On Toxicant-Induced Succession in Periphyton Communities: Effects of Single Chemicals and Chemical Mixtures

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Abstract: This thesis deals with the effects of single chemicals and chemical mixtures on biological communities. Ecosystems are exposed to complex mixtures of chemicals, and because combination effects can occur, it is necessary to assess the joint environmental risk from co-occurring contaminants. Two concepts are available to predict joint toxicity if the concentrations and toxicities of the single chemicals are known. Concentration Addition (CA) is believed to apply for mixtures of similarly acting chemicals, whereas the opposite concept of Independent Action (IA) was developed for dissimilarly acting chemicals. Most validation efforts of CA and IA have been performed under relatively simple biological conditions using single species tests. However, because the aim is to predict the risks of mixtures to complex and variable systems in nature, it is necessary to test the applicability of CA and IA on at least a community-level of biological complexity. Natural communities contain a tremendous biological diversity and thus a multitude of potential targets susceptible to toxicant exposure. In addition, species interact and a reduced ecological fitness of one species can thereby indirectly affect other species. This has the consequence that similarly acting chemicals on a biochemical/physiological level might be dissimilarly acting in a multispecies community, and ecological interactions alone are out of scope of both CA and IA. Natural marine periphyton communities were sampled from the environment on artificial substrata and incubated over a period of ecological succession together with single chemicals and chemical mixtures. Patterns and magnitude of toxicant-induced succession in the microalgal and cyanobacterial component of the biofilm were assessed by species counts using microscopy and/or chemotaxonomic analysis of pigment profiles. The resulting toxicant-induced changes were then used to assess similarity or dissimilarity between the chemicals in their ecological mode of action, and the precision with which CA and IA could predict effects from reference mixtures of similarly and dissimilarly acting chemicals. On a biochemical level similarly acting photosystem II-inhibiting herbicides acted similarly also in the periphyton communities, and CA provided an accurate prediction for their joint toxicity. CA was equally powerful for a mixture of non-congeneric PSII inhibitors assumed beforehand to have a less similar ecological mode of action. IA was slightly better than CA for predicting the joint effect from a mixture of dissimilarly acting pharmaceuticals and personal-care products. At a mixture concentration that caused a 50% reduction in biomass, IA deviated from the observed effect only by a factor of 1.1. However, lower concentrations of this mixture caused a stimulation of biomass, a phenomenon which is outside the scope of both CA and IA. The mixture studies further strengthens the evidence that joint toxicity needs to be considered in risk assessments even if the chemicals do not cause a discernible effect on their own. Furthermore, the findings show that both CA and IA are suitable to predict risks from co-occurring pollutants on community structure. The human pharmaceutical clotrimazole is regarded as a priority pollutant to the marine environment and its ecotoxicity was specifically evaluated. Clotrimazole is a fungicide that inhibits sterol synthesis at 14α-demethylase (14DM). Periphyton communities exposed to 50 pmol/L clotrimazole had a reduced 14DM functioning, and 500 pmol/L clotrimazole had profound effects on sterol biosynthesis which coincided with a decrease in biomass. Because up to 100 pmol/L clotrimazole has been detected in the marine environment, this indicates that the current use of clotrimazole is associated with a high environmental risk.

Keywords: community ecotoxicology, periphyton, toxicant-induced succession, chemical mixtures, concentration addition, independent action, diatoms, algal pigments, algal sterols

Till Kaj och Linda

"Argh! The laws of science be a harsh mistress!"

-Bender (Futurama)

On Toxicant-Induced Succession in Periphyton Communities: Effects of Single Chemicals and Chemical Mixtures

Tobias Porsbring

This doctoral thesis was produced as a collection of papers. The aim of the introductory part is to summarize, merge and extend the knowledge in the accompanying papers. The papers are throughout the thesis referred to by their Roman numerals, given below.

Paper I. Porsbring T, Arrhenius Å, Backhaus T, Kuylenstierna M, Scholze M, Blanck H (2007) The SWIFT periphyton test for high-capacity assessments of toxicant effects on microalgal community development. Journal of Experimental Marine Biology and Ecology 349:299-312

Paper II. Porsbring T, Blanck H, Tjellström H, Backhaus T (2009) Toxicity of the pharmaceutical clotrimazole to marine microalgal communities. Aquatic Toxicology 91:203-211

Paper III. Porsbring T, Backhaus T, Johansson P, Kuylenstierna M, Blanck H. Mixture toxicity from PSII inhibitors on microalgal community succession is predictable by Concentration Addition. Manuscript

Paper IV. Backhaus T, Porsbring T, Arrhenius Å, Blanck H. Single substance and mixture toxicity of 5 pharmaceuticals and personal care products to marine periphyton communities. Manuscript

Contents

1. Preface

A complex flow of metals and synthetic chemicals through a globalized market is a fundamental property of our economy. This is required for industry, households, electronics, pharmaceuticals, transportation, industrialized agriculture and so forth. The development of new synthetic chemicals has increased greatly over the past 50 years. Depending on the definition used, there is now between 30,000 and 100,000 chemicals on the European market. As of Dec 1 2008, about 150,000 chemicals had been pre-registered in concordance with the recently adopted European chemicals regulation REACH (European Chemicals Agency 2008). Even so, REACH only cover general industrial chemicals, and not *e.g.* pharmaceuticals, biocides and food additives.

Unfortunately, some of these chemicals end up in the environment and the ecosystems we ultimately depend upon for our survival. Chemicals can accumulate in soil, water and biota and cause toxicity if concentrations become too high. This may cause local or complete extinction of species and alter ecosystem functions and cycles. In turn this jeopardize crucial ecosystem services such as biodegradation of chemicals, primary production, food supply, supply of clean water and cycling of nutrients, as well as the capacity of ecosystems to withstand and adapt to future stressors.

The role of ecotoxicology is to acquire a better understanding of the fate and effects of chemicals in ecosystems. Practical implementation of this understanding hopefully helps society to remedy environmental problems that are already here, prevent novel ones from occurring and facilitate a more efficient monitoring of ecosystems to assess their health. This objective requires a broad and diverse platform for research. Knowledge is required on the physical-chemical properties of chemicals, their transport, transformation and degradation in ecosystems, their modes of action in organisms (molecular binding sites and mechanisms reducing ecological fitness), the diversity of targets in ecosystems, and ecosystem responses to exposure.

Most ecotoxicological research concerns the effects of a single chemical on a single species. However, we require better knowledge on the effects that multiple, co-occurring toxicants exert on ecological systems with multiple, co-occurring species (*e.g.* (Arrhenius et al. 2004, Lydy et al. 2004). The aim of this thesis was therefore to test the precision and applicability of predictive concepts for describing the joint toxicity of co-occurring chemicals, on a community level of biological organization. Natural periphyton communities sampled from the marine environment were used as test material.

List of Abbreviations used throughout the Thesis

BCDI - Bray-Curtis Dissimilarity Index

CA - Concentration Addition

HPLC - High Pressure Liquid Chromatography

IA - Independent Action

MDS - Multi-Dimensional Scaling

QSAR - Quantitative Structure Activity Relationship

TIS - Toxicant-Induced Succession

2. Background

2.1. Complex Environmental Exposure

Ecosystems receive mixtures of structurally and toxicologically diverse chemicals through atmospheric deposition, run-off from agricultural and urban areas, direct emissions, sewage-treatment-plant effluents etc. For example, co-occurring pesticides in receiving streams from agricultural discharge are the rule rather than the exception. A survey conducted by the U.S. Geological Survey revealed that more than 50% of all stream samples from surface waters throughout the continental United States contained five or more pesticides (U.S. Geological Survey, 1998). In a targeted investigation for 13 common herbicides and their transformation products in surface waters in the corn belt of the Midwestern United Stated most samples contained a mixture of more than 10 different compounds (Battaglin et al. 2003). A total of 57 different pesticides were detected by (Adielsson et al. 2006) in the surface-waters of seven Swedish streams from different agricultural areas; no single sample contained less than two (detectable) pesticides, and individual samples from streams in the county of Skåne contained between 10 and 22 different pesticides (Adielsson et al. 2006).

Discharge from livestock rearing, storm water and sewage-treatment-plant effluents from urban, industrialized zones means additional contamination of surface waters with a diverse cocktail of chemicals. A survey on the occurrence of human and veterinary drugs (including antibiotics), natural and synthetic hormones, detergents, plasticizers, insecticides and fire retardants in surface waters of 139 streams in the United States showed that half of the streams contained at least seven of these chemicals, while one-third of the streams contained 10 or more (U.S. Geological Survey, 2002). Roberts and Thomas (2006) detected seven out of 13 surveyed pharmaceuticals in the surface waters of the lower Tyne catchment (Roberts & Thomas 2006). Also, toxicants released from antifouling paints directly contaminate marine coastal and estuarine areas where they co-occur with other chemicals of urban and agricultural origin (Konstantinou & Albanis 2004).

Once present in ecosystems these chemical mixtures are in contact with a profoundly rich and complex biological environment. Coastal ecosystems for example contain exceptionally diverse communities of prokaryotes, microalgae, macroalgae, protozoans, macroinvertebrates, fish and mammals. The high diversity can be attributed to the heterogeneous and variable conditions that are created by the contact with a shallow littoral

zone characterized by light peneration to the bottom and land that provide an input of organic and inorganic material and nutrients from rivers and runoff.

The genetic diversity of the biosphere is much greater than believed only a few years ago. Some insights in the diversity of for example microbiota have recently become possible thanks to large-scale analysis of genetic material. Based on data from so-called shotgun sequencing of genetic material from environmental samples collected from across the worlds oceans, a total of 10,205,380 proteins were predicted which add tremendous diversity to known protein families (Yooseph et al. 2007). Furthermore, the overall encountered diversity is steadily increasing. In a recent analysis of a microbial community sampled from the Pacific Ocean, it was found that approximately 50% of the detected genes were new and unique compared to those detected in previous ocean metagenomic surveys (Frias-Lopez et al. 2008).

2.2. Community-Level Ecotoxicology

Experimental toxicity testing is a cornerstone in ecotoxicology, and can be performed with an almost limitless number of potential strategies. However, the strategy used, *i.e.* type of organism(s), endpoint and duration of exposure is best decided from the question(s) being asked. In one extreme end whole lakes have been manipulated to study the effects of stressors on ecosystem structure and function (Schindler 1996, Pelley 2003). Although this approach obviously provides direct evidence on the reactions of perturbed ecosystems and the involved processes, whole ecosystems are too large, too slowly responding and too complex to allow any extensive testing of toxicants or hypotheses (and there may not be enough lakes available that the public would be willing to sacrifice). Ecotoxicological research is therefore devoted to more "convenient", less complex subcomponents of the biosphere. For example, single species tests with organisms representative of various trophic levels is the current standard approach for obtaining toxicity data for ecological risk assessments.

Community-level studies are performed with systems where several species are simultaneously present and interacting. The interactions can be more or less direct, and involves *e.g.* predator-prey interactions, or competition for resources such as space, light and nutrients. The activities of each organism also influence the physical-chemical environment of the community, and thereby affect other community members. Although ecosystems are the collective result of interactions between much more diverse populations than can be included in a community-level study, communities are good samples of ecosystems because they contain ecosystem properties such as a high biodiversity, species interactions and fundamental ecological processes such as nutrient cycling and flows of energy (Pratt & Cairns 1996).

The inclusion of several species in the study has two major implications. Firstly, the outcome after exposure is the integrated response of a whole range of species, often from several organism groups and representative of a whole range of species sensitivities. Compared to tests with single species, this much larger sample of nature thus provides a more accurate indication on the sensitivity of the natural system(s) that the aim is to protect (Cairns et al. 1990). Secondly, the communities display a whole-system response to exposure in which interactions between species and indirect effects influence the outcome over the course of an ecological succession. Structural changes thereby occur and can be observed without directly knowing which metabolic process that was targeted by the toxicant or which species that was the most sensitive. The high ecological realism means that observations can be extrapolated to

the real environmental situation with a comparably high precision. The nature of change in structure and function provides information on toxic effects on valuable ecosystem properties such as ecosystem function, species richness and biodiversity. The outcome indicates whether the community has an inherent functional redundancy to the stressor, *i.e.* capacity to uphold function because sensitive species may be replaced by others.

Examples of aquatic community-level tests include Mixed Flask Cultures, which combine microbial communities from a variety of natural sources (Shannon et al. 1986)), semi-gnotobiotic standardized aquatic microcosms (SAMs) which contain defined algal and animal species from stock cultures with their associated microorganisms (Taub et al. 1986). Experimental ponds and streams with added sediment and macrophytes (Landner et al. 1989, Belanger 1997, Wendt-Rasch et al. 2003), in situ enclosures in aquatic ecosystems; either top-open so-called limnocorrals (Kaushik et al. 1986, Wängberg et al. 1991), or mesocosms that completely enclose a portion of the water column (Landner et al. 1989, Larsen et al. 2003).

2.3. Toxicant-Induced Succession

Ecological succession is a central concept in ecology, and provides a theoretical framework for describing the process by which the species structure and the flows of nutrients and energy in biological communities change as they progress through developmental stages. That species changes in *e.g.* forest clear cuts and bogs follow reproducible patterns was described by natural scientists from as early as the 17th century, but it was the Danish botanist Eugene Warming (considered by many as one of the "fathers" of ecology as a science) that first formulated a number of "laws of succession" in his work Plantesamfund in 1891. Pioneering and important work for refining the concept was later made in the late 19th and early 20th century by *e.g.* Henry Cowles and Frederick Clements.

Both Cowles and Clements argued that communities are self-organizing entities. On one hand, the physical environment at the site influences which species that may endure, and the rate and pattern with which succession may occur. On the other hand, the organisms of the community themselves modify their microenvironment, thereby altering the preconditions, creating new niches and allowing for novel species to enter. Each developmental stage of the community is therefore inevitably followed by another as the community ages.

A rapidly progressing ecological succession can be readily observed in microbial communities, including periphyton. Dennis C. Cooke (Cooke 1971) reviewed numerous investigations performed with "microecosystems" of microbial communities sampled from *e.g.* sewage oxidation ponds. If nitrogen and thiamine were added to the communities, a rapid photoautotrophic succession started. If proteose peptone was added, a bacterial bloom occurred with the induction of a heterotrophic succession, eventually followed by a phototrophic succession within days. As postulated by *e.g.* Eugene Odum (Odum 1969), fundamental shifts in energy flows occur in any ecosystem as succession proceeds (Fig. 1). In early stages of succession, primary production exceeds the rate of respiration. The system thereby has a high net production, which causes an accumulation of biomass. However, as the community "ages" and accumulates biomass, the rate of respiration increase and thereby energy is re-directed to maintenance. The energy cost of standing biomass increases, and the overall activity of the organisms in the community is redirected towards a more efficient

recycling of matter. From this bioenergetic aspect, a succession in a microbial ecosystem is very similar to a much slower succession *e.g.* in a larger forest ecosystem (Fig. 1). The periphyton communities used for this thesis were sampled after 7 to 9 days colonization, and incubated with toxicants for another 4 days. Hence, these communities were tested in the early stages of development (Fig. 1).

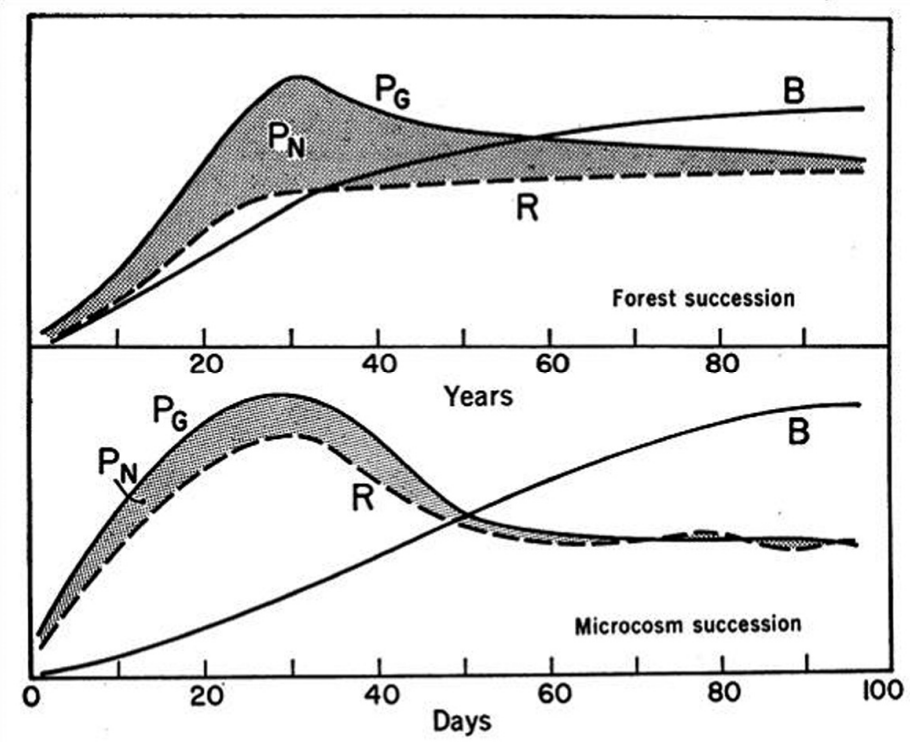


Figure 1. Comparison of the energetics of succession in a forest and a laboratory microcosm, PG, gross production; PN, net production; R, total community respiration; B, total biomass. From Eugene P. Odum (1969) The strategy of ecosystem development. Science 164:262-269. Reprinted with permission from The American Association for the Advancement of Science.

Sensitivities to environmental variables and other stressors vary between species, which is the major cause of species replacement over a natural succession. An organism can only persist, *i.e.* compete for resources and reproduce, if it can tolerate the conditions it is subjected to. The sensitivity to toxicants among microalgal species can differ several orders of magnitude (Blanck et al. 1984, Nystrom et al. 1999), and no single species is consistently most sensitive or most tolerant to different toxicants (DeLorenzo et al. 2001). Toxicant exposure can therefore cause a considerable change in a microalgal community, whereby the number of tolerant species indirectly increases because the abundances of sensitive ones decrease. The succession of the exposed community is thereby shifted into one which becomes governed by the toxicant, a Toxicant-Induced Succession (TIS) (Blanck 2002). Increasing concentrations of the toxicant increase its selective force relative to all other factors that influence the community, and cause the structure of the exposed community to become increasingly dissimilar over time from that of an unperturbed community. The pattern of species change will be determined according to the direct selection profile of the toxicant in the community, as well as indirect effects resulting from changes in species interactions.

3. Problems & Aims

Most environmental risk assessments are made only for single chemicals one at a time. Although the environmental concentrations of most individual chemicals are generally low enough not to be of concern, the overall chemical presence is of concern because of risks for combination effects (Cairns et al. 1986, Calamari & Vighi 1992, Burton Jr 1999, Backhaus et al. 2000a, Backhaus et al. 2000b, Clements et al. 2001, DeLorenzo et al. 2001, Faust et al. 2001, Walter et al. 2002, Boxall et al. 2003, Cleuvers 2003, Faust et al. 2003, Vighi et al. 2003, Arrhenius et al. 2004, Eggen et al. 2004, Lydy et al. 2004, Richards et al. 2004, Asp & Kreuger 2005, Junghans et al. 2006, Backhaus et al. 2008, Altenburger & Greco 2009).

The environmental fate of chemicals depends on their physical-chemical properties, which governs their distribution between different environmental compartments, transfer in the food-chain, speciation, transformation (biotic and abiotic) and ultimately degradation. Along with continuously varying release rates of different chemicals to the environment, this has the implication that environmental mixtures never are static in their concentrations or compositions. The possible number of different types of environmental mixtures over space and time is therefore enormous, and the testing of each and every one for prospective hazard assessments is impossible.

One way to circumvent this problem is instead to predict the toxicity of a mixture using concentrations and toxicities of the individual toxicants as input. Concentration Addition (CA) and Independent Action (IA) are two concepts that have received much attention as appropriate tools for this purpose (*e.g.* (Boedeker et al. 1992, Deneer 2000, Junghans et al. 2006, Belden et al. 2007, Altenburger & Greco 2009). Concentration Addition is believed to apply for mixtures of toxicants with a similar mechanism of action, whereas IA is believed to apply for dissimilarly acting toxicants (see sections 2.9 and 2.10 for further details).

The large majority of studies on the ecotoxicological applicability of CA and IA has been made with single-species tests, involving endpoints such as physiology, lethality or reproduction. (Altenburger et al. 1993) reviews 28 mixture studies with two or more compounds, of which only one was made at the community-level (perihyton) (Cairns et al. 1990). Numerous additional studies have since then been made on a single species level (*e.g.* (Faust et al. 1993, Faust et al. 1994, Posthuma et al. 1997, Altenburger et al. 2000, Backhaus et al. 2000b, Faust et al. 2001, Fernandez-Alba et al. 2002, Lock & Janssen 2002, Walter et al. 2002, Faust et al. 2003, Junghans et al. 2003, Altenburger et al. 2004, Altenburger et al. 2005, Brian et al. 2005, Olmstead & LeBlanc 2005, Belden et al. 2007, Koutsaftis & Aoyama 2007)), with main focus on various types of chemical mixtures or different test assays.

However, most single-species tests lack what has been coined ecological and pollutant realism (Blanck et al. 1978) (as cited in (Cairns 1981)). That is, organisms in exposed ecosystems do not live in isolation, and stressor effects on ecosystem structure and function is therefore the integrated response of many different species and the resulting changes in their interactions. Single-species tests are criticized (*e.g.* (O´Neill et al. 1982, Cairns 1984, Cairns et al. 1986, Joern & Hoagland 1996, Ahlers & Diderich 1998, Burton Jr 1999, Chapman 2002, Solomon & Sibley 2002, Eggen et al. 2004, Schmitt-Jansen et al. 2008))

because they cannot reproduce the complex effects from pollutants on the ecological fitness of organisms in structured ecosystems, or the metabolism and transport of toxicants. Also, the toxicity of the chemical may differ from that of the real environmental situation because of an unrealistic exposure regime in the test, and the sensitivity of the chosen test organism may be a poor representation of all the organisms in nature. Because the ultimate aim is to perform risk estimates for variable and complex systems in nature, it has been pointed out that it is necessary to do mixture studies under more ecologically realistic conditions, with endpoints coupled to the ecological fitness of organisms (Barata et al. 2007), or in a multispecies setting (Calamari & Vighi 1992, Newman et al. 2000, Backhaus et al. 2003, Vighi et al. 2003, Arrhenius 2005, de Zwart & Posthuma 2005, Belden et al. 2007). Natural communities change along spatiotemporal gradients, and such factors as species interactions, trophic state, structural heterogeneity and history of pollution are all of importance for the outcome of perturbations (Clements et al. 2001, Wendt-Rasch et al. 2004).

As summarized in Table 1, a range of complexities which have the potential to impair the precision and applicability of both CA and IA are introduced when an ecotoxicological study is transferred from the single-species level to the community level (see also section 4.5 for further discussion).

Table 1. Factors that contribute to ecological realism in community ecotoxicology, but potentially may impair the precision in mixture toxicity predictions.

1. Biotic and abiotic interaction between toxicants
2. A considerable number and diversity of potential targets
3. A high diversity of biotransformation and degradation processes.
4. Variations in community structure over spatiotemporal scales.
5. A toxicant-induced succession, the outcome of which is determined by direct and indirect effects from changes in species-interactions, as well as community structure at the onset of exposure.

Studies that specifically addressed the performance of CA and IA on a community level include (Nirmalakhandan et al. 1997), who demonstrated that the joint toxicity from a range of synthetic organic chemicals were additive in a gnotobiotic test system with 12 microbial strains. (Arrhenius et al. 2004) and (Backhaus et al. 2004a) used natural microalgal communities sampled from the environment and assessed both CA and IA with reference mixtures of similarly and dissimilarly acting toxicants respectively. For the mixture of similarly acting toxicants, the deviation of the CA-predictions never exceeded a factor of 2 (at EC50, *i.e.* 50% inhibition of photosynthesis), regardless if the predictions were made in one season and the mixture tested in another season or even year. In fact, this precision of CA predictions is comparable to that achieved in single species tests (Arrhenius et al. 2004). IA accurately predicted the toxicity of the mixture of dissimilarly acting toxicants. However, despite that CA conceptually would be the wrong model for this case, it overestimated the toxicity by less than a factor of 4 at the 50%-effect level of the mixture. This precision has been considered sufficient for hazard assessments (Backhaus et al. 2004a). Also, (Knauert et al. 2008) concluded that the toxicity from a mixture of photosystem II inhibitors on phytoplankton photosynthesis was in concordance with CA after exposure in mesocosms.

These studies were performed with relatively short exposure times that excluded the influence of ecological processes such as competition to influence the outcome. Also, in a short-term test the endpoint measured (*e.g.* photosynthesis, respiration) needs to be coupled to the physiological process(es) that are targeted by the chemical, which limits the scope of the study. However, no study has yet investigated whether CA and IA have a general validity for describing mixture effects on the succession of species in communities. For that, structural endpoints such as species composition and growth are required. The general aims of this thesis were to evaluate the predictive concepts of CA and IA on a community-level of biological organization, using chronic exposures over an ecological succession.

This involved the following specific objectives;

1. Development of a novel high-capacity test approach (the SWIFT test) for periphyton communities that included an ecological succession.
2. Development of a methodology for rapid measurements of pigment profiles as a surrogate to microscopic species counts.
3. Development of methodologies for quantitative and qualitative descriptions of toxicant effects on community structure (species composition and pigment profiles).
4. Evaluation of the concept of CA with reference mixtures of similarly acting toxicants.
5. Evaluation of the concept of IA with mixtures of dissimilarly acting toxicants.

These aims were intended to fill knowledge gaps as indicated in Figure 2.

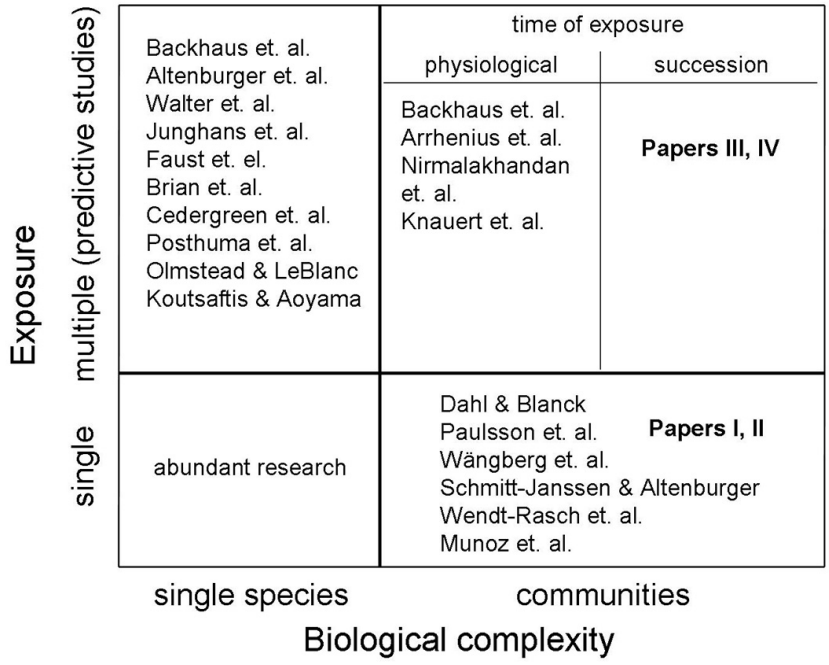


Figure 2. Overview of ecotoxicological research with emphasis on mixtures and/or communities.

4. Approaches

4.1. Investigated Toxicants

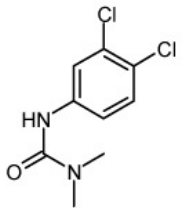
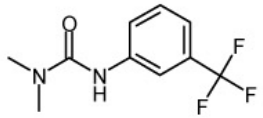
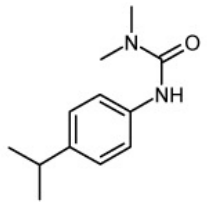
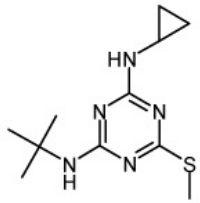
A total of 12 chemicals covering a broad spectrum of different mechanisms and modes of action were investigated for this thesis (Table 2). The list comprised a useful basis for designing sets of reference mixtures of similarly or dissimilarly acting toxicants. Such mixtures are designed to fulfill the presumptions of the CA and IA concepts and can thus be used to evaluate their predictive power. In addition, many of the chemicals are used in society for various applications and can be expected to occur as part of complex environmental mixtures in aquatic ecosystems. The chemicals thereby constitute a designed subsample of the complex exposure scenarios that occur in reality.

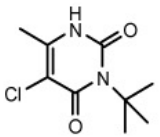
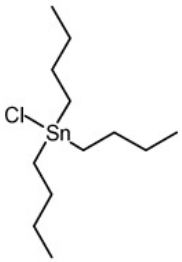
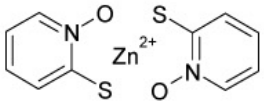
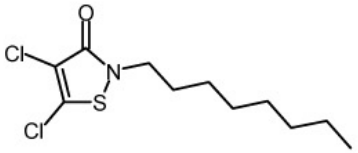
The compounds can roughly be categorized into six different groups according to their use: antifouling agents, pharmaceuticals, personal-care products, biocides, herbicides and pesticides. Some have a broad use or represent a class that has a broad use and can therefore be encountered in more than one category. The common denominator is that they all are designed to affect biological targets and provoke a physiological effect in organisms. Thereby they influence a specific physiological process for therapeutic reasons (pharmaceuticals), or induce a toxic response (*e.g.* death or growth inhibition) either through a specific or multiple, unspecific mode of action.

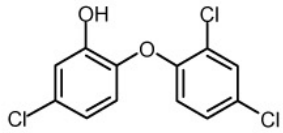
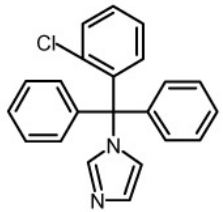
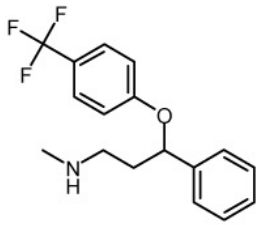
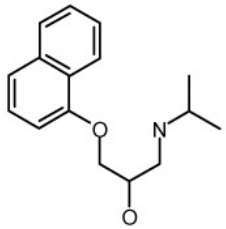
Diuron, fluometuron, irgarol, isoproturon and terbacil are photosystem II (PSII) inhibitors which is a common class of agricultural herbicides. These compounds bind to the D1-protein of PSII and thereby inhibit photosynthesis (Moreland 1980, Bowyer et al. 1991, Jansen et al. 1993). Drainage from agricultural areas can exert a significant ecotoxicological pressure on estuaries (Steen et al. 1999). Out of 57 pesticides encountered in the surface water of Swedish agricultural streams in 2005, 12 were PSII inhibitors (Adielsson et al. 2006). Isoproturon was the compound that most frequently exceeded its water quality standard (1.5 nmol/L), being detected at a maximum concentration of 10 nmol/L (Adielsson et al. 2006). Irgarol and diuron are used as so-called booster biocides in antifouling paints. Both compounds have been identified as posing a severe hazard to non-target organisms (e.g. (Dahl & Blanck 1996b, Lambert et al. 2006, Blanck et al. 2009) and as a result their use is restricted in many countries (Konstantinou & Albanis 2004).

Clotrimazole, fluoxetine (Prozac) and propranolol are human pharmaceuticals and are emitted to the marine environment via municipal sewage-treatment plant effluents. Pharmaceuticals have in the last few years been identified as potentially hazardous to the environment (Daughton & Ternes 1999, Boxall 2004, Ferrari et al. 2004, Fent et al. 2006). This is mainly because: (1) they are designed to induce a pharmacological response in organisms, (2) they are designed to have a low biodegradability in order to resist degradation in the human digestive system, and can therefore often pass sewage treatment plants relatively unaffected, and (3) they are used continuously, in practice subjecting receiving waters to a chronic, low-dose exposure. However, it should be noted that release of pharmaceuticals in effluents at manufacturing sites can be as high as to subject receiving waters to acute, therapeutic levels. In an effluent connected to pharmaceutical manufacturers in the Patencheru region, India, Larsson and co-workers (Larsson et al. 2007) detected several drugs of which 11 were present in concentrations exceeding 100 ug/L; the antibiotic ciprofloxacin

Table 2. Chemical structure and (known) ecotoxicological properties of the pesticides, herbicides, biocides, pharmaceuticals and personal-care products tested in this thesis.

Compound	CAS-Nr.	Molecular structure	Use	Mechanism of Action	Paper #
diuron (phenylurea)	330-54-1		herbicide, antifouling agent	inhibits electron transport at D1-protein of PSII[1]	III
fluometuron (phenylurea)	2164-17-2		herbicide	inhibits electron transport at D1-protein of PSII[1]	III
isoproturon (phenylurea)	34123-59-6		herbicide	inhibits electron transport at D1-protein of PSII[1]	III
irgarol (*s*-triazin)	28159-98-0		antifouling agent	inhibits electron transport at D1-protein of PSII[1]	I, III

Compound	CAS-Nr.	Molecular structure	Use	Mechanism of Action	Paper #
terbacil (uracil)	5902-51-2		herbicide	inhibits electron transport at D1-protein of PSII[1]	
TBT	1461-22-9		antifouling agent, wood preservative	affects chloroplast and mitochondrial function, uncoupler[6] (denotes a shuttling of protons across energy transducing membranes which collapse the chemiosmotic gradient. This uncouples electron transport from oxidative phosphorylation by adenosine triphosphate (ATP) synthase), interferes with cytochrome P450- dependent processes[7]	I, III
zinc-pyrithione	13463-41-7		antifouling agent, fungicide in anti-dandruff shampoo	affects chloroplast and mitochondrial function, uncoupler[8]	IV
DCOIT	64359-81-5		antifouling agent, members of the same chemical group used as preservatives in personal-care products	reacts with thiol-containing proteins[9], might cause cascades of radicals[9]	I, III

Compound	CAS-Nr.	Molecular structure	Use	Mechanism of Action	Paper #
triclosan	3380-34-5		antibacterial agent in a range of household and personal-care products	inhibits lipid biosynthesis in bacteria (at the enoyl-acyl carrier protein reductase (ENR))[10], suggested multiple mechanisms of action in algae including uncoupler[11]	IV
clotrimazole	23593-75-1		human pharmaceutical (antifungal)	Inhibits cytochrome P450 dependent sterol synthesis at 14α-demethylase[2], inhibits brassinosteroid synthesis[3]	II, IV
fluoxetine	59333-67-4		human pharmaceutical (antidepressant)	*inhibits serotonine re-uptake[4], inhibits cytochrome P450 activity in humans[5]	IV
propranolol	525-66-6		human pharmaceutical (blood pressure lowering agent)	*beta-adrenergic receptor antagonist[4]	IV

CAS = Chemical Abstracts Service. *unknown mode of action in algae. References to mechanisms and modes of action: Taylor et al. 1983[2], Collier et al. 1990[9], Bowyer et al. 1991[1], Otton et al. 1993[5], Winter et al. 1997[3], Gi et al. 1998[7], Heath et al. 1999[10], Patrick 2001[4], Hunziker et al. 2002[6], Bragadin et al. 2003a[8], Bragadin et al. 2003b[6], Franz et al. 2008[11].

was detected in concentrations up to 31 g/L, and a one day discharge at this concentration is equivalent to the total amount used of this compound in all of Sweden over 5 days (Larsson et al. 2007). The consequences are severe for the aquatic life and human health, and the risk for the development of multi-resistant pathogens is obviously evident (Babu & Yadav 2007, Larsson et al. 2007). Considering that the products are to a large extent exported, refined and marketed e.g. within the EU, this clearly highlights that it is not sufficient in the current globalized economy to regulate only the end product and only with respect to the home market.

Clotrimazole is classified as persistent and toxic, and the OSPAR-commission (the Convention for the Protection of the marine Environment of the North-East Atlantic) has registered it for priority action, meaning that its use and environmental risk is under continuous review (OSPAR-Commission 2006). The compound belongs to the class of azole fungicides. In addition to their uses as antimycotics in medicine or personal-care products, azoles are applied as fungicides in huge amounts in agriculture (in average 10 mg/m^2 plant surface annually) and many are long-lived in soil and water (Hof 2001). Clotrimazole for example has an estimated half-life in the environment of more than 60 days, and is therefore considered to be persistent in a regulatory sense (OSPAR-Commission 2005). Clotrimazole was specifically investigated in Paper II of this thesis. The biosynthesis of algal sterols was found to be remarkably sensitive to this chemical, and was affected at a clotrimazole concentration of 50 pmol/L. The modes of action in algae of propranolol and fluoxetine are unknown, since these chemicals should only affect organisms with a central nervous system (table 1). However, fluoxetine also inhibits cytochrome P450-mediated metabolism of xenobiotics in human liver cells (Otton et al. 1993). The cytochrome P450-superfamily is ubiquitous in nature and has a highly conserved function of phase 1 oxygenation of both exogenous and endogenous organic compounds (Lewis et al. 1998), and might thus constitute the site of fluoxetine action in algae.

Triclosan and zinc-pyrithione are used in personal-care products. Triclosan is a broad-spectrum bactericide in *e.g.* household-products and toothpaste, whereas zinc-pyrithione is an active ingredient in antidandruff shampoo. These compounds are therefore also released into the environment via sewage-treatment plants. In addition zinc-pyrithione is also used in antifouling paints and is thereby directly emitted into the marine environment (Voulvoulis et al. 2002). The exact mode of action of triclosan in algae is unclear, but multiple modes of action have been suggested (Franz et al. 2008).

TBT and DCOIT are antifouling agents. The use of TBT in antifouling paints is banned globally from September 2008 according to the International Convention on the Control of Harmful Anti-fouling Systems on Ships because of its adverse effects on non-target organisms. However, TBT is very persistent and TBT-contaminated sediments will constitute a high risk to the marine and estuarine environment for years (Antizar-Ladislao 2008). DCOIT is the main antifouling ingredient of Sea-Nine 211™, and belongs to the class of Kathon biocides which also are used as preservatives in personal-care products (so-called *isothiazolinones*).

4.2. Periphyton Communities

Aquatic microbial communities are of a high ecological importance as they make substantial contributions to primary production, nutrient cycling (Paerl 1998) and the removal of pollutants from the water column ((Sabater et al. 2002) and references therein). Periphyton is a common name for microbial communities that develops on surfaces, and consists of a range of autotrophic and heterotrophic species associated with organic material (foremost polysaccharides) excreted from the microalgal component and other matter sorbed from the water column.

Periphyton communities have since long been regarded as a sensitive and ecologically relevant indicator of water quality (Patrick 1979), which still is manifested in the outlined monitoring strategy of European rivers under the Water Directive (Prygiel & Haury 2006). The diatoms of natural periphyton communities sampled in streams usually display a canonical distribution (Patrick 1979). This means that a few species are very dominant, some more species have intermediate abundances, whereas most species only have low abundances and are only infrequently encountered in samples (Preston 1980). Deviations from this distribution may be caused by perturbations, and have been coupled to *e.g.* exposure to pollutants (Patrick 1979).

To utilize surfaces is a niche common to many aquatic microorganisms, and substrates that are deployed in streams or at sea quickly become colonized. The colonization usually proceed in a particular, but not necessarily obligatory sequence (Hudon & Bourget 1981, Hoagland et al. 1982, Nayar et al. 2005): Bacteria usually are the first colonizers (earliest within hours), and with a strong dependency on depth, water movement, light conditions, temperature, and water quality (*e.g.* nutrients, salinity, pH) photoautotrophic, oxygenic microalgae and cyanobacteria follow within days or weeks.

The development of the microalgal community is spatially and temporally dynamic throughout its colonisation and growth, and is analogous to the succession of higher plant communities (Hoagland et al. 1982). Early stages contain many "horizontal" species situated close to the surface of the substrate, but as the biofilm density increase, "vertical" species become more dominant (Hudon & Bourget 1981, Hoagland et al. 1982). These vertical species possess *e.g.* mucus stalks that allow them to rise above the biofilm surface, indicating that space and light availability are governing the development of the community (Hudon & Bourget 1981, Hoagland et al. 1982). Depending on algal and bacterial densities the periphytic biofilm eventually also becomes inhabited by a species-rich community of obligatory heterotrophic protists such as ciliates and amoebas (Cairns et al. 1979), and also metazoan meiofauna like nematodes and crustaceans.

Interactions in the biofilm between different species, trophic levels and the surrounding environment cause a directional change in the relative abundances of species, and the periphyton community thereby display an ecological succession (see section 2.3) (Hoagland et al. 1982). The rate of succession (*i.e.* the rate of species change) is high since the vast majority of the biota is microorganisms with short generation-times (see section 2.3). Hence, periphyton communities are good experimental models for ecologists and ecotoxicologists. This is not only because of their microscopic size which makes large containers redundant, but also because they show critical events of communities such as

species change and change in flows of matter and energy that would take far too long to be observed in systems with longer-lived organisms (Hoagland et al. 1982).

Ecotoxicological investigations with periphyton are diverse. Tests where community structure (*i.e.* species composition, biomass) has been used as an endpoint include the use of continuous flow-through systems (Dahl & Blanck 1996b, Paulsson et al. 2000, Eriksson et al. 2009) static or semi-static microcosms (Wendt-Rasch et al. 2004, Schmitt-Jansen & Altenburger 2005), closed or open model stream ecosystems (Belanger 1997, Munoz et al. 2001), artificial substratum treated with toxicants and deployed in situ in the ecosystem (Dickman 1969), and limnocorrals (Wängberg et al. 1991).

Periphyton communities tested in SWIFT (see section 4.3) are acquired by passive sampling on artificial substrata (1.5 cm^2 glass discs) deployed at sea or in streams for 7 to 9 days. The strategy to have whole communities develop in the ecosystem for subsequent manipulation in the laboratory has its own standing in community-level ecotoxicology. For example, John Cairns Jr used polyurethane foam deployed in ponds as substrate and tested the capacity of the protozoan community to colonize new surfaces under toxicant exposure under a static exposure regime in aquaria (Cairns et al. 1986). (Munoz et al. 2001) used glass substratum for sampling stream communities that were transferred to artificial laboratory streams.

The sampling technique used for SWIFT was outlined by (Blanck 1985) inspired by the diatometer approach (Patrick 1967) and the sampling of protozoan communities by Cairns (Cairns et al. 1979), and later developed to its current state by (Blanck & Wängberg 1988, Molander & Blanck 1992, Blanck & Dahl 1996). It has previously been used to acquire natural periphyton communities for short-term tests with arsenate (Blanck & Wängberg 1988), herbicide mixtures (Arrhenius et al. 2004) and antifouling agents (Arrhenius et al. 2006, Eriksson 2009) and to sample periphyton in a gradient of environmental contamination to assess Pollution-Induced Community Tolerance (PICT) from *e.g.* TBT (Blanck & Dahl 1996), irgarol (Dahl 1996, Blanck et al. 2009), Zn (Admiraal et al. 1999, Blanck et al. 2003), and Zn and Cd (Lehmann et al. 1999).

Although a periphyton community holds a number of organism groups, the phototrophic, oxygenic microalgal and cyanobacterial component was used for the studies. The phototrophic community was characterized by means of microscopy to determine species composition and analysis of pigment profiles that reflect taxonomy, physiological status and biomass. It should however be noted that effects on other organisms in the community, *e.g.* grazers and bacteria might indirectly affect the response of the phototrophic community, *e.g.* through an altered grazing pressure and/or metabolism of the test compounds.

4.3. The SWIFT Periphyton Test

The rationale of the SWIFT test is outlined in Figure 3 and comprises four phases: (1) passive sampling of periphyton communities on glass substratum (1.5 cm^2) deployed for 7 to 9 days at sea, (2) semi-static incubation with concentration series of toxicants over 4 days during which the periphyton communities undergo an ecological succession. (3) Characterization of community structure and physiological status, and (4) descriptions of concentration-effect relationships and patterns of toxicant-induced change.

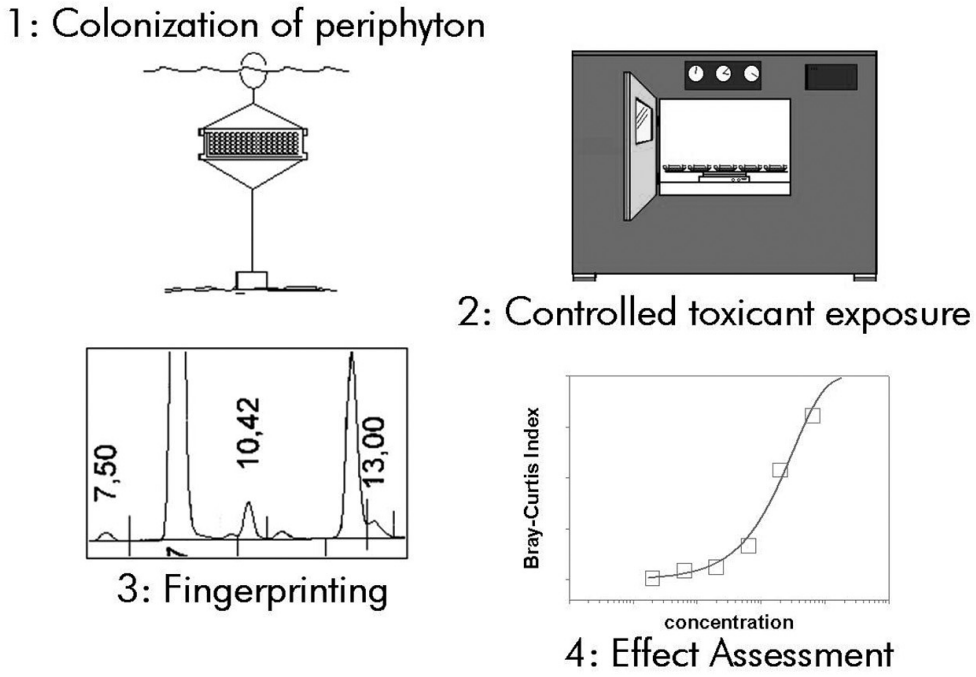


Figure 3. Schematic overview of the SWIFT test procedure.

The succession of the communities over the SWIFT test phase means that important consequences of toxicant exposure can be observed, and the reasons to include a TIS in the test were several. Firstly, TIS is sensitive because it occurs at toxicant exposure high enough to affect the ecological performance of the most sensitive species. That also means that the TIS is ecologically relevant, because it integrates the responses from organisms that at the same time are subjected to a range of additional, natural stressors such as grazing and competition for *e.g.* nutrients, light and space in a natural multispecies setting. Furthermore, TIS is independent of the mode of action of the toxicant, in the sense that it occurs regardless of which biochemical or ecological process(es) that are affected, as long as susceptible receptors are present. Finally, the resulting community structure after a period of TIS provides information on the pattern of toxicant-induced change, as dependent on which species that were targeted.

The methodology of the SWIFT test was attained by modifying a more elaborate and time-consuming test system with flow-through microcosms (Blanck & Wängberg 1988) (Table 3). SWIFT was developed in order to allow for a higher experimental capacity. This was required for repeated testing of several single chemicals and chemical mixtures, across a range of concentrations.

To accomplish a high test capacity SWIFT had to have a comparably short test duration. Even though periphyton communities display a rapid succession typical of microbial communities, one week of colonization at sea and 4 additional days of toxicant exposure only represent a limited part of their development (see Fig. 1). A cornerstone in the SWIFT test strategy was therefore to enrich the test medium with nutrients (phosphate and nitrate), because a high activity and a high microalgal growth rate is probably a prerequisite for changes in community structure to occur and a TIS to be detected over the 4 days. The nutrient enrichment maintain microbial communities in a stage of high net production and with an

increased rate of phototrophic succession (Cooke 1971, Fairchild & Lowe 1984). Also, the use of relatively young and thin periphyton biofilms probably minimizes spatial constrains on growth, thus allowing for various structural responses to be well expressed.

Table 3. Difference in rationale between the SWIFT test and the flow-through microcosms.

Property	SWIFT	Flow-through microcosms
test duration	4 days	several weeks
test medium	seawater + nutrients	seawater
toxicant delivery	daily renewal	continuous
periphyton community succession	communities pre-colonized in ecosystem, no invasion of species during test	continuous colonization and invasion of species throughout entire test
volume	0.3 litre	22 litre
max. periphyton discs (replicate communities) per treatment	15	170
light regime	continuous	day/night
labour requirements	low	high
capacity	high	low
flexibility	high	low

Analysis of species composition through species counts provide detailed information on the pattern of toxicant-induced change in the microalgal and cyanobacterial community. However, this is a very time-consuming analysis that also requires a skilled and experienced taxonomist. The late Dr. Mats Kuylenstierna performed the analyses of community composition for Papers I and III. HPLC (High-Pressure Liquid Chromatography) analysis of community pigment profiles was therefore investigated and adopted as an alternative, much faster surrogate measure of community structure that integrate species composition (Mackey et al. 1996), physiological status (Schluter et al. 2000) and biomass.

The performance of the SWIFT test was assessed in Paper I by comparisons to results obtained in the flow-through microcosms. The validation comprised four distinct properties of the test, in relation to its desired properties: (1) the biological complexity of the periphyton communities established in the ecosystem for testing, (2) the influence of the controlled, semi-static incubation on the succession of the communities, (3) the responsiveness of the communities to toxicant exposure with respect to their sensitivity, and with respect to the pattern of toxicant-induced changes in species composition, and (4) the sensitivity and resolution of pigment analysis to detect changes in community structure, and detect similarity and dissimilarity between toxicants in their ecological mode of action.

4.4. Concepts for Mixture Toxicity Predictions and Assessment

4.4.1. Concentration Addition

The theoretical foundations of CA dates back to the work by (Loewe & Muischnek 1926, Loewe 1927) in the 1920s (Altenburger et al. 1990). CA has gained widespread recognition because of it plausible pharmacological understanding (*e.g.* (Faust et al. 2001)). The concept is believed to constitute a good reference for the joint toxicity of similarly acting toxicants, which in a strict sense can be interpreted as the toxicants having a common biochemical target site (Boedeker et al. 1992).

As implied by its name, the toxicity of the mixture is determined by the sum of the concentrations of the single toxicants. However, the toxicants are first scaled to a common effect level to denote a so-called toxic unit (TU), which is the ratio of the concentration (c) of a single toxicant in the mixture to the concentration required to provoke an effect of x% (EC_x) if it had been applied singly:

$$TU = \frac{c}{EC_x} \qquad (1)$$

If the toxicity of the mixture conforms to CA, the TU of the mixture equals the sum of the TUs of the single components

$$\sum_{i=1}^{n} TU_i = TU_{Mix} \qquad (2)$$

or

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = \frac{c_{Mix}}{EC_{xMix}} \qquad (3)$$

where $c_{Mix} = \Sigma c_i$. Accordingly, c_{Mix} equals $EC_{x_{Mix}}$ in a situation where the mixture provokes x% effect and the joint toxicity agrees with CA, in which case the right hand side of eq. 2 and 3 reduces to 1 (eq. 4).

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad (4)$$

That is, a mixture component can in theory be replaced by an equal fraction of an equi-effective concentration (*i.e.* an equal TU) of another without altering the overall effect of the mixture, since the sum of the TUs will still be 1. If however the TU of the mixture turns out to be <1, the joint toxicity of the single components is said to be greater than additive. That is, a lower mixture concentration than expected by CA is required to provoke x effect. Conversely, the joint toxicity is said to be less than additive if the TU of the mixture >1.

The summation of the TUs of the single components has the implication that every toxicant of the mixture contributes to a joint toxicity, even if it is present in a concentration lower than its individual NOEC (No Observed Effect Concentration) (Boedeker et al. 1993).

This corresponds to the very strict mechanistic interpretation that all toxicants bind to the same molecular target, so that it is the overall binding to the target site that determines the effect.

To predict the toxicity of a mixture according to CA it is necessary to determine the toxicity of each single toxicant to a specific effect level (see Equations 1 - 4). The way to do this is to test several concentrations producing low to high effects and fit this data with a mathematical function to yield concentration-response curves (Fig. 3, phase 4). EC*x* values can then be estimated on the basis of the position and slope of the curves (Boedeker et al. 1992).

Two types of reference mixtures of similarly acting PSII-inhibitors were tested in order to evaluate CA. One mixture comprised the phenylurea herbicides diuron, fluometuron and isoproturon, and the other consisted of diuron along with the *s*-triazine irgarol and the uracile terbacil. The compounds were selected on the basis of chemometric methods and QSAR analysis on structural and pharmacological similarity and algal toxicity data (Gramatica et al. 2001). The first set of mixture compounds is congeneric, *i.e.* they share a high structural similarity because they are based on the same type of parent compound and the QSAR analysis indicated a high toxicodynamic similarity as well, *i.e.* a similar mode of action (Calamari & Vighi 1992). CA has also previously been shown to provide a good prediction for the joint toxicity of such congeneric inhibitors of PSII (Faust et al. 2001, Arrhenius et al. 2004, Backhaus et al. 2004b). The second set of compounds are non-congeneric inhibitors of PSII, and were therefore expected to show a lower similarity in their affinities or modes of actions as indicated by QSAR analysis (Gramatica et al. 2001). CA might thereby have a lower predictive power for this mixture, particularly in a periphyton community with a high diversity of potential targets (see further discussions in sections 3 and 4.5).

4.4.2. Independent Action

A series of studies in the 1930s to 50s refined the CA-concept further and also defined the opposing concept of IA (Altenburger et al. 1990). IA originates from quantal response analysis. One analogy to describe the logic behind this concept is "multiple Russian roulette". That is, the overall probability that Christopher Walkens character Nick in the 1978 movie Deer Hunter actually would have taken a bullet increased in an accumulative fashion for each new round of Russian roulette that he entered. IA might also be conceived of as "multiple-barrel Russian roulette", in which each round has the capacity to kill off *e.g.* 50% of the contestants. Two consecutive shots (chemicals) thereby total in a 75% kill of the original pool, since the 2nd shot only target those 50% that remain standing from the first round.

According to IA, a defined exposure concentration (c) of toxicant A has a $p^D(c_A)$ probability of leaving the organism dead after exposure. The probability for death after simultaneous exposure to a second toxicant B is thereby:

$$p^D(c_{Mix}) = 1 - ((1 - p^D(c_A)) \times (1 - p^D(c_B))) \quad (5)$$

The accumulated probability of dying from exposure to an n-compound mixture is thereby:

$$p^{\mathrm{D}}(c_{\mathrm{Mix}}) = 1 - \prod_{i=1}^{n} \left[1 - p^{\mathrm{D}}(c_i)\right] \quad (6)$$

In a population a graded response can be observed as increasing concentrations of the mixture will increase the number of fatalities. In the case that the sensitivities of the individuals to the toxicants are statistically independent (*i.e.* where two or more individuals do not have the same relative sensitivity to all toxicants), IA can be formulated as:

$$E_{\mathrm{Mix}} = 1 - \prod_{i=1}^{n} \left[1 - E(c_i)\right] \quad (7)$$

As described in (Faust et al. 2003), where E_{Mix} is the expected effect of the mixture, n is the number of mixture components and $E(c_i)$ is the effect that the ith component would provoke if applied singly in concentration c_i. Hence, in contrast to CA a single toxicant only contributes to a combined effect if it is present in a concentration that would have provoked an effect if applied singly.

As opposed to CA the idea behind IA is that the toxicants have dissimilar modes of action and affect different physiological processes (Boedeker et al. 1992). At least, IAs Russian roulette (probabilistic) type of independent joint action rules out a common molecular target site, *i.e.* a similar mode of action. However, the pharmacological inference of IA especially on an integral level of toxicity (*e.g.* death) is not well established. Considering that organisms are structured entities and contain interwoven physiological processes, a complete independence of effects from the toxicants appears unlikely ((Backhaus et al. 2000a) and references therein). Still, IA has been shown to accurately predict mixture effects from dissimilarly acting toxicants on the bioluminescence of the marine luminescent bacterium *Vibrio fischeri* (Faust et al. 2003) and the growth of *Scenedesmus vacuolatus* (a green alga) (Walter et al. 2002).

IA was not investigated with a reference mixture consisting of toxicants with a well-known mode of action. Instead, the predictive powers of both IA and CA were in Paper IV evaluated for a mixture of pharmaceuticals and personal care products comprising clotrimazole, fluoxetine, triclosan, propranolol and zinc-pyrithione. As evident in Table 2, these compounds represent a broad range of modes of action in various target organisms and use patterns, and they are also of environmental relevance (see section 4.1). This mixture can thus be regarded as a good example of a realistic type of complex environmental mixture.

4.5. On the Modes and Mechanisms of Action of Chemicals

Toxicity is caused by the interference of a chemical with the biochemical and physiological processes in an organism. The direct interaction with a biological structure in a cell is often referred to as the (biochemical) mechanism of action of a chemical, whereas its mode of action refers to the impairment of major physiological process(es) such as photosynthesis, sterol biosynthesis or cell division. There are several ways by which chemicals can exert their toxicity, and a complete list is out of scope for this thesis. However, three basic mechanisms of toxic action can be identified (Escher & Hermens 2002): baseline toxicity, specific mechanism of action and multiple mechanisms of action. Baseline toxicity is a mutual property for all organic chemicals, and denotes an unspecific partitioning of the chemical in

biological membranes which impair their function and integrity. Specific mechanism of action refers to a binding to a distinct molecular receptor or structure, while a chemical with multiple mechanisms of action bind to several distinct types of molecular receptors or structures. For example, phenylureas specifically bind to the Q_B niche of the D1 protein in photosystem II (Devine et al. 1993), whereas TBT interfere with energy-transduction of mitochondria and chloroplasts via multiple mechanisms involving both uncoupling and energy transfer inhibition (Hunziker et al. 2002).

Because all organisms contain membranes they are susceptible to baseline toxicity, although their relative sensitivity will still depend on factors such as life stage, fat content and detoxification mechanisms. Chemicals with a specific mechanism of action are more likely to have a more narrow action spectrum, *e.g.* to affect only bacteria or only photosynthetic organisms because only they contain the relevant target receptor(s) (Escher & Hermens 2002).

In an ecological context the biochemical and physiological mechanisms and modes of action remain important but ecological dimensions need to be added. (Blanck et al. 2005) argued that toxicants with a similar *ecological* mode of action affect the same set of species, either by decreasing or increasing their abundances. Along the same lines of reasoning, dissimilarly acting toxicants then affect different sets of species. This provides a conceptual basis for transferring both CA and IA to the level of community structure. Following the basic interpretations of CA and IA, the toxicity of mixtures of toxicants with a similar ecological mode of action should hence be predictable by CA, and the toxicity of mixtures of dissimilarly acting toxicants predictable by IA.

However, there are a number of confounding factors which might limit the applicability of CA and IA on this level of biological organization. For example, knowledge or estimates (*e.g.* QSAR analysis (Escher & Hermens 2002, Altenburger et al. 2003)) on similarity or dissimilarity in biochemical mechanism of action is and will most likely continue to be an important and primary source of information for selecting appropriate models for applied environmental risk assessments of mixtures (see discussions in (Escher & Hermens 2002, Altenburger et al. 2003, Vighi et al. 2003)). However, the large diversity of molecular receptors and phenotypes distributed in nature implies that a high or relatively high similarity between two chemicals on a biochemical level will not necessarily be translated into the same degree of similarity in ecological mode of action. Two examples can be used to illustrate this. Firstly, although they do bind to the same site on the same molecular receptor different toxicants may bind at slightly different domains, which is the case for non-congeneric inhibitors of PSII (Nakajima et al. 1996, Gramatica et al. 2001). Depending on the structural heterogeneity of the receptor pool in a community and the corresponding sensitivity distribution (affinity) to the toxicants, different species might thereby be targeted in spite of a high similarity in biochemical mechanism of action. Secondly, both TBT (tributyltin) and TPT (triphenyltin) interfere with the function of ATP synthase, *i.e.* the same biomolecular target (Hunziker et al. 2002). However, TBT does this by direct inhibition of ATP synthase as well as through uncoupling, whereas TPT only inhibit ATP synthase directly (Hunziker et al. 2002). TBT and TPT hence have overlapping, partly similar and partly dissimilar mechanisms of action but a very similar mode of action. It might be speculated that all species in a community are targeted through the slightly broader mechanism of action of TBT, whereas TPT only target structurally susceptible receptors. That is, TBT and TPT might exhibit more

dissimilar ecological modes of action in a community compared to their similar biochemical mode of action in a single organism.

Furthermore, compared to an exposed single species chances are much higher that a toxicant will encounter a specific target receptor in a community because it comprises a set of different organisms with sometimes fundamental differences in their physiology. A community hence contains a considerably higher variety of receptors, both variants of the same receptor type as well as fundamentally different receptors. This implies that community level ecotoxicity of a chemical is most likely caused by effects on a whole range of different receptors and species, whereas only one or a few receptors of a specific type will be directly targeted in a single organism. This might have consequences for the behavior of similarly and dissimilarly acting toxicants on a community level. Since differences in sensitivity between microalgal species to a toxicant vary (Blanck et al. 1984, DeLorenzo et al. 2001) an increased exposure will target an increasing proportion of the species. This suggests that similarity or dissimilarity in ecological mode of action might be effect-level dependent. That is, increasing exposure from dissimilarly acting toxicants will affect an increasing number of species differently and thus at least hypothetically render the toxicants more dissimilar from each other quantitatively as well as qualitatively. On the other hand, an increasing number of affected species might also mean that there is a higher probability that a particular species will be affected by biochemically dissimilarly acting toxicants. Such an overlap in ecological mode of action would contribute to making the toxicants more similar. Whether this hypothesized effect-level dependency of similarity or dissimilarity in ecological mode of action is in reality a more relevant phenomenon on a community level compared to a single species level can only be speculated at this point. The same goes for its consequences for the applicability of CA and IA under different exposure scenarios. Nonetheless, considering that most environmental mixtures are made up of several chemicals that are present in only low concentrations and effects it would be a relevant subject for research.

Finally, as opposed to its biochemical mechanism of action in a single species, the ecological mode of action of a chemical is determined by the abiotic and biotic conditions in the ecological context in which the exposure takes place. Effects on the ecological fitness of individuals, *i.e.* their reproductive capacity, capacity to compete for and assimilate nutrients and avoid predation and grazing, will be determined by the physiological effect of the toxicant - but only in conjunction with other ecological stressors such as competition and predation. This will also lead to indirect effects when species interactions change, and the ecological mode of action of a toxicant will thereby depend on the specific community circumstances (available targets, their richness, distribution, susceptibility and functional connectivity to other species (see further discussions in (Calow 1995, Baird et al. 2001, DeLorenzo et al. 2001)). This suggests that different chemicals will display varying degrees of similarity or dissimilarity in their ecological mode of action not only along a toxicant-concentration scale, but also across spatiotemporal scales.

4.6. Multivariate Analysis of Toxicant-Induced Succession and Ecological Modes of Action

A biological community can be characterized by the species that are present and their frequency distribution. Using that information, two communities can be compared with each other to quantitatively describe how similar or (dissimilar) they are. Several such indices of (dis)similarity have been developed not only for biological data, but the Bray-Curtis index (Bray & Curtis 1957) is preferred by many ecologists for several reasons: (1) because joint absences of species are not mistaken for a similarity between the considered communities, (2) that dominant species have a higher influence on the outcome which is intuitively appealing, and (3) the index is based on the identity and not only numbers of species so replacement of one species with another is accounted for (Field et al. 1982, Clarke 1993). The Bray-Curtis index integrates structural community characteristics such as the distribution of species abundances, species richness, and absolute differences in species abundances. Hence, it is more sensitive than species richness which does not respond until species are completely eradicated from the community.

The Bray-Curtis index is calculated according to the following formula:

$$BCDI = \frac{\sum_{i=1}^{n} \left| X_{A,i} - X_{B,i} \right|}{\sum_{i=1}^{n} \left(X_{A,i} + X_{B,i} \right)} \qquad (8)$$

Where $X_{A,i}$ is (depending on the observed endpoint) either the relative abundance of species i or the amount of pigment i in community A (*e.g.* in a control) and $X_{B,i}$ the corresponding value of species or pigment i in community B (*e.g.* in a toxicant-exposed community).

For this thesis, patterns of toxicant-induced change were further characterized with the use of non-metric Multi-Dimensional Scaling (MDS). Using MDS, communities are projected in two dimensions according to their rank-order (dis)similarity. That means that they are ranked according to information of the kind that "community *A* is more similar to community *B* than it is to community *C*..." and so forth. The input to the analysis is a matrix of Bray-Curtis distances for pair-wise comparisons of communities. In the MDS algorithm, communities are first placed at random, and then an iterative procedure re-organizes their positions so the rank order of the distances between communities agrees as closely as possible with the rank order in the original matrix (Clarke 1993). In this way, communities that are similar to each other end up in close proximity. A completely accurate preservation of the original relationships is never possible, and the resulting distortion is indicated by a stress value.

As an example of the performance of MDS, Figure 4 shows the outcome after an analysis of a matrix of distances between Swedish towns that can be found in an ordinary road atlas. Despite the re-organization of towns according to ranked distances only, the result is a strikingly accurate visualization of a map of Sweden.

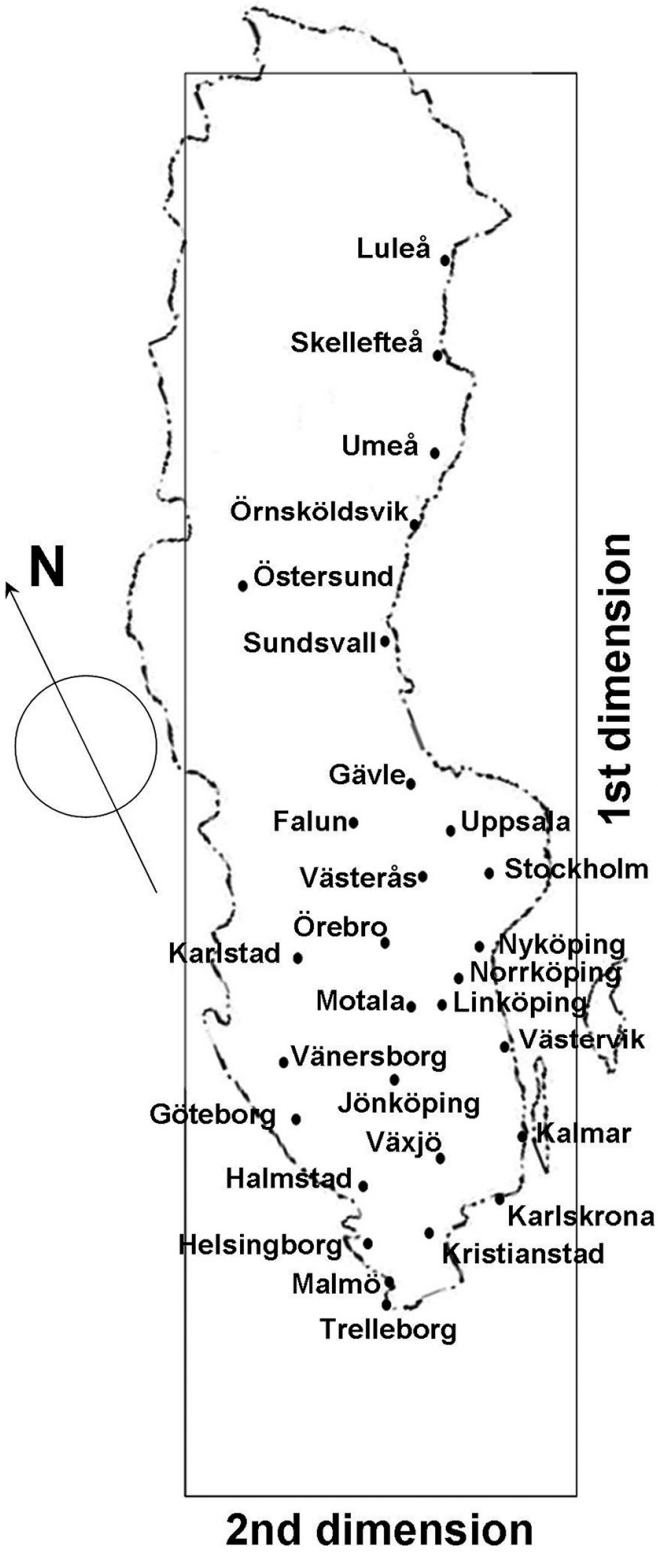


Figure 4. MDS ordination of some of Sweden's towns. A matrix of geographical distances was used as input. The outline of Sweden has been superimposed on the ordination as a measure of conformity. A Swede remembering basic school geography would say that it looks like an almost perfect map. That the original distances are only slightly distorted in the ordination is further indicated by the low stress value (0.02). Clarke (1993) performed the same exercise using the distances between 33 major cities of the world. Because the earth is a sphere, the projection of such much greater circumferential distances onto a two-dimensional plane increased the distortion and gave a stress-value of 0.13 (Clarke 1993). The relative distances between most cities were however still convincingly preserved.

With MDS, changes in community structure can be graphically illustrated as a *trajectory* of community development over time (Fig. 5). If left unexposed to any toxicant, a community will go through a succession over time (Fig. 5, *start community → control*) in a certain direction which is driven by the succession of the individual species. Communities exposed to toxicants *A*, *B* and *C* in concentrations high enough to alter the ecological performance of any of the species will go through a TIS and develop in a toxicant-dependent direction (Fig. 5, *start community → A, or B, or C*). Since toxicants with a similar ecological mode of action target the same species, they should shift the succession of a community in the same direction (Fig. 5, *A and B*), whereas communities exposed to dissimilarly acting toxicants progress in different directions (Fig. 5, *A* and *B vs. C*).

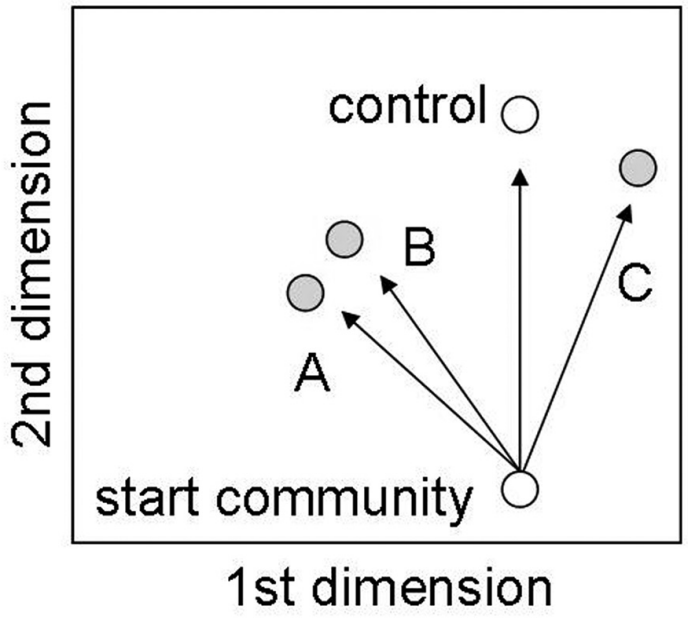


Figure 5. Hypothetical trajectories of normal succession and TIS. Arrows indicate degree and direction of community development. A total of 5 (hypothetical) communities (circles) have been mapped according to their structural similarity (species composition) so that the closer any two are situated the higher is their similarity, and vice versa.

However, the TIS trajectories are also dependent on toxicant concentration, because the degree of influence from the toxicant on the succession of the community will determine the severity and types of effects that occur. For example, a gradually increased concentration will affect an increasing number of species, thus increasing the distance from the control. Similarity or dissimilarity between toxicants in their ecological mode of action therefore needs to be judged at the same effect level or along an effect trajectory across a range of concentrations that have a low to high influence on community succession (Fig. 6). A high degree of overlap in effect trajectories would indicate that the toxicants have a similar ecological mode of action (Fig. 6).

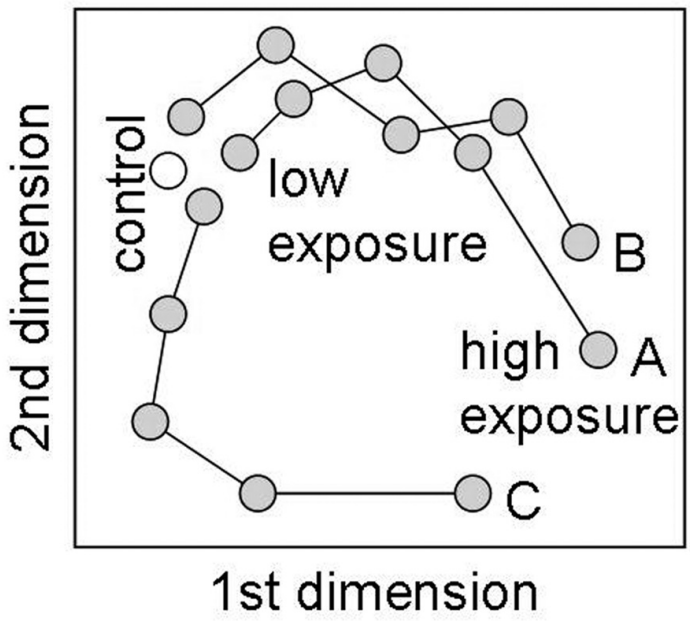


Figure 6. Hypothetical effect trajectories of different toxicants. The communities have hypothetically been tested in three concentration series of toxicants *A*, *B*, and *C*, and sampled after a period of succession. In the Figure they are connected from low to high toxicant exposure to display their effect trajectories. A gradual increase in concentration has shifted the communities a farther distance away from the unperturbed control. The overlapping effect trajectories from toxicants *A* and *B* suggest that they have a similar ecological mode of action. The ecological mode of action of toxicant *C* is dissimilar to that of *A* and *B*, and its effect trajectory becomes increasingly different from *A* and *B* at intermediate toxicant concentrations. At very high concentrations where most species are severely affected, the ecological mode of action of the three toxicants becomes more similar again and their effect trajectories begin to converge.

Conceptually, it is relatively straight-forward and intuitively appealing that ecologically similarly acting compounds should yield similar and overlapping effect trajectories (*i.e.*, they act as if they were dilutions of the same compound) (Fig. 6, toxicants *A* and *B*). This hypothesis is also testable using structurally congeneric toxicants with the same biochemical mechanism of action, such as the phenylureas studied in this thesis **(Paper III, Figs. 2 and 5)**. A low similarity between the compounds will render the effect trajectories less similar in their form, progression and directionality.

A maximum degree of dissimilarity would theoretically cause effect trajectories to progress in such different directions as allowed by the spatial constraints of the two MDS dimensions and the set of data plotted therein. Obviously, tests can be made with compounds from different chemical classes, with different toxicological properties (Papers I and IV). However, the possible numbers of different outcomes (possible changes in species and pigment compositions) is so large that it is improbable that two compounds will have exactly opposite effects in the communities, and their ecological modes of action might overlap to some extent. An attempt to illustrate the effect trajectories from completely dissimilarly acting chemicals can instead be made in a thought experiment in which different single pigments (or species) are assumed to be affected by an exposure to the different compounds, while the remaining pigments are left unchanged. Figure 7 shows the resulting effect trajectories from such a reduction of fucoxanthin, chlorophyll a, or diatoxanthin from the pigment profile of a

SWIFT control community. The three effect trajectories form a rather symmetrical shape in three directions, and hence their directionalities are as different as is possible in a two dimensional plot (Fig. 7).

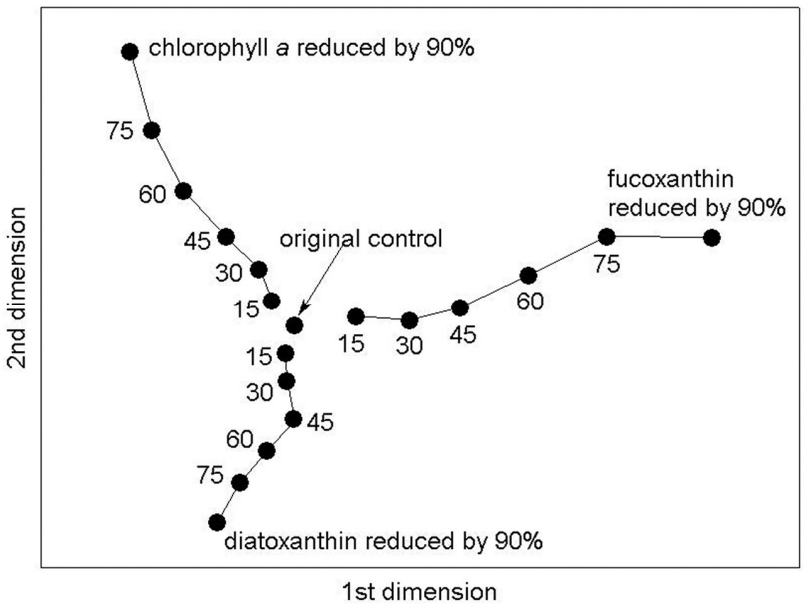


Figure 7. Three completely dissimilar effect trajectories. The content of either chlorophyll *a*, fucoxanthin or diatoxanthin in the original control pigment sample have been manually reduced by 15, 30, 45, 60, 75 or 90% as indicated. Stress: 0.05. The effect trajectories would look equally dissimilar also if species data had been used for the exercise.

Figure 8 depicts SWIFT pigment data from three different groups of chemicals: PSII inhibitors (Fig. 8A), antifoulants (Fig. 8B) and pharmaceuticals/personal-care products (Fig. 8C). The similar form and overlap of the effect trajectories from the PSII inhibitors (Fig. 8A) clearly indicates that although these chemicals are non-congeneric in their molecular structure (*i.e.* they belong to different chemical classes), they have a similar ecological mode of action. Hence, a high similarity in their biochemical mechanism of action (binding to the D1 protein of PSII, but at slightly different domains ((Jansen et al. 1993, Gramatica et al. 2001) and references therein) translated to a high similarity also at the community level. Furthermore, in Paper III it is shown that CA provided an accurate prediction of their joint toxicity **(Paper III, Fig. 1F)**, which also should be a direct consequence of their high similarity. However, to what extent the compliance with the CA prediction was because of a high similarity in either the biochemical mechanism of action or the ecological mode of action is unknown.

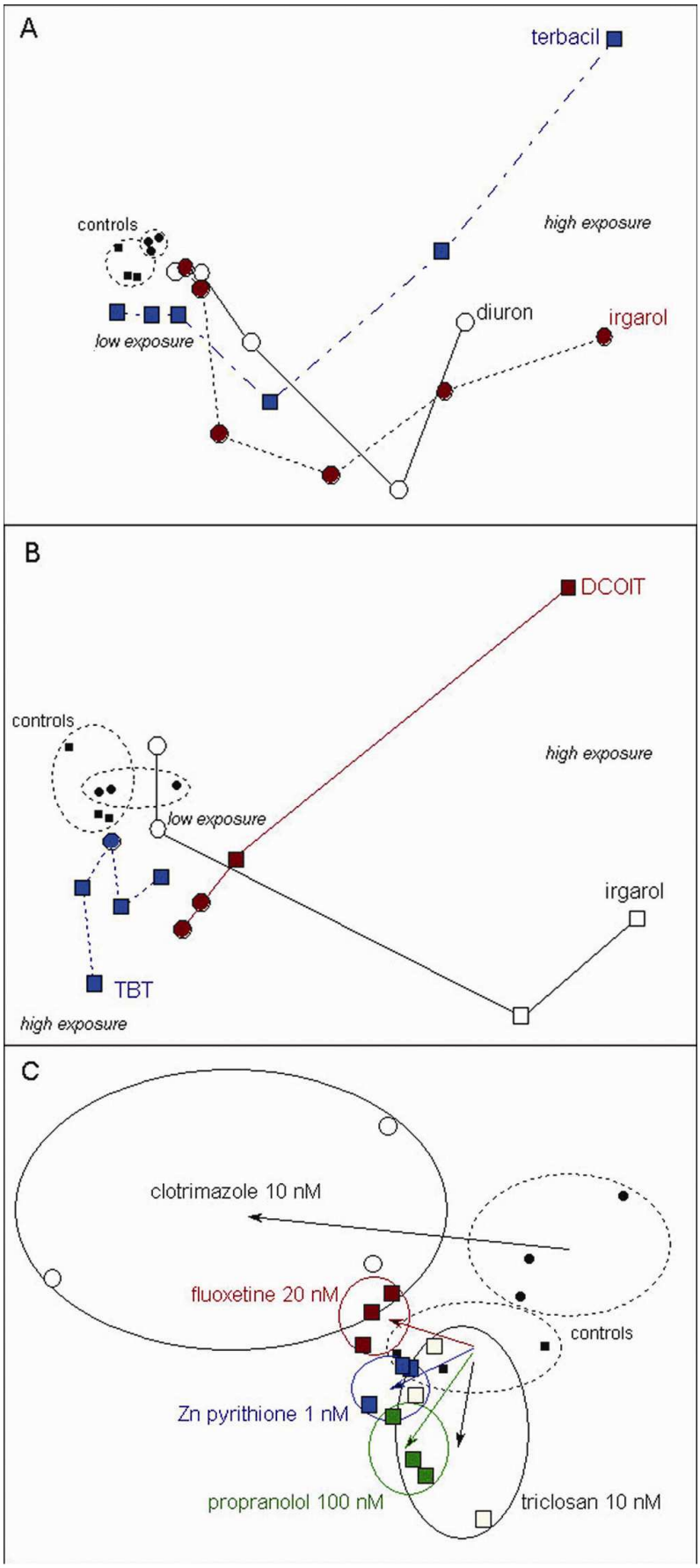


Figure. 8. MDS ordinations of pigment data after exposure in SWIFT. **A:** Effect trajectories from three different non-congeneric PSII inhibitors, stress 0.09. **B:** Effect trajectories from three different antifoulants, stress 0.07. **C:** Set of five different pharmaceuticals and personal-care products tested at their respective NOEC (No-Observed Effect Concentration) for total pigment content. Each compound was tested in triplicate, and the data have been encircled for the sake of visual clarity. Arrows indicate the degree and directionality of effects. Stress 0.12. For every test treatments were distributed over two growth chambers each holding three controls that are encircled in the Figures. Symbol shape denotes chamber origin.

In contrast to the similar effect trajectories of the PSII inhibitors the effect trajectories from the antifoulants progress in very different directions (Fig. 8B). A comparison to the completely dissimilar effect trajectories depicted in Figure 7 suggests that these chemicals are quite dissimilarly acting in the community, and this picture holds also for species composition **(Paper I, Fig. 3)**. Their dissimilarity corresponds well to different mechanisms of action also on a biochemical level (Table 2).

The set of 5 pharmaceuticals and personal-care products in Figure 8C is plotted at a concentration that corresponds to their respective NOEC (No-Observed Effect Concentration) for total pigment content. The observed effects ranged between 5% and 10% although not statistically different from the controls. Hence, they are compared within the same low effect range. Judging from the different TIS trajectories displayed by these compounds, they are not all similarly acting which again corresponds to their different biochemical mechanisms of action (Table 1). The biochemical mechanisms of action of propranolol and fluoxetine in algae are not known, but it seems that also for these chemicals at least their ecological modes of action are dissimilar (Fig. 8C). IA provided a good estimation for the joint effect that was observed when these compounds were mixed at their respective NOEC values. The observed inhibition of total pigment content was 28%, IA predicted 30% **(Paper IV, Fig. 3)**. Still, it should be remembered that an accurate prediction from one concept does not rule out that also the other concept would have been as accurate or almost as accurate (Faust et al. 2003, Backhaus et al. 2004b, Junghans et al. 2006). In fact, based on individual substance toxicity data recorded in previous tests, CA also predicted 30% effect from this mixture **(Paper IV, Fig. 3)**.

Taken together, it thus seems as if also on a community level relative similarity or dissimilarity between toxicants in their ecological mode of action indicates how well CA and IA describe the joint action of chemicals. Furthermore, for all chemicals displayed in Figure 8, similarity or dissimilarity in biochemical mechanism of action is mirrored in their ecological mode of action. This finding might be more surprising for similarly acting chemicals than for dissimilarly acting chemicals. That is, it does not seem likely that dissimilarly acting chemicals on a biochemical level will target the same species in a community and thereby have a similar ecological mode of action. Conversely, as indicated earlier in section 4.5 it is plausible that chemicals with a similar biochemical mechanism of action can have dissimilar ecological modes of action. This implies that more groups of similarly acting toxicants than the PSII inhibitors should be tested before any general conclusions on the relationship between biochemical mechanism of action and ecological mode of action can be drawn. For example, inhibitors of 14α-demethylase such as the azole fungicide clotrimazole have a high similarity in their biochemical mechanism of action because they bind to the same enzyme, but they can still have a high specificity to only certain types of 14α-demethylases in nature, *e.g.* only plant-specific or fungi-specific ones (Bellamine et al. 2004, Lepesheva & Waterman 2007). The spectrum of activity of azole fungicides in a periphyton community might therefore be anything from quite narrow to very broad, dependent on the specificity of the toxicant and the diversity of 14α-demethylases in the community.

5. Methodological Considerations

5.1. Periphyton Species Composition

The relative abundance of species was determined by estimating their frequency of occurrence across 50 analyzed fields, each 150 µm in diameter per periphyton disc (representing 0.03% of the total disc area). This approach was used because it is often too tedious or impossible to count all algal cells on a periphyton disc or in subsamples as the biofilm may be too complex, *e.g.* layered with dense conglomerates of organisms. In addition to the Bray-Curtis index, species richness and the Shannon-Weaver diversity index (Magurran 2004) were used in Paper I to assess the biological complexity of the periphyton communities sampled at sea for testing, and the influence of the semi-static 4 days incubation on their growth and succession.

5.2. Periphyton Pigment Profiles

Algal pigments were extracted from 7 pooled periphyton discs per treatment by freezing (-18°C) in methanol for a maximum of three weeks. Pigment profiles were then determined using HPLC. The abundance of each pigment was estimated as its peak area in the chromatograms at a wavelength of 436 nm.

Typically the pigment profiles have a very uneven distribution, with a few very dominant pigments with peak areas two to three orders of magnitude higher than most other pigments. These major pigments, *e.g.* chlorophyll *a*, fucoxanthin, diadinoxanthin and diatoxanthin always recur in samples and are indicative of diatoms (Rowan 1989), which is to be expected considering their high dominance in our samples (Blanck & Wängberg 1991, Dahl & Blanck 1996a, Porsbring et al. 2007, Porsbring et al. 2009). To decrease the high influence of these major pigments and thus increase the dominance of minor pigments which are likely to carry a higher taxonomic specificity, pigment values were square-root transformed prior to further analysis. In addition, prior to analysis of effect trajectories using MDS, each pigment was normalized to the total pigment content of the corresponding pigment profile (see papers I, II and III for further details). This treatment excludes the influence of biomass differences between communities, which enhances the resolution of the MDS analyses.

5.3. Periphyton Sterols

Sampled periphyton communities were stored at -80° C until extracted with a two-phase chloroform/methanol method outlined by (Bligh & Dyer 1959). The sterols were then analysed using GC/MS, and identified by analysis of fragmentation patterns and retention times (see Paper II for further details).

5.4. Experimental Design and Analysis of the Mixture Experiments

Basically two approaches were used for assessing the interaction between chemical mixtures and periphyton communities, and the applicability of CA and IA. Pattern analysis of effects on species and pigments were done using MDS to assess similarity or dissimilarity between the toxicants in their ecological mode of action (see section 4.6). Also, the effect data from the single toxicants on total pigment content or pigment profiles (the latter endpoint analysed with the Bray-Curtis index) were fitted to yield concentration-response curves

(CRCs) from low to high effect. This information was then used to predict a full CRC of the mixture according to CA and/or IA against which the actually observed effect of the mixture was compared.

The periphyton communities established in the environment display a seasonal change in species composition. Also, communities at the sampling site become increasingly tolerant to irgarol over the boating season (Blanck et al. 2009). This may alter their sensitivity to the various test chemicals, and thus impair the assessment of the validity of CA and IA. The single compounds were for that reason always re-tested in at least three concentrations in the same experiment as the mixture, to eliminate that deviations between the tests confounded the conclusion on CA and IA applicability. However, testing the predictive capacity of CA and IA in a strict sense of the word obviously also required that the single substance toxicity data used for the predictions were recorded in a different experiment with a different batch of start communities.

The molar ratios of the toxicants in the mixture were chosen on the basis of their individual NOEC or EC50 values, as determined in preceding tests. The aim was to obtain a fairly balanced contribution from the single toxicants, to the overall combination effect. This did not reflect the true environmental situation, but provided a better case for assessing the performance of the concepts.

Two major approaches to research on the toxicological behaviour of chemical mixtures can be identified in the scientific literature. Either the molar ratios of the toxicants in the mixture are kept constant and the mixture concentration is varied (a so-called fixed-ratio approach; *e.g.* (Altenburger et al. 2000, Backhaus et al. 2000a, Faust et al. 2003, Junghans et al. 2003, Arrhenius et al. 2004)), or the molar ratios of the toxicants in the mixture are varied, sometimes also along with the concentration of the mixture. The latter so-called isobologram (*e.g.* (Altenburger et al. 1993)) or concentration-response surface analysis (*e.g.* (Jonker et al. 2005)) methodologies are applied to search for concentration-ratio dependent deviations between predicted and observed mixture toxicity. While the fixed-ratio approach allows the testing of mixtures composed of several toxicants, experiments on dose-response surface analysis quickly become very large as more than two toxicants are added. The mixtures in this thesis were tested with the fixed-ratio approach. As indicated, this maximized the number of components in the mixtures. However, the downside is then that toxicant interactions that might occur at other toxicant ratios are missed.

5.5. Confounders Inherent to the use of the Bray-Curtis Index

During the work with this thesis a number of complications with the use of the Bray-Curtis index have emerged. Firstly, for descriptions of concentration-effect relationships toxicant-treated communities were not compared to every individual control of the test. That is because that would result in an apparent pseudo-replication where *e.g.* unreplicated samples would be represented by n = control number of values. To circumvent this, samples were related to a single, average control community (ACC). The ACC is put together from the arithemetic means or medians for each pigment or species in the controls. The ACC thus has the average abundance of each pigment or species in the control communities.

However, slight variations in structure that is always present even in replicate communities mean that any two samples are never exactly similar. Therefore, even a single control which is used for estimating the structure of unperturbed succession will have a Bray-Curtis value >0. Conceptually it would be tempting to define the control situation, and hence the level of no-effect, according to the Bray-Curtis values of the individual controls. However, because these values are calculated in relation to an ACC, individual controls automatically become more similar to this construct than any other sample because they were used for defining it.

Secondly, the Bray-Curtis index is non-linear. That is, there is not a constant proportion between this measure and the relative abundance of species or pigments. An increase from *e.g.* 0.1 to 0.2 in the Bray-Curtis index does not represent the same change in relative abundances as an increase from 0.2 to 0.3, and so forth. This has the implication that the Bray-Curtis index is an inappropriate effect measure in IA predictions. IA indicates the joint probability that compounds will exert an effect according to Eq. 7; section 4.4.2). Because probabilities function on a strictly linear scale, IA predictions on a non-linear scale are inherently erroneous. However, the CA concept is not erroneous on the Bray-Curtis scale. This is because toxic units of the single toxicants are used as input to the CA predictions (see section 4.4.1). The toxic units indicate the concentration required of each toxicant tested singly to provoke a given toxicity, and this information is used to predict a concentration of the mixture required to provoke the same effect. Hence CA is mathematically equally valid on a non-linear effect scale as it is on a linear scale. In order to test the predictive capacity of IA for a mixture of dissimilarly acting toxicants, total pigment content was used as effect indicator in Paper IV.

Thirdly, concentration-response curves on Bray-Curtis analyzed species data are often bi-phasic, and can be difficult to fit mathematically. This is probably because changes in species composition often are more dynamic and multidirectional compared to changes that occur *e.g.* in the pigment profile of a community. Such patterns are obviously more difficult to analyze with only a one-dimensional measure.

Hence, the use of a one-dimensional Bray-Curtis index scale for predictive purposes, *i.e.* as input to the CA and IA equations is still in a developmental phase. An ideal effect indicator would be: (1) mathematically useful for predictions and descriptions of concentration-response curves according to both CA and IA, (2) as sensitive as the Bray-Curtis index that considers the identities of species and pigments, and (3) informative to what changes in species structure that occur, *i.e.* with an additional variable that indicates the directionality of species change.

6. Main Results and Discussion on Most Significant Findings

6.1. Performance of the SWIFT Test

The major requirement of SWIFT was that the test phase would have to be long enough to cover a period of periphyton growth and succession. This is a prerequisite for: (1) exposure in an ecologically relevant context, under conditions (*e.g.* competition, grazing) that are of importance for the long-term ecological performance of microalgal cells, (2) a high sensitivity, because community structure changes when the most sensitive component is adversely affected, regardless of the mechanism of action of the toxicant, and (3) insights into the pattern of toxicant-induced change, later operationally defined as an indication of the ecological modes of action of toxicants on microalgal communities.

The validation of SWIFT´s ecotoxicological performance was made against the flow-through microcosms used as a blueprint for its design (see Table 3; section 4.3). The validation was mainly made with respect to the biological complexity of the tested periphyton communities, their succession in SWIFT and their responses to exposure from the antifouling agents irgarol, DCOIT and TBT (Paper I). This revealed that:

1. The biological complexity was high in the photoautotrophic microalgal and cyanobacterial community that had been established for 8 days in the marine environment for the SWIFT tests. Species richness and Shannon-Weaver diversity index were similarly high to that of communities developed for 14 days in the flow-through microcosms **(Paper I, Table 2)**.

2. The incubation under semi-static conditions in SWIFT, with daily renewal of the test medium and with added nutrients and continuous light did not decrease community diversity. Growth of individual species was high and no major changes in the relative dominance of species occurred **(Paper I, Table 2, Fig. 1)**.

3. Species composition in SWIFT changed at levels of exposure for irgarol and TBT that were comparable to the flow-through microcosms **(Paper I, Fig. 4)**. However, periphyton communities in SWIFT were approximately one order of magnitude less sensitive to DCOIT. It was hypothesized that this depended on the high degradation rate of this compound. The exposure in SWIFT is semi-static compared to continuous in the flow-through microcosms, so the exposure in SWIFT is in reality lower for readily degradable compounds in spite of the daily renewal of the test medium.

4. MDS analysis of species data indicated that toxicant-specific changes in community structure had occurred in SWIFT from exposure to DCOIT, irgarol and TBT in a similar manner as in the flow-through microcosms. The three toxicants clearly had different selection profiles in the communities and thus dissimilar ecological modes of action **(Paper I, Fig. 3)**. However, the pattern was less pronounced in SWIFT. The species structure of communities converged at high concentrations of the toxicants. This was in contrast to the flow-through microcosms were communities became increasingly more dissimilar from each other with increasing concentrations of the different toxicants **(Paper I, Fig. 3)**. It was hypothesized that the continuous immigration of new species in the flow-through microcosms allowed a broader window of possible responses, in contrast to SWIFT which is a closed system without immigration during the test. Furthermore, communities tested in SWIFT are already

established at the onset of exposure, and are therefore likely to show a higher structural inertia to perturbations. To minimize this inertia it is advisable to use comparably thin biofilms for testing where the different species have a higher potential for growth.

The duration of the SWIFT test only covers a part of the communities´ succession (see Fig. 1). Each and every potential response in a periphyton community can probably not be expressed over this limited time scale, and only some of the dynamics of toxicant effects on community succession are therefore captured. However, the outcome after the TIS in SWIFT provides a surprisingly accurate indication of more chronic effects. For example, dissimilar modes of actions from DCOIT, irgarol and TBT were detected by both pigments and species in SWIFT after 4 days exposure, which corresponded to dissimilar effects from these toxicants also in the flow-through microcosms after two and three weeks exposure **(Paper I, Fig. 3)**.

6.2. The use of Pigment Profiles as Ecotoxicological Endpoint

The use of HPLC-measurements of algal pigment patterns was evaluated in Paper I in addition to the SWIFT approach *per se*. The pigment data were compared with species data to assess the sensitivity of the pigments for detecting a TIS, and the resolution with which it reflected effects on species composition. In short, this revealed that:

1. The multivariate pigment response pattern allowed differential effects from the three antifouling agents to be identified using MDS. This also corresponded to the toxicants effects on community species composition **(Paper I, Fig. 3)**.

2. For irgarol and DCOIT pigment analysis allowed changes in community structure to be detected with a sensitivity close to that of species counts using microscopy. However, for TBT the pigment profiles only responded at a concentration ten times higher than that which caused changes in species composition **(Paper I, Fig. 4)**.

3. Pigment profiles could distinguish groups of communities tested at different seasons and with differing species composition **(Paper I, Fig. 5)**. However, because many species have the same pigments, the responses of individual pigments to toxicant exposure were much less dynamic than that of individual species **(Paper I, Fig. 2)**.

4. The pigments were responsive to changes in the physiological status of the algae. This physiological signal was correlated to the biochemical mode of action of the toxicant. Irgarol, which interfere with the photosynthetic machinery through binding to the D1 protein of photosystem II (PSII), induced the most evident effects of the three toxicants tested **(Paper I, Fig. 6)**.

The pigment profiles integrate species composition and physiological status. Hence, toxicant-induced changes in pigment profiles resulted from each of these community characteristics, but in a ratio that varied depending on the biochemical mode of action of the toxicant, and quite possibly also depending on the identities of species. This is because not only the taxonomic, but also the physiological signal to a given environment or stress is likely to vary between microalgae. In addition, the pigments responded to changes in total algal biomass, and this was expressed as changes in the total pigment content of communities.

The physiological signal in the pigments caused by PSII-inhibitors is probably exceptionally strong, in comparison to all other toxicants tested in this thesis. Their binding to

the D1 protein reduces the rate of electron transport in the thylakoid membrane, and the homeostatic response to a reduced photosynthetic capacity includes two specific changes in the pigments: (1) a shift between the pigments diatoxanthin and diadinoxanthin which comprise the diatom xanthophyll cycle, and (2) a sometimes increased content of major light-absorbing pigments such as chlorophyll a and fucoxanthin. The shift between the xanthophylls is indicative of an increased allocation of absorbed light energy to photosynthesis, and in concordance major light absorbing pigments can sometimes also respond to what the algae perceives as a low-light situation (the so-called "greening effect" *e.g.* (Molander & Blanck 1992)).

6.3. Effects of the Pharmaceutical Clotrimazole

Clotrimazole is a sterol synthesis inhibitor, and binds to the 14α-demethylase enzyme. This cause an accumulation of sterol precursors with a methyl group at 14α, and a simultaneous decrease in normal sterols **(Paper II, Fig. 1)**. Sterols are important structuring components in membranes, and toxicity in fungi is believed to be related to an impaired cell wall integrity (Sud & Feingold 1981). In addition, changes in the sterol composition in higher plants also affect the function of membrane-bound receptors, pumps, and channels, and can have negative consequences for plant growth, development and reproduction (Rademacher 2000, Schaller 2004).

1. Periphyton communities tested in SWIFT were very susceptible to clotrimazole. Analysis of their sterols indicated that 14α-demethylase activity was impaired after exposure to 50 pmol/L clotrimazole. This was manifested through an accumulation of the sterols obtusifoliol and norlanosterol, which both are substrates to 14α-demethylase **(Paper II, Fig. 1)**. Increasing clotrimazole exposure caused an increasing impairment of sterol synthesis **(Paper II, Fig. 2)**. A clotrimazole concentration of 500 pmol/L had caused a 36% decline in the total content of normal sterols while the sterol desmosterol had declined by 80% **(Paper II, Fig. 4)**.

2. Three independent tests were performed. A clotrimazole concentration of 32 nmol/L, which was sufficient to almost completely inhibit sterol synthesis **(Paper II, Fig. 2)**, had actually caused an increase in community biomass (estimated as total pigment content) in two of the tests **(Paper II, Fig. 3)**. Because other inhibitors of 14α-demethylase have been found to stimulate the growth of some microalgal species tested singly, it was speculated that the occasional stimulatory effects in SWIFT was caused by a stimulated growth of some species not always present from the start.

3. Community biomass was negatively affected by 500 pmol/L clotrimazole, but a concentration-dependent monotonous decrease in biomass did not occur until concentrations exceeded between 10 and 100 nmol/L. This suggests that low to intermediate concentrations of clotrimazole restructured the community, but that higher concentrations were required to affect also the most tolerant species and cause a general reduction in community growth.

4. The changes in periphyton growth might be related also to effects on brassinosteroid synthesis (BR-synthesis), an additional biosynthesis chain inhibited by clotrimazole (Winter et al. 1997). The brassinosteroids is a group of plant hormones synthesized from the sterol campesterol, and their regulatory effect is imperative for normal plant development and growth (Fujioka & Yokota 2003). The possible relationship between clotrimazole effects on

BR-synthesis and periphyton growth was manifested in the data as a consistently negative relationship between the content of campesterol and chlorophyll a content. This pattern might be a logical consequence of BR-synthesis inhibition, according to the hypothesis that an accumulation of the brassinosteroid precursor campesterol mirrors an impaired BR-synthesis and hence also an impaired growth, measured as inhibition of chlorophyll *a* content.

5. Clotrimazole has been detected in U.K. estuaries in concentrations up to 100 pmol/L (0.0001 µmol/L) (Roberts & Thomas 2006), and predicted concentrations in the marine environment range between 66 and 603 pmol/L (OSPAR-Commission 2005). The results thus indicate that clotrimazole concentrations predicted to be in the environment or actually encountered in the environment can affect both the sterol metabolism and the succession of marine periphyton communities. The current use of clotrimazole therefore appears to be associated with a high environmental risk. However, its environmental risk needs to be assessed against a background contamination of other similarly acting azole fungicides which are used *e.g.* in agriculture in much higher amounts (see section 4.1.). The high compliance of CA with mixtures of similarly acting toxicants encountered in this thesis (see section 6.4.) as well as several other studies suggests that CA should be an appropriate tool for assessing a combined environmental risk from the use of azoles.

6.4. Predictability and Toxicity of Mixtures of Similarly Acting Chemicals

Similarly acting inhibitors of photosystem II were tested singly and in mixtures to specifically address the performance of CA. One type of mixture comprised non-congeneric inhibitors of PSII, and the other type of mixture congeneric phenylureas. In short, it was found that:

1. CA could describe mixture effects on periphyton community pigment profiles (condensed through the Bray-Curtis index) with a high accuracy and for the full range of effects (low to high) **(Paper III, Fig. 1D-F)**.

2. The CA predictions were equally good for a mixture of non-congeneric inhibitors of PSII as it was for a mixture of strictly congeneric phenylurea herbicides **(Paper III, Fig. 1D-F)**.

3. Predictions by CA on the basis of single substance toxicity data could be readily extrapolated to describe a mixture effect in another experiment **(Paper III, Fig. 1D-E)**, despite differences in the structure of pre-established communities between tests **(Paper III, Fig. 2)**.

4. MDS analysis of pigment profiles indicated that all single toxicants and mixtures had caused similar changes. Step-wise increase in concentrations shifted communities in a similar direction away from the controls in the MDS ordination and the pigment-effect trajectories of the PSII inhibitors overlapped **(Paper III, Fig. 2)**.

5. In concordance with the pigments, MDS analysis of species data also indicated that the species-effect trajectories of the PSII inhibitors were similar **(Paper III, Fig. 5)**.

6. The predictive capacity of IA for these mixtures could not be evaluated. That is because IA is incompatible with the Bray-Curtis index used as effect measure (see section 5.5). An alternative to the Bray-Curtis index is total pigment content, used in Paper IV. However, an

increase in total pigment content was caused by the greening of the communities, and such negative effects are mathematically out of scope of IA (see section 6.5 for further details).

Paper III clearly demonstrates that the concept of CA is valid also on the level of community structure. Because the effect trajectories of the single toxicants and mixtures overlapped, any toxicant could therefore theoretically have been replaced fully or in part by an equal fraction of an equi-effective amount of another, without altering the overall effect. This is a central principle of CA (Altenburger et al. 1993). Also, the similarity in ecological mode of action of the toxicants implies that they all contributed to a joint toxicity, even at a concentration where they might not have caused an effect singly. This stresses the point that the joint environmental risk from co-occurring pollutants needs to be considered.

6.5. Predictability and Toxicity of a Mixture of Dissimilarly Acting Chemicals

Clotrimazole, fluoxetine, propranolol, triclosan and zinc-pyrithione comprised the reference for a mixture of dissimilarly acting toxicants.

1. A first test only included a single concentration of the mixture, tested in 9 replicates. The concentrations of the single toxicants in this treatment corresponded to their NOEC-values, *i.e.* they were each present in a concentration which would not have provoked a statistically significant effect if applied singly. The individual toxicants were also tested singly at their respective NOEC in the same experiment. The combination provoked a clear effect of 28% inhibition of total pigment content compared to controls, which was accurately predicted by IA as well as CA **(Paper IV, Fig. 3)** (the CA prediction was based on single substance toxicity data recorded in previous experiments).

2. The second and third tests included a range of mixture concentrations. The concentration-response curve of the tested mixture was bi-phasic at both occasions. High mixture concentrations caused a general decrease in pigment content, whereas lower concentrations had increased biomass **(Paper IV, Fig. 2)**.

The hormesis-like (*i.e.* stimulating) effects provoked by the mixture could not be adequately modeled by either CA or IA. Predictions on joint effects according to IA are based on probabilistic reasoning, and probabilities are obviously limited to values between 0 and 1. On an effect scale over total pigment content hormesis effects translate into negative values which hence are conceptually out of the scope of IA. CA might have a conceptual bearing for this situation but requires that toxicity data from all single toxicants are available for the considered effect level. In this case, the hormesis effect was only observed for the mixture.

3. IA provided a rather good prediction in the region where a decrease in biomass was observed whereas the prediction from CA slightly overestimated the effect of the mixture **(Paper IV, Fig. 2)**. Considering the dissimilar modes of action of the toxicants IA should be more appropriate, but since CA only slightly overstimated mixture toxicity this provides additional evidence to the proposal that CA provides a reasonable, worst-case assumption for any type of chemical mixture (*e.g.* (Berenbaum 1985, Boedeker et al. 1992, Junghans et al. 2006)).

4. The EC50 of the mixture on total pigment content was 356 nmol/L in the first experiment and 2560 nmol/L in the second **(Paper IV, Fig. 2)**. This corresponded to a shift also in the toxicity of the chemicals tested singly. The change in the periphyton community response coincided with a pronounced change in the pigment profiles of the start communities used for the respective test. The relative dominance of the diatom marker-pigment fucoxanthin was clearly elevated in the start communities of the second test, suggesting that the difference in sensitivity was due to an increased dominance from diatoms. In concordance, diatom species are the least sensitive to at least triclosan and zinc-pyrithione **(Paper IV, Table 3**).

7. Outlook

On the basis of the results from this thesis several suggestions for future research can be made, either to fill current knowledge gaps or to pursue novel issues.

Biochemical Mode of Action vs. Ecological Mode of Action

In paper III it is demonstrated that on a biochemical level similarly acting inhibitors of photosystem II also displayed a similar ecological mode of action in the periphyton community, which clearly warrants the use of CA for predictions on their joint toxicity. Knowledge on toxicants mode of action is obviously advantageous when choosing the appropriate model for environmental risk assessments of mixtures. Therefore, additional groups of environmentally relevant contaminants should be assessed, in order to examine whether also they show a high within-group similarity in their ecological mode of action.

Mode of Action and Toxicity of Azole Compounds

There were indications that clotrimazole inhibited not only 14α-demethylase but also brassinosteroid-synthesis (BR-synthesis) in the periphyton. However, it is currently not known whether a growth-regulating BR-synthesis chain exist in microalgae, although brassinosteroids have been detected (Tarakhovskaya et al. 2007). Hence, BR-function in microalgae should be assessed, along with its sensitivity to exposure from clotrimazole and other azole-type fungicides. This is because brassinosteroids have a hormonal function, which means that only minor interference with their functioning is likely to have consequences for *e.g.* microalgal morphology, growth and reproduction.

Sterols as Chemotaxonomic Markers

The periphyton communities contained a high diversity of sterols, even though they were dominated by only a single algal class (diatoms). Sterols are also recognized as useful chemotaxonomic markers in microalgal communities (Volkman et al. 1998). 19 different sterols were encountered in 14 analysed diatom species (Barrett et al. 1995) and the distribution of the sterols was quite species-specific. For example, the diatom *Cylindrotheca closterium*, one of the most common species on the periphyton communities used for this thesis contained only one sterol, desmosterol (Barrett et al. 1995). Hence, sterols as chemotaxonomic markers are likely to display a quite high resolution and sensitivity for changes in species composition, a potential application for ecotoxicological studies with periphyton which clearly warrants further exploration.

POtential for Community Tolerance (POCT)

Blanck and co-workers (Blanck et al. 1988) formulated the PICT (Pollution Induced Community Tolerance) concept, arguing that it can be an ecologically relevant and sensitive tool to detect pollutant effects. The PICT approach has been applied in numerous studies involving heavy metals and pesticides in contaminated terrestrial and aquatic environments (see review by (Blanck 2002)). According to PICT, toxicant exposure high enough to alter the ecological performance of single species will eventually restructure the whole community through TIS. Tolerant species will be favored and their dominance will increase, hence rendering the community as a whole more tolerant to the stressor in question. An increase in community tolerance along a gradient of contamination is detected in short term tests where toxicant effects on a metabolic process, *e.g* photosynthesis are quantified.

A re-structuring of toxicant-exposed communities in SWIFT might also lead to an increased dominance from tolerant species. Crucial properties of the community such as biomass and primary production are thereby upheld despite an exclusion of sensitive species. The occurrence of such a redundancy within the community depends on two factors: the degree of toxicant influence on the succession, and the original distribution of species sensitivities at the onset of toxicant exposure. That is, a high presence of tolerant species at start means that the community has a high POtential for (Community) Tolerance (POCT), and might thereby express a high redundancy to toxicant exposure.

A POCT study in SWIFT might be used to amplify a PICT-signal from a contaminated location, where the selection from the toxicant might have been too low to provoke a clear PICT in a short term test only. Also, toxicant-induced effects are in SWIFT manifested as a change in community structure, regardless of the mode of action of the toxicant. Hence, POCT from a diverse range of toxicants can be investigated, whereas a PICT study in the field utilizing only short term exposure requires a match between the physiological endpoint measured and the mode of action of the toxicant, in addition also that the involved toxicokinetic processes involved are fast enough to provoke measurable effects.

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Populärvetenskaplig Sammanfattning

Vårt samhälle och dess invånare har blivit kraftigt kemikalieberoende. Syntetiska kemikalier och metaller finns i våra möbler, kläder, hus, bilar, leksaker och hemelektronik. Våra kök och garage, mediciner, tvättmedel, rengöringsmedel och schampon. Vår mat kan inte produceras utan bekämpningsmedel och veterinärmediciner, och våra trähus och verandor måste impregneras med konserveringsmedel för att inte ruttna. Vissa kemikalier som läkemedel, bekämpningsmedel för jordbruk och hushåll samt båtbottenfärger är designade för att orsaka en särskild biologisk effekt i utsatta organismer. Andra kemikalier som till exempel flamskyddsmedel, nanopartiklar, mjukgörare för plaster, doftämnen i parfymer och allmänna industrikemikalier har en mer ospecifik och oförutsedd påverkan. Tyvärr hamnar en hel del av kemikalierna i miljön, där de i kombination med annan mänsklig påverkan förändrar Jordens ekosystem. Detta riskerar i sin tur att påverka ekosystemens biologiska mångfald. Samt dessas förmågor att tillhandahålla rent vatten, föda, kapacitet att bryta ner gifter och cirkulera näringsämnen.

Miljöriskbedömningen av kemikalier utförs som regel endast för en kemikalie i taget. Detta trots att ekosystemen exponeras för flera kemikalier samtidigt som genom kombinationseffekter bidrar till att påverka arters ekologiska kapacitet. Det finns därför ett behov av verktyg för att kunna uppskatta riskerna från kemikalieblandningar. Den här avhandlingen bidrar till utvärderingen av två sådana verktyg; "Koncentrationsaddition" (Concentration Addition, CA) och "Oberoende Verkan" (Independent Action, IA). CA antas gälla för blandningar av likverkande kemikaler (d.v.s. kemikalier som påverkar samma biokemiska processer). IA antas gälla för olikverkande kemikalier. CA och IA har tidigare visat sig fungera väl under förhållandevis okomplicerade biologiska förhållanden. Exempelvis i tester med enskilda arter. Men ekosystemen innehåller en oerhörd biologisk och ekologisk komplexitet, d.v.s. en mångfald av biokemiska receptorer för kemikalier att binda till samt en mångfald av olika effektvägar när kopplingar mellan arter påverkas. Konsekvenserna av kemikalieexponering på den här nivån kan med andra ord ta sig många olika uttryck. Till exempel med avseende på vilka arter som påverkas och hur. CA och IA är egentligen inte alls utformade för att fungera under sådana förhållanden. Men eftersom målet är att använda CA och IA för att uppskatta effekterna av blandningar i miljön, så är det är nödvändigt att testa deras relevans i flerartssystem.

För den här avhandlingen så har jag använt mig av så kallade perifytonsamhällen. Perifyton är en fastsittande biofilm av mikrobiella organismer såsom bakterier, mikroalger och protozoer, och de förkoloniserades ute i den marina miljön på glasplattor. Eftersom samhällena består av snabbväxande mikrober, så genomgår de en snabb ekologisk succession. Detta betyder att deras artsammansättning förändras i snabb takt när populationer tillväxer och livsbetingelserna för olika arter ändras. Ett gift som påverkar en organisms förmåga att konkurrera om resurser och reproducera sig orsakar därför i förlängningen en avvikelse i samhällets struktur, d.v.s. artsammansättning och biomassa.

Med hjälp av en ny snabb testmetod (SWIFT) så utsattes perifytonsamhällena för kemikalier enskilt och i blandningar över en 4 dagar lång succession. Deras struktur och fysiologi undersöktes sedan med hjälp av analyser av mikroalgsamhällets artsammansättning samt pigmentsammansättning. Av teoretiskt intresse för just CA och IA, så användes den informationen sedan för att avgöra hur likartade effekterna var mellan olika kemikalier, d.v.s. hur likartade deras ekologiska verkningsmekanism var. Men informationen var naturligtvis också användbar för att avgöra samhällenas känslighet för olika kemikalier, liksom för att undersöka hur väl CA och IA rent kvantitativt kunde uppskatta effekterna från olika blandingar.

Avhandlingen består av fyra vetenskapliga artiklar. Den första artikeln beskriver och utvärderar SWIFT-metodiken. Särskild vikt lades vid att utvärdera den biologiska komplexiteten i perfytonsamhällena koloniserade ute i ekosystemet, samt deras succession inne i laboratoriet, med eller utan gifter. Även användandet av pigmentsammansättning som ett sammanfattande mått på mikroalgsamhällets artsammansättning, fysiologi och biomassa utvärderades.

Den andra artikeln beskriver och diskuterar effekterna av läkemedlet clotrimazole. Clotrimazole slår mot sterolsyntesen och används mot hudsvamp, men har identifierats som en särskilt miljöfarlig substans då den är svårnedbrytbar och hamnar i den marina miljön via utsläpp från reningsverk. Sterolsyntes är vanlig i naturen, och flera andra grupper av organismer än svamp är känsliga. Steroler behövs för att reglera genomsläppligheten i cellväggar, och för funktionen av en rad proteiner. Steroler har också mer eller mindre indirekt en tillväxtreglerande funktion; Växter med nedsatt eller ändrad sterolsyntes får en onaturlig tillväxt, utveckling och reproduktionsförmåga. Clotrimazole påverkade också perifytonsamhällenas sterolsyntes och succession i SWIFT. Detta redan vid halter som faktiskt uppmätts i miljön, vilket innebär att miljörisken från användandet av clotrimazole är ännu högre än vad som tidigare antagits.

Clotrimazole testades vidare i en referensblandning bestående av fem olikverkande kemikalier som återfinns i andra läkemedel och hushållsprodukter (4e artikeln). Blandningen testades i en koncentration i vilken halterna av de enskilda kemikalierna var för låga för att ge några synliga effekter i sig själva. Blandningen gav en tydlig negativ effekt, och IA beskrev denna effekt väl. Då blandningen testades i en koncentrations-serie kunde både CA och IA beskriva de negativa effekter som uppkom ganska bra. Låga koncentrationer av blandningen stimulerade samhällets tillväxt, och sådana effekter kan inte beskrivas av varken CA eller IA.

Effekterna av en referensblandning av likverkande kemikalier bekrivs i den 3e artikeln. Blandningen utgjordes av en vanligt förekommande grupp av växtbekämpningsmedel som slår mot fotosyntesen, så kallade fotosystem-2-inhibitorer. Dessa ämnen hade liknande påverkan också på perifytonsamhällets succession. CA kunde med god precision användas för att beskriva effekterna från blandningen. Med avseende på blandningar kan två viktiga slutsatser dras från detta avhandlingsarbete. Att kombinationseffekter från samtidigt förekommande föroreningar måste beaktas i riskbedömningar, samt att både CA och IA är lämpliga för att uppskatta kombinationseffekter från kemikalier i miljön.

"These chemicals have taken over my life! I don ´t have a life! I have a half-life!"

-The Toxic Avenger