences between periphyton communities exposed to prometryn and unexposed control communities could be related to different tolerance levels of the communities (Sans-Piché, 2011). As metabolomics enables the detection of stressor influences on many biochemical pathways, it might be a promising tool to reveal stressor-specific indicators on the one hand and more general impacts of various stressors on phototrophic or heterotrophic communities on the other hand.

As shown in this thesis, PICT is an effect-based monitoring tool which causally links toxicant exposure to community-level effects and has therefore the potential to be diagnostic tool within environmental risk assessment. However, a whole battery of short-term tests for stressors with different mode of action has to be developed to broaden the field of application of PICT studies. With such a (short-term) test battery, PICT could be used to identify the causes for the “bad” ecological status of many rivers. Communities sampled at sites with a “bad” ecological status and a “good” chemical status could be exposed to different “key” toxicants with different modes of action, in order to identify the type of stressor causing effects. For instance, atrazine could be used to detect effects of PSII inhibitors, norflurazon to detect effects of inhibitors of the carotenoid synthesis, methaxachlor to detect effects of lipid biosynthesis inhibitors and paraquat for oxidative stressors. This approach might be able to establish a diagnosis for adverse effects on aquatic environments.

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Curriculum Vitae

Name	Stefanie Rotter
Date of Birth	17th of July 1983
Place of Birth	Leipzig
Citizenship	German

Professional career

University

10/02 to 01/08	Study of Biology at the University of Leipzig Main subjects: soil ecology, botany, microbiology Minor subject: biochemistry
07/06 to 09/06	Project thesis at the University of Lund (Sweden) Department of Microbial Ecology ”Effects of eleven different antibiotics on bacterial growth in low and high pH soils”
04/07 to 01/08	Diploma thesis at the Helmholtz-Centre for Environmental Research Department of Effect Directed Analysis in Cooperation with the Department of Bioanalytical Ecotoxicology ”Einfluss von algentoxischen Verbindungen auf Biofilmgemeinschaften in Fließgewässern“

Project funded PhD

04/08 to 10/13	External PhD of the Technical University Bergakademie Freiberg, realisation of work at the Helmoltz-Centre for Environmental Research, Department of Bioanalytical Ecotoxicology. ”Pollution-induced community tolerance of periphyton as a tool for environmental effect assessment“

Projects:

04/08 to 01/10	EU Integrated Project: "Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on Marine and Freshwater Ecosystems and Biodiversity - MODELKEY" (511237 - GOCE)
11/11 to 05/12	Project of Exxon Mobil: "Informations- und Dialogprozess der Exxon Mobil über die Sicherheit und Umweltverträglichkeit der Fracking Technologie für die Erdgasgewinnung“
11/12 to 04/14	Project of the Environmental Agency – UBA: "Erarbeitung eines Konzepts der vergleichenden Bewertung von Pflanzenschutzmitteln unter besonderer Berücksichtigung des Vergleichs der Umweltrisiken“ (FKZ:371267406)

List of Publications

Research articles and book contributions

Rotter, S., Heilmeier, H., Altenburger, R., Schmitt-Jansen, M., 2013. Multiple stressors in periphyton – comparison of observed and predicted tolerance responses to high ionic loads and herbicide exposure. J. Appl. Ecol. 50, 1459-1468.

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Eidesstattliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.
Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:

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Weitere Personen waren an der Abfassung der vorliegenden Arbeit nicht beteiligt. Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Weitere Personen haben von mir keine geldwerten Leistungen für Arbeiten erhalten, die nicht als solche kenntlich gemacht worden sind.

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Datum, Unterschrift

“It isn't expectations that carry us forward, it's our desire to go on.”

~ Paulo Coelho, Brida