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Impact of Antifouling Compounds on Photosynthesis, Community Tolerance and *psbA* Genes in Marine Periphyton

Doctoral thesis in Environmental Sciences

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ABSTRACT

Toxicants can act as selective pressures in the environment, eliminating sensitive genotypes or species and favouring tolerant ones. Such toxicant-induced selection can be detected in natural communities using the Pollution-Induced Community Tolerance (PICT) concept. Mechanisms of tolerance to toxicants in communities can potentially be studied using metagenomic approaches in which the pool of genes or genomes of all community members are analysed. Such approaches have the potential to unravel how toxicants interact with molecular targets, and combined with phylogeny, they can also unravel what tolerance mechanisms different organisms are compelled to use. Combining PICT and traditional measures of community function and structure with metagenomic and phylogenetic approaches, can potentially enable integrated studies of how toxicants interact with biological entities from the molecular to the community level, including important ecological interactions.

Antifouling compounds are toxicants which by their toxicity prevents attachment and growth of organisms on ship hulls and underwater installations. The major part of this thesis (Paper II-IV), concerns selection pressure from the antifouling compound irgarol on periphyton communities in Swedish coastal waters. It is shown that community tolerance to irgarol developed slowly over the years from 1994 to 2004, and that PICT was dependent on the contamination pattern over the boating season. Although not statistically significant in our studies, a small tolerance increase was observed at all sites investigated, indicating that irgarol might affect organisms adversely over larger areas in Swedish coastal waters.

PICT to irgarol was verified in flow-through microcosm experiments. Clone libraries of *psbA*, the gene coding for the target protein of irgarol - D1 - was made from communities highly and moderately tolerant to irgarol. Irgarol caused a clear shift in *psbA* haplotypes, D1 protein types and morphologically distinct species. None of the previously known mutations, conferring tolerance to compound with the same mechanism of action as irgarol, was found in any of the libraries. However, another region of D1, corresponding to the so-called PEST region, was identified as important for irgarol tolerance. Since the PEST region is suggested to regulate the degradation of the protein, a mechanism of increased degradation and turnover of the target protein is proposed. Tolerant communities were less diverse at the gene, protein and species levels, and the dominance of diatoms and cyanobacteria increased. Phylogenetic analysis enabled the determination of diatoms as the taxonomic group in which the proposed tolerance mechanism is important, whereas the cyanobacteria were identified as a group that probably use other tolerance mechanisms.

Irgarol seems to exert a specific selection pressure in the Swedish coastal marine environment, with the potential to restructure the distributions of genes, proteins and morphologically distinct species and thereby induce community tolerance.

In addition, this thesis evaluates the capacity of short-term photosynthetic endpoints in detecting toxicity of five additional antifouling compounds. The use of such endpoints when testing compounds with mechanisms of action not directed towards photosynthesis might underestimate toxicity. Since short-term toxicity tests are crucial for PICT detection it was tested whether prolonging the exposure time, thereby allowing for toxic effects to be propagated to photosynthesis, increased the performance of the photosynthetic endpoints.

Keywords: PICT, antifouling, periphyton, photosynthesis, D1, *psbA*, tolerance, resistance

Marie, Albin och Moa

-Den här är eran lika mycket som min

-Man kan inte se allting.
Albin Eriksson 2.5 år

-Ibland tror man att det skall bli läskigt, men så blir det inte det.
Moa Eriksson 3 år

LIST OF PAPERS

A doctoral thesis at a university in Sweden can be produced as a collection of papers. The aim of the introductory part is to summarise, merge and extend the knowledge in the accompanying papers.

This thesis is based on the Papers listed below. They are referred to in the text by their Roman numerals.

I. Eriksson K.M., Göransson A. and Blanck H. Toxicodynamic responses of periphyton community photosynthesis to six antifouling compounds. (2008) Manuscript.

II. Blanck H., Eriksson K.M., Grönvall F, Dahl B., Martinez K., Birgersson G and Kylin H. (2009) A retrospective analysis of contamination and periphyton PICT patterns for the antifoulant irgarol 1051, around a small marina on the Swedish west coast. Marine Pollution Bulletin. In press.

III. Eriksson K.M., Clarke A.K., Franzen L.-G., Kuylenstierna M., Martinez K. and Blanck H. (2009). Community level analysis of *psbA* gene sequences and irgarol tolerance in marine periphyton. Applied and Environmental Microbiology. Accepted.

IV. Eriksson K.M., Antonelli A., Nilsson R.H., Clarke A.K. and Blanck H. (2008) A phylogenetic approach to detect selection on the target site of the antifouling compound irgarol in tolerant periphyton communities. Manuscript submitted to Environmental Microbiology.

POPULÄRVETENSKAPLIG SAMMANFATTING

Moderna samhällen producerar, använder och släpper ut stora mängder kemikalier i miljön. En del av dessa är giftiga och påverkar organismerna i ekosystemen negativt. Eftersom kemikalieanvändningen innebär risker för ekonomiska, etiska och hållbarhetsmässiga värden i samhället, bör risker med kemikalieanvändningen utvärderas och användningen regleras. Inom traditionell riskanalys av kemikalier utvärderas ofta effekter av varje enskild kemikalie på enskilda arter av testorganismer som har odlats under laboratoriemässiga förhållanden. Detta är fördelaktigt ur synpunkten att kemikaliernas giftighet blir jämförbar för att alla testats på samma sätt och med samma testorganismer, men det speglar inte så bra vad som pågår, eller vad som kan komma att hända i ekosystemen. I detta arbete har istället ett annat angreppssätt använts. Jag har studerat effekter av gifter på naturliga samhällen av organismer, d.v.s. använt mig av alla arter som finns i en viss del av ekosystemet på samma gång. Detta innebär att gifterna testas på organismer som faktiskt finns i miljön, d.v.s. de organismer som riskanalysen syftar till att skydda. Det innebär också att ekologiska interaktioner, som att olika arter faktiskt konkurrerar med varandra eller äter upp varandra, är inkluderat i riskanalysen. I ett sådant sammanhang kan ett gift också betraktas som (nästan) vilken miljömässig parameter som helst. Precis som organismer skiljer sig åt genom att dom bara tål vissa förhållanden i miljön, t.ex. så tål isbjörnar kyla väldigt bra medans orkideér inte gör det, så kan olika arter tåla gifter olika bra. Detta gör att när organismerna i ett ekosystem exponeras för att gift kommer vissa att klara detta sämre, och antingen dö av direkt förgiftning eller förlora i konkurrensen med andra organismer som klarar giftet bättre. Genom att känsliga organismer försvinner, gynnas mer toleranta organismer. Hela processen innebär att toleransen mot giftet generellt ökar i samhället. Jag har använt mig av detta fenomen, som kallas Pollution-Induced Community Tolerance (PICT), för att uppskatta om gifter som finns i miljön påverkar naturliga samhällen.

Om man sänker ner något objekt i havet så kommer detta snart att vara täckt av små organismer som koloniserat denna yta. Det samlade namnet på alla dessa organismer är perifyton. Enkelt uttryckt så är perifyton det som man brukar halka på när man går på klippor eller stenar för att gå i och bada på sommaren. Så småningom kommer också större organismer, som havstulpaner och musslor, att sätta sig på det nedsänkta objektet. Detta är ett stort problem bl.a. för sjöfarten eftersom det kraftigt ökar motståndet för båtar i vattnet. Detta tekniska problem har lösts genom att måla skrovet med en giftig färg som skyddar mot påväxt men som tyvärr också läcker ut gifter i vattnet. Dessa gifter förgiftar organismerna som annars skulle kunna sätta sig fast och tillväxa där. Jag har använt mig av perifyton samhällen för att uppskatta effekter av sådana s.k. påväxtmedel som finns i båtbottenfärger.

I den första studien (Paper I) har effekter på två fotosyntetiska processer i perifyton av sex sådana gifter studerats. Kort-tids-effekter, d.v.s. effekter som man kan mäta kort tid efter giftexponeringen börjar, är en viktig del av PICT-metodologin. Jag kunde dra slutsaterna att resultaten stämmer med vad som tidigare är känt om hur dessa gifter verkar, och delvis om hur snabbt de bryts ned. Resultaten visar också att om man mäter på en för liten del av livsprocesserna riskerar man att missa eller åtminstone underskatta giftigheten av vissa ämnen.

I de övriga studierna har effekter av ett påväxtmedel (irgarol, eller triazin som det står på färgburken) som idag är tillåtet på svenska västkusten studerats mera i detalj. Perifyton-samhällenas tolerans mot irgarol har följts under 10 år. När irgarol började

användas var det extremt giftigt och det fanns inga toleranta organismer som kunde ta över. Man kunde inte uppmäta någon PICT på sommaren 1994. Efter år av irgarolanvändning har detta emellertid ändrats, och mikroalger och cyanobakterier har sedan år 2000 mekanismer för att hantera irgarol. Under de senare åren har toleransmönstret varierat under säsongen men höga toleransnivåer under perioder med mycket båtar och irgarol i vattnet. Detta tyder på att det kostar på för organismerna att bli toleranta. Dessutom finns det tecken på att toleransen generellt ökar under dessa 10 år, även vid den provstation som ligger allra längst ut i kustbandet. Även om denna ökning är liten indikerar detta att irgarol kanske inte bara påverkar hamnar och marinor, utan kanske hela det västsvenska marina ekosystemet. Det är därför lämpligt att detta utreds ytterligare.

Den metodologiska utvecklingen inom molekylärbiologin har möjliggjort att vi nu kan sekvensera alla gener från alla organismer i ett ekosystem. För att ta reda på orsaken till den irgarol-tolerans som beskrivs i Paper II sekvenserade jag den gen som kodar för det protein som irgarol binder till. Jag använde en metodologi för att kunna sekvensera denna gen från alla organismer i perifyton. Inom jordbruket används bekämpningsmedel som har samma verkningsmekanism som irgarol. Dessa sprutas på åkrarna för att få bort ogräs. Dock har det har visat sig att mutationer i denna gen i ogräsen har lett till att de har blivit toleranta för bekämpningsmedlen. DNA-sekvenserna från de toleranta perifyton-organismerna visade sig inte innehålla någon av dessa tidigare kända mutationer. Däremot visade sig en annan region av proteinet vara viktigt för irgarol-tolerans. Man tror att denna region reglerar nedbrytningen och därmed omsättningen av proteinet. Toleransmekanismen för irgarol inom perifyton kan alltså vara att snabbare omsätta det protein som irgarol binder till.

Eftersom DNA innehåller information både om funktionen av proteiner och om släktskap mellan organismer, så kunde irgarols påverkan på den genetiska mångfalden i perifytonsamhällena uppskattas. Det visade sig för det första att mångfalden i perifyton samhällen är mycket stor. T.ex. så finns det organismer inom perifyton som med avseende på denna gen är mera olika varandra än vad en tall-art (Contorta-tall) är jämfört med en primitiv planktonisk cyanobakterie. Det visade sig också att det var möjligt att identifiera kiselalger som den grupp av organismer som kan tänkas använda sig av toleransmekanismen med ökad proteinomsättning av det irgarol-bindande proteinet. Den andra stora gruppen av fotosyntetiserande organismer i perifyton var cyanobakterier. Dock verkar det som dessa organismer använder sig av någon annan, hittills okänd mekanism för att tolerera irgarol.

Avslutningsvis kan man säga att användningen av irgarol verkar påverka frekvenser av olika gener, proteiner och arter i perifytonsamhällen så att dessa blir mer toleranta mot irgarol. I den studerade marinan var perifytonsamhällena toleranta och därmed kraftigt påverkade av irgarol. Det finns indikationer på att denna tolerans är förknippad med en kostnad för den fotosyntetiska delen av samhället, och att irgarol-föroreningen även kan påverka större marina områden på den svenska västkusten

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SCOPE, AIM AND APPROACH

This thesis deals with effects of antifouling compounds on marine periphyton communities. Antifouling compounds are toxic substances used as additives in antifouling paint, which by their toxicity prevent attachment and growth of organisms on man-made constructions in the aquatic environment. Typical applications of these paints are hulls of boats and subsurface parts of oil rigs or other marine installations. In order to be effective these compounds are very toxic and since many of them also are relatively persistent they become environmental problems when they affect non-target organisms in the environment. A well-known example is the induction of imposex (pseudo-hermaphroditism) in gastropods by the antifouling compound tri-butyl-tin (TBT) (Thain and Waldock, 1986; Bryan *et al.*, 1987; Gibbs *et al.*, 1987). These problems and the adverse effects from TBT on other organisms (e.g. Fent and Meier, 1992; Mercier *et al.*, 1994; Cooper *et al.*, 1995; Blanck and Dahl, 1996; Dahl and Blanck, 1996b) the International Maritime Organisation (IMO) decided to ban TBT by September 2008 (IMO, 2001). The process of substituting TBT resulted in the use of a variety of compounds (Voulvoulis *et al.*, 1999; Ranke and Jastorff, 2000; van Wezel and van Vlaardingen, 2004). The organic compounds identified as most common and of most concern in Europe (Table 1) were selected in the project "Assessment of Antifouling Agents in Coastal Environments" (ACE, 2002).

The failure to predict environmental effects of TBT is an illustrative example of some problems in ecological risk assessment of chemicals. Unfortunately such failures are not uncommon. It has been shown that environmental impact assessments of major development projects in Australia, including release of toxicants, were only accurate in 44% of the cases, with impacts usually being more severe than predicted (Buckley, 1991). Still, repeated observations of the need to perform more ecologically realistic risk assessment of chemicals have continuously been made (*e.g.* see Cairns *et al.*, 1978; Cairns, 1983; Kimball and Levin, 1985; Pratt *et al.*, 1997; Moore, 2002; Schmitt-Jansen *et al.*, 2008).

In this work, more ecologically realistic aspects of antifouling toxicity is aimed for through the use of communities sampled in their natural environment. The use of the ecotoxicological tool Pollution-Induced Community Tolerance (PICT) offers possibilities to detect ecologically relevant effects from toxicants on natural communities (Blanck *et al.*, 1988; Blanck, 2002; Boivin *et al.*, 2002). Since inherent characteristics of PICT include toxicant specificity and causality of exposure, it is especially suited for detecting adverse effects of toxicants in such complex entities as ecosystems. PICT can be used in retrospective risk assessment to determine effects of ongoing contamination (Paper II), or in predictive risk assessment to quantify hazard of novel toxicants to communities. Molecular approaches, often used in the field of environmental microbiology, can favourably be used in combination with PICT. These approaches include studying diversity and/or selection using

neutral or functional molecular markers, by gel-electrophoresis-based methods or sequencing of clone libraries (reviewed by Dahllöf, 2002; Dorigo *et al.*, 2005). Sequencing of functional genes that are related to the mechanism of action of the toxicant enables the study of both toxicant-induced decreases in diversity, and selective advantages of specific tolerant genotypes. Thus, both community structure and community function can be studied in an ecotoxicological context.

Since the ability of PICT studies to accurately estimate effects of toxicants is dependent on the coupling between the mechanism of action of the toxicant and the detection method used, evaluations and development of PICT detection methods are needed. If the detection method is not matched to the mechanism of action of the studied compound the detection capability of the method is limited. In Paper I, two methods were evaluated for their capacities to detect toxicity of toxicants with various mechanisms of action. Since both methods estimate effects on photosynthesis we evaluated whether a prolonged exposure time can improve their detection of toxicity for compounds with mechanisms of action outside photosynthesis.

Of the six compounds identified in the ACE project only irgarol has been approved for use in Sweden. Since irgarol has been used in Swedish coastal waters, it was possible to make a retrospective analysis of community-level effects of irgarol in the Swedish coastal environment. This study, reported in Paper II, follows the development of PICT over a wide range of environmental irgarol contamination, over several boating seasons during 10 years. It demonstrates a slow PICT development in periphyton for irgarol.

The conclusions from Paper II were supported by the results from an experimental microcosm study. In this study, a molecular approach was developed in order to test the hypothesis that slow PICT development was due to a tolerance mechanism involving a modification of a conserved target protein – the D1 protein of photosystem (PS) II. This protein is encoded by the *psbA* gene and a community-level approach for studying this gene was developed. Such approaches, commonly termed *environmental genetics* or *metagenomics*, treat all community members as a single genomic pool (Kowalchuk *et al.*, 2007). The results of this study are given in Paper III.

Whereas Paper III describes functional genetical aspects of irgarol tolerance mechanisms, Paper IV describes the structure and diversity of the gene putatively involved in such mechanisms in periphyton. The gene coding for the irgarol target protein, from communities with high and low irgarol tolerance was put in a broad phylogenetic context. The work in Paper III and IV also gave the possibility to compare the traditional microscope-based identification of species to that of a metagenomic and phylogenetic approach, thus comparing effects of irgarol on the species level to effects on the gene level. Consequently, effects of irgarol have been studied over different time scales and at different levels of biological organisation.

The general aim of this thesis was to develop means to study toxicant-induced selection processes in contaminated ecosystems and in particular try molecular approaches to improve the resolution in such studies.. The specific objectives were to:

- evaluate the abilities of photosynthetic endpoints to detect toxic effects of antifouling compounds with various mechanisms of action
- describe the relation between irgarol contamination of the marine coastal environment and the development of community tolerance to irgarol in natural marine periphyton communities
- identify amino-acid sequence regions in the irgarol target site important for tolerance in periphyton communities
- describe the phylogenetic context of irgarol-tolerant and -sensitive target sites

BACKGROUND

Ecotoxicology, chemical risk assessment and scientific proof

Ecotoxicology as a science deals with organising knowledge about the fate and effects of toxicants based on explanatory principles (Newman, 1996). It has become increasingly apparent that contamination of the environment has unwanted effects on economical, ethical, human health and sustainability values. Therefore society has a need to assess the risks associated with chemical use and eventually regulating it. In addition to purely scientific goals, ecotoxicology thus has an important function to help society by supplying the techniques, ideas and data required to perform and develop risk assessment of toxicants. This dual nature can lead to confusion among people within ecotoxicology, environmental regulation and also among people outside these fields, about the aims of ecotoxicology, effectiveness of environmental regulation and justification and funding of different approaches within these areas (Depledge, 1993). Therefore, it is important to be aware of common goals as well as differences between ecotoxicology as a science and the application of ecotoxicology for ecological risk assessment of toxicants.

It first needs to be pointed out that the important function of ecotoxicology in performing risk assessments is in fact in contradiction to that of deductive science. Authorities, corporations and also common people are most interested in whether the use of a toxicant will have adverse effect on human health and/or the environment. The question is –*Will something bad happen if we use this toxicant*? In deductive science, however, a hypothesis can not be verified,

but can only be corroborated if we fail to falsify the opposite of the hypothesis. A well-known example is that of the black swans (Popper, 1959). A statement like “There are no black swans” is valid only as long as no black swans have been found. The statement is based on induction, which in practice means that we believe that what we normally see and find is “the truth”. However, as soon as someone finds a black swan this statement is not true anymore. Thus, inductive evidence is only temporarily ”true”. The criticism against inductive reasoning has a long history from the classical Greek philosopher Sextus Empiricus over David Hume (1711–1776) to modern scientists like Karl Popper (1959) and David Miller (1994). The consequence for ecotoxicology is that it is not possible to prove that a toxicant is environmentally “safe”, only that we so far have failed to falsify the hypothesis that the toxicant is hazardous. This means that we can not deductively validate environmentally ”safe” levels of toxicants. In ordinary life this may seem irrelevant; most people believe that gravity exists, irrespectively if it is deductively validated, since every time they drop something it falls to the ground. However, in ecotoxicological risk assessment of chemicals it means that ecotoxicology, in a strict sense, can not provide what is asked for. This phenomenon is of course not restricted to ecotoxicology only, but is valid in all natural sciences. However, since the aim of the risk assessment is to prevent something ”really bad” and perhaps irreversible to happen this problem is underlined in toxicology, ecotoxicology and risk assessment of chemicals. This means that the difficulties of predicting adverse effects of toxicants in ecosystem is not only linked to limited resources, or ability of scientists to perform good risk assessments, but is also an inherent difficulty in risk assessment. No matter how much resources and skills we have, it is *scientifically impossible* to guarantee that something ‘really bad’ won’t happen. The words of Ulrich Beck (1992) paraphrase this problem, “*The destructive forces scientists deal with in all fields today impose on them the inhuman law of infallibility. Not only is it one of the most human of all qualities to break this law, but the law itself stands in clear contradiction to science's ideals of progress and critique*.” Therefore, society should perhaps act even more precautionary in chemical risk assessment in order to avoid adverse effects to human and environmental health.

Community and single species ecotoxicology

The approaches currently used in risk assessment of chemicals are not good reflections of processes in the environment. Ecotoxicological tests are routinely performed with standardised laboratory protocols and single laboratory-cultured species. There are advantages with this approach, such as a high test capacity and high reproducibility. However, it is known that sensitivities of different species can differ with several orders of magnitude (Blanck *et al.*, 1984; Vaal *et al.*, 1997). Moreover, these differences are not arranged in an ordered fashion, which leaves us with the inconvenient truth that there is no

way of knowing beforehand whether a certain species is sensitive or tolerant to a certain chemical. What is also inconvenient from a scientific perspective is that the choice of test species is not made with any scientifically sound justification. Such justification could be considering species characteristics, for example the commonness in and among ecosystems or its position in the food web, when determining its appropriateness as test species. Rather this choice is highly influenced by practical aspects such as whether the species is readily cultivable in laboratories and whether it is suitable when determining an easily measured endpoint. This is understandable from a practical point of view; but from a scientific perspective this approach is very unsatisfactory. If we want ecotoxicological tests to predict anything about effects outside Petri dishes and 96-well plates, *i.e.* to predict effects in the natural environment, other approaches are necessary.

An alternative approach used in this thesis, is to use multi-species communities sampled from the natural environment. I use the definition of a community as a group of interacting populations that overlap in time and space (Clements and Newman, 2002). There are some disadvantages with this approach, like lower test capacity and low reproducibility. Stringently speaking; if one considers reproducibility as being the ability to repeat an experiment with the same morphologically distinct species, the community approach actually has zero reproducibility since it is impossible to sample exactly the same community twice from a natural environment. From the viewpoint of ecological relevance and robustness of the risk assessment, however, the community approach is very appealing. This approach elegantly avoids the problem associated with the differential sensitivity of species. Since the periphyton communities used in this thesis are very diverse, they are likely to contain a wide range of sensitivities. This is satisfactory since it means that sensitive species to any type of chemical is likely to be included. Another appealing characteristic is that we can choose the appropriate environment to sample when estimating effects of a certain chemical, and there exists no ambiguity whether the test species is ecologically relevant since they are the actual ones that should be protected. Therefore, the community approach includes inherent quality by sampling the actual entities that we aim to protect and then let the ecosystem select which species are relevant for each environment. Sampling the environment to obtain test communities might even be easier than culturing test organisms. However, care must be taken to ensure that the sampled communities are not tolerant to the tested compound.

Selection and Pollution-Induced Community Tolerance (PICT)

Charles Darwin (1859) introduced the concept of natural selection as "*preservation of favourable* [biological] *variations and the rejection of injurious* [biological] *variation*". This preservation and rejection is caused by differential fitness of the organisms (the biological variations). In the standardised single-

species approach natural selection during cultivation can be seen as a problem, since it selects only the genotypes that are successful during cultivation and thus continuously drives the population away from its natural niche. This is in sharp contrast to the community approach used here where natural selection is used as a means to refine ecotoxicological relevant information after toxicant exposure. Such refinement is used within the concept of Pollution-Induced Community Tolerance (PICT) outlined by Blanck *et al.* (1988) and reviewed by Blanck (2002) and Boivin *et al*. (2002).

Using the terminology of Blanck (2002), PICT can be divided into two phases; a selection phase in which the long-term toxicant exposure acts a selection pressure, and a detection phase in which the effects of this selection is quantified. During the selection phase the toxicant will be one of the environmental conditions that affect the fitness of the organisms in the community. The range of environmental conditions under which an organism can exist is called its fundamental niche. This has also been called the hypervolume, *i.e.* the multi-dimensional space, defined by the set of environmental conditions, under which an organism can exist and prosper. The fundamental niche is always diminished by ecological interactions (*e.g.* competition, predation) to the realised niche, which is the range of environmental conditions under which an organism can exist in interaction with others (Hutchinson, 1957). Exposure to a toxicant can be viewed as one of the environmental conditions that affect the size of the realised niche (Fig. 1). Due to differences in toxicant sensitivities among species, a community will go through a Toxicant-Induced Succession (TIS) upon exposure. Species or genotypes that are sensitive to the toxicant will be eliminated, which in turn will cause changes in the ecological interactions within the community. The more tolerant species thus have possibilities to increase in abundance due to *e.g.* lowered competition or predation, which results in increased tolerance of the community as a whole. In communities exposed to higher toxicant concentrations, the selection pressure of the toxicant will become increasingly important. The concentration at which community tolerance starts to deviate from unexposed communities should therefore represent the threshold where the direct or indirect selection pressure from the toxicant is ecologically relevant. It has also been shown experimentally that PICT responds at approximately the same concentration that eliminates the sensitive species in periphyton communities (Blanck and Wängberg, 1988; Molander *et al.*, 1990; Molander and Blanck, 1992; Dahl and Blanck, 1996b; Schmitt-Jansen and Altenburger, 2005) and in other communites (Gustavson and Wangberg, 1995; Wangberg, 1995; Pennanen *et al.*, 1996; Larsen *et al.*, 2003). This means that PICT is approximately as sensitive as the most sensitive species in the community and the problem of differential sensitivities among species is avoided altogether.

The tolerance detection phase involves short-term experiments, where communities are exposed to a series of concentrations of the toxicant, and their tolerances quantified. It is only the compound that has exerted a selection pressure during the selection phase, or a process inflicting the same biochemical stress on the community members, that will give increases in community tolerance. Since all the detection experiments are identical, a decreased sensitivity in some experiments for some communities must depend on the etiology of those communities. This renders PICT both causality and specificity.

Assuming that all selective pressures from the toxic compound have had maximal effect in all species in the community, a PICT response has integrated over all levels of biological complexity, from molecular interactions to direct or indirect ecological interactions. This means that selection will eliminate the most sensitive species or process, and PICT will allow us to determine the concentration of the toxicant that affects these inherently sensitive processes. This renders the PICT approach inherent sensitivity.

Thus, TIS refines ecotoxicologically relevant information that can be quantified as PICT. The advantage of refining ecotoxicological relevant information in this way also has important implications for future development of ecotoxicology as a science, which will be discussed in the section Future directions.

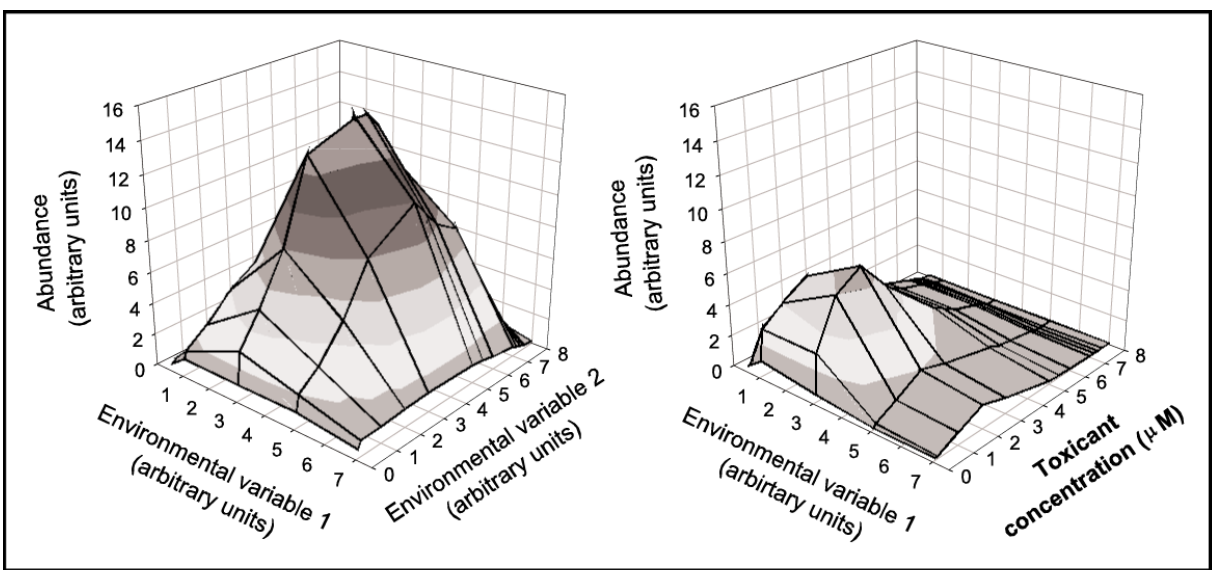

Figure 1. Abundance of organisms in a hypothetical realised niche described with two arbitrary environmental variables, where organisms are not exposed to a toxicant (left graph) and where organisms are exposed to a toxicant (right graph). In this example, the toxicant affects organism abundance, but also changes the capabilities of the organisms to withstand environmental variable 1.

Modern, especially western world, societies release an enormous multitude of chemicals into the environment. The CAS Registry database (CAS, 2008), which keep a worldwide record over all chemicals produced, had by the 11th December 2008 registered 40,861,971 organic and inorganic substances. The causality and specificity of PICT gives it at least a potential to establish cause-effect relationships between exposure of a toxicant and its effects in a complex ecosystem with a multitude of toxicants present. Crucial to this potential is the phenomenon of multiple tolerance and co-tolerance. In the former, exposures to several toxicants lead to multiple tolerances, while in the latter case exposure to one toxicant lead to increased community tolerance also to other toxicants. Co-tolerance are likely to occur when compounds have similar biochemical mechanisms of action, interacts with the same structures or compartments or are transported or degraded along similar routes (Blanck, 2002). Multiple tolerance, *i.e.* exposure to multiple toxicants that leads to multiple tolerances, can theoretically be viewed like any other combination of selection pressures that leads to a smaller realized niche. For example, "environmental variable 1" in Fig. 1 could be viewed as a second toxicant. In practice, however, it can be difficult to separate co-tolerance from multiple tolerances without extensive ecotoxicological testing and chemical analysis.

Since it is out of the scope of this thesis, I will not discuss all aspects of co-tolerance. However, some of the findings in Paper II and III have implications that require a deeper understanding of the co-tolerance between PS II inhibitors. However, first we need to review the mechanism of action of irgarol and the mechanisms of irgarol tolerance.

psbA, the D1 protein and effects of irgarol

Irgarol binds with high affinity to the Q_B-niche of the D1 protein within PS II, where plastoquinone otherwise accepts electrons. This results in at least three toxic effects: (i) blockage of photosynthetic electron transport, which in turn leads to hindered ATP and NADPH production (Fedtke, 1982; Draber et al., 1991), (ii) oxidative stress due to production of reactive oxygen species (ROS) at PS II (Ridley, 1977; Rutherford and Krieger-Liszkay, 2001; Fufezan *et al.*, 2002) and (iii) blockage of D1 turnover (Kyle *et al.*, 1984; Mattoo *et al.*, 1984; Trebst *et al.*, 1988; Jansen *et al.*, 1993). D1 is a protein which turns over rapidly (Mattoo et al., 1981; Gaba et al., 1987). Since it is situated in the reaction centre of PS II it is probably under constant pressure of light and ROS damage, even during non-herbicidal conditions. However, this turnover is also a regulating mechanism of electron flow and activation/inactivation of PS II as a mechanism of light adaptation, *e.g.* during photoinhibition (Mattoo et al., 1981; Schuster et al., 1988; Oquist et al., 1992; Critchley and Russell, 1994; Schnettger et al., 1994). Thus, D1 turnover is involved in regulation of fundamental processes in photosynthesis.

D1 is encoded by the *psbA* gene which is evolutionary very conserved among cyanobacterial, cyanophage and chloroplast genomes (Zurawski *et al.*, 1982; Svensson *et al.*, 1991; Trivedi *et al.*, 1994). Since these genomes are haploid, the term "haplotype" is used to describe a unique *psbA* sequence, *i.e.* "haplotype" is analogous to the term "genotype" in diploid genomes. In cyanobacteria there are 2–5 *psbA* genes; *psbA1* – *psbA5*. However, these only encode two different forms of D1 proteins; D1:1, encoded by *psbA1* and D1:2, encoded by *psbA2* – *psbA5* (Curtis and Haselkorn, 1984; Mulligan *et al.*, 1984; Vrba and Curtis, 1990). These two forms give different functional characteristics to PS II and are means for light adaptation in cyanobacteria (Campbell *et al.*, 1996).

Even though there are exceptions, most eukaryotic organisms only have one *psbA* gene in each chloroplast genome (Palmer, 1985; Dwivedi and Bhardwaj, 1995). However, there can be several copies (one to hundreds) of the genome within one chloroplast (Lee and Haughn, 1980; Bendich, 1987; Birky and Walsh, 1992) and these genome copies may be polymorphic (Frey *et al.*, 1999). Depending on species, there can also be several chloroplasts within one eukaryotic cell. One interesting example of chloroplast genome organisation, that further demonstrates the variable nature of chloroplast genes, is found among the dinoflagellates. Their chloroplast genomes are reduced to only 16 functional genes, including *psbA*, and divided into so-called minicircles, containing only one to three genes each (Zhang et al., 1999; Hackett et al., 2004; Barbrook et al., 2006). Just as the copy number of chloroplast chromosomes in other species, the copy number of minicircles is variable (Koumandou and Howe, 2007).

This variability in *psbA* gene organisation and copy number among different organisms gives a complex relationship between number of cells/individuals and number of genes. Therefore it is tempting to view these two levels of biological organisation as discrete entities. However, the variability also reminds us that the genomic context in which different *psbA* genes occur is very different. Selective processes on *psbA* are dependent on selection on other genes, or traits, which differ among organisms. Therefore, selection on one level is coupled to that of a higher level, and we need to be aware of this continuum of layered selection.

Tolerance mechanisms for PS II inhibitors

Among the toxicants studied in this thesis, PICT has been estimated for irgarol only. Therefore, the discussion is focused on tolerance mechanisms for compounds with similar mechanism of action as irgarol. However, parts of this discussion are valid also for other compounds.

Tolerance mechanisms to photosystem (PS) II inhibitors may involve decreased uptake, compartmentalisation, increased excretion, increased degradation, or an altered target site (Holt *et al.*, 1993; Oettmeier, 1999; Reade

et al., 2004; Downs and Downs, 2007). The most common and well-known tolerance mechanism to PS II inhibitors in terrestrial weed species are mutations in the chloroplast *psbA* gene resulting in the amino acid change $Ser_{264}{\rightarrow}Gly$ in the Q_B-binding niche of the D1 protein, which is the target site of these herbicides (reviewed by Oettmeier, 1999; and Devine and Shukla, 2000). The D1 protein is one of the two large apoproteins within photosystem (PS) II. The Q_B-binding niche of D1 is the site where plastoquinone accepts electrons during photosynthetic electron transport. The blockage of this site by PS II inhibitors thus arrests this electron transport. The $Ser_{264}{\rightarrow}Gly$ substitution is thought to reduce the affinity between the toxicant and the target site. However, it has been shown that in atrazine-resistant *Brassica napus* (Sundby et al., 1992) and *Synechococcus sp.* (Ohad et al., 1990a) $Ser_{264}{\rightarrow}Gly$ mutants also have an increased D1 turnover rate. In Paper III we suggest that increased D1 turnover itself might be a tolerance mechanism for these compounds. In these examples both the effect of the reduced toxicant affinity and the effects of increased D1 turnover might contribute to the atrazine tolerance and it is difficult to decipher which one is most important.

An interesting example of tolerance to PS II inhibitors and mutations in the *psbA* gene is that of the atrazine-tolerant cyanobacterium SG2, which was isolated from the wastewater treatment system of the Syngenta atrazine production facility in St. Gabriel, Louisiana, USA. Although the authors did not measure the atrazine concentration in the wastewater, it is likely that the atrazine exposure was extreme. In spite of the strong evolutionary conservation of *psbA*, the isolated strain had a very dissimilar *psbA1* gene compared both to *psbA2* and *psbA3* from the same strain and compared to *psbA* genes from other species. The *psbA1* had a $Ser_{264}{\rightarrow}Glu$, instead of the common $Ser_{264}{\rightarrow}Gly$ mutation, but also a 5-amino-acid insertion after amino acid 265 and 12 additional amino-acid substitutions between position 259 and 288. It thus seems likely that the tolerance, at least partly, is due to a lowered affinity for atrazine to the D1 protein, but it might also be due to other properties of this altered amino acid sequence. In this case no inhibition of growth rate and only minor inhibition of O_2 evolution (10%) were evident in spite of very high tolerance levels. It is tempting to speculate that an extreme toxic selection pressure has given rise to an extraordinary haplotype, well adapted to its specific environment.

An increased degradation rate of PSII inhibitors is also a common tolerance mechanism in various weed species. Atrazine and chlortoluron are metabolised by phase I enzymes through hydroxylation and dealkylation (Hall *et al.*, 1995; Cherifi *et al.*, 2001; Menendez *et al.*, 2006), followed by phase II enzymes like glutathione-S-transferase (GST) catalysing conjugation (Anderson and Gronwald, 1991; Gray *et al.*, 1996). Activity of the so-called phase III enzymes is another tolerance mechanism, mostly known from medicine where it results in unsuccessful chemotherapy of human tumour

cells (Ecker and Chiba, 1997) and ineffective antibiotic treatment of infectious bacteria (Bolhuis *et al.*, 1997), but is now also getting increased attention in environmental sciences. In an ecotoxicological setting the phenomenon is called multi-xenobiotic resistance and has been found in a great variety of species in the environment (Kurelec, 1992; Minier *et al.*, 1999). These enzymes keep internal concentrations of toxicants at low levels, by pumping them out of the cell. This is done with low substrate specificity and hence the mechanism gives tolerance to toxicants with different mechanism of action. Such a mechanism was shown to be an acute response to irgarol in the coral *Madracis mirabilis* (Downs and Downs, 2007).

Sulmon and co-authors (2004; 2006; 2007) showed that exogenous sucrose conferred tolerance to atrazine in *Arabidopsis thaliana*. This tolerance could not be ascribed to carbohydrate metabolism compensating for lowered photosynthesis. Even though the exact mechanism by which sucrose induce this tolerance is not explained, the authors find interesting correlations between tolerance and increased levels of Reactive Oxygen Species (ROS) scavenging enzymes and also with increased levels of *psbA* mRNA transcripts and of D1 proteins. This latter correlation is consistent with the suggested tolerance mechanism in Paper III.

Co-tolerance and supersensitivity of PS II inhibitors

There are essentially three chemically distinct classes of PS II inhibitors: the triazine and triazinone compounds, the urea compounds and the phenolic compounds. These all bind to the herbicide-binding niche of the D1 protein and thereby block the electron transport. However, they do not bind identically to D1 but to overlapping sites at the herbicide binding niche (Pfister *et al.*, 1979; Jansen *et al.*, 1990; Trebst *et al.*, 1993; Nakajima *et al.*, 1996a). This implies that a mutation giving tolerance to one type of PS II inhibitor can (i) give co-tolerance, (ii) result in no change of sensitivity or (iii) give increased sensitivity to another PS II inhibitor. For example, the $Ser_{264} \rightarrow Gly$ mutants of *Amaranthus hybridus* are very tolerant to triazines but show no tolerance to urea compounds and show increased sensitivity to phenolic compounds (Oettmeier, 1999). From the pattern of tolerances and increased sensitivities of mutants it seems like the phenolic compounds are the most diverging group compared to the other PS II inhibitors, which lead Trebst (1987) to define two different binding areas for phenolic and triazine/urea compounds. The phenolic compounds are also different from the others since they do not inhibit D1 turnover. In contrast, they actually seem to induce cleavage of D1 (Nakajima *et al.*, 1995; Nakajima *et al.*, 1996b) This is most interesting in the light of the findings of Paper III, where a tolerance mechanism of increased D1 turnover is suggested. It leads to the hypothesis that the irgarol tolerant periphyton described in Paper III should, to some degree, have been co-

tolerant to urea type PS II inhibitors, but much less co-tolerant to phenolic PS II inhibitors.

This hypothesis was tested by Blanck and Molander (1991) who found a high level of co-tolerance between urea and triazine compounds but less so for phenolic compounds in marine periphyton communities. However, the authors noted that the pattern of co-tolerance in the communities is different compared to the co-tolerance pattern given by the different amino acid substitutions in the D1 protein (Thiel and Böger, 1984; Erickson *et al.*, 1985; Brusslan and Haselkorn, 1988). In addition to the urea and triazine tolerance the communities showed tolerance to the phenolic compound ioxynil. This was supported by the description of ioxynil binding as intermediate between triazines and other phenols (Laasch *et al.*, 1982) and the co-tolerance observed between ioxynil and diuron in (Haworth and Steinback, 1987). In this context, and in the light of Paper III, it is interesting to note that mutants with deletions in the PEST region showed tolerance towards both triazines (atrazine), ureas (diuron) and ioxynil (Kless *et al.*, 1994). Also for these mutants supersensitivity was detected instead of co-tolerance towards the phenol compound bromonitrothymol. It is also interesting to note that Blanck and Molander (1991) suggested that "*it is more likely that the periphyton cotolerance pattern results from a mixture of several D1 genotypes present in the community*". Hence, the proposed tolerance mechanism in Paper III actually seems to fulfil some criteria for being responsible for the observed increase in community tolerance to urea, triazine and ioxynil (Blanck and Molander, 1991). It is important to note that the community tolerance for diuron (Molander and Blanck, 1992), and the co-tolerance to triazine and triazinone compounds in diuron tolerant communities (Blanck and Molander, 1991), were detected before irgarol became a heavily used antifouling compound. Since the use of irgarol started in 1992, and replaced diuron as an antifouling compound in 1994, it is reasonable to believe that the environmental concentration of triazines was very low when Molander and Blanck performed their studies in 1988-1990. Diuron, however, was at that time present in the coastal environment (Molander and Blanck, 1992). The environmental contamination thus seems to have shifted from a diuron-type to a triazine-type of PS II inhibitor. Albeit in low concentrations, diuron could have selected for tolerant cyanobacterial or microalgal genotypes that subsequently were enriched and produced the community tolerance and co-tolerance patterns in the experiments of Molander and Blanck (1991, 1992). Thus, the selection pressure of PS II inhibitors, affecting the energy conversion at PS II, might have been similar over the years, but the shift from diuron to irgarol contamination lead to a slightly different selection at the molecular level. The fact that co-tolerance to triazines were detected in diuron tolerant communities (Blanck and Molander, 1991), and that it subsequently seemed very difficult to become irgarol tolerant (Dahl and Blanck, 1996a; Paper II), indicates that in spite of the seemingly

similar mode of action of these compounds, very small differences at the molecular level might gives very different responses viewed at longer temporal scales at the community level.

There have, however, also been reports of co-tolerance between seemingly very different types of stress. Navarro *et al.* (2008) found co-tolerance to cadmium in periphyton communities after increased exposure to ultraviolet radiation (UVR), and argued that common tolerance mechanisms might occur through induction of antioxidant enzymes. Even though there is no consensus regarding the mechanism of action of cadmium, the argument seems valid since oxidative stress is reported as one feature of cadmium toxicity (DalCorso *et al.*, 2008). In the light of Paper III in this thesis, it is interesting to note that the co-tolerance detected by Navarro *et al.* could originate from an optimised D1 turnover in the communities, since D1 protein turnover has been shown to protect both against cadmium exposure (Geiken *et al.*, 1998; Franco *et al.*, 1999) and UVR (reviewed by Bouchard *et al.*, 2006). The hypothesis is also interesting in relation to the UVR-induced shift in community composition from diatoms to cyanobacteria detected by Navarro *et al.* (2008). This observation coincides with the fundamental differences of these groups in *psbA* gene organisation and in strategies for light adaptation. Among other things it has been shown that gene expression of *psbA* in response to UVR is different in cyanobacteria and chlorophytes. Whereas an up-regulation is observed in cyanobacteria exposed to UVR (Campbell *et al.*, 1996; Mate *et al.*, 1998; Tyystjarvi *et al.*, 2002), a down-regulation is evident in chlorophytes (Jordan *et al.*, 1991; Chaturvedi and Shyam, 2000). Although no reports of UVR-induced alteration of *psbA* gene expression in diatoms are available, it supports the view that pro- and eukaryotes have different strategies for UVR-adaptation.

Communities in equilibrium or in continuous change

Communities are often portrayed as being quite stable units, "in equilibrium" or "in balance". Environmental regulation has adopted this idea of a stable, undisturbed community as indication of environmental health, from which deviations can be estimated and adverse effects can be inferred. Processes like recovery, *i.e.* the process where a community returns to a pre-disturbed state, and resilience, the rate of this process, is central in this paradigm. There have also been attempts to predict what effects stress, *e.g.* toxicant exposure, will have on communities (Odum, 1985; Rapport *et al.*, 1985). Although some processes or functions of communities might be stable, for example due to functional redundancy (Pratt and Cairns, 1996), the perception that communities behave in a predictive manner has been questioned (Schindler, 1990; Pratt and Cairns, 1996; Moore, 1998). This perception was also questioned by Landis, Matthews and Matthews by the Community Conditioning Hypothesis (Landis *et al.*, 1996; Matthews *et al.*, 1996). These

authors suggest that communities are dynamic entities which continuously respond to disturbances, and that each have a historical record that determines their response to a new stressor. Moreover, the hypothesis states that since communities are products of their unique etiology there can never be two identical communities, and that information from events that change the function and/or structure of a community can be stored at different levels of biological complexity. The hypothesis was further supported by Landis et al. (2000) and Landis (2002). The hypothesis also has fundamental consequences for concepts as resilience and recovery, since it implies that communities are in constant change instead of returning to a pre-disturbed state, or as Landis *et al.* (2000) puts it "*The search for the recovery of an ecological structure is meaningless in terms of the ecological system*". Moreover, the term "reference site" might also need revision in the community conditioning context. If no communities are ever alike, and they therefore develop differently irrespective of pollution, the question is if comparisons between sites are ever valid. The use of "reference sites" is also challenged by the increasing knowledge of global contamination (Schindler *et al.*, 1995).

These ideas really complicate the concept of ecotoxicological effects and emphasises the need for different ecotoxicological approaches. In this regard Paper II is probably quite unique since it describes spatial and temporal patterns of community tolerance in natural communities over a time frame as long as 10 years. In this study, site 6 at least *a priori* was regarded as a ″reference site". Here it is important to make some clarifications: (i) The PICT study in Paper II uses a spatial contamination gradient over time and does not compare a polluted site to a "reference site" only. (ii) PICT will only be detected if factors that increase community tolerance vary between the sites. Hence, comparing sensitivities of communities to a toxicant through the PICT approach is partly uncoupled from other factors that affects or condition communities. (iii) Even though PICT is uncoupled from these other factors, and site 6 could be considered as ″pristine″ or as a ″reference site″, we detected a consistent pattern of increasing community tolerance at this site (Fig. 4B, Paper II). Even though the increase is quite small it confirms that the ″reference site″ concept should be regarded with caution.

Toxicity over time

In this thesis, effects of irgarol on periphyton communities have been studied over time frames from 2 minutes to 10 years. Effects at different temporal scales are coupled to different levels of biological complexity. It is, for example, not appropriate to study toxicant-induced changes of community composition before selection has eliminated any individuals or species, or to study irgarol-induced increase in fluorescence induction several weeks after irgarol addition. Processes at different levels of biological complexity proceed

at different rates. The relative importance of different ecotoxicological processes is depicted in Fig. 2.

For a toxic effect to occur the chemical needs to be taken up by an organism. Uptake rate of the toxicant is a process that depends on many factors, like concentration, chemical properties of the toxicant or characteristics of the exposed organism just to mention a few. When the toxicant reaches its target, which can be a specific target like a protein or receptor or a general target like a membrane, it elicits the start of a physiological chain/chains of events that eventually results in toxicity. At low toxicant doses normal homeostasis mechanisms might be enough to avoid adverse effects. When the dose is slightly increased physiological responses in the form of compensatory and repair mechanisms are induced (Depledge, 1989). These mechanisms are likely to involve metabolic costs that can have effects on higher levels of biological complexity, *e.g.* population growth rate (Sibly, 1996).

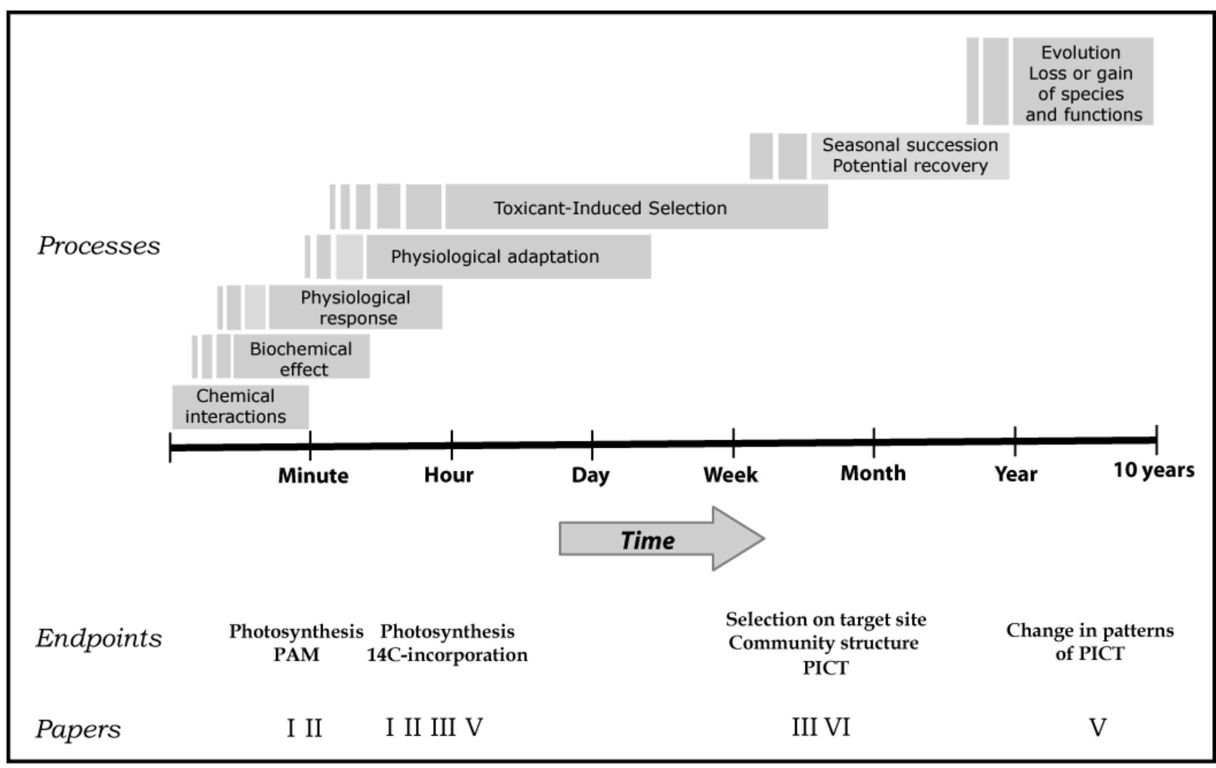

Figure 2. Schematic illustration of the time dependency of processes at different organisation levels, examples of endpoints used to detect changes in these processes and the Papers in this thesis dealing with the processes. The time frames of the processes are only outlined as conceptual. The scale on the time axis is categorised.

An example of a compensatory mechanism to sublethal effects of PS II inhibitors is the so-called greening effect, which appears as an increase in the amount of accessory pigments (Fedtke, 1982; Hatfield *et al.*, 1989; Koenig, 1990). This increase is a response to the lowered photosynthetic electron transport. Similar to a shade-adaption response, it increases electron transport

by leading more energy into PS II. This can, however, be devastating during PS II inhibitor exposure since the extra energy funnelled into PS II can not be transformed into electron transport, but instead the energy is transformed to reactive oxygen species (Fedtke, 1982; Rutherford and Krieger-Liszkay, 2001). There are possibilities to measure chlorophyll content with PAM methodology, based on the minimum fluorescence level (Fo) of a dark-adapted sample. Such measurements could reveal the greening effect. Unfortunately, the experimental design of the PAM experiment in Paper I does not permit an analysis of minimum fluorescence level (Fo) of dark-adapted samples. However, a minimum fluorescence level of light-adapted samples, detected just prior to the saturating pulses (Fo′), is possible to analyse for the irgarol and diuron experiments. In the experiments with the other compounds the gain settings varied too much for such an analysis. The results are not included in Paper I but are shown in Fig. 5. The initial increase in Fo′-level caused by the PS II inhibitors was very rapid, since higher values in exposed communities occurred already in the first measurement. This increase in Fo′ is directly caused by the binding of the compounds, which redirects the incoming energy from the saturating light pulse to fluorescence. As such, it is a reflection of the chemical interaction between toxicant and target, and not a physiological response of the organisms. However, increases from the first Fo′ level in each treatment would indicate the physiological greening effect. There seems to be a trend of such a response in the irgarol-exposed periphyton communities, since exposure to 0.32, 3.2 and 10 nM leads to increased Fo′-values over time. For periphyton exposed to the highest concentration there is instead a decrease in Fo′ over time, indicating that this high level of exposure results in degradation of chlorophyll. For diuron there is no obvious trend of increasing Fo′-values. Since diuron is known to be persistent (Callow and Willingham, 1996), and its effect on photochemical efficiency of PS II (ϕ_P) is persistent throughout the incubation in Paper I, it is unlikely that the absence of Fo′ increase in the diuron experiment is due to diuron degradation. The detection of a greening effect for irgarol but not for diuron is interesting since they bind to only slightly different domains of D1 (see section Co-tolerance and supersensitivity of PS II inhibitors). The analysis of minimum fluorescence levels was based on measurement on light-adapted communities that received saturation pulses. The light-adapted state of photosynthesis means that the organisms might have different redox status of their electron transport components (*e.g.* reaction centers and plastoquinone pool) and the Fo′ levels might therefore not correspond to the actual chlorophyll content in the communities. Thus, these Fo′ studies only provide an approximate measure of the greening effect.

In addition to compensatory and repair mechanisms, degradability of the toxicant is important during development of toxicity. If the toxicant is degraded into less toxic degradation products, and the effects caused are

reversible, functional recovery may occur. An example of such a functional recovery is the increase in photochemical efficiency of PS II (ϕ_P) after prolonged exposure to dichlofluanid (Fig. 2, 4-5, Paper I).

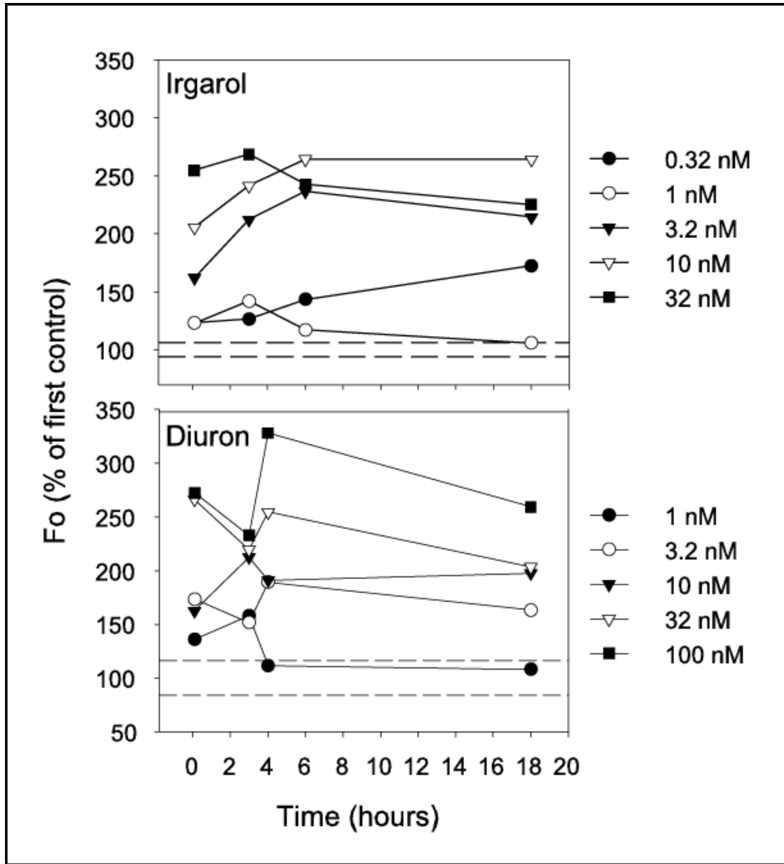

Figure 5. Effect of irgarol and diuron exposure on Fo values over the incubation period. Dashed lines represent 95% confidence limits of the first control measurement.

If the exposure is prolonged, the physiological responses are continuously needed and physiological adaptation may be selected for among individuals. These adaptations may be different among species and inter-species selection becomes increasingly important. Selection will force the community through a toxicant-induced succession (TIS), potentially resulting in an altered community composition and a PICT response. If a system has limited diversity and/or immigration of new species/genotypes, the PICT potential is also limited. However, marine ecosystems are diverse, open and dynamic, with high immigration and emigration rates. The dynamics and high PICT potential of periphyton communities is evident from the season-dependent community-tolerance patterns observed in Paper II (Fig. 3, Paper II). If exposure becomes permanent, or reoccurring as for irgarol (Fig. 1A, Paper II), over large geographical areas, there are risks of adverse irreversible effects such as extinction of species/genotypes. Schindler (1996) identified this as an area of concern for ecotoxicology. The ongoing release and the long-distance transport of persistent toxicants lead to contamination of even distant ecosystems (Schindler *et al.*, 1995). For example, Douglas *et al.* (1994) have showed that extinction of several diatom species in isolated and seemingly pristine

Canadian ponds co-occurred with the industrial revolution. In this context it is alarming that the minimum tolerance levels to irgarol seem to increase at all stations in Paper II. Even though marine ecosystems are open and dynamic, the minimum tolerance level increase at all stations during all seasons of the year. This could indicate that irgarol-sensitive species, haplotypes or genotypes are threatened or have gone extinct over large geographical areas.

Selection from toxicants can also lead to loss of diversity within populations, as described by the "genetic erosion" hypothesis (reviewed by van Straalen and Timmermans, 2002). These authors list four mechanisms by which toxicants can affect genetic diversity in populations: (i) by increasing mutation rates, (ii) by directional selection on tolerant genotypes, (iii) by causing bottleneck events, and (iv) by altering migration. A change in PICT pattern over time will detect the consequences of all these mechanisms in all populations originally present in the communities. Even though it is likely that tolerant communities have lower diversity, an altered PICT pattern can not be interpreted as a loss of diversity *per se* since PICT may also include immigration or favouring of formerly low-abundant tolerant species/populations. An altered PICT pattern over time indicates that the communities under study are restructured with regard to species, population and/or genotype compositions. Therefore diversity estimations are suitable complements to PICT studies.

Periphyton

The use of periphyton in environmental monitoring and in ecotoxicological research has a long tradition. Early studies, *e.g.* by Seligo (1905), termed periphyton "Aufwuchs" and the first to use the term "periphyton" was probably Behning (1924). The term periphyton means growing around, or overgrow (Railkin, 2004). There are many definitions of periphyton. For example the one given by Young (1945): "*By periphyton is meant that assemblage of organisms growing upon free surface of submerged objects in water and covering them with a slimy coat. It is that slippery brown or green layer usually found adhering to the surface of water plants, wood, stones or certain other objects immersed in water and may gradually develop from a few tiny gelatinous plants to culminate in a woolly, felted coat that may be slippery, or crusty with contained marl or sand.*" Although this definition might not be the most convenient one, it is very illustrative. The issue separating most of these definitions is whether the term strictly applies to organisms growing on artificial surfaces or if it also applies to organisms growing on natural surfaces. This separation is not important for the work presented here since only communities growing attached to artificial surfaces were used. Thus, several definitions of periphyton could be used in this thesis, for example the one by Behning (1924) *organisms growing attached to artificial objects in water*, or the one by (Wetzel, 1975) *microbial community found on submerged substrata*.

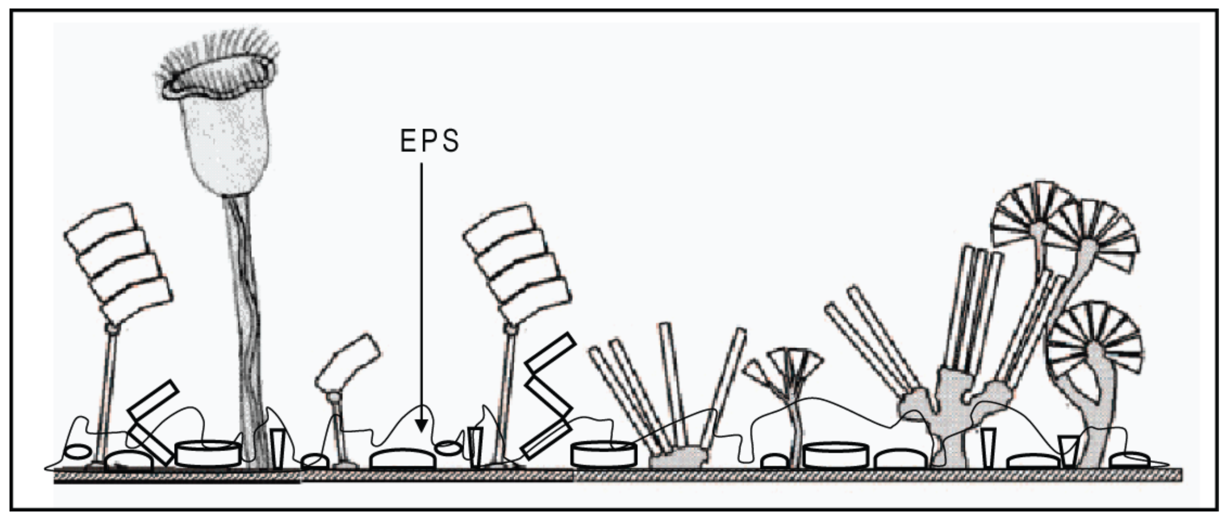

Figure 3. Schematic of a periphyton community. Various organisms are attached to a glass surface or embedded in the matrix held together by extracellular polymeric substances (EPS).

These communities consist of a food-web of interacting organisms from a diverse set of taxonomic and functional groups. Even though a common term for periphyton is "biofilm", these organisms produce a three-dimensional structure with organism habitats in different layers (Railkin, 1998; Molino and Wetherbee, 2008). In this way they are similar to many other communities, *e.g.* to a terrestrial forest community. They typically contain three dimensional structures like channels or tunnels, which increase the surface area and facilitate transport of *e.g.* substrates, nutrients, metabolites and oxygen between inner and outer parts of the biofilm (Lawrence *et al.*, 1991; Debeer *et al.*, 1994; Stoodley *et al.*, 1994; Massoldeya *et al.*, 1995; Battin *et al.*, 2003). These structures also diversify the biofilm by increasing the number of habitats, both for small single-cell organisms like bacteria and microalgae and larger organisms like nematodes and invertebrate larvae. Within these communities ecological interactions, *e.g.* competition, predation, parasitism and mutualism, are present (James *et al.*, 1995; Burchard and Sorongon, 1998; Railkin, 2004). Most of the relevant anabolic and catabolic pathways and nutrient cycles are also present, and therefore periphyton communities can be considered as small but autonomous entities that are well suited for ecologically relevant ecotoxicological experimentation (Sabater *et al.*, 2007). This complex three-dimensional biofilm also consists of extracellular polymeric substances (EPS), like carbohydrates, extracellular proteins, lipids and nucleic acids. EPS have several functions in the community like enabling adhesion, organism motility, colony formation and habitat stabilisation (see reviews by Hoagland *et al.*, 1993; Wuertz *et al.*, 2004; Branda *et al.*, 2005; Sabater *et al.*, 2007; Molino and Wetherbee, 2008). EPS can also bind toxicants, thereby lowering the bioavailable fraction and thus lowering the exposure to toxicants (Loaec *et al.*, 1997; Pistocchi *et al.*, 1997; Samrakandi *et al.*, 1997; Admiraal *et al.*, 1999).

Moreover, communities have been shown to respond to exposure to various metals and 2,4-dichlorophenol by increased production and changed composition of EPS (Sheng *et al.*, 2005) As periphyton species grow attached to surfaces, they are per definition potential fouling organisms.

Fouling and antifouling compounds

The problem of fouling on ship hulls and marine installations has been known for a long time and the use of antifouling systems is not a modern phenomenon. The common solution to the problem of fouling has been to cover the surface with some toxic compound that leak out from the surface and by its toxicity prevent attachment of organisms. Even before 2000 BC man used copper sheathings to protect ships from fouling (Omae, 2003). Although it is not only a phenomenon on artificial surfaces, the definition of biofouling by Yebra *et al*. (2004) as "*the undesirable accumulation of microorganisms, algae and animals on artificial surfaces immersed in seawater*", is appropriate in an antifouling context. Colonisation of surfaces by organisms is a universal and fundamental characteristic of the biota in marine environments. The economic implication of this characteristic is, however, of major importance for any activity performed in marine waters. Fuel consumption of ships increase, which in turn decrease ship speed and increase CO_2 discharges to the atmosphere, when fouling is present. For example, Schultz (2007) predicted that fouling consisting of so-called slime (*i.e.* periphyton) only, could increase the total resistance with 16-20%, corresponding to increases of shaft power with 16-21%. The same authors also predicted that heavy calcareous fouling could increase total resistance of a frigate with 55-80%, which corresponded to a required shaft power increase of 59-86%. The maintenance costs for marine installations and ships also increase substantially as fouling results in increased corrosion, dry-docking time, and manpower (reviewed by Gitlitz, 1981; Yebra *et al.*, 2004; Chambers *et al.*, 2006).

It has been estimated that commercial and recreational vessels use antifouling paint to control the growth of 4,000–5,000 plant and animal species (Evan and Smith, 1975). However, as they also affect non-target organisms in the environment they become an environmental problem. This has been recognised in the last years and the International Maritime Organisation's ban of tri-butyl-tin (TBT) is the most apparent example of this recognition (IMO, 2001). The compounds studied in this work are all used as ingredients in antifouling paints. Legislation in different countries has resulted in different usage of the compounds in different geographical areas. Of the studied compounds, only irgarol is presently allowed to be used in Sweden, and only on the Swedish west coast. Ecotoxicological information about the compounds is shown in Table 1.

Table 1. The names, structures, CAS numbers, mechanisms of action, degradation characteristics, highest concentrations detected in the environment and additional usage of the antifouling compounds studied in this thesis.

Substance name	Full name	CAS no.	Mechanism of action	Degradation	Highest environmental concentrations detected	Additional usage
Irgarol	2-methylthio-4-tert-butylamino-6-cyclopropylamina-s-triazine	28159-98-0	Photosystem II inhibitor, blocking electron transport in D1 in photosystem II. [1]	Persistent: t1/2 = 201 days [1], 350 days [16]. Photodegradation of >95% in 6 months [17].	16.7 nM [26]	No other use reported
Diuron	N-(3,4-dichlorophenyl)-N,N-dimethyl urea	330-54-1	Photosystem II inhibitor, blocking electron transport in D1 in photosystem II [2-3].	Persistent: <1% reduction during 42 days [16].	21 nM [27]	Herbicide in agriculture [29]
DCOIT	4,5-dichloro-2-N-octyl-4-isothiazoline-3-one	64359-81-5	Reacts with thiol-containing proteins [4]. Inhibiting succinate dehydrogenase in mitochondria [5-6]. Might cause cascades of RS· radicals [7-9].	Biodegradable: t1/2 < 1 hour [19], = 1.9 days [16].	12 nM [28]	No other use reported
Chlorothalonil	2,4,5,6-tetrachloro-1,3-benzene dicarbonitrile	1897-45-6	Reacts with thiol-containing proteins. Depletes glutathione reserves. [10-11]	Biodegradable: t1/2 = 2.8 days [16], 8 weeks [19], 4.4-150 hours [20]. Photodegradable: t1/2 = 1-48 hours [21].	5.2 nM [28]	Fungicide in agriculture and wood preservation [30-31]
Dichlofluanid	N-dichlorofluoromethylthio-N′,N′-dimethyl-N-phenylsulfamide	1085-98-9	Inhibits thiol-containing proteins by forming disulfide bridges. Stimulates Ca^{2+} efflux from mitochondria [12].	Very rapid hydrolysis [22]. Photodegradable: t1/2 = 8-83 hours [23]. Total degradation: t1/2 = 0.8 days [16].	1.8 nM [28]	Fungicide in agriculture and wood preservation [29, 31]
Zinc Pyrithione	bis(2-pyridylthio)zinc-1,1′-dioxide	12463-41-7	Interacts with (and possibly disrupt) biological membranes [13-14]. Causes intracellular ATP losses [15].	Photodegradable: t1/2 = >2-17.5 minutes [24], 8.3 minutes [25].	No reports available.	Fungicide in anti-dandruff shampoos and metal chelating agent in mining industry [13]

[1] Hall *et al.* (1999) [2] Moreland (1980) [3] Chesworth *et al.* (2004) [4] Fuller *et al.* (1985) [5] Chapman and Diehl (1995) [6] Bragadin *et al*. (2005) [7] Collier *et al.* (1990a) [8] Collier *et al.* (1990b) [9] Collier *et al.* (1991) [10] Tillman *et al.* (1973) [11] Long and Siegel (1975) [12] Hertel *et al.* (1981) [13] Dinning *et al.* (1998a) [14] Al-Adham *et al.* (1998) [15] Dinning *et al.* (1998b) [16] Thomas *et al.* (2002) [17] Okamura *et al.* (1999) [18] Jacobson and Willingham (2000) [19] Callow and Willingham (1996) [20] Davies (1988) [21] Sakkas *et al.* (2002) [22] Schouten *et al.* (2005) [23] Sakkas *et al.* (2001) [24] Turley *et al.* (2000) [25] Maraldo and Dahllof (2004) [26] Basheer *et al.* (2002) [27] Thomas et al. (2001) [28] Konstantinou and Albanis (2004) [29] Voulvoulis *et al*. (2000) [30] Gallagher *et al.* (1991) [31] KemI (1991)

METHODOLOGICAL CONSIDERATIONS

Sampling

Small glass discs (1.5 cm^2) were used for periphyton sampling. All discs were cleaned by boiling them in concentrated nitric acid for at least 10 minutes to oxidise any remaining organic matter, and then thoroughly rinsed in distilled water. In the field sampling these discs were mounted in polyethylene holders, placed in polyethylene racks as shown in Figure 4. Just prior to placing the rack into the water, the discs were rinsed in 70% ethanol for 10 minutes. The discs were then colonised by the indigenous biota present at the sampling site for 2-4 weeks. The sampling sites used in Paper II are shown in Fig. 5. Site 5 shown in this figure is also the sampling site used for all tests in Paper I.

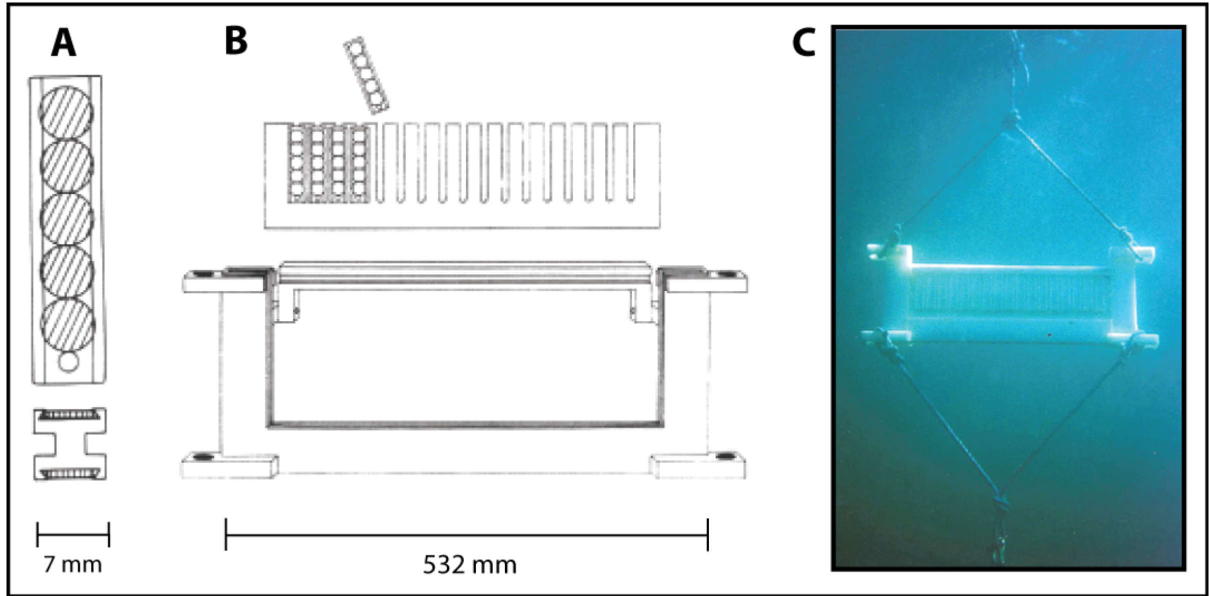

Figure 4. Periphyton field sampling equipment. (A) Holder for periphyton glass discs. Five periphyton glass discs are mounted on each side in each holder. (B) Rack for 17 holders (top) is mounted in a frame (bottom). The rack is held in place by a crossbar that is locked by screws. (C) A periphyton sampling unit hanging in the water, connected to a buoy at the surface and a stone at the bottom. All devices shown, except for the glass discs and the rope, are made in polyethylene. (A) and (B) are redrawn from Dahl (1996). Picture in (C) is taken by Frederick Grönwall.

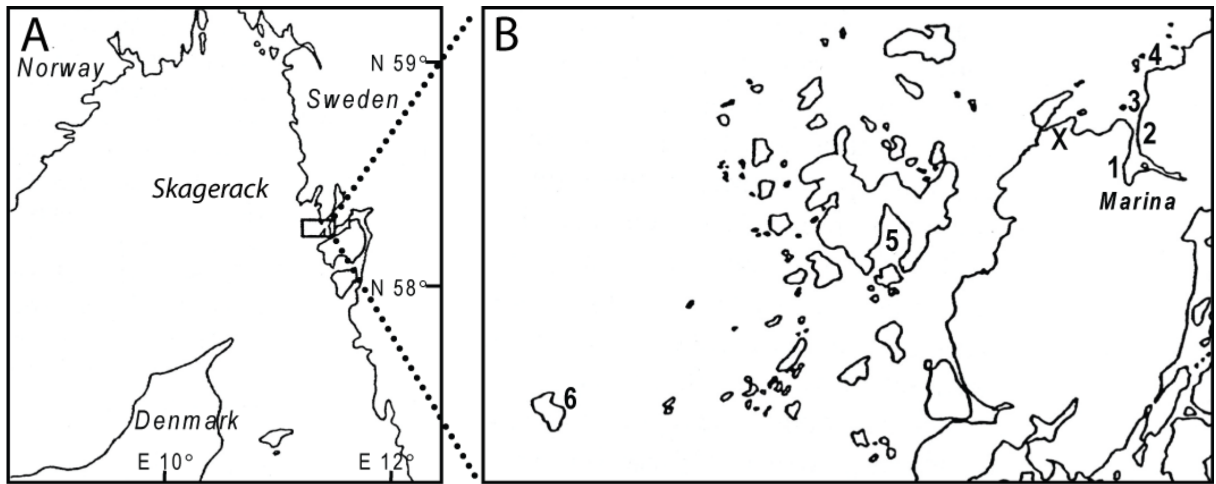

Figure 5. Map showing sampling sites in Paper II. (A) Geographical area of the Skagerack sea. Small rectangle is enlarged in (B) that shows the sampling area. Sites are numbered 1-6 from high to low irgarol contamination levels. The characteristics of the sites are described in Table 1 in Paper II. Site numbers are the original ones given by Blanck and Dahl (1996). Site 2 was not used in this thesis. The location of the research station were all the experiment were performed is marked with a X. Redrawn from Dahl (1996).

In the microcosm experiments the same procedure for cleaning the discs was used. Colonisation of periphyton was enabled in these experiments by continuously pumping natural seawater from approximately three meters depth into an in-door microcosm system. Simultaneously, toxicant solutions and distilled water were pumped into the exposed and unexposed microcosms respectively, with the aim to achieve stable levels of the desired test concentrations of the toxicants. Each microcosm was equipped with a light source, set to the current light/dark regime, and a stirring device to ensure thorough mixing of the water. The discs were colonised by the periphyton species able to cope with the environment in the microcosms. The potential immigration in the microcosm communities was thus identical, but the actual immigration in the communities was influenced by the toxicant concentrations. Colonisation and growth was allowed for 3-4 weeks in the microcosm studies.

Photosynthesis

Both functional and structural measures of toxicity have been used in this work. The two functional measures ^{14}C-incorporation and Pulse Amplitude Modulation (PAM) fluorometry are both estimating rates of photosynthetic processes. The use of the ^{14}C-incorporation method is well established in ecotoxicology (Blanck, 1985; Lewis, 1995; Falkowski and Raven, 1997). When used in this thesis it measures the incorporation of radiolabelled carbon into acid-stable molecules in periphyton. As such it is an integrated measure of processes of both light and dark reactions in photosynthesis. PAM fluorometry measures induction of *in vivo* chlorophyll *a* fluorescence. By applying light of

different intensities and duration to the sample the fluorescence signal at different time points can be used to calculate the photochemical efficiency of PS II (ϕ_P). The scheme of PAM measurements of ϕ_P is shown in Figure 7. Govindjee (1995) estimated that approximately 90% of the total fluorescence signal comes from photosystem (PS) II. The signal from PS I is low at ambient temperatures because photochemistry and other processes, like thermal dissipation and triplet formation, are particularly efficient in that system (Strasser and Butler, 1977). Thus ϕ_P is an estimation of photosynthesis that reflects the status of photosystem (PS) II mainly. As PAM fluorometry is an easy-to-use and non-destructive method it is suitable for ecotoxicological time-course experiments.

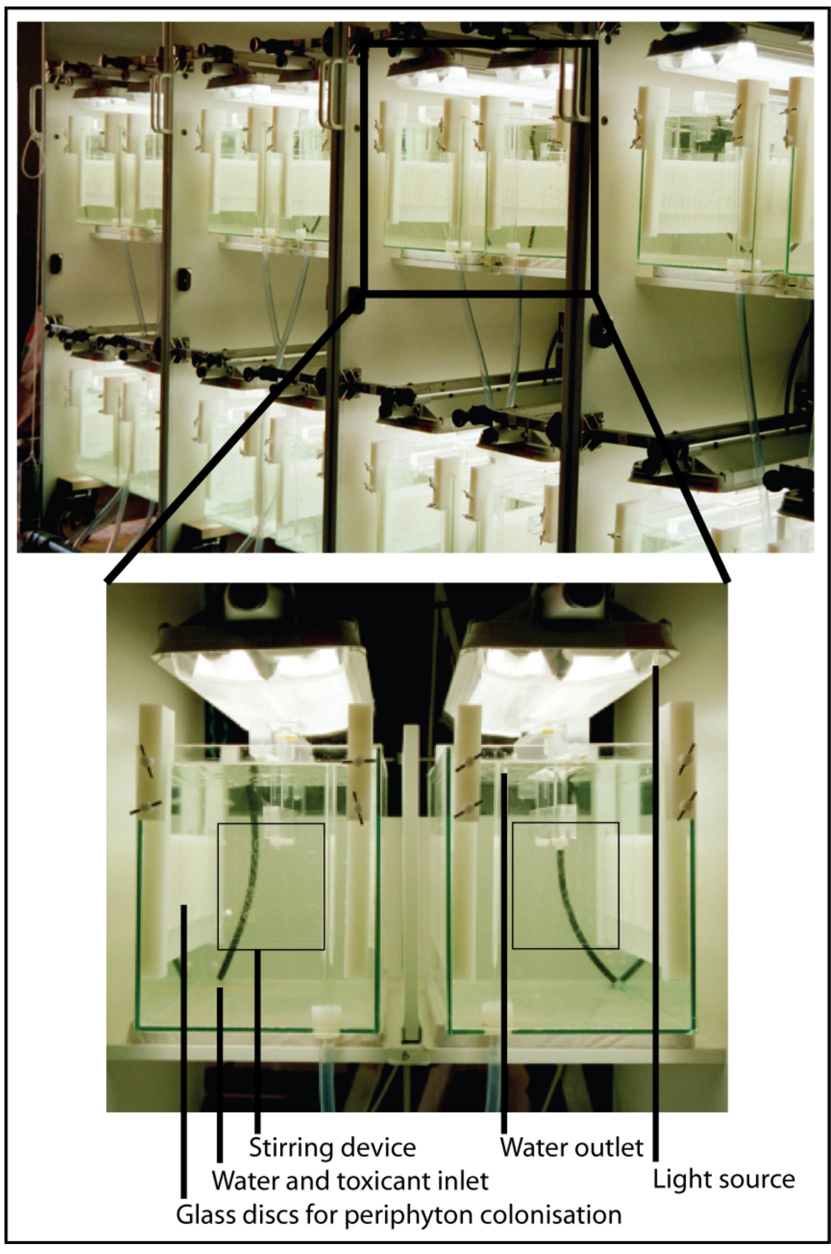

Figure 6. Microcosm system used for long-term exposure experiments with irgarol (Paper III, IV and the section Significant Findings). Sea water from 3 m depth is pumped into an indoor water-delivery system that supplies the 22 L aquaria with a continuous water flow of 220 mL min^{-1}. Toxicant solution, or distilled water, is simultaneously pumped into the tubing of the water inlet for each aquarium. The mean water residence time is 100 minutes. The outlet is placed at the surface in the opposite corner of the aquaria. Each aquarium is also equipped with a light source of two luminescent tubes, and a stirring device made of four acrylic glass plates which constantly move back and fourth. Two periphyton racks per aquarium are placed by the long sides. Pictures taken by K. Martin Eriksson.

Measures of photosynthesis have been shown to be less sensitive than more integrating measures, like population growth, for various chemicals (Delistraty, 1986; Lewis and Hamm, 1986; Turbak *et al.*, 1986). The lower ability of photosynthesis endpoints in detecting effects from compounds with mechanisms of actions not directed towards photosynthesis is reasonable, since toxic effects elsewhere in the metabolism may allow photosynthesis to proceed undisturbed. Eventually the consequences of such a toxic effect may propagate throughout the cell and may thus reach photosynthetic processes and be detected. The ^{14}C-incorporation method has similar sensitivity as PAM fluorometry for estimating effect of PS II inhibitors, but has higher sensitivity for estimating effect of other investigated compounds (Paper I).

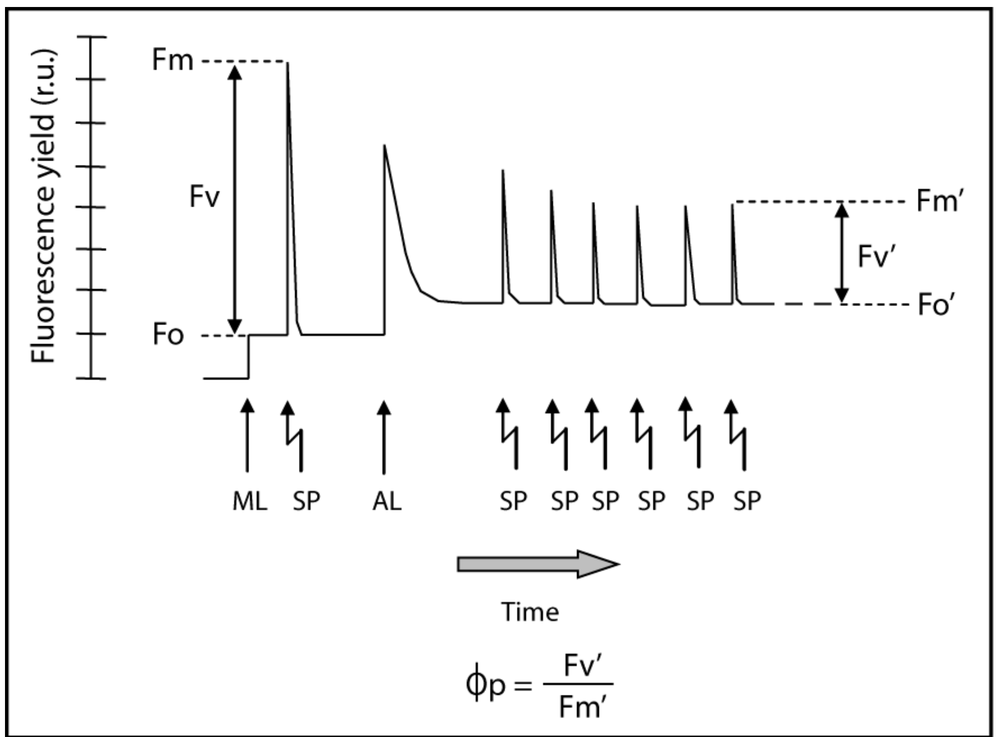

Figure 7. *In vivo* chlorophyll fluorescence induction in Pulse Amplitude Modulation (PAM) fluorometry. Measuring light (ML) is applied to a dark-adapted sample at an intensity not driving photosynthesis, and results in emission of the minimum fluorescence intensity (Fo). A short saturating pulse of high intensity white light (SP) is given, which fully reduces photosystem (PS) II and gives rise to a maximal increase in fluorescence (Fm). From these parameters the variable fluorescence of a dark-adapted sample (Fv) can be calculated, as Fm – Fo. Actinic light (AL) that drives photosynthesis of the sample is applied with a concomitant increase in fluorescence. This fluorescence is subsequently quenched because photosynthesis starts to convert the light to chemically bound energy. The quenching of fluorescence is known as the "Kautsky effect". When a stable fluorescence level under actinic light illumination (Fo') is attained, saturating pulses are given and the maximal fluorescence of the light-adapted sample (Fm') is recorded. The parameters Fo' and Fm' is used to calculate the variable fluorescence of a light-adapted sample (Fv'). In the calculations of the photochemical efficiency of PS II (ϕ_P), 15 saturating pulses were given and the average Fv'/Fm' values of the last five pulses were used.

This is logical since ^{14}C-incorporation is dependent on other metabolic processes than photochemical efficiency of PS II (ϕ_P). This is an important advantage of ^{14}C-incorporation as compared to PAM. However, the ^{14}C-incorporation tests might be insensitive compared to other endpoints for compounds with mechanisms of action directed towards non-photosynthetic processes. Both in short-term toxicity tests in general and in PICT detection, the endpoint should preferable be matched to the mechanism of action of the compound. If the mechanism of action is unknown, a more integrating endpoint is preferred.

Taxonomic analysis of cyanobacteria and algae in periphyton

As described previously, structural effects of toxicant in communities are among the most sensitive measures in community ecotoxicology. However, analysis of the complete species composition in natural periphyton communities is time-demanding. Therefore, a semi-quantitative approach was adopted, in which 50 randomly chosen microscopy fields were analysed for the presence or absence of morphologically distinct species. In such an analysis the increase in accumulated number of species rapidly approaches zero after 35-40 analysed fields (Dahl and Blanck, 1996a). I have had the privilege of working together with the very skilled periphyton taxonomist, the late Dr. Mats Kuylenstierna. Periphyton microalgae and cyanobacteria were identified to the level of species or genus whenever possible. Some organisms with unknown identity, but with morphologically distinct appearance, were tentatively classified and labelled according to Dr. Kuylenstierna´s periphyton reference library.

Clone libraries

Clone libraries of a fragment of the *psbA* gene were built from the metagenome of communities with background and elevated irgarol tolerance levels in the microcosm study made in 2001 (Paper III, Fig. 8). The use of a functional gene, *i.e.* a non-neutral marker, enabled direct study of selective processes on the target protein of irgarol. We made clone libraries by scraping off the periphyton biofilm, extracting total community DNA, performing degenerate polymerase chain reaction (PCR), cloning the PCR product, and sequencing. Some of these steps may introduce biases and artefacts.

When taking the samples for the DNA extraction, the periphyton was carefully scraped off the glass surface with a scalpel. This step could potentially introduce bias against small and firmly attached species. There was, however, no clear indication of such problems in Paper III. After centrifugation and removal of excess water the communities were snap-freezed in liquid nitrogen and later thoroughly grinded with a mortar and pestle in liquid nitrogen. The alternative method of bead-beating was evaluated. The very powerful bead beater Precellys 24, capable of

homogenising materials like teeth and wood (Ekstrand Söör, 2006), was used. However, there were clear biases in breakage of different periphyton organisms. In order to break all cell types it seems like freezing in liquid nitrogen is needed to make the cells fragile. After grinding in liquid nitrogen there was no obvious bias in breakage of cells as judged by microscopic inspection. The DNA-extraction procedure was made according to the well developed method with DNAzol™ (Invitrogen) and chloroform, and no systematic biases are suspected in this step. PCR, however, is associated with problems.

Community, or multi-template, PCR is widely used, especially for genes encoding ribosomal RNA (rDNA genes like 16S and 18S). However, many studies have demonstrated problems with this approach, and some has suggested severe limitations (Wang and Wang, 1996; Thompson *et al.*, 2002; Markmann and Tautz, 2005). Therefore this topic needs some specific attention. The problems can be divided into bias and artefacts.

Bias can appear during PCR as skewed template-to-product compositions. This means that the distribution of different genotypes within the extracted DNA becomes altered during the PCR. This bias can be divided into PCR drift and PCR selection (Wagner *et al.*, 1994). PCR-drift is defined as stochastic variation in the early cycles in the reaction, when amplification still proceeds largely from the genomic template. Its outcome should therefore not be reproducible in replicate PCR amplifications, which means that separate PCR experiments should not produce the same bias. Thus, problems of PCR drift can be minimised by pooling separate PCR products (Wagner *et al.*, 1994; Polz and Cavanaugh, 1998). PCR selection, however, should be reproducible, as it consists of mechanisms which inherently favour the amplification of certain sequences. This can originate from properties of the template; such as differences in GC content between genes or larger parts of the genome and/or differential accessibility of genes due to secondary structures (Dutton *et al.*, 1993; von Wintzingerode *et al.*, 1997; Hansen *et al.*, 1998; Polz and Cavanaugh, 1998). It can also originate from the properties of the primers used. Although primers are not completely complementary to a template they might anneal sufficiently to give amplification of some genotypes, but with a reduced efficiency. The use of degenerate primers complicates this even more. The reason for incorporating degeneracy in PCR primers is to broaden the annealing capacity to a more diverse set of templates, which enables amplification of more diverse sequences. The degenerate approach in PCR has the advantage of enabling amplification of "unknowns", *i.e.* amplification of more distantly related orthologous gene fragments. However, it also results in a complex pattern of different binding affinities between primers and templates. Some authors have recommended that degeneracies should be avoided due to their potentially biased amplification (Polz and Cavanaugh, 1998). However, avoiding degeneracy in the primers does not guarantee that

primer-induced PCR selection will not occur. Moreover, degeneracy in the template also causes PCR selection, and such degeneracies are unknown. Therefore, PCR product distributions can be biased in an *a priori* unpredictable way, even if degeneracies in the primers are avoided.

Since PCR is an exponential process we could theoretically suspect that it will produce more of the common sequences compared to the rare ones. In practice, however, it has been shown that the opposite occurs, *i.e.* that rare sequences are preferably amplified (Suzuki and Giovannoni, 1996). These authors showed that in controlled mixtures of templates a bias towards increasing numbers of rare genes in the PCR product is observed. The mechanism behind this bias is that common sequences produced in the PCR have higher probability to re-anneal to each other than rare sequences during late cycles in the PCR. Such template-template duplexes will not be amplified and therefore the rare sequences, which have a higher relative probability of forming template-primer duplexes, will have a relatively higher amplification rate in later cycles. The rare sequences will thus ”catch up” the common ones.

PCR can also introduce artefacts of three different kinds. These are (i) formation of chimerical sequences (ii) formation of heteroduplex sequences and (iii) DNA polymerase errors (Liesack *et al.*, 1991; Thompson *et al.*, 2002; Kanagawa, 2003; Acinas *et al.*, 2005). A chimerical sequence, or simply a chimera, originates from two different DNA sequences. It can be produced during a template switch with half the sequence replicated from one template (one genotype) and half replicated from another template (another genotype) (Kanagawa, 2003). Thus, chimeras are artificial sequences not originally present in the community, but produced in the PCR tube. They have been reported to be produced in substantial amounts when 16S rDNA have been amplified (Wang and Wang, 1996; Ashelford *et al.*, 2005; Markmann and Tautz, 2005). Chimeras can also be produced when the DNA polymerase encounters a secondary structure of the DNA template, *e.g.* a hair-pin structure, which causes the polymerase to fall off and the resulting incompletely amplified sequence to act as a template during subsequent cycles. (Shuldiner *et al.*, 1989; Paabo *et al.*, 1990; Judo *et al.*, 1998). It is quite possible that the PCR production of chimeras is especially common for rDNA (*e.g.* 16S and 18S), since the rRNA produced from these genes are meant to produce such secondary structures in ribosomes. This is not the case for functional genes like *psbA* and the production of *psbA* chimeras may therefore be only a marginal phenomenon. For rRNA genes several methods have been developed for chimera detection, such as the Mallard program (Ashelford *et al.*, 2006), Chimerabuster (Ceraj, 2008), the Chimera Detection program (Cole *et al.*, 2003), Chimera and Cross-Over Detection and Evaluation (Ccode) program (Gonzalez *et al.*, 2005) and the Bellerophon server (Huber *et al.*, 2004). However for other genes, and especially for short fragments, there seem to be no such tools developed.

Heteroduplex sequences are also hybrid molecules but has DNA strands of different origin (Thompson *et al.*, 2002; Kanagawa, 2003). Although not completely complementary, the two strands may anneal. When cloned into a bacterial cell, the bacterial host mismatch repair system will convert the heteroduplex into a homoduplex and the artefact is never detected. However, these artefacts do not produce new artificial diversity since the parent sequence was already present in the community. Rather it obscures the actual distributions of the genotypes in the community. The third type of artefact is polymerase error (Kanagawa, 2003; Acinas *et al.*, 2005). The commonly used Taq polymerase is theoretically expected to produce errors at a rate of 2×10^{-5} per nucleotide and duplication, which can lead to substantial amounts of PCR artefacts (Acinas *et al.*, 2005).

We developed a strategy to minimize problems of PCR-related artefacts in the clone libraries. By sequencing both the coding and complementary strand, and excluding non–complementary forward and reverse sequences, we minimised sequencing errors. The polymerase errors were minimised by excluding sequences that differed with only one nucleotide from any other sequence and that only occurred once within each library. Since it is highly unlikely that the same polymerase error would occur at exactly the same position in two sequences, this was considered conservative enough. Chimeras were excluded by manually identifying possible recombination points in sequences in each library, and checked whether any sequence could originate from other sequences within that library. Putative heteroduplex sequences could not be corrected for, but as described previously this would not have produced any artificial diversity. This strategy gave a reduction in the number of unique haplotypes from 95 to 72, but it also resulted in a conservative library with a very low probability of containing erroneous sequences. Bias in template-to-product composition caused by the PCR is a very complex phenomenon and could not be estimated in this thesis. However, here it is important to note that the reduction in the number of sequences that our strategy gave did not change the distributions of the haplotypes, the protein sequence types or the PEST sequence types (see Significant findings below).

The ligation of the PCR product into the vector and the subsequent transformation of the bacterial cell during the cloning are not suspected to produce any bias. There are no suggested mechanisms that produce bias during A-T overhang ligation. Neither is there any reason to suspect that vectors with some specific inserts (gene sequences) should be preferentially taken up by the bacterial cells during cloning. Rainey *et al.* (1994) reported that different cloning systems gave different distributions of taxon-specific clones. However, it is not stated which cloning systems the authors used and neither do they quantify the difference. Therefore it is difficult to judge the importance of this study, and since other reports of similar bias are very scarce, the ligation and transformation are not suspected to produce any bias. During

sequencing errors may occur, but as described previously this can be controlled and corrected for. No bias in composition of haplotypes will be produced during sequencing.

Phylogenetic inference

The sequences in the clone libraries of *psbA* gave us information about the functions of the D1 proteins in the periphyton community. By using phylogenetic inference we were also able to get information about community structure at the *psbA* gene level.

In this thesis, phylogenetic inference is only used to describe the composition of *psbA* haplotypes, and the irgarol-induced change in this composition in periphyton communities. The dataset used for phylogenetic inference in Paper IV is only based on one gene fragment of 285 base pairs. Thus, it is not appropriate to draw conclusions about evolutionary processes among the phototrophic species studied. For an evolutionary analysis, more characters would be needed. However, it is encouraging to note that in spite the need of this stringent attitude there are many lineages and groups in the phylogenetic tree in Paper IV that correlates well with other evolutionary studies of the *psbA* gene (Morden *et al.*, 1992; Zhang *et al.*, 2000; Yoon *et al.*, 2002; Zeidner *et al.*, 2003), of other chloroplast genes (Chesnick *et al.*, 1996; Chesnick *et al.*, 1997; Tengs *et al.*, 2000) and combinations thereof (Zhang *et al.*, 1999; Yoon *et al.*, 2005; Wang *et al.*, 2008). Both Bayesian and maximum parsimony analysis were used and the two approaches gave reasonably similar results.

Comparing species and genes

As we in Paper III and IV described diversity of periphyton communities both as morphologically distinct species and *psbA* haplotypes, a comparison of detection and identification between microscopy on the one hand and clone libraries and phylogenetic inference one the other, was possible. There were consistencies, as well as inconsistencies, between the data sets. The general composition of the communities, harbouring the two major groups cyanobacteria and diatoms, are the same for the two data sets. There are also some examples of correspondence at lower taxonomic levels, but unfortunately the microscopy and/or phylogenetic identification was not satisfactorily made to the level of species in these cases. However, we should perhaps not anticipate a good correlation between such data sets, since there are fundamental differences between the levels of genes and species. The methodological limitations of the two approaches results in discrepancies. For example, cyanophages cannot be detected with a microscope but their DNA can readily be extracted, amplified and cloned. Discrepancies also originate from inherent differences between the two levels of biological organisation. There are examples where clones of the same morphologically distinct species

show considerable genomic variation (Muller *et al.*, 2005; De Martino *et al.*, 2007) or variation in specific genes (Auinger *et al.*, 2008). Genes are normally coupled within genomes of organisms. Whereas one cell can be identified in a microscope, its genome may contain many *psbA* genes, which may or may not be similar to each other. Different cells may also contain different numbers of *psbA* genes (Curtis and Haselkorn, 1984; Golden *et al.*, 1986; Bendich, 1987; Koumandou and Howe, 2007; Ravi *et al.*, 2008). When we perform PCR and produce clone libraries the couplings of genes within genomes of organisms are lost and paralogous genes will be treated individually. Therefore it is simply not the same thing to measure gene and species frequencies.

SIGNIFICANT FINDINGS

Both endpoint and exposure time are important when estimating effect parameters for toxicants with different mechanisms of action

The ability of PICT to detect adverse effects from toxicants is dependent on the quality of the short-term detection tests. If the mechanism of action of the studied compound is directed towards a target or process not directly linked to the endpoint used to quantify PICT, there is a risk of not detecting an existing community tolerance. In such situations it is important to know whether a prolonged exposure time in the detection tests can compensate for the uncoupled endpoint. Since the toxicity to photosynthesis was followed during 18-19 hours in this study, it includes the possibilities of recording delayed toxicity and/or functional recovery. It is important to note here that the exposure time in tolerance quantification tests should not be long enough to incorporate selection, which would obscure a PICT signal. Therefore effects of the six antifouling compounds to periphyton were not followed longer than 18-19 hours. The study also specifically evaluates the capacity of two different photosynthetic endpoints to detect effects of toxicants that act on different targets.

We used the two endpoints ^{14}C-incorporation and photosystem (PS) II efficiency, in the latter estimated through variable *in vivo* chlorophyll fluorescence (Fig. 7). The results show that the sensitivities of these endpoints, in detecting effects from toxicants not inhibiting photosynthesis explicitly, were only partly increased by prolonging the exposure (Fig. 2 in Paper I). Since processes like degradation of the toxicant and physiological adaptations to the exposure, occurs simultaneously as propagation of the toxic effect, the possibilities of sensitive indirect detection of toxic effects is obscured.

However, the endpoints were proven sensitive when estimating effects of PS-II-inhibiting toxicants. This is not so surprising since the metabolic distance between the inhibited pathway and the process used as endpoint for these

toxicants is extremely short. It is also noteworthy that the effect concentrations of the PS II inhibitors in this study, detected even after the shortest exposure time (strictly acute toxicity), compares relatively well with effect concentrations of integrated measures like population growth rate. This means that for these compounds simple measurements of PS II efficiency using variable *in vivo* chlorophyll fluorescence, after very short exposure times, is equally sensitive as whole population level effects measured during several days.

Thus, the photosynthetic endpoints ^{14}C-incorporation and inhibition of PS II efficiency is recommended for detecting effects of PS II inhibitors, but the latter endpoint can not be recommended for detecting effects from toxicants with other mechanisms of action. Also, using the time-to-effect approach for various endpoints can be an essential tool for testing hypotheses on the mechanism of action of compounds.

Patterns of community tolerance in natural environments reveal dynamic responses of communities to toxicants

Paper II is a unique study in the sense that it monitors PICT patterns in natural communities over a time frame as long as 10 years (Fig. 8). The absence of PICT to irgarol in periphyton communities in 1994, both in the field study (Paper II) and in the microcosm experiment by Dahl and Blanck (1996a), was clear but somewhat surprising at the time. It was in sharp contrast to effects of other contaminants in the same system, such as TBT (Blanck and Dahl, 1996; Dahl and Blanck, 1996b) or arsenate (Blanck and Wängberg, 1988, 1991). Therefore it seemed as there were inherent properties of irgarol or its mechanism of action that prohibited tolerance development in any species. However, after years of contamination of the coastal waters, the response pattern of periphyton had changed (Fig. 4 in Paper II). The tolerance seem to be associated with high costs since (i) PICT was absent in the microcosm experiment in 1994 and during the whole field season the same year, (ii) it developed only slowly over the years and (iii) it roughly followed the contamination pattern during the season in later years. If PICT levels are well correlated with contamination levels it suggests that there is a cost associated with the tolerance.

Since the mechanism of action of irgarol is directed towards photosynthesis, and the proposed tolerance mechanism (Paper III) might have fundamental effect on the performance of PS II, the cost is likely to result in a lowered primary production. For higher plants, tolerance to triazine herbicides (same mechanism of action as irgarol and diuron) has been shown to result in biomass reductions (total dry weight) of 22%-66% compared to non-tolerant biotypes (reviewed by Holt, 1990). Moreover, the cost of the tolerance-

conferring mutation Ser_{264}→Gly in the D1 protein have been shown to have 23% lowered photosynthetic quantum yield in higher plants. These mutants are also more sensitive to photoinhibition and heat-stress (reviewed by Oettmeier, 1999), which would be of significance in an ecological perspective. Even though the overall majority of reports show that there is a cost associated with tolerance to PS II inhibitors, there is one report where no cost could be correlated to tolerance (Schonfeld *et al.*, 1987).

Although it seems clear that tolerance to PS II inhibitors, including those with alterations of the D1 protein, comes with reductions in primary production (Jasieniuk *et al.*, 1996), it is out of the scope of this thesis to estimate reductions in primary production associated with the community tolerance to irgarol in periphyton. What is interesting and maybe alarming, is that the minimum level of sensitivity seem to have increased over the years (Fig. 4B in Paper II). This trend of increased community tolerance at all sampled sites is quite small, approximately an increase from 1 to 3 nM and not statistically significant, but seems to be steady. Since site 6 is located just by one of the outermost islands in the Swedish archipelago, the absence of the really sensitive communities could indicate that the selection pressure from irgarol affects not only hotspots like marinas, but in fact the whole Swedish coastal archipelago.

Microcosm experiments support the field studies and reveal a new putative tolerance mechanism

Besides the four field studies included in Paper II, three microcosm experiments studying long-term effects of irgarol in periphyton were performed in 1994, 2001 and 2003. The results from the experiment in 1994 is published by Dahl and Blanck (1996a) and results from the experiment in 2001 is reported in Paper III and Paper IV. My results from the 2003 experiment are unpublished but are presented in this thesis and used in the discussion. Since PICT appeared and was clearly detected in 2000, some periphyton species must have acquired some mechanism of tolerance. This, of course, made us curious about the nature of this mechanism. What trait had now been acquired that made it possible to tolerate irgarol in spite of high costs? To screen for changes in the target protein was an obvious starting point since mutations in the *psbA* gene, encoding the target protein (D1), had been shown to be a tolerance mechanism (Hirschberg and McIntosh, 1983) and had been found in PS-II-inhibitor-tolerant weeds all over the world (reviewed by Holt, 1990; Holt *et al.*, 1993; Oettmeier, 1999; Devine and Shukla, 2000). The microcosm experiment performed in 2001 (Paper III) simultaneously tested whether the community tolerance was inducible and if amino-acid alterations in the D1 protein of the community members were correlated with tolerance.

In contrast to the experiment made in 1994, we detected clear PICT signals both in 2001 and 2003. Here it is important to note that the 1994 and the 2003 experiments were made in April, when the irgarol concentrations in the coastal waters were low, whereas the 2001 experiment was made in July-August when boating activity and irgarol concentrations were high. When comparing community tolerance results from the three experiments a clear pattern of change over the years but also over the season can be seen (Fig. 9). The absence of a PICT signal in 1994, and the detection of PICT in 2001 and 2003, is consistent with the development over the years seen in the field studies (Fig. 4A and 5 in Paper II). Interestingly, the change over season seen in the field studies (Fig. 3 in Paper II), is also reflected in the microcosm experiments as different background tolerance levels in April (2003) compared to July-August (2001) (see dashed lines in Fig. 9). Also, the long-term irgarol concentrations required to induce an increase in EC50 values are higher, and the increase in EC50 values is steeper in July-August 2001 compared to April 2003 (Fig. 9).

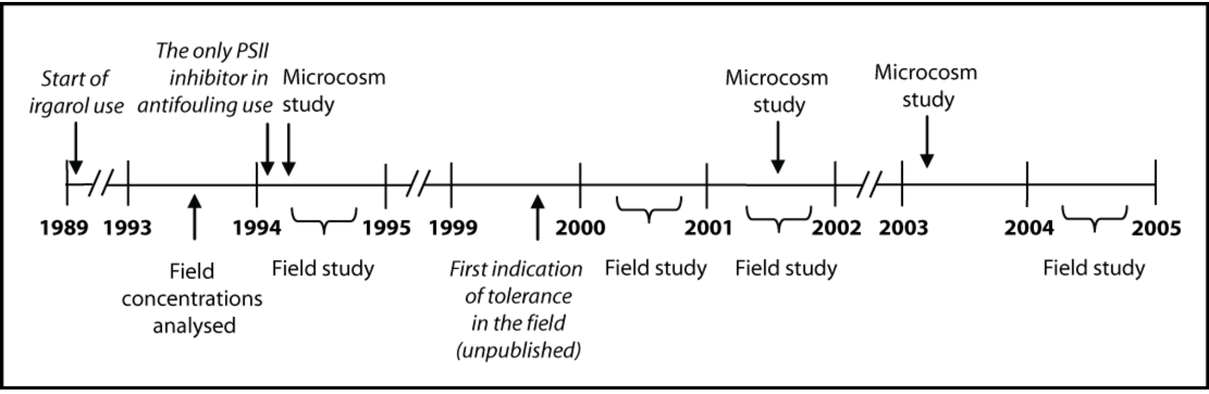

Figure 8. Historic depiction of irgarol usage and experimental work of irgarol effects in periphyton communities. All experimental work, except the microcosm study in 1994 (Dahl and Blanck, 1996a) and the first indication of irgarol tolerance in 1999 (Nihlén), is included in this thesis.

This pattern is interesting in the context of the Community Conditioning Hypothesis (Landis *et al.*, 1996; Matthews *et al.*, 1996; and described previously), which suggests that information about historical events can be stored in communities and will influence their response to subsequent stress. In this context, a further example of such stored information, analogous to a "memory" or "footprint", in communities is the study by Frey (1999). He showed that mutations, giving tolerance to triazine herbicides, is not lost during triazine-free periods in spite of associated fitness costs, but instead kept in low frequency among chloroplast genomes. This is a very interesting observation in the light of Paper II-III and the unpublished 2003 microcosm experiment. From Paper II we can conclude that (i) a tolerance mechanism to irgarol appeared sometime between 1994 and 2000 and (ii) after its appearance

it was only present at times of severe irgarol-induced selection. According to the Community Conditioning Hypothesis, information about these periods of irgarol-induced selection can be stored in the communities, and since Frey (1999) showed that a tolerance mechanism like the one proposed in Paper III can be stored in chloroplast genomes over substantial time periods, a mechanism for the tolerance patterns can be seen.

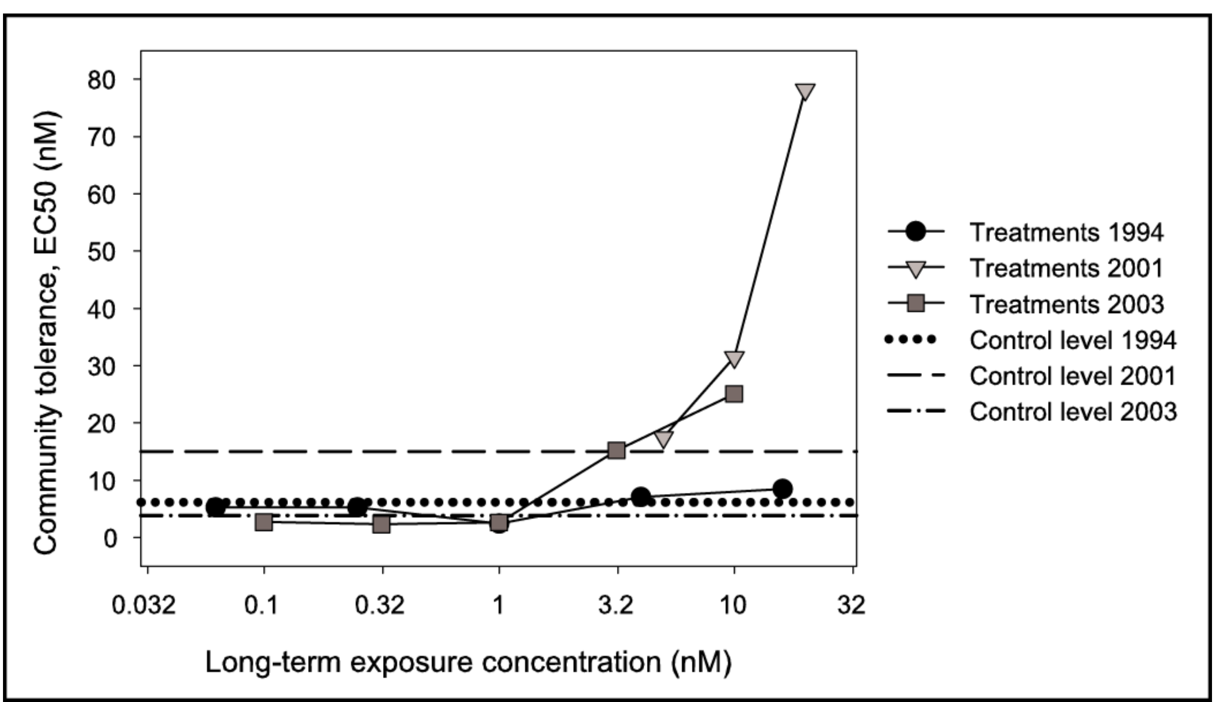

Figure 9. Induction of community tolerance to irgarol in microcosm studies made in 1994, 2001 and 2003. Since irgarol is used as an antifouling agent in Swedish coastal waters, hence present in low concentrations in the incoming microcosm water, no true control can be obtained. The levels of community tolerance in microcosms with no experimentally added irgarol, *i.e.* the background levels, are shown as lines as indicated.

PICT can be induced irrespective of season, but the effect concentration and also response pattern might be dependent on the season. The very high EC50 value of 78 nM in the experiment in July-August 2001 is thus the results of a three-step selection process consisting of selection over the years, over the season and over the course of the experiment. From this microcosm, and the background microcosm, we extracted community DNA and produced clone libraries of the *psbA* gene.

Within the clone libraries we searched for sequences that contained previously described mutations giving tolerance to PS II inhibitors. The slow development of PICT to irgarol is consistent with the hypothesis of periphyton species acquiring this trait (the tolerance) through fixation of a mutation, similar to what happened in tolerant land plants that grew in areas contaminated by PS II inhibitors and acquired tolerance-conferring mutations (Hirschberg and McIntosh, 1983; Holt *et al.*, 1993; Jasieniuk *et al.*, 1996;

Oettmeier, 1999; Devine and Shukla, 2000). However, none of these mutations were found. When we then extended our search to any systematic differences between the background and the control sequences, we found one region that clearly showed differences in diversity on the amino acid level. This region was identified as the PEST region of D1, which is involved in regulating the degradation of the protein (Rogers *et al.*, 1986; Rechsteiner and Rogers, 1996). The pattern of reduced diversity in the PEST region of D1 is shown in Figure 2C in Paper III. Our interpretation of this difference is that long-term selection from irgarol has resulted in elimination of species with certain D1 degradation characteristics. At the same time this selection pressure has favoured species having other characteristics of their D1 degradation. The identification of the PEST region as important during irgarol-induced selection is a novel discovery. Interestingly it is consistent with early proposals that increased D1 turnover (degradation, synthesis and assembly) would act as a tolerance mechanism (Trebst *et al.*, 1988; Draber *et al.*, 1991; Trebst, 1996). Experimental support has also been given for this hypothesis (Kless *et al.*, 1994; Pace *et al.*, 2001). There are also studies that have emphasised D1 turnover as a general stress-compensating mechanism (Godde and Buchhold, 1992; Lutz *et al.*, 1992; Giardi *et al.*, 1996; Geiken *et al.*, 1998; Franco *et al.*, 1999). The fact that this putative tolerance mechanism was found in natural communities, structured also by ecological interactions like competition and predation, strengthens its ecological relevance and its role for the fitness of natural periphyton in irgarol-contaminated environments. It is worth noting that the irgarol-favoured PEST sequences were present also in the background community. This is consistent with the fact that the control community was exposed to background contamination concentrations of irgarol and with the relatively high tolerance level in the background community. Thus, the change in composition of PEST sequences in the community may be the result of selection for optimised D1 degradation during irgarol exposure.

The PEST-sequence mechanism for increased irgarol tolerance is seen in Figure 2C in Paper III as two PEST sequence types being clearly irgarol-favoured. In that analysis we only focus on the PEST region (Fig. 2C in Paper III) and ignore the rest of the protein sequence in the proteins. However, we also need to consider in which proteins the irgarol-favoured PEST sequences occur. Therefore we checked in which proteins the two irgarol-favoured PEST sequence types were present and found that they had different patterns of occurrence. Both the irgarol-favoured PEST sequence types were found in irgarol-favoured proteins. However, PEST sequence type 9 was also found in proteins that were *not* present in the irgarol-exposed community, but exclusively found in the background community (Fig. 4 in Paper III). The conclusion must then be that PEST sequence type 10 is more likely to contribute to the tolerance than PEST sequence type 9.

Phylogenetic analyses pinpoints in which taxonomic groups the proposed tolerance mechanism is active

Since we used a metagenomic approach, *i.e.* sequencing *psbA* directly from the community and not from isolated and cultured clones, the *psbA*- sequence data set is uncoupled from the data of morphologically distinct algal and cyanobacterial species. Therefore we used a phylogenetic approach to try to identify the periphyton *psbA* haplotypes to lower taxonomic levels like genus or species. The periphyton haplotypes were analysed together with a large number of *psbA* genes of known species identity, from the very diverse realm of phototrophic organisms. This included as diverse groups as viral cyanophages, cyanobacteria, various eukaryotic micro- and macroalgae and land plants. Identification of periphyton haplotypes proved difficult and several clades were shown to be strictly periphytic, which shows that periphyton species is poorly represented in international sequence databases. There were, however, some interesting groupings of periphyton haplotypes with known sequences. For example the grouping of 10 background haplotypes with cyanophage sequences. The fact that there are no irgarol haplotypes in this clade is consistent with previous observations in virology, where factors that inhibit photosynthesis have been shown to simultaneously inhibit phage production (*e.g.* Sherman and Haselkorn, 1971; Allen and Hutchison, 1976; Sherman, 1976). Moreover, the diversity of *psbA* in perihyton communities was shown to be very high. Various photosynthetic pro- and eukaryote groups, and viral cyanophages, colonised and grew on the glass discs (Fig. 1, Paper IV).

The phylogenetic analysis also gave us the opportunity to analyse whether phylogenetic affiliation was indicative of sensitivity or tolerance, and allowed us to describe the diversity of periphyton *psbA* genes in a broad taxonomic context. Combined with the analysis of in which proteins PEST sequence type 9 and 10 occurred, we could show that the proposed tolerance mechanism of PEST sequence optimisation was present in a specific taxonomic part of the irgarol-tolerant community. *psbA* genes from Bacillariophyta (diatoms) increased both in abundance and in diversity (Fig. 2D, Paper IV), and all haplotypes within this group had the PEST sequence type 10. Thus, species within Bacillariophyta were favoured in the analysis of morphologically distinct species (Fig. 1, Paper III), *psbA* abundance and *psbA* diversity (Fig. 2D, Paper IV). This is consistent with the suggestion of a PEST-sequence-dependent tolerance mechanism present in this group. The other taxonomic group favoured by irgarol is the cyanobacteria (Fig. 1, Paper III and Fig. 2B, Paper IV). In this diverse group there was no PEST sequence type 10 present. Instead PEST sequence type 9 occurred in several cyanobacterial clades, including the big clade 6 (Fig. 2B, Paper IV), which suggests that cyanobacteria in the irgarol community do not tolerate irgarol via PEST-sequence

optimisation. Organisms in this group probably have some other mechanism of tolerance. This observation coincides with the different organisation these two groups have for light harvesting and light adaptation (Larkum and Howe, 1997; Malkin and Niyogi, 2000). For example, diatoms have light-harvesting complex II as antenna and have mechanisms like altered pigment composition (Müller and Wilhelm, 1997) or down-regulation of light-harvesting complexes (Larkum and Howe, 1997), for light regulation. In contrast cyanobacteria have phycobilisomes associated to PS II for light harvesting and transcribe different *psbA* copies, producing D1 proteins with different characteristics, as means for light adaptation (Campbell *et al.*, 1996). What importance these differences have in the context of irgarol tolerance is not easy to disentangle, but it is interesting to note that fundamentally different strategies of light harvesting and light regulation coincide with differences in irgarol tolerance mechanisms.

The analysis of periphyton *psbA* genes suggests that these communities harbour extensive diversity. Most likely they harbour *psbA* genes from as divergent groups of organisms as viral cyanophages, different cyanobacteria and eukaryotic groups like Haptophyceae and Bacillariophyta. One illustration of the periphytic diversity is that the similarity between the studied fragment of the *psbA* gene (Paper III) of the terrestrial conifer *Pinus contorta* and the marine planktonic cyanobacteria *Prochlorococcus marinus* (MIT 9515) is 69.4%, while some *psbA* sequences within the background periphyton community show as little as 57.1% similarity.

Why not the common Ser_{264}→Gly mutation?

The fact that periphyton species have not acquired the tolerance-conferring mutation common in terrestrial systems (Ser_{264}→Gly), might depend on several factors, such as geo-physico-chemical differences in habitat, differences in the biology of inhabiting organisms or in the toxicant-exposure situation. As described previously in section *psbA*, the D1 protein and effects of irgarol, terrestrial plant cells can have thousands of chloroplast genome copies (Bendich, 1987). Frey (1999) showed that the tolerance-conferring mutation Ser_{264}→Gly can be kept in low abundance among chloroplast genomes for many generations. Moreover, selection on polymorphism at this position results in changes in chloroplast gene frequencies within the life-span of an individual, and thus represents a rapid genetic response to selection from PS-II-inhibiting toxicants. Also Taylor and Feyereisen (1996) suggest that larger amount of deleterious mutations can be kept in polyploid genomes, which leads to rapid evolution of tolerance during selection from toxicants. Unfortunately there is only scarce information about the genome copy numbers in diatoms. Coleman and Goff (1991) indicate that algal genome copy numbers are similar to those of land plants, which could indicate that similar

retention and selection of Ser_{264}→Gly could occur in microalgal communities. However, many other factors might influence these processes and the relevance of genome copy number for tolerance mediated via the Ser_{264}→Gly mutation or mediated via PEST sequence mechanism is purely speculative.

Another explanation to the presence of PEST sequence optimisation instead of Ser_{264}→Gly mutation in periphyton might simply be that the PEST sequence optimisation is enough. Even though irgarol concentrations can be very high in marinas during some periods, it is reasonable to argue that the toxic selection pressure can be higher in the terrestrial environment since the mobility of the herbicide is lower. The bioavailability of the compound in a soil sprayed with a herbicide may be a critical issue. However, it is likely that organisms in such environments are consistently exposed to high herbicide concentrations for longer periods, compared to a marina where currents, tide and wind-driven transport of water constantly transport and dilute irgarol. Therefore, extreme tolerance might be needed for land plants in such an environment. The Ser_{264}→Gly mutation can give more than 1000-fold increase in tolerance in some weed species (reviewed by Oettmeier, 1999) but is, on the other hand, accompanied with substantial costs in the form of lowered photosynthesis and growth reductions (Jasieniuk *et al.*, 1996). This is consistent with the suggestion by Taylor and Feyereisen (1996) who postulates that that tolerance mechanisms involving structural mutations of targets probably results in higher tolerance levels than mechanisms involving overproduction of targets. Moreover, alterations in the PEST region have been shown to give only moderate tolerance but do not seem to be associated with any growth reductions (Kless *et al.*, 1994). Thus, optimisation of D1 degradation via the PEST sequence might be better suited in the marine environment since this environment likely has lower and less persistent selection pressure from this type of compound.

OUTLOOK

During the years of a PhD one learns a lot. Actually, I dare to say that the knowledge presented in the papers of this thesis is only a minor fraction of all knowledge I have gained during this period. Therefore I have allowed myself to write this perhaps more personal and speculative section. Except for all knowledge and experience of a more personal nature, some of the substantial amount of knowledge within the realm of science and ecotoxicology, not presented in the papers, now urge our attention.

The development of molecular techniques such as high-through-put sequencing, and tools within bioinformatics, have revolutionised biology as a science. This new era of genomics (including transcriptomics and metabolomics), metagenomics and bioinformatics represents a true shift in paradigm that will, or to some extent already have had a profound impact on our understanding of how this world is organised and how it functions. The progress of DNA sequencing has lead to an overwhelming amount of sequence data. Already in 1999, before the revolution of shotgun sequencing of entire metagenomes, Boguski wrote in a historical overview about the increasing number of DNA sequences in GenBank and the relative lack of published annotations of genes, *i.e.* identification of functions of genes, something he termed "the gap". Boguski noted about 5 million DNA sequence records in GenBank in 1998. 10 years later (February 2008) this number has increased to almost 83 million in the traditional GenBank division, which also is complemented with approximately 27 million records in the Whole Genome Shotgun Sequences division (GenBank, 2008). The statement by Boguski in 1999, "*it is now widely accepted that we cannot realistically expect traditional experimental methods to scale up and have a substantial impact on bridging this gap*" is even more valid today.

Ecotoxicogenomics have commonly dealt with gene expression of exposed and unexposed organisms. Techniques such as microarray experiments, production of Expressed Sequence Tags (EST) libraries or 2D-gel separation proteins have been used (reviewed by Snell *et al.*, 2003; Snape *et al.*, 2004; Watanabe and Iguchi, 2006). We can view this approach as measuring gene expression of all genes from one organism – "*all from one*". Even though microarrays are becoming more common in community ecotoxicology (Eyers *et al.*, 2006; Scholten *et al.*, 2007), various fingerprinting methods, *i.e.* PCR amplification coupled with different gel electrophoresis methods (*e.g.* T-RFLP, DGGE/TGGE or SSCP), have been more used (reviewed by Dahllöf, 2002; Dorigo *et al.*, 2005). The PCR-based fingerprinting approach aims to amplify one gene from all community members – "*one from all*". The new era of metagenomics might make an "*all from all*" approach possible.

Large scale sequencing of whole microbial metagenomes started with sequencing of a microbial biofilm from a acid mine drainage (Tyson *et al.*,

2004). This community had low diversity and the focus of the study was to reconstruct microbial genomes. Later the same year the already classic Sargasso Sea study by Venter *et al.* (2004) was published. These authors found 1.2 million previously unknown protein-coding genes in the bacterial fraction of 1730 litres of sea water. The Global Ocean Sampling (GOS) survey (Rusch *et al.*, 2007) that followed up the Sargasso Sea study, was much more extensive and raised this number to 6.12 million predicted protein-coding genes (Yooseph *et al.*, 2007). In hindsight it is interesting to note that Amann (2000) in his pre-metagenomics study from 2000, dealing with microbial diversity, asked *"Who else is out there?"*. What then are the potential impacts of this development on ecotoxicology?

To try to answer, or at least speculate over this issue, an analogy with more traditional measures comes handy. Arrhenius *et al.* (2005) used the community approach when estimating effects of three different toxicants and mixtures of the three. As already mentioned, by allowing toxicant-induced selection to refine ecotoxicologically relevant information in a community, this study avoids non-scientific assessment of what is an ecotoxicologically important variable to measure. In this study, using the same type of marine flow-through microcosms as used in Paper III, 129 morphologically distinct algal species were found. This means that we are dealing with a 129-dimensional response variable, which, at least within traditional community ecotoxicology, must be regarded as quite complex. Let us now consider what would happen if we instead of counting the algal cells within a community by microscopy, would sequence all genomic information of all species in the same community. Unfortunately, there are no large-scale, complete-genome metagenomic studies of marine periphyton communities. Even though we can not directly compare the data from Arrhenius *et al.* to the metagenomic datasets of the GOS survey since among other things, the data comes from different parts of these microbial communities (mainly algal and mainly bacterial), different ecosystems (periphyton and plankton) and different geographical regions. However, from an indirect comparison it is obvious that shifting from more traditional measures to large-scale metagenomics would increase the dimensionality of our response variable tremendously.

We can now ask ourselves what the fundamental differences are between traditional species data and large-scale, complete-genome metagenomic data. The first observation might be that one cell seen in the microscope has many genes. Ignoring for a moment the fact the DNA sequences themselves contain information, and only considering them as present or absent (compare to species counts), metagenomic data still gives us much more information about the community members. The obvious question is then –*How much*? and the next question is of course –*How much of this information is ecologically or ecotoxicologically relevant?*, i.e. what is the amount of redundancy in such data? The first question can be roughly answered by referring to metagenomic

studies like the ones cited above. An attempt to multiplying the number of species detected with some estimation of the average number of proteins within a microbial species is futile, since the differences in genome size and numbers of protein coding genes among microbial species are huge. Microbes can have very small genomes, for example *Pelagibacter ubique* (possibly the most numerous bacteria in the world (Morris *et al.*, 2002)) that have a genome of 1.3 megabases (Mb) (Giovannoni *et al.*, 2005). However, there are also microbial species with enormous genomes, for example some dinoflagellate species have 215,000 Mb (Hackett *et al.*, 2004). As a comparison, the diploid human genome size is 6360 Mb and the hexaploid genome of wheat is 16,000 Mb. Thus, we are dealing with a tremendous complexity, which makes any attempt to extrapolate metagenomic data to the species level pointless (at least presently). The next question about the fraction of ecological or ecotoxicological relevant data, and the amount of redundancy, is even harder to answer. First we have to exchange the traditional view that species diversity might, or might not, influence community function or community stability, to the metagenomic view of protein diversity having an influence, or not, on community function or community stability. Although there is no complete scientific consensus about the relationship between community structure and function in traditional community ecology (Mooney, 2002; Allison and Martiny, 2008), functional redundancy is often seen in traditional ecotoxicological experiments (Schindler, 1987; Pratt, 1990; Dahl and Blanck, 1996b; Griffiths *et al.*, 2000). This means that even though sensitive species are eliminated by toxicants, more tolerant ones can increase in abundance and thereby uphold the major functions of the community. If we now exchange species for proteins, functional redundancy would mean that even though proteins from sensitive species are eliminated by toxicants, proteins belonging to a more tolerant species will uphold the functions of the community. This reasoning, however, is probably too simplistic, since proteins are actually coupled to each other within organisms and when an organism dies all its proteins are degraded. Thus, the proteins of a certain protein family can be degraded even though they are not sensitive to a toxicant, but instead due to their occurrence in an organism which have other proteins that are sensitive to the same toxicant. Therefore we should not view different biological organisation levels as completely discrete entities, but more as a continuum.

The transition of ecotoxicology from species level to metagenomic level can perhaps be seen as an increase in number of pixels in a picture. We obtain a higher degree of resolution; a fine-grained pattern can be produced. If the traditional method of counting species is viewed as a large picture but with low resolution, clone libraries could perhaps be seen as a torch that lights up only a small part of the picture. Whether or not large-scale, complete-genome metagenomics corresponds to a large beautiful, clearly visible and high-resolution picture remains to be proven, but the potential of it is obvious.

There are, of course, things that hampers achieving the goal of the beautiful, clearly visible and high-resolution picture. Hitherto, metagenomics have been used in a descriptive way in discovery-driven science. However, ecotoxicology is often an experimental exercise where we may need many treatments and replicates. Although the sequencing costs have been reduced dramatically in the last years, even a modest experiment with a few treatments and replicates would require a substantial funding. Such experiments would also produce extraordinary amounts of data which will require substantial efforts within bioinformatics and computing. We need to find "the needles in the metagenome haystack" as Kowalchuk *et al*. (2007) elegantly puts it. Ecotoxicology can find its needles by an approach that refines the ecotoxicological relevant information before the sequencing begins. Such an approach could be to refine the variables that are important by selection from the toxicant (as discussed previously). The metagenomic libraries from communities in a long-term ecotoxicological experiment would contain information from relevant toxicological events during selection on the gene, protein, organelle and individual (cell) level.

The Sargasso Sea and the following GOS study used shotgun Sanger sequencing. Sanger sequencing is a sequence-termination-based method which at the time of invention revolutionised molecular biology (Sanger *et al.*, 1977). The shotgun approach (Staden, 1979; Anderson, 1981) circumvents the laborious piece-by-piece sequencing (the chromosome-walking approach) and instead sequencing of random, but multiple overlapping pieces of DNA is performed, and complemented with bioinformatic tools to assemble the sequence reads into longer DNA sequences (contigs and scaffolds). The shotgun approach enabled a powerful boast of the Human Genome Project (Venter *et al.*, 1998). New sequencing techniques like pyrosequencing (Ronaghi *et al.*, 1998), commercialised as massively parallel sequencing or 454 sequencing (454 Life Sciences Corporation), is capable of sequencing 100 times faster than the current Sanger-sequencing platforms (Goldberg *et al.*, 2006). This technique produces sequence data by real-time monitoring of enzymatic complementary DNA synthesis to DNA strands immobilised to small beads. The drawback is that, relative to Sanger sequencing, it produces shorter sequence reads (around 200-500 bp). Depending on the objective of the study, this can be a big disadvantage in metagenomics. Since short fragments of highly repetitive DNA sequence are impossible to assemble, it becomes more difficult to assemble genes and genomes. However, if we primarily are interested in comparing similarities between controls and treatments, and not in assembling genes or genomes, 454 sequencing might be advantageous since it rapidly can produce much more data.

The next generation sequencing technique is probably SMRT™ sequencing (Pacific Biosciences). This technique is similar to pyrosequencing since it also monitors DNA synthesis, but in SMRT the DNA polymerase is immobilised to

the bottom of very small holes (tens of nanometers) in a metal film, and the DNA synthesis proceeds in 20 zeptolitres (10^{-21} litres) reaction volumes. It has the potential to produce long sequence reads (thousands of bp) and even though it does not exist as a functional platform yet, it has been appointed as the technique that possibly could be used to sequence genomes of individual human beings. Due to its high speed and low cost we might soon be able to SMRT-sequence ourselves!

Even though some reflexions over future development sometimes seem ridiculous or pointless, we can, similar to the development of computers and computing, look back on the development of biology into molecular biology and –omics and be amazed. From our present horizon, things like sequencing your own genome, or metagenomic sequencing of large ecotoxicological experiments, might only seem futuristic. Still, we can imagine ourselves living during a time period when computers never were considered to be used by common people, and then transcend into real time when we write things like this on our PCs, or download huge amounts of information via www whenever we like. My belief is that we will soon read publications describing effects of toxicants on the metagenomes or proteomes of natural communities. Perhaps they are already "*In press*".

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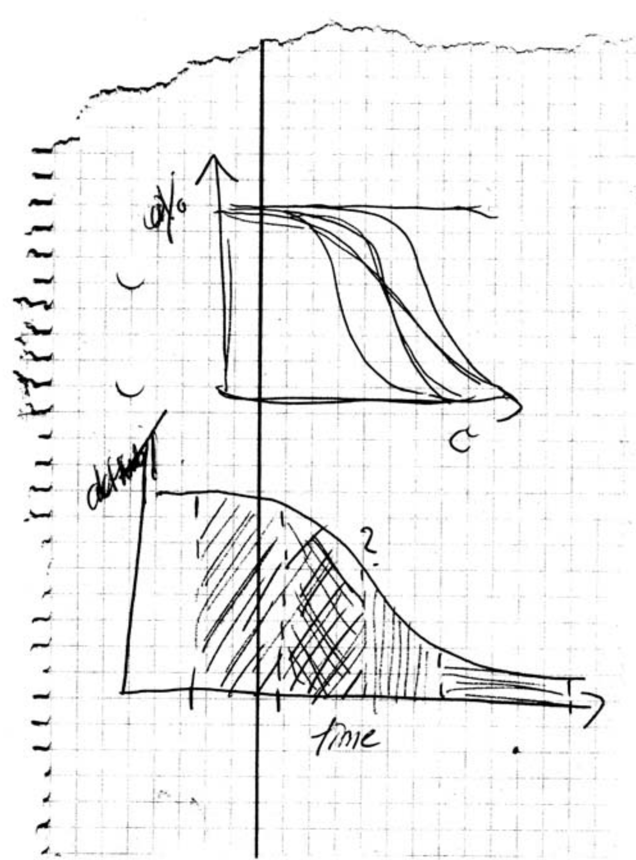
Paper I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TOXICODYNAMIC RESPONSES OF PERIPHYTON COMMUNITY PHOTOSYNTHESIS TO SIX ANTIFOULING COMPOUNDS

Manuscript

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ABSTRACT

This study analyses the development of toxicity of the antifouling compounds irgarol, diuron, DCOIT, chlorothalonil, dichlofluanid and zinc pyrithione to photosynthetic processes in periphyton communities. Effects on photosynthesis were followed over time, with the two methods incorporation of radiolabelled carbon (^{14}C incorporation) and Pulse Amplitude Modulation (PAM) fluorometry. PAM fluorometry specifically estimates effects on the efficiency of photosystem (PS) II, whereas ^{14}C incorporation gives a more integrated measure of photosynthesis. Since a time-to-effect approach was used for both specific and integrated effect indicators, information about the mechanism of action and the degradation/detoxification of the studied compounds could be retrieved. The approach gives opportunities to judge whether the methods are appropriate for short-term toxicity testing with periphyton communities. The most toxic compounds were the photosystem (PS) II inhibitors irgarol and diuron, with the lowest EC50 values of 1.9 and 7.3 nM respectively. These compounds were also the two most rapidly acting ones. The two methods were approximately equally sensitive in estimating effects of irgarol and diuron and estimated similar responses over time. DCOIT, zinc pyrithione and dichlofluanid showed intermediate toxicity and chlorothalonil was the least toxic compound. For these compounds ^{14}C incorporation was more sensitive than PAM fluorometry, and the lowest EC50 values were 110, 140, 320 and 560 nM respectively. Exposure to these compounds also gave different time-to-effect patterns for the two methods. Dichlofluanid and zinc pyrithione seems to induce effects on photosynthesis more rapidly than DCOIT and chlorothalonil. Our results are consistent with previously suggested mechanisms of action of the compounds. They are also consistent with the suggested rapid hydrolysis of dichlofluanid, since photosynthetic recovery was observed after 6 hours. No recovery was evident for any other compound. For PS II inhibitors PAM fluorometry seems appropriate for toxicity assessment. However, for toxicants with other mechanisms of action this method can not be recommended for assessing toxicity, even after prolonged exposure times up to 18 hours.

INTRODUCTION

The use of natural communities in toxicity testing avoids some important limitations associated with single species tests. These limitations include the lack of ecological relevance and the uncertainty whether the test species is sensitive or tolerant to the tested compound (Cairns, 1983; Blanck, 1984; Kimball and Levin, 1985; Pratt *et al.*, 1997; Schmitt-Jansen *et al.*, 2008). Single species can provide discrete pieces of ecotoxicological information, while a community deals with the whole set of organisms in their ecological context. Concentration response curves in acute ecotoxicological tests thus indicate the sensitivity distribution over a broad range of species. If toxicant exposure is prolonged the community will go through a Toxicant-Induced Succession (TIS), where sensitive species will be replaced by more tolerant ones. This results in a changed sensitivity distribution of the community and increased community tolerance to the toxicant, according to the Pollution-Induced Community Tolerance (PICT concept (Blanck *et al.*, 1988). When using PICT as an ecotoxicological tool a range of long-term toxicant exposures are needed, either in a microcosm experiment or in the environment. After the communities have gone through TIS they are sampled and short-term toxicity tests are made to detect PICT. This detection involves the change in sensitivity distributions of the communities seen as a right-shift of the concentration response curves. The detection of PICT is thus entirely dependent on the short-term toxicity tests.

Common methods for PICT detection are measurement of specific metabolic processes, such as protein synthesis (e.g. Hjorth *et al.*, 2006; Demoling and Bååth, 2008) or photosythesis (*e.g.* Blanck and Wängberg, 1991; Schmitt-Jansen and Altenburger, 2005). These processes are linked but not directly coupled to other metabolic processes and pathways in an organism. A method for a specific metabolic process is likely to be sensitive when used for estimating effects of toxicants that affect targets and pathways that are quantified with the method. However, the same method may be insensitive when quantifying effects of toxicants affecting other processes than that under measurement. Such a mismatch between endpoint and mechanism of action may limit the ability to detect PICT. For example, photosynthesis is a sensitive endpoint for assessing effects of photosystem (PS) II inhibitors, since they specifically inhibits photosynthetic processes (Moreland, 1980b; Chesworth *et al.*, 2004), but it is less sensitive when used to quantify toxic effects of zinc (Admiraal *et al.*, 1999), which do not inhibit photosynthesis specifically. A long metabolic distance and/or few connections between the processes affected by the studied compound and measured by the endpoint can thus result in poor and/or slow coupling between exposure and detected effect.

A key question is whether increased exposure time and resulting effect propagation can compensate for poor and/or slow coupling between the

mechanism of action and the endpoint measured. The initial effects of the toxicant, for example inhibition of photosynthesis, will eventually propagate to other metabolic processes, such as protein incorporation, and endpoints measuring these other processes may therefore gain in sensitivity over time. If so, increased exposure time would give a better estimate of toxicity in spite of such mismatches, and would improve PICT detection. We also need to remember, however, that there are additional important ecotoxicological processes that proceed during such effect propagation. For PICT detection the short-term test should not be long enough to induce adaptation or selection to the compound, which would obscure PICT detection. Also, if the test compound is readily degradable the exposure concentration might be lowered before the toxic effect has propagated to the pathway used in the measurement. This will lead to an underestimation of toxicity in the short-term tests and an erroneously determined threshold for induction of community tolerance.

We used two measures of photosynthesis to follow the toxicity of six antifouling compounds on periphyton communities over time. The compounds have various mechanisms of action (Table 1). Antifouling compounds are designed to prevent attachment and growth of organisms onto surfaces in the aquatic environment. Typical applications of these compounds are additives in paint for boat hulls or marine installations. They leak out from the paint and by their toxicity they prevent accumulation of organisms onto the surface. The periphyton communities we used are composed just of such sessile surface-growing organisms. They consist of a very diverse set of populations, from bacteria and single-cell eukaryotic organisms to multicellular animals. Thus, sensitive species are very likely to be included in the community. Since they also contain many different guilds, producing communities with important ecological interactions (*e.g.* competition and predation) present, they are also ecologically relevant. Moreover, the sampling of natural biota ensures that the organisms are adapted to a natural environment and not adapted/acclimated to an artificial laboratory environment (Cairns *et al.*, 1992).

The two methods used were incorporation of radiolabelled carbon (^{14}C incorporation) (see for example review by Falkowski and Raven, 1997) and Pulse Amplitude Modulation (PAM) fluorometry (for example reviewed by Schreiber, 2004). ^{14}C incorporation measures the amount of incorporated radiolabelled carbon into macromolecules for a certain time, and is thus an integrated measure of photosynthetic rate, including both light-dependent and light-independent reactions. PAM fluorometry measures the variable *in vivo* chlorophyll *a* fluorescence from photosystem (PS) II at different excitation states. It is estimated that 90% of the total fluorescence signal comes from PS II (Govindjee, 1995). The fluorescence from PS I is low at ambient temperatures because photochemistry and other processes, like thermal dissipation and

triplet formation, are particularly efficient in that system (Strasser and Butler, 1977). The energy of incoming light brings PS II to an excited state which then can be deactivated by four pathways: photosynthetic energy conversion via electron transport, triplet formation, heat dissipation and fluorescence. This means that there is an inverse relationship between fluorescence and photosynthetic energy conversion. The PAM technique uses a flash of light to saturate electron transport and fully reduce PS II. At this state photosynthetic energy conversion is zero and fluorescence reaches a maximum level (Fm). The increase in fluorescence from the original level (Fo) to Fm is the variable fluorescence (Fv). The ratio Fv/Fm for a dark-adapted sample (ϕ_{PO}) is termed the photochemical (or quantum) yield of PS II and represents the potential energy conversion of PS II, whereas the ratio for a light-adapted sample, photochemical efficiency of PS II (ϕ_P), reflects the current photosynthetic electron transport (Genty *et al.*, 1989; Hofstraat *et al.*, 1994). Assuming that heat dissipation and triplet formation are processes of equal importance in samples of the same type, ϕ_P is a good measure of the relative photosynthetic energy conversion at PS II. Since ϕ_P is a specific measure of efficiency of electron transport from PS II, it is less integrated compared to ^{14}C incorporation and will only indirectly detect effects on other components in photosynthesis, *e.g.* on enzymes in the Calvin cycle.

By following the development of toxicity with methods differing in specificity it is possible to obtain information about the mechanism of action of the studied compound, and judge whether a method is sensitive and appropriate for PICT detection for the studied compound. Newman and McCloskey (1996) proposed time-concentration-effect models that monitor effects of both exposure intensity (concentration) and exposure duration. Such models produce several consecutive concentration-response curves that can be used to form a concentration-time-response surface. The shape of such surfaces describes toxicodynamic processes.

This study aims to describe the development of toxicity of the antifouling compounds irgarol, diuron, DCOIT, chlorothalonil, dichlofluanid and zinc pyrithione to photosynthesis of natural marine periphyton communities over times scales of a few minutes to 18-19 hours. Since we also compare the response of a specific photosynthetic function to that of a more integrated measure of photosynthesis, we can judge whether the methods seem appropriate for toxicity estimations and also test whether previous suggestions of mechanisms of action and degradability of the compounds are consistent with the photosynthetic response of periphyton communities.

Table 1. Names, molecular structures, CAS registry numbers, mechanisms of action and highest reported environmental concentrations of the studied antifouling compounds.

Substance name and structure	Full name	CAS no.	Mechanism of action	Highest environmental concentrations
Chlorothalonil	2,4,5,6-tetrachloro-1,3-benzene dicarbonitrile	1897-45-6	Reacts with thiol-containing proteins. Depletes glutathione reserves. [1, 2]	5.2 nM [17]
DCOIT	4,5-dichloro-2-N-octyl-4-isothiazoline-3-one	64359-81-5	Reacts with thiol-containing proteins [3]. Inhibiting succinate dehydrogenase in mitochondria [4-5]. Might cause cascades of RS· radicals [6-8].	12 nM [17]
Dichlofluanid	N-dichlorofluoromethylthio-N´,N´-dimethyl-N-phenylsulfamide	1085-98-9	Reacts with thiol-containing proteins, stimulates Ca^{2+} efflux from mitochondria [9]. Induce lipid peroxidation [10].	1.8 nM [17]
Diuron	N-(3,4-dichlorophenyl)-N,N-dimethyl urea	330-54-1	Photosystem II inhibitor, blocking electron transport in D1 in photosystem II [11-12].	21 nM [17]
Irgarol	2-methylthio-4-tert-butylamino-6-cyclopropylamina-s-triazine	28159-98-0	Photosystem II inhibitor, blocking electron transport in D1 in photosystem II. [13]	16.7 nM [19]
Zinc Pyrithione	bis(2-pyridylthio)zinc-1,1´-dioxide	12463-41-7	Interacts with (and possibly disrupt) biological membranes [14-15]. Causes intracellular ATP losses [16].	No reports available.

[1] Tillman *et al.* (1973) [2] Long and Siegel (1975) [3] Fuller et al. (1985) [4] Chapman and Diehl (1995) [5] Bragadin et al. (2005) [6] Collier *et al.* (1990a) [7] Collier et al. (1990b) [8] Collier *et al.* (1991) [9] Hertel *et al.* (1981) [10] Suzuki *et al.* (2004) [11] Moreland (1980a) [12] Chesworth *et al.* (2004) [13] Hall *et al.* (1999) [14] Dinning *et al.* (1998a) [15] Al-Adham et al. (1998) [16] Dinning *et al.* (1998d) [17] Reviewed in Konstantinou and Albanis (2004) [18] Thomas et al. (2001) [19] Basheer *et al.* (2002)

MATERIALS AND METHODS

Sampling and handling of periphyton

Periphyton communities were sampled in the shallow bay of Kalvhagefjorden (58° 13' 43.36" N, 11° 24' 13.26" E) situated in the outer archipelago of the west coast of Sweden. The ^{14}C-incorporation and PAM tests were made during the summer of 2002 and 2004 respectively. Periphyton communities were sampled by natural colonisation of artificial substrata. The sampling units were circular glass discs (1.5 cm^2) mounted on polyethylene holders as described by Blanck and Wängberg (1988). The periphyton sampling devices, connected to buoys, were hanging approximately 1.5 m from the water surface for 2-4 weeks. During this period the glass discs were colonised by the indigenous species at the sampling site. The communities were protected from temperature changes and strong light when transported to the laboratory. Periphyton discs were gently cleaned on all but the colonised side and atypical ones discarded.

Test media and toxicant solutions

Filtered (Whatman GF/F) natural seawater, stored at 4°C for less than a week, was used as incubation media and for toxicant solutions. Irgarol, diuron and DCOIT stock solutions were prepared in methanol or acetone and stored at – 20 °C. The toxicant solutions were prepared by adding stock solutions to glass flasks, letting the methanol evaporate and then add the filtered seawater. They were left at least 12 hours in darkness and room temperature for the toxicant to dissolve completely. The water solubility of chlorothalonil, dichlofluanid and zinc pyrithione is low. Therefore it is not possible to make toxicant solutions of these compounds at higher concentrations than the test concentrations in sea water. Thus the toxicant solutions of these compounds were made in acetone and added directly to the incubation media.

^{14}C-incorporation tests

Periphyton discs were incubated in scintillation vials, at the *in situ* temperature and a light intensity of about 125 μmol photons m^{-2} s^{-1}. The light source during the incubation was fluorescent tubes (Osram Lumilux Daylight L18W/11). The samples were gently shaken during the tests. Incubations with toxicant were made for 30 minutes, 3 or 4 hours before addition of radiolabelled carbon and then further incubated for either 15 minutes or 1 hour, giving total incubation times of 30 + 15 minutes, 3 hours + 15 minutes and 4 hours + 1 hour. Each test had 10 control samples, 6-7 toxicant concentrations with 5 samples for each concentration, and 3 samples for abiotic carbon fixation. The abiotic carbon fixation was estimated by the addition of 50–100 µL formaldehyde (37%), which inhibited biotic activity, before incubation with radiolabel. For irgarol 1051, diuron and DCOIT 2.5 ml media and 2.5 ml toxicant solution was added, giving exposure concentrations

half that of the toxicant solutions. For chlorothalonil, dichlofluanid and zinc pyrithione 5 ml media and 5 µl toxicant solution was added, giving exposure concentrations 1000-fold lower than the toxicant solutions, and an acetone concentration of 0.1 %. For these toxicants, 5 controls without addition of acetone were used to control for a possible acetone effect. The solution of ^{14}C-labelled sodium bicarbonate was prepared by diluting a stock solution of 1 mCi/mL (37 MBq/mL) (DHI, Hoersholm, Denmark) in GF/F-filtered seawater, giving an activity of 40 µCi/mL (1.48 MBq/mL). 50 µL aliquots of this solution were added to each sample, giving a final activity of 2 µCi (0.074 MBq) in each sample. Incubation was terminated by addition of 50–100 µL formaldehyde (37%) to each sample. After incubation, the media were removed from the samples, which were acidified with 1 ml acetic acid and dried at 60°C under a gentle stream of air. To each sample, 1 ml dimethyl sulfoxide and 8 ml of Ready gel ™ scintillation cocktail (Beckman Inc., USA) was added and the radioactivity of the samples was measured by liquid scintillation counting (LS 5000 TD, Beckman Inc., USA). Disintegrations per minute (DPM) were calculated from counts per minute (CPM) based on the correction factors for the sample quench characteristics and the machine efficiency.

PAM fluorometry tests

Periphyton was incubated in glass dishes (Ø 3 cm) under the same conditions as in the ^{14}C-incorporation tests concerning temperature, light and gentle shaking. Each dish contained four periphyton discs, which after measurement were removed from the dish. Measurements were made with a multiwavelength-excitation PAM fluorometer (Phytopam) equipped with the Emitter-Detector-Fiberoptics Unit for periphyton measurements (Waltz Mess- und Regeltechnik). In order to subtract the background fluorescence from other components than the periphyton, such as stray fluorescence from the dish and/or glass discs or from humic acids in the media, a so-called Zero Offset (Zoff) determination was made. Background fluorescence from clean glass discs and the same media as in the other measurements was subtracted from the periphyton fluorescence signal. The Phytopam is capable of exciting pigments at the four wavelengths 470, 520, 645 and 665 nm, corresponding to pigments present in different groups of microalgae and cyanobacteria. If a proper reference excitation spectrum is used, the four channel signal enables potential separation of fluorescence from the three groups cyanobacteria, green algae and diatoms/dinoflagellates. However, the optical properties of the glass dishes affected Zoff values of channels 1 (470 nm) and 2 (520 nm) in an irregular way, which increased the variability of reading for these channels. Since this represents non-biological variability, and since we found no differential sensitivity to the toxicants among the excitation channels, the data from channels 1 and 2 were not further analysed. All calculations and analyses

are therefore made on responses from excitation channel 3 (645 nm) and 4 (665 nm) only.

For irgarol, diuron and DCOIT the discs were placed in 7.5 mL media, and for chlorothalonil, dichlofluanid and zinc pyrithione they were placed in 15 ml media. The irgarol, diuron and DCOIT tests were started by adding 7.5 ml toxicant solution to the samples. The tests with the other compounds were started by adding 15, 30 and 45 µL of toxicant solution for chlorothalonil, dichlofluanid and zinc pyrithione respectively.

Two kinds of measurements were made: estimation of photochemical efficiency of PS II (ϕ_P) and rapid light curves (RLC). Whereas the ϕ_P measurements tested if the photochemical efficiency at a fixed light intensity was affected by the toxicants, RLC measurements tested whether the light adaptation to increasing light intensities was disturbed by the toxicants. The Phytopam is equipped with an indicator of a stable fluorescence signal and all ϕ_P and RLC measurements were made after stable fluorescence readings were achieved.

Effects on ϕ_P were measured in a time series with four observations. Each test had 5 control samples and 5-6 toxicant concentrations with 5 samples each. Before the incubations started, a RLC was made (see below) to determine the light intensity (I_k) to which the communities were adapted. A intensity just below the I_k value was then used as the actinic light intensity in the ϕ_P measurements. 15 consecutive saturating light flashes were given with five seconds interval during actinic irradiation. ϕ_P values were calculated with the PhytoPAM Win-Software V 1.45 (Walz GmbH), and the average of the last five saturating light flashes was used as the ϕ_P value of one replicate. Toxicants were added at approximately the same time to the replicate samples of a concentration and since one measurement took about 2 minutes, a time scale over the first 12 minutes for the 5 replicates was obtained. The incubation times for the five replicates are therefore approximate and we estimate that they are at most ± 40 seconds those given in Fig. 3. Complete concentration-response-curves were made for irgarol, diuron, dichlofluanid and zinc pyrithione after 0.1, 3, 6, 10 and 18 hours of incubation and for DCOIT and chlorothalonil after 0.1, 4, 6, 10 and 19 hours of incubation.

A RLC was produced by applying saturating pulses after intervals of increasing actinic light intensity. The first saturating pulse was applied to a semi-dark-adapted sample (only dark-adapted for minutes, until a stable fluorescence reading was reached) and later pulses were applied after 40 seconds of actinic light. The value of Fv/Fm for each pulse was multiplied by the actinic light intensity, giving the relative electron transport rate (ETR). ETR values were then plotted against light intensity. Analysis of the RLC curve provides estimates of the parameters α, which is the initial slope of the curve, the relative maximum ETR (ETR_{max}) and the predicted irradiance for maximum ETR (I_k), which is calculated from the intersection of the lines

defined by α and ETR_{max} (Falkowski and Raven, 1997; Runcie and Riddle, 2004). Effects of the toxicants on RLC were estimated after 3 and 19 hours of exposure. All other conditions were as for the ϕ_P measurements.

Calculations

EC50- and ET50 values were estimated by interpolation in concentration- and time-response curves with the mean values of the controls set to 100%.

Table 2. EC50 values (nM) detected with the two methods PAM fluorometry, as photochemical efficiency of PS II (ϕ_P), and ^{14}C-incorporation (^{14}C) after different incubation periods with the six antifouling compounds.

	Exposure time (hours:minutes)	EC50 values (nM)					
		Irgarol	Diuron	DCOIT	Chlorothalonil	Dichlofluanid	Zinc Pyrithione
ϕ_P	0:06	6.2	10	N.A.	N.A.	N.A.	N.A.
	3:00	1.9	7.3	-	-	1500	520
	4:00	-	-	4700	2100	-	-
	6:00	1.9	7.3	-	-	2100	610
	7:00	-	-	2800	2400	-	-
	18:00	1.9	7.5	-	-	3200	520
	19:00	-	-	2400	2300	-	-
^{14}C	0:45	4.3	27	110	4000	320	270
	3:15	-	-	426	-	470	251
	5:00	3.3	29	400	560	440	140

N.A. indicates that EC50 values were non-available due to too low inhibition.

- indicates that no EC50 was determined for respective exposure time.

RESULTS

The effect of the tested antifouling compounds depended both on exposure concentration and exposure duration. The most toxic compounds to photosynthesis of periphyton communities were the PS II inhibitors, irgarol and diuron (Table 2, Fig. 1). This is not surprising since the mechanism of action of these compounds is inhibiting photosynthetic electron transport at PS II. Although there were no dramatic differences between the ^{14}C-incorporation and PAM tests for these compounds, the PAM method gave slightly lower EC50 values (Table 2). These compounds were also the two most rapidly acting ones. Within two minutes the photochemical efficiency of PS II was lowered almost to the level where it stabilised for the next 18 hours (Fig. 3 and Fig. 4).

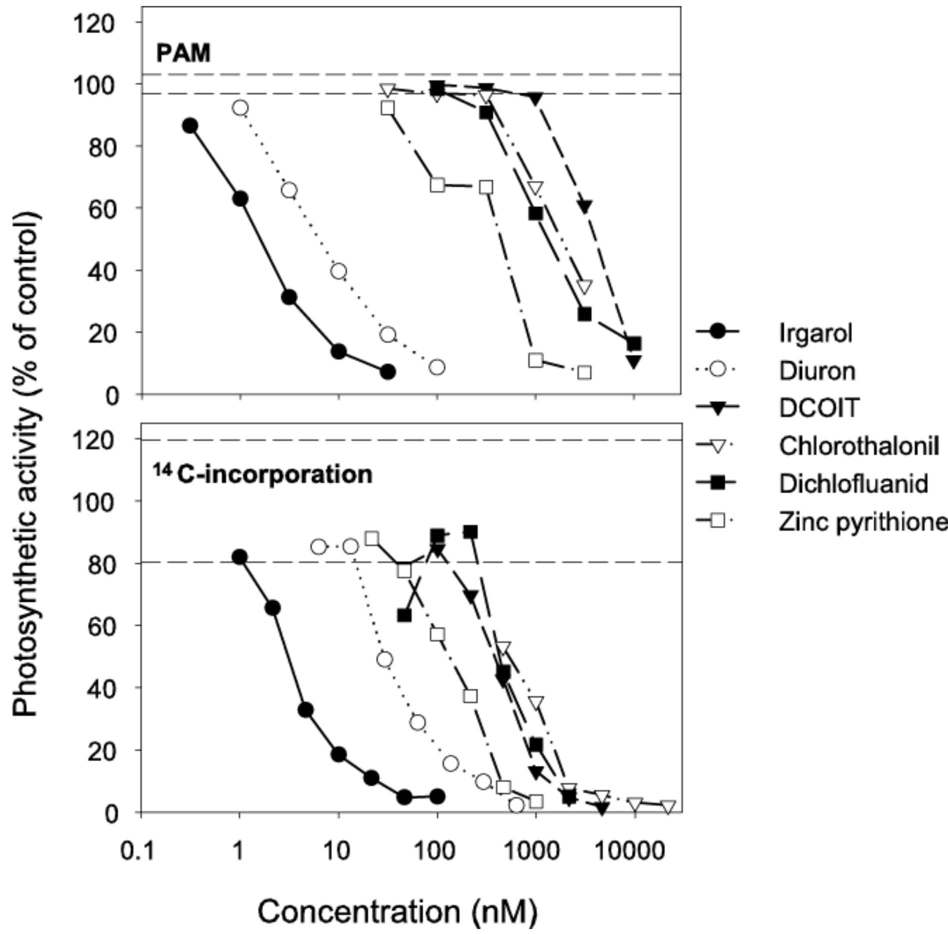

Figure 1. The highest observed toxicities to photosynthesis of the six antifouling compounds. Inhibition of photosynthetic reactions was detected with PAM fluorometry during 18-19 hours (upper panel) and with ^{14}C-incorporation during 5 hours (lower panel). In the PAM tests the exposure time for the shown concentration response relationships was 3 hours for irgarol, diuron, dichlofluanid and zinc pyrithione and 4 hours for DCOIT and chlorothalonil. In the ^{14}C-incorporation tests the exposure time for the shown concentration response relationships was 5 hours. The dashed line indicates the lower limit of the average 95% confidence interval of the control samples in the tests.

The third most toxic compound was zinc pyrithione. The difference between the methods in sensitivity was larger for zinc pyrithione than for the PS II inhibitors and the relation between them was inverted, ^{14}C-incorporation being the most sensitive (Fig. 2). Zinc pyrithione seems to affect photochemical efficiency of PS II quite quickly, with 40% inhibitory effect already after 9 minutes (Fig. 3). The more integrative measure, $^{14}CO_2$-incorporation gave almost as low toxicity estimation after 45 minutes as after 5 hours, indicating that toxicity of zinc pyrithione appears rather quickly and that no recovery occurs. Even though the PAM measurements gave approximately twice as high EC50 values as the ^{14}C-incorporation measurements, the absence of recovery was evident for PAM measurements as well (Fig. 2). This is an

important observation since it has been suggested that this compound is rapidly photodegraded (Maraldo and Dahllöf, 2004b). The biphasic nature of the concentration response curve of zinc pyrithione (Fig. 1) was evident also after 7 hours, and has been found in ^{14}C-incorporation tests as well (data not shown).

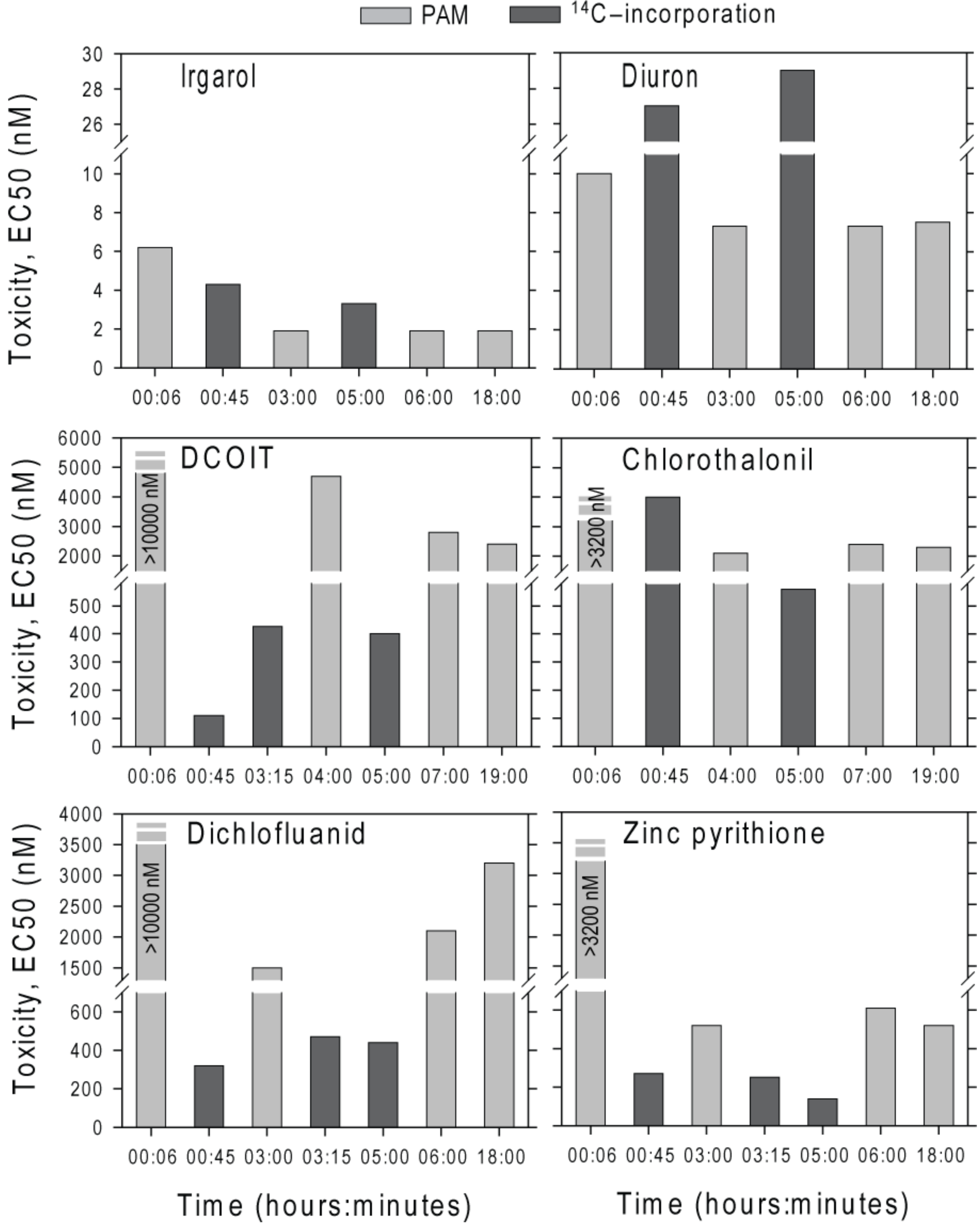

Figure 2. EC50 values of the six antifouling compounds, determined with ^{14}C-incorporation (dark grey bars) and PAM fluorometry (light grey bars), after different exposure times as indicated.

It was also shown as the somewhat gibbous concentration and time response surface for zinc pyrithione in Figure 4. This could indicate that zinc pyrithione, or its degradation products, has two mechanisms of action which affect photosynthesis at different concentrations. In some aspects dichlofluanid toxicity is similar to that of zinc pyrithione. The ^{14}C-incorporation method is more sensitive than PAM for detecting effects of dichlofluanid on photosynthesis (Fig. 2). Similar to zinc pyrithione, dichlofluanid seems to affect photochemical efficiency of PS II rather quickly (Fig. 3). However, in contrast to zinc pyrithione there is a trend for recovery of photochemical efficiency of PS II after 3 hours of dichlofluanid exposure (Fig. 2, 4–5).

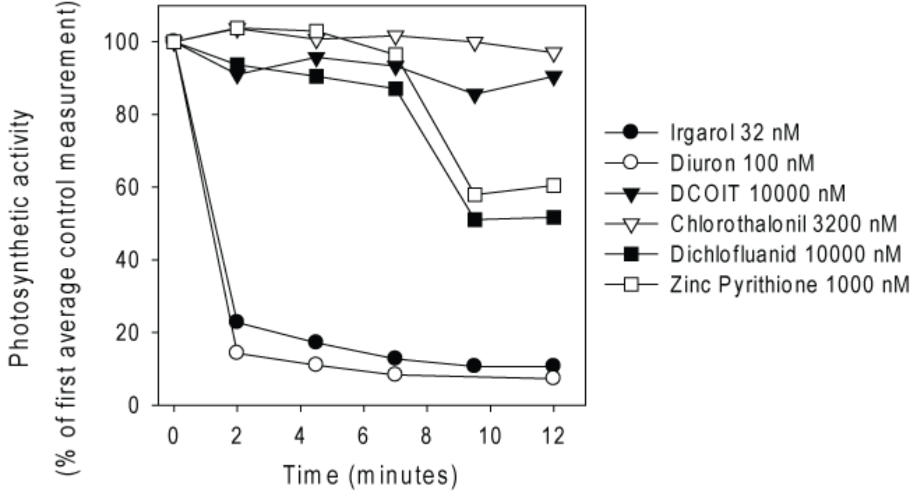

Figure 3. Inhibition of photochemical efficiency of PS II by the six antifouling compounds during the first 12 minutes of exposure. Values are single measurements of PS II photochemical efficiency.

There are similarities between the toxicities of chlorothalonil and DCOIT as well, although there are some important differences. They are the least toxic compounds to periphyton photosynthesis, although their toxicity ranking is not the same for the two methods (Fig. 1). Judged from EC50 values, Chlorothalonil is the least toxic compound when estimated with the ^{14}C-incorporation method, whereas DCOIT takes that position when estimated with PAM fluorometry. There are no signs of early effects on photochemical efficiency of PS II for any of them (Fig. 3), but the time-to-effect patterns are very different after the first 12 minutes. For chlorothalonil the ^{14}C-incorporation EC50 values decrease substantially from 4000 to 560 nM, whereas the PAM EC50 values stay at a higher but fairly constant level (2100–2400 nM) (Table 2, Fig. 2). Compared to chlorothalonil the toxicodynamic response of periphyton photosynthesis to DCOIT is completely different. EC50 values determined with the ^{14}C-incorporation method increase after 45 minutes, whereas they slowly decrease when determined with the PAM

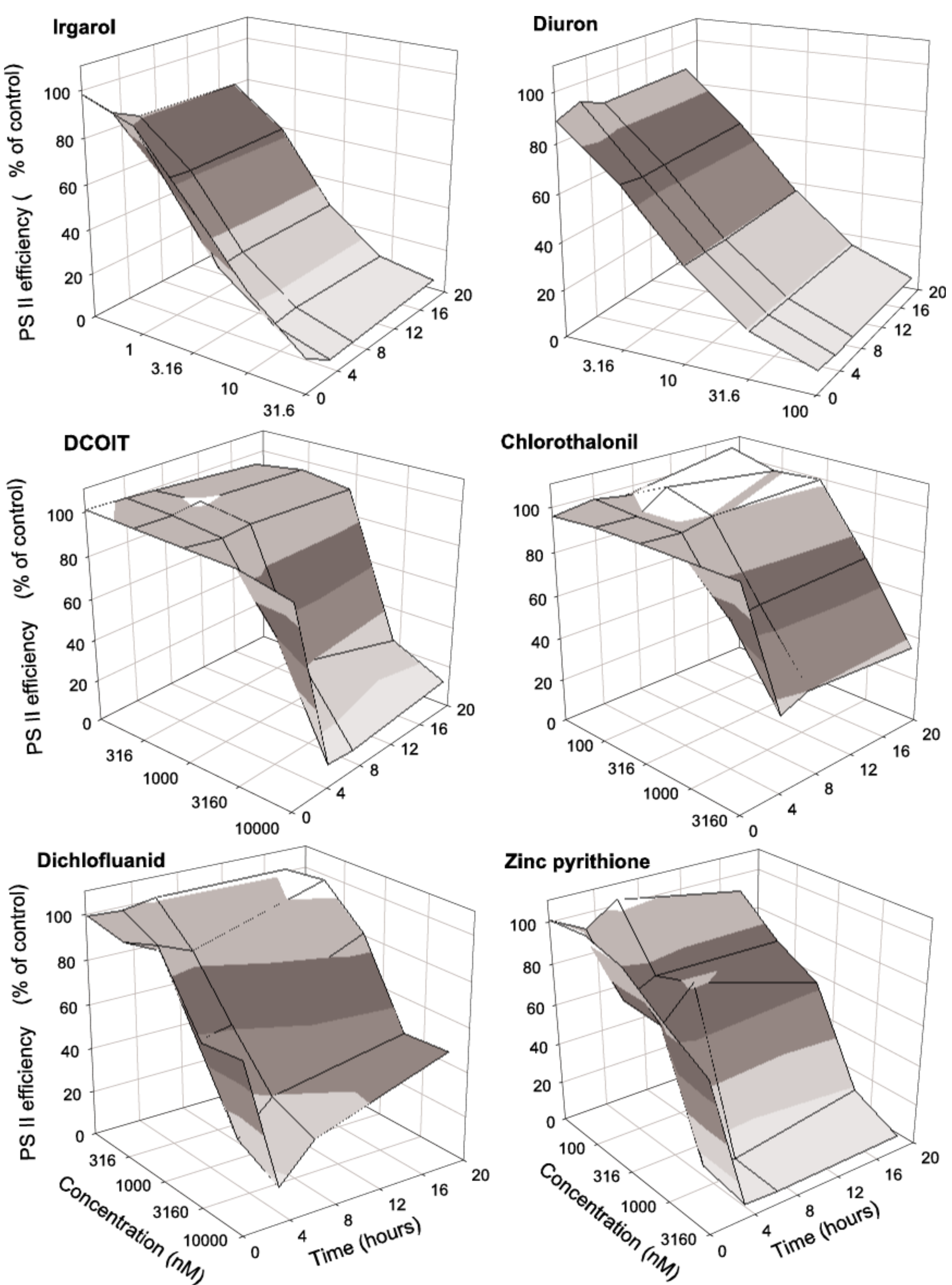

Figure 4. Concentration and time dependent response surfaces for the six antifouling compounds determined as inhibition of photochemical efficiency of PS II (ϕ_P).

method (Table 2, Fig. 2). Since the lowest PAM EC50 value still is approximately a factor of 5 higher than the high ^{14}C-incorporation EC50 values, it is possible that there is a slight recovery of overall photosynthesis at the same time as toxic effects slowly reaches PS II through effect propagation. DCOIT as well as chlorothalonil seem to have other targets than the

photosynthetic electron-transport chain, since the ^{14}C-incorporation method is much more sensitive than the PAM method.

In order to describe how the toxicity to photochemical efficiency of PS II developed in a robust but also detailed way, all responses, *i.e.* both concentration and time responses that passed below 50% of average control values were plotted against time (Fig. 5). Either the values of EC50, coupled to a specific incubation time, or the ET50, coupled to a specific concentration, were calculated by interpolation and plotted. Thus, the plot shows how the development of the 50% inhibition level is dependent on both time and concentration. For the PS II inhibitors all 50% inhibition levels are EC50 values since even measurements after the first incubation time resulted in inhibition below 50%. Clearly, these compounds most rapidly affect photochemical efficiency of PS II as the EC50 values measured after the first incubation time are almost as low as for the later measurements, producing almost horizontal straight lines (Fig. 5). The flat curves for chlorothalonil and DCOIT show that these compounds only slowly affect photochemical efficiency of PS II, and their EC50 values continue to decrease over the 19 hours. The complex concentration and time dependent response to zinc pyrithione (Fig. 4) was also reflected in Fig. 5, where the more or less stable 50% inhibition level after approximately 7 hours, was followed by a second decrease in EC50. The previously mentioned recovery of the photochemical efficiency of PS II during the dichlofluanid exposure is also evident (Fig. 5).

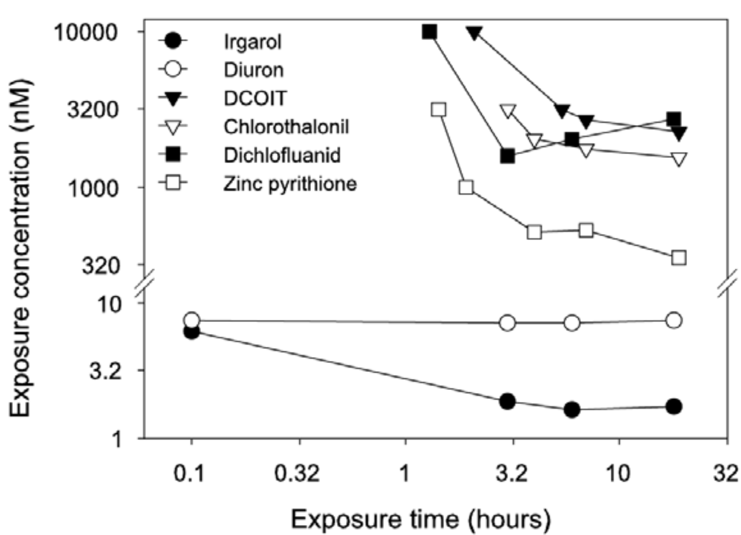

Figure 5. Concentration and time dependence of the 50% inhibition levels of photochemical efficiency of PS II (ϕ_P) after exposure to the six antifouling compounds. Each value is coupled to an exposure concentration and an exposure time. The 50% inhibition levels are thus interpolated from either the exposure concentration or the exposure time responses.

No new and important information about toxicity was obtained from the RLC tests. RLCs showed a pattern of decreased α, ETR_{max} and I_k values for all compounds, but the corresponding EC50-values for these parameters were more variable and less sensitive than EC50-values for photochemical efficiency.

DISCUSSION

The sensitivity of the endpoint is dependent on the mechanism of action of the studied compound. It is almost a truism to say that photosynthesis measurements should be sensitive in detecting effects from compounds that inhibit photosynthesis. However, the outcome of photosynthesis measurements is not so obvious for compounds with other mechanisms of action, and is, at least partly, dependent on exposure time. The time-course approach used here can give important information about mechanisms of action of different compounds, since toxic actions distant from the pathway under study take longer time to reach a part of the metabolism where it can be detected. Thus, the approach can help to disentangle toxic effects on different parts of the metabolism. Moreover, the use of longer incubation times with several measurements of effect can indicate whether an exposure has reached maximum effect or if the toxicity is underestimated.

Consistent results have been obtained when using ^{14}C-incorporation and PAM fluorometry for estimating photosynthesis (Hartig *et al.*, 1998; Barranguet *et al.*, 2000) and both methods have been used extensively in ecotoxicological research. As shown here and suggested previously (Dorigo and Leboulanger, 2001; Guasch *et al.*, 2003; Schmitt-Jansen and Altenburger, 2007), ^{14}C-incorporation and photochemical efficiency of PS II give approximately equal sensitivity to PS II inhibitors. In this study and in the study of Guasch *et al.* (2003) PAM was found to be slightly more sensitive, whereas the studies of Dorigo and Leboulanger (2001) and Schmitt-Jansen and Altenburger (2007) found the inverse relationship. However, the differences were very small and actually suggest that the two methods are very similar in their ability to detect PS II inhibitor toxicity. The severe inhibition from irgarol and diuron on both ^{14}C-incorporation and photochemical efficiency of PS II is consistent with the well established knowledge about mechanism of action of these compounds (Moreland, 1980b; Pfister *et al.*, 1981; Fedtke, 1982; Vermaas *et al.*, 1983). Moreover, the rapid response (Fig. 3) and absence of recovery (Figs. 2, 4–5) is consistent with previous reports of very rapid uptake (Conrad *et al.*, 1993; Dorigo and Leboulanger, 2001; Macedo *et al.*, 2008) and slow degradation (Callow and Willingham, 1996; Liu *et al.*, 1997; Okamura *et al.*, 1999; Giacomazzi and Cochet, 2004). It is noteworthy that photochemical efficiency of PS II has been shown to be equally sensitive as endpoints that detect effects on growth rate (Nystrom *et al.*, 2002; Macedo *et al.*, 2008). This implies that for these compounds it is possible to extrapolate from effects detected with simple fluorescence measurements to characteristics of entire populations. However, it is also important to note that periphyton communities exposed for even longer time periods, under more ecologically realistic exposure regimes, community level endpoints such as Pollution-

Induced Community tolerance and/or species composition is even more sensitive than photosynthesis or population growth endpoints (Dahl and Blanck, 1996; Schmitt-Jansen and Altenburger, 2005; Blanck *et al.*, 2008; Schmitt-Jansen and Altenburger, 2008). Many studies of irgarol and diuron toxicity to photosynthesis of various organisms and communities have been made, and the effect levels of irgarol and diuron for periphyton photosynthesis reported here are in the lower range of these studies (*e.g.* see Molander *et al.*, 1992; Dahl and Blanck, 1996; Nystrom *et al.*, 2002; Berard *et al.*, 2003; Jones and Kerswell, 2003; Devilla *et al.*, 2005; Blanck *et al.*, 2008).

Whereas ^{14}C-incorporation and PAM have approximately equal sensitivities in detecting effects of PS II inhibitors, important differences between these methods are evident when used with other toxicants. Furthermore, these differences may in turn be dependent on incubation time. The compound that gives the smallest differences in toxicity estimation between the methods is zinc pyrithione. The difference in EC50 values between the two tests were only a factor of approximately two. Apart from the initial PAM EC50, the levels in EC50 values of both methods also seem roughly stable over time (Fig. 2). This could be interpreted as a quite rapid uptake of zinc pyrithione and that the substance affects processes quite close to photosynthetic electron transport. This is supported by the 40% inhibition of ϕ_P already after the first 7 minutes (Fig. 3). It has been shown that zinc pyrithione causes loss of membrane integrity (Gibson *et al.*, 1985; Al-Adham *et al.*, 1998), and Dinning (1998b) suggested that this loss is due to chelation of the metal cations present at the outer leaflet of membranes and interaction between the Zn moiety with phospholipid head group. Zinc pyrithione may exist in a monomeric or dimeric form. The interaction between the Zn moiety and the phospholipid head group can lead to a split of the dimeric form into two monomers, which are both capable of reacting with phospholipid head groups. In this interaction, chelation of the zinc atom by the phospholipid head group can transform the monomer into two pyrithiolate anions. If the membrane configuration is disrupted these anions might diffuse into the cell and interact with other components (Dinning *et al.*, 1998b). Thus, it seems like the mother compound by its toxic action could be transformed into two toxic pyrithiolate anions molecules and a zinc ion. The complex pattern of transformation and toxic effects of the individual components might be responsible for the biphasic concentration-response-curves recorded for zinc pyrithione (Fig. 1 and 4).

The disruption of membranes will also cause cells (or organelles) to leak (Al-Adham *et al.*, 1998) and cause breakdown of membrane gradients, such as the proton motive force which in turn will cause ATP deficiency (Dinning *et al.*, 1998c). A breakdown of thylakoid-membrane gradients would uncouple photosynthetic electron transport from ATP production and probably generate more reactive oxygen species that would decrease the efficiency of PS II.

Another possible scenario is that the pyrithiolate anions formed after the interaction with the phospholipid head groups, bind univalent cations and shuttle them over membranes. Zinc pyrithione would thus function as an uncoupler. This hypothesis does not exclude the membrane activity of zinc pyrithione, but merely suggests that the observed ATP-deficiencies might be a result of uncoupling rather than the destabilisation and leakage. In both these scenarios ϕ_P should be a fairly sensitive effect indicator. The higher sensitivity of ^{14}C-incorporation is reasonable since that process is dependent also on other processes that could be affected by loss of membrane integrity, for example lowered activities of Calvin cycle enzymes due to change in pH (Malkin and Niyogi, 2000). However, it has also been argued that zinc pyrithione causes breakdown of membrane gradients by specific inhibition of the primary H^+-pumping ATPase rather than a general breakdown of membrane permeability (Ermolayeva and Sanders, 1995). Our study does not support this argument, since the proton gradient over the thylakoid membrane is not accomplished by a H^+-pumping ATPase but through oxidation of water and photosynthetic electron transport. Since the concentration of ϕ_P inhibition was quite close to that of ^{14}C-incorporation (Fig. 2) it is likely that thylakoid membranes were also depolarised by zinc pyrithione, which instead favours the hypothesis of a general loss of membrane integrity.

The roughly stable level of toxicity over time (Fig. 2) indicates that there is no fast recovery from static zinc pyrithione exposure in this study. This may seem contradictory to results of photodegradation studies (Turley et al., 2000; Maraldo and Dahllöf, 2004b; Okamura et al., 2006). Zinc pyrithione is photodegraded by short-wave length visible light and ultraviolet radiation (UV) (KemI, 1999; Turley *et al.*, 2000; Okamura *et al.*, 2006). Although zinc pyrithione seems to be degraded by UV to a greater extent, photodegradation in artificial light, excluding UV, have been shown to give a half-life as short as 17.5 minutes (KemI, 1999). In our incubation the light intensity at these wave lengths is only a small fraction of the total intensity, and not as high as in the report by KemI (1999). This indicates that even though some photodegradation might occur during our incubation, the extent of it is probably less than previously reported. Thus, the absence of periphyton photosynthetic recovery can partly be due to low degradation rate, but can also be due to toxic degradation products. It was reported that fourteen different degradation products were formed after photodegradation only Kemi (1999). Although pyridine sulphonic acid dominated (accounted for 70% of the added zinc pyrithione) it is not known which of the degradation products that might exert the highest toxicity. The absent recovery in this study is consistent with other effect studies, in the sense that high toxicities have been recorded also after incubations in light (Karlsson and Eklund, 2004; Maraldo and Dahllöf, 2004a; Hjorth *et al.*, 2006).

The recorded EC50 values for periphyton ^{14}C-incorporation (140–270 nM, Table 2) are slightly higher than those reported for phytoplankton communities, 95 nM (Hjorth *et al.*, 2006) and 2-60 nM (Maraldo and Dahllöf, 2004a). However, inner parts of periphyton biofilms can be protected from higher exposure concentrations (Lehmann *et al.*, 1999; Barranguet *et al.*, 2000) since produced extracellular polymeric substances (EPS) can bind the toxicants (Loaec *et al.*, 1997; Pistocchi *et al.*, 1997; Sheng *et al.*, 2005), thus lowering the average exposure concentration within the community. This is consistent with the findings of Bonilla *et al.* (1998), who showed that phytoplankton communities were more sensitive to simazine and paraquat compared to periphyton communities, and that of Guasch *et al*. (2003) who showed that suspended periphyton are more sensitive to zinc than attached periphyton and that loosely attached algae are more sensitive to atrazine than attached periphyton. Therefore, the discrepancy in zinc pyrithione sensitivity between phytoplankton and periphyton communities seems reasonable, probably a consequence of the attached life strategy.

Exposures to the other three compounds all gave different toxicodynamic response patterns. For DCOIT there seems to be contrasting results for the development of toxicity with the two methods (Fig.2). There are some indications of recovery when using the ^{14}C-incorporation method, as EC50 values are higher for the 3:15 and 5 hours incubation compared to 45 minutes of incubation. However, the opposite pattern is evident when using the PAM method, since EC50 values continue to decrease up to 19 hours of incubation (Fig. 2). These contrasting patterns should, however, be viewed in the context of the mechanism of action and effect propagation of DCOIT. Kathon compounds with high similarity to DCOIT are believed to interfere with thiol-containing enzymes (Fuller *et al.*, 1985), and especially with certain enzymes in the respiratory chain in mitochondria (Chapman and Diehl, 1995; Bragadin *et al.*, 2005). When reacting with thiols, these compounds can be transformed to a ring-opened form, which then itself contains accessible thiols that can further react with another biocide molecule. These ring-opened molecules are also capable of tautomerisation to a thio-acyl chloride molecule, which is highly reactive to various targets (Collier *et al.*, 1990c). We again seem to deal with a molecule that by its mechanism of action is transformed and thus may produce several different toxic mechanisms. These toxic mechanisms will affect many targets and pathways in periphyton organisms. Since ^{14}C-incorporation, compared to ϕ_P, has more and closer links to other parts of the metabolism, it is more likely to be dependent on inherently sensitive metabolic processes. Therefore the ^{14}C-incorporation method could be expected to be more sensitive. Since the EC50 values for ϕ_P are approximately 20 times higher than for ^{14}C-incorporation (average of the two last ^{14}C-incorporation EC50 values compared to the 4 hour ϕ_P EC50, Fig. 2) and the effects on ϕ_P slowly increases with time, the effects of DCOIT on ϕ_P are likely to be indirect. The

measured effect on ^{14}C-incorporation could also be indirect since suggestions of more sensitive targets have been made (Chapman and Diehl, 1995; Bragadin *et al.*, 2005) and reported toxicities of more integrative endpoints are higher. Examples of these higher toxicities include EC50 values of 25 nM for lethality of haemocytes (Cima *et al.*, 2008), 10.6 nM, 10.6 nM and 3.5 nM for *Vibrio fischeri* bioluminescence, growth of *Selenastrum capricornotum* and motility of *Daphnia magna* respectively (Mezcua *et al.*, 2002) and 50 nM for rainbow trout lethality (Okamura *et al.*, 2002). An alternative explanation to the lower sensitivity of periphyton in this study is that the bioavailability of DCOIT might be lower in natural filtered sea water, since it contains higher concentrations of dissolved organic substances compared to artificial media. Similarly to the time-dependent effect of DCOIT detected in this study, delayed inhibition by similar isothiazolone compounds have been recorded for bacteria and yeast (Collier *et al.*, 1990a) and even in simple enzyme assays (Collier *et al.*, 1991).

In spite of certain similarities in the mechanism of action (Table 1), the periphyton responses to chlorothalonil and dichlofluanid exposure were not similar to that of DCOIT. The only common characteristic among these compounds is that they do not seem to primarily inhibit photosynthetic light reactions, as ϕ_P was insensitive compared to ^{14}C-incorporation (Fig. 2). This is consistent with the proposed mechanism of action of chlorothalonil as inhibiting glycolytic and Krebs cycle enzymes involved in energy metabolism (Tillman *et al.*, 1973; Long and Siegel, 1975; Baier-Anderson and Anderson, 2000b, 2000a). This inhibition is again thought to occur via interference with thiol groups in these enzymes, which is supported by observations of glutathione depletion upon exposure in various organisms (Tillman *et al.*, 1973; Gallagher *et al.*, 1991; Suzuki *et al.*, 1997). This depletion is also consistent with the suggestion that free radicals are involved in toxicity and with the observation of lipid peroxidation upon chlorothalonil exposure (Suzuki *et al.*, 1997; Suzuki *et al.*, 2004). Results of Yamano and Mortia (1995) also supported the hypothesis of chlorothalonil interfering with thiol groups, but they did not find effects on mitochondrial metabolism. Chlorothalonil only seems to affect photosynthesis slowly, with EC50 values for ^{14}C-incorporation decreasing from 4000 nM after 45 minutes down to 560 nM after 5 hours (Fig. 2). There is no initial inhibition of ϕ_P during the first 12 minutes of incubation (Fig. 3) and after 4 hours the efficiency of PS II seems to be stable. At low exposure concentrations ϕ_P is actually slightly stimulated after 7 and 19 hours (Fig. 4), but only by 5–6% and not statistically significant. Delayed effects on the growth of the algae *Selenastrum capricornutum* was also reported by Fernándes-Alba *et al.* (2002) with EC50 values lowered from 159,000 nM after 30 hours to 25 nM after 72 hours. However, the first value has to be regarded with some scepticism, since it is above the water solubility of chlorothalonil. Also for *Daphnia magna* the toxicity seems to increase with time from an EC50 of 260

nM at 24 hours to 110 nM at 48 hours (Fernandez-Alba *et al.*, 2002). Development of chlorothalonil toxicity over time has also been recorded in isolated rat hepatocytes (Suzuki *et al.*, 2004). In their study lipid peroxidation reaches maximum effect first after 90 minutes. Other reports of toxicity to algae include an EC50 value for inhibition of *Dunaliella tertiolecta* growth (96 hours) of 240 nM (DeLorenzo and Serrano, 2006), of *Selenastrum capricornotum* growth (72 hours) of 26 nM (Mezcua *et al.*, 2002), and of *Chlorella pyrenoidosa* and *Scenedesmus obliquus* growth (96 hours) of 377 nM and 30,300 nM respectively. The high EC50 value for *Scenedesmus obliquus* is above the water solubility of chlorothalonil. Since these toxicity measurements were made after longer exposure times, the toxicity estimates for periphyton reported here seems reasonable.

Dichlofluanid, on the other hand, seems to affect photosynthesis rather quickly, as there was an initial ϕ_P inhibition of almost 50% after the first 7 minutes (Fig. 3). Moreover, the highest inhibition of ^{14}C-incorporation was found after 45 minutes and subsequent EC50 values were higher (Fig. 2). This rapid effect is in contrast to effects of chlorothalonil and DCOIT even though the mechanisms of action as thiol agents are similar. It is believed that also dichlofluanid inhibits thiol-containing enzymes, and induces free radical formation which depletes glutathione and cause lipid peroxidation (Yamano and Morita, 1993; Suzuki *et al.*, 1997; Suzuki *et al.*, 2004). Suzuki *et al.* (2004) followed dichlofluanid-induced lipid peroxidation in rat hepatocytes over time and found that substantial effects were not observed after 30 minutes, but after 60 minutes of exposure. They also showed that lipid peroxidation from dichlofluanid was induced earlier than that of chlorothalonil, which is consistent with our results. There are indications of recovery from dichlofluanid exposure. In the ^{14}C-incorporation tests there is a trend of increasing EC50 values with incubation time and in the PAM tests this trend is even more clear (Fig. 2, 4–5). This pattern of recovery is consistent with observations that dichlofluanid can be rapidly hydrolysed (van Wezel and van Vlaardingen, 2004; Schouten *et al.*, 2005). van Wezel and Vlaardingen reviewed various reports and concluded that dichlofluanid concentrations in toxicity tests rapidly can drop below the detection limit and that the observed toxicity instead comes from the stable degradation product dimethylaminosulfanilide (DMSA). Assuming that DMSA toxicity is lower than that of dichlofluanid, it is likely that the pattern of recovery (Fig. 4) represents the lowering of toxicity when dichlofluanid is degraded into DMSA.

Following the development of toxicity in concentration-time-response surfaces, is indeed something desirable in ecotoxicity testing. If non-destructive methodology (methods where the effect can be measured several times on the same sample, *e.g.* PAM fluorometry) can be used, much more ecotoxicological information can be gained with relatively little extra effort. However, if destructive methodology (each sample can only estimate effect at

one specific time point) is used, more resources are needed to produce a concentration-time-response surface as compared to a concentration-response relationship. Still, it can be important to follow toxicity over time. Guasch et al. (2003) showed that zinc toxicity to periphyton communities, measured as inhibition of ϕ_P, was almost doubled when estimated after 6 hours compared to 90 minutes. In this case, the authors suggested that slow penetration of the toxicant into the periphyton biofilm was the reason for the delayed toxicity, which seem probable since it has been shown that metals can bind to EPS produced by biofilms (Loaec *et al.*, 1997; Pistocchi *et al.*, 1997; Sheng *et al.*, 2005). Detection of increased toxicity after longer incubation periods do not, however, seem to be restricted to metals. In spite of using a detection method (^{14}C-incorporation) matched to the mechanism of action (PS II inhibition), and the fact the PS II inhibitors act rapidly, Gustavson *et al.* (2003) detected an almost 10-fold decrease in EC50 value for freshwater periphyton photosynthesis when prolonging the exposure time for isoproturon from 1 to 24 hours. We did not find any similar decrease in EC50 values for the two PS II inhibitors irgarol and diuron when exposure time was prolonged to 18 hours (Fig. 2). However, it is likely that factors such as environmental conditions, colonization time, biomass and species composition influence the response of periphyton to PS II inhibitors (Guasch *et al.*, 1997). If such differential response is time-dependent as well, lowering of EC50 values over time might well occur even for tests using photosynthesis endpoints for effects of PS II inhibitors.

In conclusion, the time-to-effect approach has proved its value in estimating increasing, as well as decreasing, toxicity to periphyton. PAM fluorometry is a suitable technique for this approach since it is easy to use and non-destructive. It is suitable for estimating effects of PS II inhibitors. However, in spite of prolonging exposure times to 18-19 hours, PAM fluorometry was not suitable for detecting effects of toxicants with mechanisms of action not directed towards photosynthesis. Therefore, its value in ecotoxicological testing and PICT detection of such toxicants is questionable. ^{14}C-incorporation seems to have a greater capability of detecting effects of toxicants with different mechanisms of action. Our results support the previously proposed mechanisms of action of the studied compounds. In addition the results support suggestions of rapid hydrolysis of dichlofluanid, but rapid photodegradation of zinc pyrithione could not be verified under the light conditions used in this study.

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Paper II

"That they do not know, and yet act as if they did, is the annoying and dangerous thing, as well as the fact that they continue to insist on their impossible "knowledge" even where they should have known better long ago"

Ulrich Beck (Risk Society, 1992), concerning the behaviour of ecotoxicologists.

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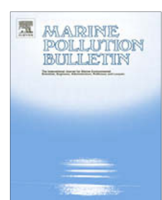

A retrospective analysis of contamination and periphyton PICT patterns for the antifoulant irgarol 1051, around a small marina on the Swedish west coast

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ABSTRACT

Irgarol is a triazine photosystem II (PSII) inhibitor that has been used in Sweden as an antifouling ingredient since the 1990s. Early microcosm studies indicated that periphyton was sensitive to irgarol at concentrations regularly found in harbours and marinas. However, field studies of irgarol effects on the Swedish west coast in 1994, using the pollution-induced community tolerance (PICT) approach, failed to detect any effects of the toxicant in the field. A PICT study involves sampling of replicate communities in a gradient of contamination, and a comparison of their community tolerance levels, with an increase being an indication that sensitive species have been eliminated and replaced by more tolerant ones. Typically, short-term assays are used to quantify the community tolerance levels. Later PICT studies in the same area over a 10 year period demonstrate that irgarol tolerance levels have increased, although the contamination pattern has been stable. Our results support the hypothesis that that the PICT potential was low initially, due to a small differential sensitivity between the community members, and that a persistent selection pressure was required to favour and enrich irgarol-tolerant species or genotypes.
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1. Introduction

The *s*-triazine algicide, irgarol 1051 (2-(*tert*-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine), is used as a booster biocide in antifouling paint, to prevent settling of aquatic plants on ship hulls. Irgarol is a so-called PS II inhibitor, since it inhibits photosynthesis by interfering with chloroplast electron transport in photosystem II, through binding to the membrane-bound D1 protein (Moreland, 1980; Pfister et al., 1981; Vermaas et al., 1983; Hall et al., 1999; Oettmeier, 1999). This binding interferes with the electron and proton transport mediated by the shuttling of plastoquinone between D1 and the cytochrome b complex.

Irgarol was first reported as an environmental contaminant in the Mediterranean Sea (Readman et al., 1993) and in UK coastal waters (Gough et al., 1994). It was tentatively approved for use in Swedish waters in 1992 and is since 1994 the only booster biocide allowed on pleasure craft on the west coast of Sweden. Irgarol was detected in the vicinity of a small marina at Fiskebäckskil on the Swedish west coast in 1993/94 in the range of 0.12–1.6 nM, the highest values being recorded at the peak of the pleasure craft season in late June (Dahl and Blanck, 1996a). At the same time a microcosm study showed significant effects on periphyton communities at concentrations in the range of 0.16–0.79 nM (analyzed concentrations; Dahl and Blanck, 1996a). Since then, irgarol has regularly been detected in coastal waters all over the world at levels approaching or exceeding the effect levels on microalgae (see e.g., Hall et al., 1999; Thomas, 2001; Lamoree et al., 2002; Gardinali et al., 2004; Harino et al., 2005; Lam et al., 2005; Gatidou et al., 2007). The highest concentrations are found during the peak of the boating season, when boats are re-painted and in areas with high density of pleasure crafts (Gatidou et al., 2007). The highest concentration reported in water is 4.2 μg/l (16 nM) (Basheer et al., 2002).

Irgarol is not easily degraded in seawater although *N*-dealkylation leads to the formation of the main metabolite M1 (2-methylthio-4-*tert*-butylamino-*s*-triazine) (Liu et al., 1997; Okamura et al., 1999). After the ban of irgarol 1051 in UK in September 2000, irgarol levels have decreased by 10–55% (Cresswell et al., 2006). However, levels as high as 136 ng/l (0.5 nM) were occasionally found as long as three years after the ban (Gatidou et al., 2007). This is exactly the level estimated to affect 10% of the algal species based

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on a probabilistic risk assessment of both fresh-water and marine algae (Hall et al. (1999), suggesting that biological recovery might take a long time.

Community-level studies with a variety of species are likely to be more sensitive than single species tests, because the probability of including sensitive species in the tests is higher. The first report on environmental effects on algae (Dahl and Blanck, 1996a) concluded that irgarol is likely to damage algal communities in contaminated coastal marine environments. It was based on observed effects on marine periphyton communities at sub-nanomolar concentrations (0.16–0.79 nM, 44–215 ng/l), in a flow-through microcosm experiment in April 1994. These low effect levels have later been confirmed. Nystrom et al. (2002) demonstrated that phytoplankton photosynthesis from Lake Geneva had an estimated threshold for inhibition at 8–80 ng/l (0.03–0.3 nM). Growth studies of phytoplankton (*Synechococcus* sp. and *Emiliana huxleyi*) showed effects at 160–250 ng/l (0.5–1.0 nM) (Devilla et al., 2005b). Exposure of a phytoplankton community to irgarol at 500–1000 ng/l (2–4 nM) affected prymnesiophytes and prasinophytes while chlorophytes and dinoflagellates were more tolerant and the chlorophytes actually expanded (Devilla et al., 2005a). It appears that effects on the most sensitive algal species are detectable at sub-nanomolar concentrations both in periphyton and phytoplankton.

Due to its ability to discriminate between selection pressures exerted by different chemicals, the pollution-induced community tolerance (PICT) approach can be used to establish causative links between toxicants and their effects (Blanck et al., 1988; Blanck, 2002). Another advantage is that PICT field studies also account for the ecologically relevant sensitivity that organisms show in their natural habitat, due to biotic interactions and abiotic stress. The utility of the approach was demonstrated in a series of field and microcosm studies of the negative effects of the antifoulant TBT on marine periphyton in coastal waters of Sweden before and after the ban on pleasure craft (Blanck and Dahl, 1996, 1998; Dahl and Blanck, 1996b). A PICT study involves a selection phase where a series of communities are exposed to toxicants in natural or experimental ecosystems. Toxicant stress that affects the fitness of any sensitive species in their community context will lead to the elimination of these sensitive species and replacement by more tolerant ones, thus increasing the fraction of tolerant organisms, and therefore the tolerance of the community as a whole, to that particular compound. For PICT to develop it is thus essential that differential sensitivity is present in the community. The PICT potential of a community depends on the distribution of sensitivities among the community members, and is therefore based on the type and efficacy of available tolerance mechanisms. In the PICT detection phase, the community tolerance to the toxicant is quantified in a short-term test using for example EC50 of community photosynthesis. Preferably, the detection phase should employ an effect indicator matched to the mode of action of the toxicant or a superior (integrating) effect indicator able to gauge effects from all modes of action. The use of PICT in microcosm and field studies of marine periphyton was first outlined by Blanck et al. (1988) and its possibilities and limitations reviewed by Blanck (2002).

In the case of irgarol no significant PICT signal could be detected in 1994, neither in the field (Blanck and Dahl, unpublished) or in a microcosm experiment (Dahl and Blanck, 1996a), in spite of the fact that irgarol mode of action as a PSII inhibitor indeed is matched to the community-photosynthesis test used. Furthermore, several tolerance mechanisms (degradation through dealkylation, transformation through hydroxylation and conjugation, modification of target protein through point mutations) were known to render higher plants, cyanobacteria and algae tolerant to PSII inhibitors (Pfister et al., 1979; Hirschberg and McIntosh, 1983; Bettini et al., 1987; Ajlani et al., 1989; Gronwald et al., 1989; Holt, 1990; Anderson and Gronwald, 1991; Hall et al., 1995). Community tolerance to atrazine (another PSII-inhibiting *s*-triazine) was detectable in high-precision microcosm studies with river periphyton, using both community photosynthesis and sulfolipid synthesis to quantify tolerance (Nystrom et al., 2000). However, the tolerance increase was small, suggesting that the PICT potential in the community was limited. This is consistent with the irgarol-induced succession pattern observed in marine periphyton microcosms (Dahl and Blanck, 1996a, Fig. 6) where no algal species seemed to be tolerant enough to replace the eliminated sensitive species when irgarol exposure increased. A hypothesis emerged (Dahl, 1996; Blanck, 2002), suggesting that the PICT potential was low and that only small tolerance increases to irgarol was to be expected.

In the light of these findings, the irgarol community tolerance pattern around a small marina on the Swedish west coast was reinvestigated several times over a 10 year period taking both spatial and seasonal aspects into consideration. The results presented demonstrate that community tolerance to the PSII inhibitor irgarol 1051 is inducible, but also suggest that community tolerance to irgarol develops slowly and only as a consequence of a long and persistent selection pressure from the irgarol exposure.

2. Materials and methods

2.1. Sampling

Periphyton communities were sampled in a documented gradient of antifouling contamination (Blanck and Dahl, 1996) from the small marina of Fiskebäckskil to the outermost islet in the archipelago at the mouth of the Gullmar fjord on the Swedish west coast. The sampling sites are characterised in Table 1.

Periphyton communities were established on circular glass discs mounted on polyethylene holders (Blanck and Wängberg, 1988; Blanck and Dahl, 1996) that were hung from a buoy 1.5 m below the surface. Communities were sampled after 2–4 weeks and protected from temperature changes and strong light during transport to the laboratory for further experimentation. Sub-surface water samples for analysis of environmental irgarol concentrations were taken at periphyton sampling stations during the week prior to the periphyton sampling. In short, one 500–1000 ml sample was taken for each station and occasion and put on disposable 6 ml cartridge columns packed with 200 mg of Isolute ENV$^+$, from International Sorbent Technology (IST, Hengoed, UK) at a flow rate of 10 ml/min. Columns were preconditioned using methanol (HPLC grade) and MilliQ-filtered water. The dry columns were packed and stored at −18 °C until chemical analysis.

2.2. Chemical analysis

Samples from 1994 were analysed by GC/MS as described by Dahl and Blanck (1996a). Samples from 2000 and 2001 were analysed using HPLC–MS at CSIC, Barcelona, Spain (Martinez et al., 2000). Samples from 2004 were analysed by GC/MS (Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MS). The GC was equipped with a 30 m × 0.25 mm fused silica column coated with HP-1 (100% methyl siloxane, df = 0.25 μm: Hewlett-Packard, Palo Alto, CA, USA). Temperature programming was 80 °C for 2 min; 20 °C/min to 325 °C, followed by isothermal at 325 °C for 2 min (to clean the column after each injection). Injector temperature was 275 °C and the transfer line was programmed at 250 °C for 7 min and 20 °C/min to 300 °C, and kept isothermal. Helium was used as carrier gas, at 35 cm/s, and the electron impact (EI mode) mass spectra were obtained at 70 eV. Compounds were identified by their GC retention times and obtained mass spectra and compared with

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Table 1
Sampling sites in antifouling gradient

Site	Type	Antifouling contamination
Site 1: Kilen1	Inner part of shallow and muddy bay; marina site	Very high
Site 2: Kilen 2 Not sampled	Centre of shallow bay; marina site	Very high
Site 3: Färjeläget	Mouth of bay; at a small ferry berth	High
Site 4: Rödbergsskär	Lee side of small rocky shore islet	Moderate
Site 5: Kalvhagefjorden	Shallow and muddy bay; visited by pleasure craft	Low
Site 6: Bonden	Outer archipelago; lee side of outmost islet	Very low; coastal background

Site numbers are the original ones given by Blanck and Dahl (1996). Site 2 was not used in this study.

authentic samples of synthetic references. Quantification was based on prominent MS fragments in each compound, according to Garland and Powell (1981) (*m/z* 182 for irgarol, confirmed by *m/z* 253 and *m/z* 284, confirmed by *m/z* 286, for the internal standard hexachlorobenzene, respectively).

2.3. Pollution-induced community tolerance

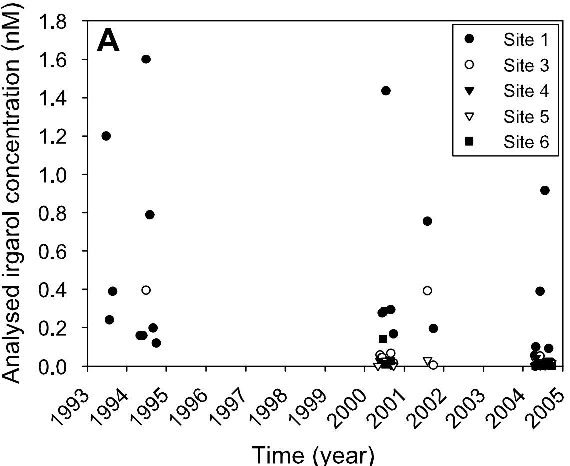
PICT was quantified as the EC50 for inhibition of community photosynthesis measured in a short-term test using a set of periphyton-colonized glass discs, as described in Dahl and Blanck (1996a). Glass discs were cleaned on all but the colonised upper side. Samples were inspected, and damaged or atypical ones discarded. Samples were incubated in scintillation vials with 2 ml of test solution, at the current temperature at the sampling site and at a photon flux density of 120 $\mu E\,cm^{-2}\,s^{-1}$. Test solutions were made from irgarol stock solutions in acetone or methanol, either by diluting the stock solution in GF/F-filtered seawater to a co-solvent concentration of <10 $\mu l\,l^{-1}$, or by evaporating the acetone or methanol and dissolving the remaining irgarol in GF/F-filtered seawater. Samples were preincubated for 30 min before addition of 50 µl aliquots of ^{14}C-bicarbonate solution with 2 µCi (74 kBq) to each vial. After 15 min of incorporation of radiolabelled carbon, photosynthesis was stopped by addition of 100 µl 37% formaldehyde solution. The water solutions were removed and the samples acidified by adding 1 ml concentrated acetic acid. The remaining CO_2 was driven off in a gentle stream of air and the samples were dried at 60 °C. The release of organic carbon from the samples was enhanced by addition of 1 ml dimethyl sulphoxide before addition of 8 ml Ready Safe™ (Beckman Coulter) scintillation cocktail. ^{14}C disintegrations per minute were measured in a liquid scintillation spectrometer using the appropriate corrections for quenching and machine efficiency. The photosynthetic activities were corrected for abiotic binding of ^{14}C in periphyton samples killed by formaldehyde before the incubation.

Concentration response curves were constructed and EC50 values, and values of photosynthesis inhibition at 1.6 nM, were estimated by log-linear interpolation.

3. Results

Analysed irgarol concentrations in seawater were generally below 0.40 nM (92-percentile), with occasional values at 0.75 to 1.6 nM (8-percentile) (Fig. 1 and Table 2). The highest values were found at the stations closest to the marina, with peak values from late June to early August, corresponding to the most intensive pleasure boat activity in Sweden. The seawater contamination pattern seems to be roughly stable for the investigated years between 1994 and 2004, when irgarol was the only booster biocide approved for Swedish pleasure craft.

In contrast, the pattern of biological response to this contamination have changed. In 1994, community tolerance to irgarol in periphyton communities was low and stable, with no significant differences between sites in the spatial gradient from the marina



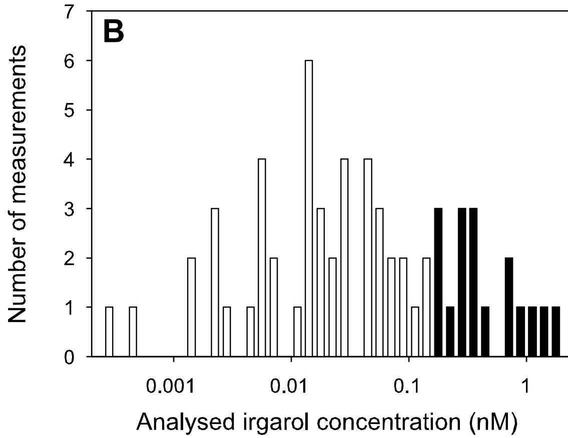


Fig. 1. Irgarol contamination in surface coastal water round a small marina in Fiskebäckskil on the Swedish west coast 1993–2004. (A) Shows the development over time and (B) the frequency distribution of recorded irgarol concentrations in seawater samples. Note that the level of detection has decreased over the study due to improved analytical techniques. The ten black-bar values exceed the lowest documented effect concentration (0.16 nM) for periphyton (Dahl and Blanck, 1996) and are therefore expected to cause damage to marine periphyton. The sampling sites are described in Table 1.

out to the outer archipelago (Fig. 2). In the following years a gradient with increasing community tolerance levels appeared, particularly at the two sites closest to the marina (Fig. 2).

The temporal community tolerance pattern also changed during the 10 year period. In 1994, community tolerance was rather stable over the season, while in later years, peaks were observed close to the marina mainly in mid- or late summer (Fig. 3). However, in retrospect a tiny tolerance peak could be discerned in late July also in 1994, both for the marina site 1 and site 5 which is a popular small-boat destination for day cruises during the Swedish holiday month of July.

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Table 2
Irgarol concentrations in water in temporal and spatial gradients round a small marina on the Swedish west coast 1993–2004

Date	Analyzed concentration of irgarol (nM)				
	Site				
	1	3	4	5	6
1993-06-23	1.2	–	–	–	–
1993-07-22	0.24	–	–	–	–
1993-08-23	0.39	–	–	–	–
1994-05-05	0.16	–	–	N.D.	–
1994-05-26	0.16	–	–	N.D.	–
1994-06-26	1.6	0.39	N.D.	N.D.	N.D.
1993-07-29	0.79	–	–	–	–
1994-08-29	0.20	–	–	N.D.	–
1994-09-29	0.12	–	–	N.D.	–
2000-05-05	–	–	–	0.001	–
2000-05-24	–	0.057	0.025	–	–
2000-06-07	–	0.045	0.015	–	N.D.
2000-06-13	0.28	0.043	0.021	–	–
2000-06-20	–	–	–	–	0.14
2000-07-18	1.4	–	0.29	0.027	0.008
2000-08-30	0.29	0.066	0.007	–	0.025
2000-09-26	0.17	0.017	–	0.002	N.D.
2001-08-03	0.75	0.39	–	0.031	–
2001-09-25	0.20	0.005	–	N.D.	–
2004-04-16	0.056	–	–	0.001	–
2004-04-28	0.099	0.042	0.042	0.002	N.D.
2004-06-08	0.39	0.051	0.013	0.004	0.002
2004-07-21	0.92	0.013	0.014	0.005	0.003
2004-08-23	0.090	0.015	0.029	0.015	0.005
2004-09-20	0.018	0.006	0.015	0.018	–
2004-09-22	–	–	–	–	N.D.

N.D. indicates that the irgarol concentration was below the limit of detection, –indicates that no sampling was made.

The distribution of community tolerance to irgarol (Fig. 4) indicate that major change has occurred in the maximum tolerance levels, which have increased more than 3-fold over the study period. A more subtle, but interesting observation is that also the minimum tolerance values have increased from 1.2 nM in 1994 to 3.4 nM in 2004 (Fig. 4B). The increase in minimum tolerance is evident for all 5 sites in the study, indicating a possible large-scale contamination of the waters in the Swedish west coastal area. There does not seem to be any extremely sensitive communities in the area any longer.

The major trend over the 10 year irgarol contamination period is the emergence of a considerable community tolerance gradient from the marina and outwards in the archipelago (Fig. 5). This spatial tolerance gradient was not present in 1994 when the baseline tolerance levels were all in the range from 1.2 to 13 nM. There has also been a clear increase in tolerance with time at the two most contaminated sites 1 and 3. Since the irgarol contamination pattern seems to have been stable since 1994 (Fig. 1, Table 2), it suggests that the biological changes underlying this between-year trend in tolerance development are slow or inefficient.

The highest irgarol concentration (1.6 nM) in our study was recorded 1994 at the marina site (Table 2). This contamination level corresponds to a season-average inhibition of photosynthesis of 18% (±17% SD). This is based on the short-term tests used to quantify community tolerance of periphyton from this site. However, at that time this inhibition did not result in any irgarol-induced community tolerance. The community tolerance levels were plotted against photosynthesis inhibition at 1.6 nM, to reveal any change in pattern over the 10 year period (Fig. 6). It is clear that from year 2000 and onwards, the periphyton communities have obtained a tolerance level that eliminates stress on their photosynthetic processes from the highest documented irgarol exposure in the region (Table 2).

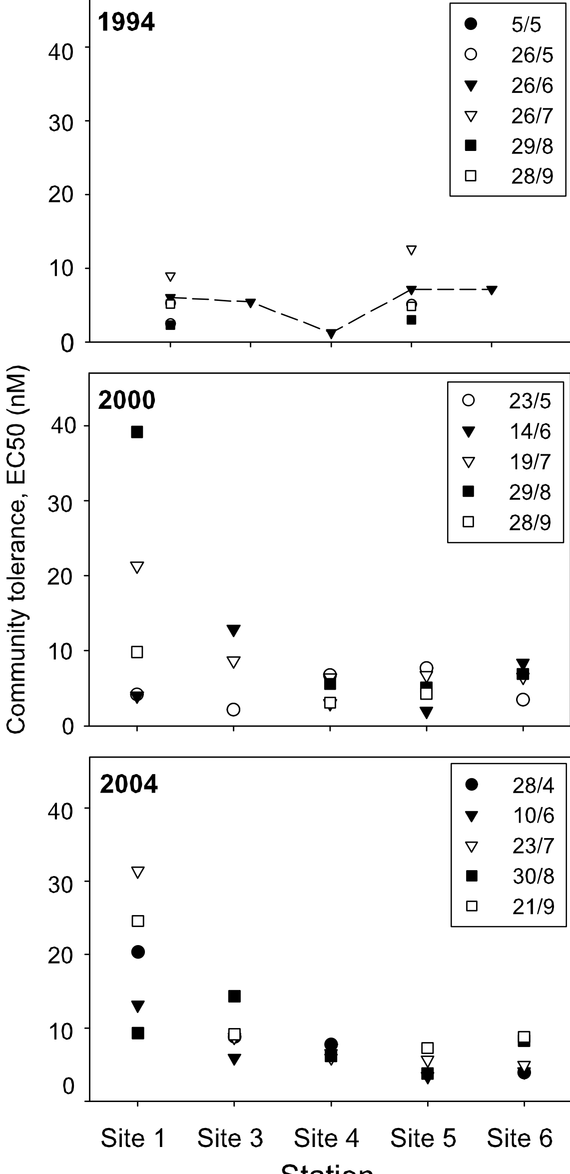

Fig. 2. Irgarol community tolerance in a spatial contamination gradient round a small marina on the Swedish west coast, sampled over the boating season in the years 1994, 2000 and 2004. The symbols represent different sampling periods over the year as indicated. The dashed line highlights the partly hidden data for spatial gradient in June 1994. The sampling sites are characterised in Table 1.

4. Discussion

4.1. Contamination pattern

The contamination pattern in the vicinity of the Fiskebäckskil marina in the Gullmar fjord area (Fig. 1, Table 2) seems to have been typical for many European marinas with maximum values of a few to some hundred ng/l (Konstantinou and Albanis, 2004). These authors argue that irgarol was the most frequently detected antifoulant worldwide, and indicated Southern East Coast of UK (Thomas et al., 2001), Côte d'Azur, France (Readman et al., 1993) and Southeast Spain (Hernando et al., 2001) as hot spots during the 90s with irgarol levels up to 1421, 1700 or 1000 ng/l, respectively. In our study area, the distribution of irgarol was rather similar from 1993 to 2004 (Fig. 1, Table 2). At the marina site there

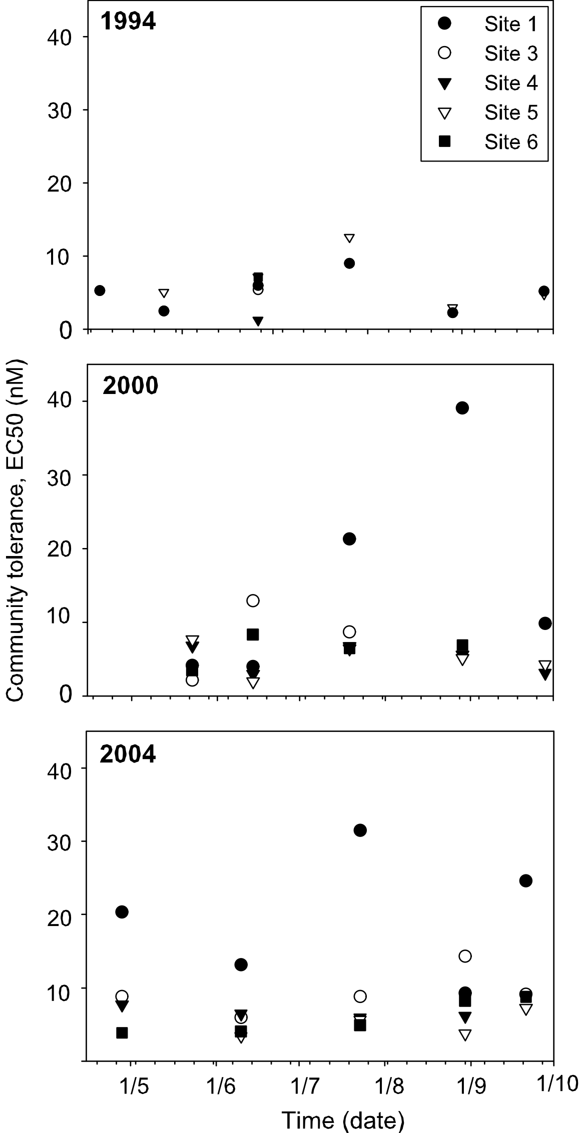

Fig. 3. Irgarol community tolerance in temporal contamination gradients over the boating season for five sampling sites round a small marina on the Swedish west coast in the years 1994, 2000 and 2004. The sampling sites are characterised in Table 1.

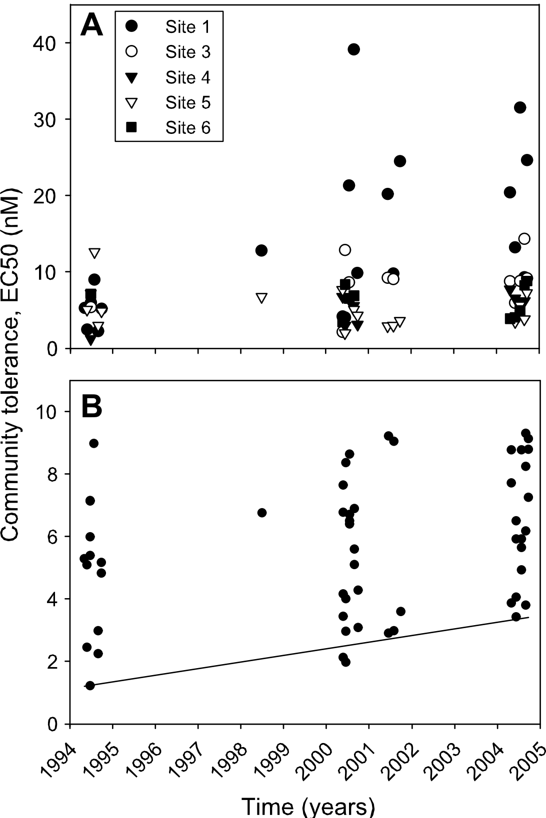

Fig. 4. Development of irgarol community tolerance around a small marina on the west coast of Sweden over the ten year period 1994–2004. (A) Shows an increasing trend for all data available while and (B) focuses on the increase in the minimum community tolerances to irgarol. Note the different *y*-axis scales in (A) and (B). The different symbols in (A) represent the five sampling sites as indicated.

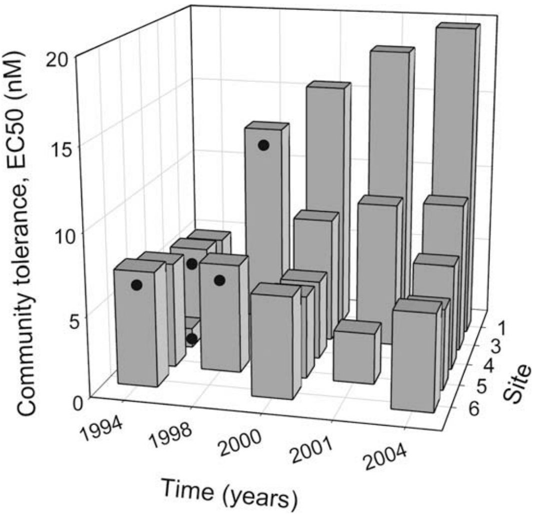

Fig. 5. Development of mean irgarol community tolerance levels in the spatial gradient over the study period 1994 to 2004. Unless indicated the tolerance levels are averaged over the seasons. Unreplicated observations are labelled with a black dot. The sampling sites are characterised in Table 1.

seemed to be some reduction over time, which may reflect changed use patterns of antifouling paint due to market changes. Although the Swedish legislation from the year 2000 did not apply directly in our study, since it banned the use of irgarol on pleasure craft only on the east, i.e., the Baltic coast of Sweden, it may still have had indirect effects on contamination patterns all along the coast. The ban of irgarol in UK seems to have decreased the irgarol contamination levels considerably (Cresswell et al., 2006).

In contrast to the contamination pattern of TBT (Blanck and Dahl, 1996), the peak in irgarol concentration did not appear in May when most boats are launched, but in late June or July when the boating activity is peaking (Table 2). We have no explanation for this except that it may be related to new paint technology with more even leaching rate over time.

During most of the 90s, irgarol was the main or sole booster biocide approved for use on pleasure craft on the Swedish west coast. Irgarol was also the major contaminant in the area with a mechanism of action as PSII inhibitor. This is of interest since it is known that some co-tolerance may occur between PSII inhibitors (Blanck, 2002). Monitoring of Swedish agricultural streams identified 12 herbicides with this mechanism of action (Adielsson et al., 2006). With one exception, these herbicides have not been detected in Swedish coastal waters. Diuron (10–100 ng/l, 0.04–0.4 nM) was detected at site 1 in 1993 and 1994 by (Dahl and Blanck, 1996a).

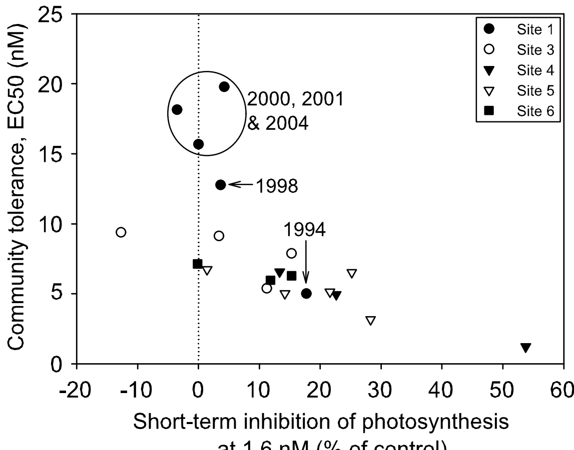

Fig. 6. Relation between inhibition of photosynthesis at 1.6 nM (the highest irgarol water concentration detected in this study; Table 2) and community tolerance (EC50) at the different field sites. The values are averaged over the whole season. The values for site 1 in 1994 and 1998 are indicated with arrows and the site 1 values in 2000, 2001 and 2004 are indicated with a circle. The dotted line indicates zero inhibition. The position of a coordinate in the plot is related to the shape/slope of the concentration-response curves between a 50% effect (the EC50) and the effects provoked by 1.6 nM of irgarol. This curve shape/slope is likely to change when community tolerance is induced.

Diuron was tentatively approved to be used as an antifouling ingredient in Sweden, but was replaced by irgarol from 1994. At that time, diuron was approved as an ingredient for marine paint in Denmark and visiting vessels or illegal use may explain the detection of diuron (up to 35 ng/l, 0.14 nM) in summer season of 2000 at site 1 close to the marina (unpublished).

4.2. Tolerance pattern

A PICT response will occur when the relation between sensitive and tolerant organisms is changed due to selection pressure that eliminates sensitive phenotypes or species. Even though the irgarol contamination remained similar or even decreased over the 10 year period (Fig. 1), a tolerance increase to irgarol took several years to develop (Fig. 5). In year 2000 the highest irgarol concentration recorded in the field was 1.4 nM (Table 2, station 1, July 18). The corresponding EC50 is >20 nM, which indeed is a PICT signal. Similarly, in 2004 the highest value is 0.9 nM (Table 2; station 1, July 21) corresponding to EC50 of >30 nM. Obviously, the conditions for a PICT response to emerge and be detected have changed between 1994 and 2000 (Figs. 4 and 5).

In 1994 irgarol seemed to provoke very little PICT response (Figs. 2 and 3) in spite of high irgarol concentrations in the vicinity of the marina (Table 2, Fig. 1). Although the concentrations exceeded the thresholds known to affect species composition and primary production in periphyton (0.25–1 nM, nominal; 0.16–0.79 nM, analysed, Dahl and Blanck, 1996a), there was no significant increase in community tolerance neither in the spatial nor in the temporal gradient (Figs. 2 and 3).

This is in contrast to our TBT studies where the community tolerance pattern was well in agreement with the contamination pattern in the seawater (Blanck and Dahl, 1996). There is one important difference between the PICT studies of TBT and irgarol. Even when the first TBT studies were made in 1987 and 1988 (Dahl and Blanck, 1990; Blanck and Dahl, 1996), TBT had been used in Sweden for many years and the TBT contamination of coastal waters was already an issue (Thain and Waldock, 1986; Bryan et al., 1987; Gibbs et al., 1987). In the irgarol case the PICT studies were made much sooner after coastal contamination started. Therefore we cannot exclude the possibility that the contrasting tolerance patterns observed for TBT and irgarol are caused by differences in contamination prehistory, and that the selection pressure from TBT had been exerted much longer.

When an increase in irgarol tolerance became detectable in year 2000 (Figs. 2 and 3) they also showed a new seasonal pattern: the tolerance developed slowly over the season and actually increased up till late August. A similar slow development was observed again in 2004 (Fig. 3), except for one occasion with early high tolerance in May 2004 at the most contaminated marina site. This is again in sharp contrast the PICT responses to TBT, where there was an immediate increase in TBT tolerance of periphyton when boats were launched in May (Blanck and Dahl, 1996). However in both cases the seasonal tolerance pattern seems to follow the seasonal pattern of contamination. In the TBT case, both the TBT concentrations and the TBT tolerance levels peaked in May (at the time of launching) and then decreased almost linearly with time. Thus there was a very good match between seawater concentrations and the tolerance levels, suggesting a good time resolution (< a month) in the adjustment of the community tolerance level to the selection pressure (Blanck, 2002). In the case of irgarol, the slower development of PICT over the season only roughly corresponds to a slower development of the peak in irgarol concentration that does not appear in May when boats are launched, but in late June or July when the boating activity is peaking (Table 2).

In a variable irgarol exposure situation, like the field study reported here, the temporal coupling between exposure and tolerance does not seem to be immediate. This may be due to recovery periods in between pulses of irgarol, since the exposure regime is current-driven and stochastic and since PSII inhibitors are known to enter and leave cells rather freely (e.g., Nikkila et al., 2001). The periphyton matrix might also offer some protection for short pulses of exposure, at least to the inner parts of the biofilm. The rough temporal coupling leads to difficulties in establishing a precise quantitative relation between irgarol exposure and the community tolerance that it induces, although there is a similarity in the general pattern of exposure and increased tolerance.

Since the PICT signal detected in the year of 2000 and onwards is not due to any major changes in irgarol exposure regime between the years it is very likely that there must be biological reasons for the slow development of community tolerance to irgarol. However, changes in exposure regime of other PS II inhibitors are also important since co-tolerance between PS II inhibitors might occur (Blanck, 2002 and references therein). Except for irgarol, the only detected PS II inhibitor in the studied area, during the studied time period, was diuron. Diuron was present at contamination levels (0.04–0.4 nM) 1000–100-fold lower than what was required to generate a PICT signal (4 nM) in periphyton in the microcosm study by Molander and Blanck (1992). It is therefore unlikely that these diuron levels would cause a significant diuron tolerance in periphyton at that time. Also, when expressing the selection pressure of irgarol as toxic units, i.e., dividing the exposure level with the toxicity, it was 160 times higher than that of diuron. This number is based on the maximum documented concentrations in the study area and on estimated values for their threshold effects on species composition and/or PICT induction. The estimated toxic units were 0.01 for diuron (0.4 nM/40 nM; exposure level from Dahl and Blanck (1996a), effect on species composition and PICT (Molander and Blanck, 1992)), and 1.6 for irgarol (1.6 nM/1 nM; exposure level from Table 2, effect on Bray–Curtis Index for species composition (Dahl and Blanck, 1996a)). Although we can not entirely rule out the possibility that diuron contamination might have contributed to the emerging PICT signal, it is very unlikely that this is a substantial contribution.

4.3. Biological responses to irgarol and PICT development

The absence of PICT in natural periphyton communities in 1994 is consistent with the results from the periphyton microcosm experiment made the same year and in the same area (Dahl and Blanck, 1996a). According to the observed toxicant-induced succession in that study (Fig. 6 in Dahl and Blanck, 1996a) several species remained actively growing in the community at low irgarol concentrations, but at 1.6 nM only three species were abundant, although all had started to decline at 2 nM of analysed irgarol. At higher irgarol exposure levels, algal biomass and primary productivity declined severely to very low levels but no irgarol-tolerant community was established. We therefore have to assume that the difference in competitive outcome under irgarol stress in 1994 were more dependent on other features than an irgarol-tolerant photosynthesis. These could be grazing tolerance, light adaptation, nutrient storage, since grazers, light conditions and nutrients are known to modify effects of PS II inhibitors (Guasch et al., 1998; Munoz et al., 2001). These aspects are not readily accounted for in a short-term assay of photosynthesis, which we used for quantifying PICT. Periphyton may also respond differently to short-term and long-term exposure regimes. For example, when control periphyton communities were exposed to 1.6 nM approximately 20% inhibition of photosynthesis was detected in the short-term tests, while there were much stronger effects on primary productivity after long-term exposure (Dahl and Blanck, 1996a). Blanck (2002) argued that the inter-species variability in tolerance to PSII inhibitors is small leading to a small PICT signal detectable only with very precise measurements (Nystrom et al., 2000). The fact that no irgarol-tolerant community was detected in the microcosm experiment in 1994 (Dahl and Blanck, 1996a) shows that the PICT potential to irgarol was low. It is very likely that the same is true for the natural periphyton communities from 1994 described here. Blanck (2002) also argued that a long and persistent irgarol exposure is required to increase the PICT potential to irgarol. This argument is verified here. Between 1994 and 2000 the PICT potential must have changed since PICT is readily detectable in 2000. In accordance with this, periphyton from the marina site showed no season-average inhibition at 1.6 nM in the short-term tests of photosynthesis inhibition in 2000, while periphyton from site 6 showed 15% season-average short-term inhibition of photosynthesis the same year (Fig. 6). This means that changes in abundance, efficiency or types of tolerance mechanisms must have occurred within the communities.

The tolerance mechanism can be directed towards affinity-reducing properties of the D1 protein to irgarol as shown for many weed species (see e.g., Erickson et al., 1989; Oettmeier, 1999; Devine and Shukla, 2000). It can also be directed towards any metabolic pathway that reduces the internal concentration of irgarol, or the fraction of inhibited D1 proteins. The observed tolerance pattern in this study (Fig. 3) led to the hypothesis that irgarol-tolerant traits are rare in clean environments due to strong evolutionary costs. Absence or low frequency of tolerant genotype(s) would explain why no PICT-conveying species was able to take over and dominate an irgarol-exposed community in 1994. We hypothesise that these genotypes are favoured and become enriched over consecutive years of persistent irgarol use until a readily detectable PICT signal emerges in 2000 (Fig. 3). We further hypothesise that the abundance of tolerant genotype/genotypes is linked to the long-term exposure concentration of irgarol over the boating season.

4.4. Concluding remarks

We have to conclude that no efficient tolerance mechanisms were present in the community when irgarol first was introduced. After some years of irgarol use, tolerant components seem to have emerged, probably as a response to a more consistent exposure to irgarol. Actually recent observations in our group (to be reported elsewhere) indicate that several periphyton species now are able to maintain a periphyton biomass also at irgarol concentrations around 10 nM which formerly were strongly affecting the community biomass in microcosm studies.

It is an important question whether these tolerant components are wild-type organisms or irgarol-tolerant mutants that have established themselves in spite of the fact that the tolerance mechanisms most likely are accompanied by a cost. The latter would mean that the use of irgarol has lead to such a powerful toxic selection pressure that otherwise inferior genotypes with low fitness in a clean environment now are favoured due to the irgarol contamination.

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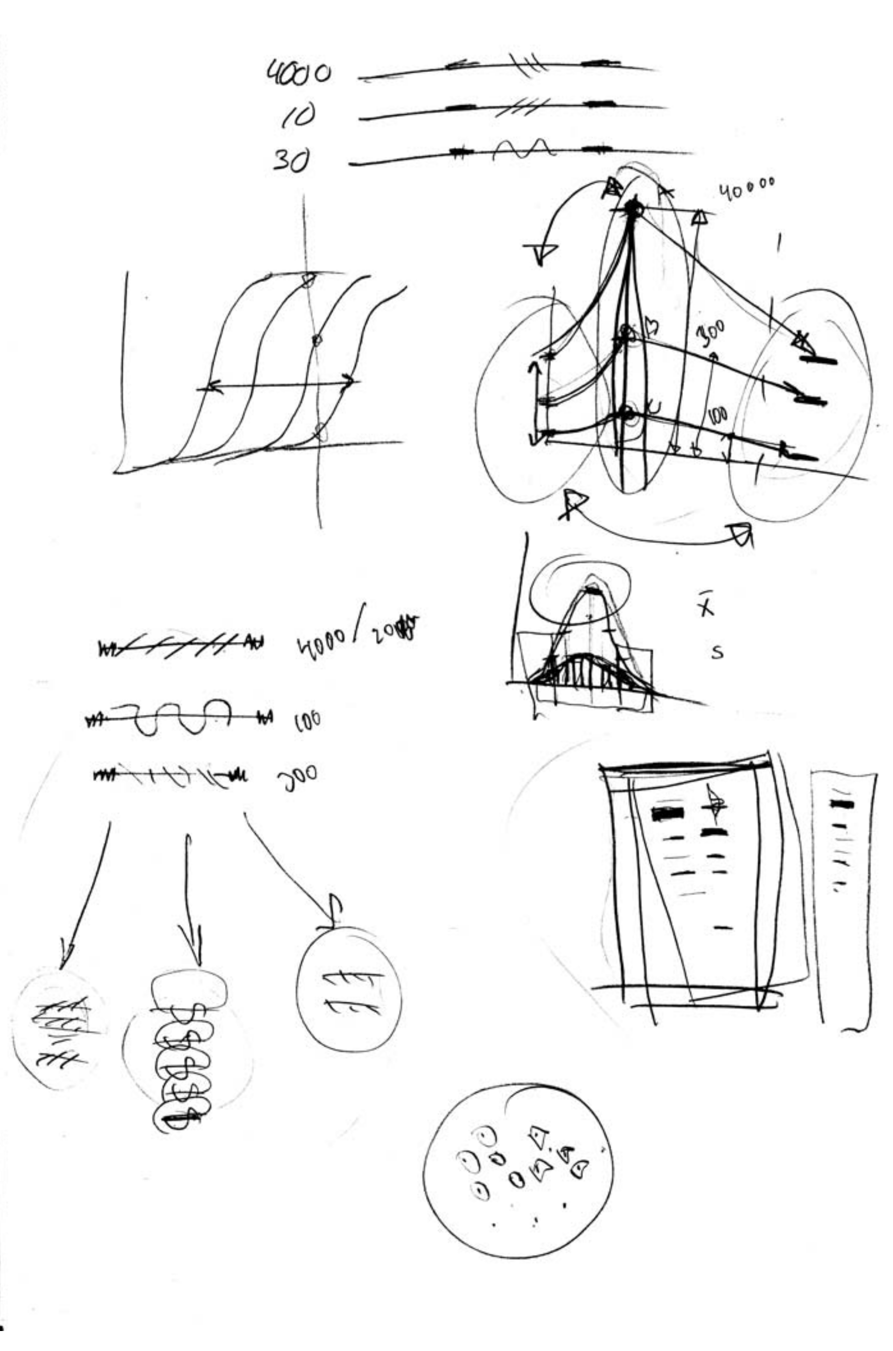
Paper III



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Community level analysis of *psbA* gene sequences and irgarol tolerance in marine periphyton

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ABSTRACT

This study analyses *psbA* gene sequences, predicted D1 protein sequences, species relative abundance and pollution-induced community tolerance (PICT) in marine periphyton communities exposed to the antifouling compound irgarol 1051. The mechanism of action of irgarol is inhibiting photosynthetic electron transport at photosystem II by binding to the D1 protein. The metagenome of the communities was used to produce clone libraries containing fragments of the *psbA* gene encoding the D1 protein. Community tolerance was quantified with a short-term test for inhibition of photosynthesis. The communities were established in a continuous flow of natural seawater through microcosms with or without added irgarol. The selection pressure from irgarol resulted in an altered species composition and an induced community tolerance to irgarol. Moreover, there was a very high diversity in *psbA* gene sequences in the periphyton, and the composition of *psbA* and D1 fragments within the communities was dramatically altered by increased irgarol exposure. Even though tolerance to this type of compounds in land plants often depends on a single amino acid substitution ($Ser_{264}\rightarrow Gly$) in the D1 protein, this was not the case for marine periphyton species. Instead the tolerance mechanism likely involves increased degradation of D1. When comparing sequences from low and high irgarol exposure, differences in non-conserved amino acids were only found in the so-called PEST region of D1, which is involved in regulating its degradation. Our results suggest that environmental contamination with irgarol has led to

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selection for high-turnover D1 proteins in marine periphyton communities at the Swedish west coast.

INTRODUCTION

Development of tolerance, or resistance, to anthropogenic toxicants released into the environment is an issue of increasing importance. Reports of tolerant organisms are increasing and tolerance towards a variety of compounds has been found (20, 44, 46, 101). This is essentially evolution in action and shows that toxicants can act as selective pressures in the environment. Even though high concentrations of toxicants can be released as episodes or pulses, the overall environmental concentrations of toxicants are often quite low, which implies that adaptation and non-lethal selection is more common than acute and/or lethal effects.

Since sensitivities to a given toxicant differ within, and even more so between species (12), a toxicant-induced succession (TIS) will occur in toxicant-exposed communities where sensitive species, individuals or genotypes are replaced by more tolerant ones, giving an increase in average tolerance in the community. This chain of events is fundamental for the Pollution-Induced Community Tolerance (PICT) concept (14). PICT studies can be made in natural or model ecosystems and have the important advantage of having a causal link between exposure and effect. It has been used to demonstrate effects of long-term selection of many toxicants in several types of communities, reviewed by Blanck (10) and Boivin et al. (15). Periphyton communities (111) are attached to submerged surfaces and consist of a foodweb of interacting organisms. Our focus in this paper is on the eukaryotic and prokaryotic oxygenic primary producers – microalgae and cyanobacteria.

Toxicants used as antifouling compounds are made to prevent growth of organisms on ship hulls and underwater installations. Irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine) is used to eliminate algae and cyanobacteria and since it acts as a triazine-type photosystem II (PS II) inhibitor it is less toxic to heterotrophic organisms (42). These inhibitors replace the native plastoquinone at the Q_B-binding niche in the D1 protein within PS II (29, 72, 86, 106). This results in various toxic effects like inhibition of photosynthetic electron transport, which in turn leads to reduced ATP and NADPH production (25, 29), and oxidative stress due to accumulation of reactive oxygen species (ROS) at PS II (29, 31, 85, 89, 91).

Several authors have previously reported increased community tolerance to irgarol in freshwater communities (8, 79) and to other PS II inhibitors in both freshwater (23, 40, 70, 92) and marine communities (7, 80, 96). It should be noted, however, that the magnitudes of the PICT responses were quite small in some of

these studies. Moreover, none of the above-mentioned studies tried to identify any tolerance mechanism active in the communities. Despite this, in terrestrial weed species tolerant to PS II inhibitors, there are two common tolerance mechanisms. One is increased detoxification of the compound by dealkylation or by hydroxylation followed by conjugation (3, 17, 38, 41, 67). The other is alteration of the target site, such as mutations in the *psbA* gene coding for D1. This gene is found in genomes of plastids, cyanobacteria, and cyanophages and is evolutionary very conserved (100, 104, 114). Mutations in *psbA* have emerged in plants growing in environments contaminated by PS II inhibitors as a consequence of their selection pressures (see review by Oettmeier (81) and Devine and Shukla (24)). The mutations result in amino acid substitutions in, or close to, the herbicide-binding region of D1 in either of two positions: Ser_{264} or Val_{219} (9, 51, 53, 55, 62, 68), which are thought to give tolerance through a reduced affinity of the herbicide to the Q_B-binding niche (45, 97). Whereas eukaryotes only have one or two copies of *psbA* per chloroplast genome (26, 27), cyanobacteria have from two up to five paralogous *psbA* genes (19, 73, 108). These encode two different forms of D1 proteins, which gives different functional characteristics to PS II and are means for light adaptation in cyanobacteria (16). Flexibility of interchanging between high- and low-light forms of D1 could be a selective trait during irgarol exposure.

In this work we have studied a highly conserved region of D1 proteins predicted from the metagenome of periphyton communities exposed to low and elevated irgarol concentrations. The region, from amino acid 197 to 291, contains most parts of helix four and five and the herbicide binding niche between these helixes. Important sites within this region are the before mentioned amino acids Ser_{264} or Val_{219}, as well as the initial cleavage site (39, 69) and the so-called PEST region (from Arg_{225} to Arg_{238}) (39). PEST regions are typically rich in Pro, Glu, Ser and Thr and are often found in proteins with high turnover rates (88, 90). Although not the actual cleavage site, they are thought to regulate the degradation of proteins (64). Apart from this metagenomic approach, this study is very similar to the study by Dahl and Blanck (21) performed in 1994 when irgarol was new on the Swedish antifouling market. At that time only a weak TIS and no clear PICT signal was observed for irgarol. Field studies of community tolerance to irgarol repeatedly made between 1994 and 2004 (11) indicated that PICT developed slowly over the years, probably through a successive enrichment of algae and cyanobacteria with more efficient tolerance mechanisms. It has been argued (Blanck 2002) that highly conserved target proteins like D1 would require a strong and persistent selection pressure to establish tolerance-conveying mutations in an algal community. Although many tolerance-conveying mutations have been described for algal and cyanobacterial

strains (reviewed by 81), it is not known how competitive they would be in a contaminated natural environment. Therefore, it is advantageous to use natural communities with many competing species, to single out those mechanisms that have the greatest fitness under environmental (-like) conditions in the field or in a microcosm system. The ecological relevance can then be evaluated. This information has so far been lacking for aquatic environments although tolerance-conveying mutations at Ser_{264} or Val_{219} have been reported to be viable in agricultural environments.

Here we show that these mutations (Ser_{264} or Val_{219}) were <u>not</u> present in irgarol-tolerant periphyton communities. Instead the tolerance mechanism seem to be coupled to the turnover of D1, since there were systematic differences in the amino acid-sequences of the PEST region, between periphyton exposed to background or elevated irgarol concentrations. To our knowledge, this study is the first attempt to find an ecotoxicogenomic mechanism behind a PICT response.

MATERIALS AND METHODS

Microcosm system. A flow-through microcosm system (13) was used to establish periphyton communities. The experiment was performed indoors at Sven Lovén Centre for Marine Sciences, Kristineberg on the west coast of Sweden during July and August in 2001. Seawater, with its indigenous microbiota, was continuously pumped with an air-driven Teflon-membrane pump (Dominator Maskin AB, Sweden) from three meters depth in the Gullmar fjord into the microcosm system. The system was composed of a water delivery system, similar to the one used by Granmo and Kollberg (37) and modified by Molander et al (71), a toxicant delivery system and four 22-L aquaria. A nylon net (1 mm mesh) was used to prevent larger organisms from entering the system. The seawater flow rate was approximately 220 ml/min through each aquarium and the mean residence time of the water was about 100 min. The irgarol water solutions were delivered from the start of the periphyton colonization at a flow rate of 2 ml/min using a peristaltic pump (Ismatech IPN 26, Ismatech AG, Switzerland). These water solutions were renewed every third day. Irgarol stock solutions were made in acetone at concentrations of 2.38, 1.19 and 0.59 mM. Irgarol water solutions were made by adding 10 ml stock solution to 10 L of de-ionised water. An equal amount of acetone and de-ionised water was added to the untreated microcosm. Flow rates of the seawater and irgarol water solutions was checked daily and adjusted when deviating more than 5% from the desired values. Each aquarium had two fluorescent tubes (Osram lumilux Daylight L 18W/12) as light source giving a photon flux density of ca. 120 µmol

photons m^{-2} s^{-1} at the water surface, and set to the light/dark regime in Sweden at this time of year. A stirring device in each aquarium ensured thorough mixing of the water.

Small glass discs (1.5 cm^2) were used to colonise the periphyton communities. These discs were mounted in polyethylene holders and placed along the sides of each aquarium. Before being submerged into the aquaria the discs were boiled for 10 min in concentrated nitric acid, rinsed in de-ionised water and rinsed again in 70% ethanol.

Measurements of photosynthetic activity. Photosynthetic activity was measured by the incorporation of ^{14}C-labelled sodium bicarbonate/carbon dioxide into acid-stable compounds. The solution of ^{14}C-labelled sodium bicarbonate was prepared by diluting a stock solution of 1 mCi ml^{-1} (DHI lab products, Hoersholm Denmark) in GF/F-filtered sea water, giving a concentration of 1.48 MBq ml^{-1} and a final activity of 0.074 MBq in each sample. The samples were incubated at the current *in situ* temperature in the aquaria. Fluorescent tubes (Osram Lumilux Daylight L18W/11), with a photon flux density of ca. 125 µmol photons m^{-2} s^{-1}, were used as light source. The pre-incubation time, *i.e*. before addition of 50 µl of the radioactive solution to each sample, was 30 min and the subsequent incubation time was 15 min. Carbon fixation activity was terminated by adding 50 µl of formaldehyde (37 %) to each sample. In order to estimate the amount of abiotic ^{14}C-carbon fixation, biotic activity was inhibited by adding 50 µl formaldehyde in three samples before the incubation. The samples were acidified with 1 ml acetic acid and dried at 60°C under a gentle stream of air. To each sample, 1 ml dimethyl sulfoxide and 8 ml of Ready gel ™ scintillation cocktail (Beckman Inc., USA) was added and the samples were thoroughly mixed. The radioactivity of the samples was measured using a liquid scintillation spectrometer (LS 5000 TD, Beckman Inc., USA). Disintegrations per minute (DPM) were calculated from counts per min (CPM) based on the correction factors for the sample quench characteristics and the machine efficiency.

Analysis of relative abundances of morphologically distinct taxa. The analyses of abundance of algal and cyanobacterial taxa were made according to Dahl and Blanck (21). Periphyton was sampled at the end of the experiment and stored in 70% ethanol at 4°C in darkness until analysed. Three glass discs per microcosm, and 50 randomly chosen fields (diameter of 252 µm) per disc, were analysed using a phase contrast microscope at 1000× magnification. The relative abundance of each taxa was estimated as the number of fields where the species was observed, thus giving a relative abundance scale from 0-50.

Chemical analysis of irgarol and the irgarol metabolite M1. Solid phase extraction (SPE) cartridges (Isolute ENV+) were conditioned with 6 ml of methanol (Lichrosolv) and 10 ml MilliQ-water. Approximately 500 ml water from each microcosm was filtered (Whatman GF/F) and passed through the cartridges at a flow rate of 10 ml/min. The cartridges were dried under vacuum for 10 min and stored at -20°C. The cartridges were eluted and analysed at CSIC, Barcelona, Spain according to Martinez et al. (61)

DNA extraction, *psbA* amplification, cloning and sequencing. The periphyton community was carefully scraped off the glass discs with a scalpel into filter-sterilised seawater and centrifuged at 6500 *g* for 10 min. Microscopy inspection of scraped discs showed a high efficiency in removing attached life forms. The pellets were snap-frozen in liquid nitrogen and stored at -80°C. DNA extraction was made according to the protocol of the Plant DNAzol® Reagent (Invitrogen, Carlsbad USA).

PCR was performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala Sweden), with 3 ng of template DNA and 4 and 4.6 µmoles of forward and reverse primers, respectively, in each 50 µl reaction. Degenerate primers were used to amplify a 285 base pair (bp) region of the *psbA* gene. The forward and reverse primer sequences were 5′GTITTYCARGCIGARCAYAAYATIYTIATGCAYCC3′ and 5′CCRTTIARRTTRAAIGCCATIGT 3′, respectively. PCR started with an initial 2 min period at 95°C, followed by 45 cycles of 95°C for 30 sec, 50°C for 45 sec and 72°C for 30 sec. There was then a final extension period of 5 min at 72 C°. Amplification of the correct-sized DNA fragment was verified by agarose gel electrophoresis, with the excised fragment then purified using the Wizard® PCR Preps DNA Purification System (Promega, Madison USA). The *psbA* fragments were ligated into the pGEM®-T Easy Vector System II (Promega, Madison USA) and transformed into competent *E. coli* cells (JM109). After overnight growth on agar plates, cells from randomly chosen bacterial colonies were collected with a toothpick and transferred to 150 µl 0.1 × TE buffer and lysed in a microwave oven for 1-2 min. The lysed *E. coli* cells were transported in sealed 96-well plates on dry ice to Genomic Ecology, Lunds University, Sweden for sequencing. In total 288 clones were investigated by DNA sequencing. The bacterial lysates (1 µl into a total reaction of 10 µl) were directly used as templates for PCR amplification of cloned fragments using standard PCR conditions and the universal primers M13 forward (5′-CCCAGTCACGACGTTGTAAAACG) and M13 reverse (5′-AGCGGATAACAATTTCACACAGG). After PCR, amplicons were purified by standard isopropanol precipitation and then redissolved in 10 µl of H_2O. PCR products (2 µl in a total reaction of 10 µl) were used for DNA sequencing in both directions using either M13 forward or M13 reverse primers and the BigDye kit (Applied Biosystem) followed by analyses on an ABI3100 instrument (Applied Biosystem) according to the manufacturer.

Sequence analysis. Many authors have shown that artefacts, (polymerase errors or chimera formation) and biases can be associated with PCR-produced clone libraries (1, 48, 57, 60, 87, 107, 109, 110). These reports have mainly concerned 16S rDNA libraries, probably because 16S rDNA is the predominant gene used in clone libraries, and tools have also been developed for detecting 16S rDNA chimeras (*e.g.* the Chimera Check program at the ribosomal database project II, the Mallard program, ChimeraBuster, Bellerophon or the Ccode program). However, for other genes, and especially for shorter fragments (< 300 bp), there are to our knowledge no such tools developed. Since

rRNA forms secondary structures, as part of its function in ribosomes, formation of chimeric sequences during PCR are likely to be more common when amplifying 16S rDNA compared to functional genes as *psbA*. Still, a strategy was developed in order to minimize the problem of PCR artefacts within our libraries. Sequencing errors were removed by excluding clones with non–complementary forward and reverse sequences from further analysis. In order to avoid artefacts from polymerase errors we excluded sequences that differed with only one nucleotide from any other sequence and that only occurred once within each library. Moreover, we manually identified possible recombination points in all sequences in each library, and checked whether any sequence could originate from other sequences within the library, *i.e.* be of chimeric origin. This strategy gave a reduction in the number of unique haplotypes from 95 to 72, but also resulted in a conservative library with a very low probability of containing chimeric sequences.

Sequences were aligned using Mafft v. 5.64 (49, 50) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The settings for Mafft were FFT-NS-i (Slow; iterative refinement method), a gap opening penalty of 1.53 and an offset value of 0.123. Similarity of sequences were analysed using the sequence identity matrix incorporated in the Bioedit software (43).

Data deposition. The *psbA* sequences are deposited at EMBL-EBI with accession numbers from AM933675 to AM933749. Additional sequences, which were excluded from further analysis and not deposited at EMBL-EBI due to their unproven non-chimeric origin, can be obtained from the corresponding author.

RESULTS

Analysed concentrations of irgarol. The seawater in the control microcosm contained environmental contamination of irgarol. Therefore, this microcosm cannot be viewed as a true zero-concentration control, but rather as one with the current background concentration of irgarol (0.02 nM). In the following we therefore use the terms “background” and “irgarol” to couple sequences or taxa to the exposure of periphyton either to background or experimentally elevated irgarol concentrations. The analysed concentrations in the microcosms dosed with irgarol were approximately half the experimentally intended, nominal, concentrations (Table 1). In all figures and tables the analysed concentrations are used. Low concentrations of the irgarol degradation product M1 was also found in irgarol microcosms (Table 1).

Table 1. Concentrations of irgarol and the irgarol degradation product M1 in the microcosms.

Sampling date [a]	Nominal irgarol concentration (nM) [b]	Analyzed irgarol concentration (nM)	Analyzed M1 concentration (nM) [c]
2001-07-31	-	0.02	0
	5	2.9	0
	10	5.5	0.008
	20	10	0.008
2001-08-16	-	0.02	0
	5	2.9	0.008
	10	5.6	0.008
	20	9.5	0.016

[a] Samples were taken at the two indicated occasions during the experiment.

[b] This is the experimentally intended concentration. The background concentration of irgarol in the incoming water was 0.02 nM.

[c] Degradation product of irgarol (2-methylthio-3-tert-butylamino-6- amino-s-triazine) also known as GS26575.

Pollution-induced community tolerance (PICT), toxicant-induced succession (TIS) and relative abundance of morphologically distinct taxa. We clearly detected both TIS and PICT to irgarol. The PICT signal is shown in Figure 1 as higher EC50 values at higher exposure levels. In the 10 nM irgarol treatment the detected EC50 value was 78 nM which is a very high value for photosynthesis in periphyton communities. This value is five times higher than the EC50 detected in the background exposure microcosm (16 nM). However, here it is also important to note that the community tolerance level in the background community is relatively high compared to sensitive periphyton communities (3-4 nM) sampled in a less contaminated nearby area the same year (Blanck et al. 2008). The PICT signal was accompanied by TIS in the form of clear shifts in distribution of morphologically distinct taxa (Fig. 1). In total we identified 32 algal and cyanobacterial taxa in the four microcosms (Table 2). In the background and the 10 nM irgarol community, 16 and 14 species were found, respectively (Fig. 2D). These numbers are, however, low compared to the number of haplotypes (*i.e.* the number of unique nucleotide sequences) found in the clone libraries.

Nucleotide sequences. Clone libraries were only built for the background community and the 10 nM irgarol community. After excluding some sequences in order to minimize PCR artefacts (see Materials and Methods) there was 68% efficiency in producing high quality sequences. The total number of high-quality *psbA* fragments was 89 for the background and 108 for the irgarol communities. Despite the *psbA* gene being overall highly conserved within phototrophic organisms, and that the region sequenced is one of the most conserved within

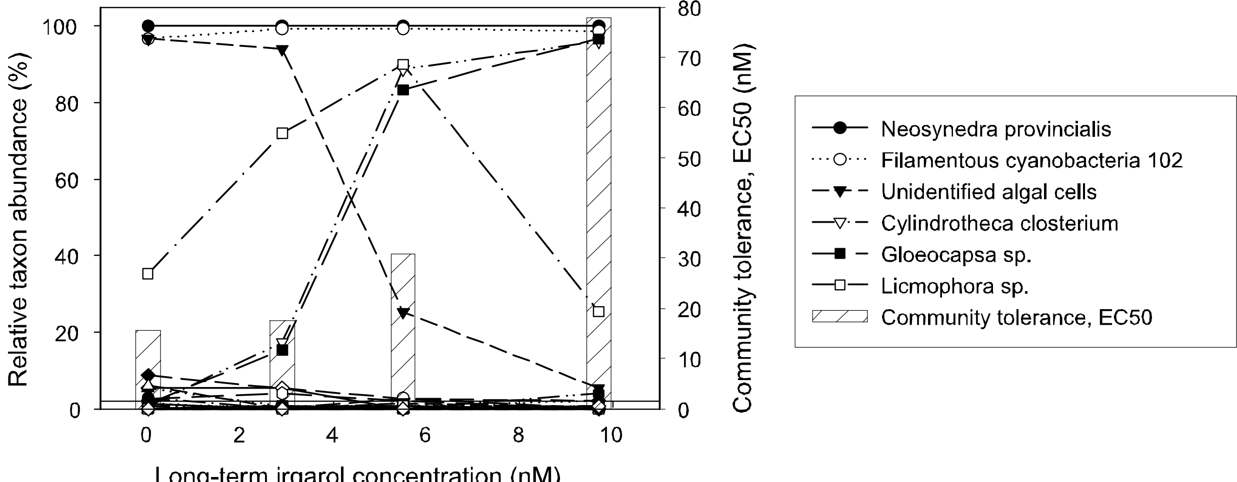

Figure 1. Response pattern of all identified morphologically distinct taxa and community tolerance in the communities. The response pattern of the different taxa is given as relative abundance, *i.e.* the fraction (%) of microscope fields in which a species was found, on the left Y-axis. The identity of the dominant species is shown in the legend. The identity of all species is given in table 2. Community tolerance, detected as EC50 values from the short-term tests, are shown as dashed bars and given in the right Y-axis.

the gene itself, we found as many as 72 unique *psbA* haplotypes in total. Surprisingly, only three haplotypes were found in both communities. Thus, the distributions of *psbA* haplotypes were drastically different, with 34 haplotypes found only in the background community and 35 haplotypes only in the irgarol community (Fig. 2A). Note that none of the previously described tolerance-conferring mutations, giving amino acid substitutions at Ser_{264} and Val_{219} (9, 51, 53, 55, 62, 68), were found in any of the *psbA* haplotypes.

Predicted protein sequences. In spite of degeneracy in the genetic code, a high level of diversity was maintained at the protein level with 40 different predicted D1 protein fragments in total. The clear differences between the background and irgarol communities remained after translation of the nucleotide sequences. There were four protein sequences present in both communities, whereas 19 were found only in the background community and 17 only in the irgarol community (Fig. 2B). As mentioned above, no protein sequences contained the amino-acid substitutions (*e.g.* Ser_{264} or Val_{219}) previously shown to confer tolerance to PS II inhibitors. However, we also searched for other differences typical for irgarol or background protein sequences. Particularly non-conserved amino-acid changes were examined since these are more likely to

affect the function of a protein than conserved amino acid substitutions. We found only one region, located between amino acid 228 and 238, where background and irgarol sequences had non-conserved amino-acid differences; this region corresponded to the PEST region of D1 (39). In both communities we found 14 different PEST region sequence types. Five of those types were found in both communities, whereas six and three were exclusively found in the background and the irgarol community, respectively (Fig. 2C). A greater diversity in PEST regions occurred in the background relative to the irgarol community in which only three types dominated (Fig. 2C). This indicates a strong irgarol selection pressure in this region of D1 and that its amino acid composition is causative for the increased community tolerance to irgarol. The amino acid sequences of the PEST sequence types are given in figure 3 (28).

DISCUSSION

There are several significant findings from this study that deserve further attention: (1) None of the mutations known to convey tolerance to PSII inhibitors in higher plants, algae and cyanobacteria, were present in the communities; (2) Despite this, irgarol caused a clear difference in composition of *psbA* haplotypes and D1 sequence types, which imply that irgarol exerted a strong selection pressure on this gene; (3) A new putative tolerance mechanism was discovered, involving the amino acid sequence in the PEST region of D1 that is likely to affect the rate of D1 degradation, and; (4) Although *psbA* has been reported to be relatively conserved, the variability and diversity of this gene within periphyton was surprisingly high.

The absence of those mutations regularly found in terrestrial environments contaminated by PS II inhibitors (*e.g.* giving the amino acid substitution $Ser_{264} \rightarrow Gly$) in the irgarol community implies that these mutations are not functional in marine periphyton species, in spite of a strong irgarol selection pressure. The reason for this remains to be clarified. A similar result within a marine environment was also found by Galgani *et al.* (33) when sequencing *psb*A from the red macroalga *Porphyra linearis* in environments with different atrazine concentrations. Despite this, the amino acid sequence of D1 still appears very important during irgarol selection, since such a clear difference was found in the composition of *psbA* haplotypes and D1 protein types between the background and the irgarol communities (Fig. 2A and 2B). However, we cannot exclude at this stage the possibility that the difference in distributions of *psbA* haplotypes and D1 protein types is a result of a changed species distribution, which in turn could be caused by other tolerance conferring traits within the community. This would imply selection on other gene/genes, or possibly on other regions of D1 than the one analysed in this study. For cyanobacteria there might also be a

selection for efficient use of their paralogous *psbA* genes. Despite such possibilities, however, the distributions of PEST sequences between the different communities (see below) does suggest that this D1 domain is an important factor in conferring irgarol tolerance.

The strong discrimination by irgarol between amino acid sequences in the PEST region (compare upper and lower panels in Fig. 2C) indicates that D1 degradation is particularly important in periphyton species during irgarol exposure. Only three PEST sequence types seem to be functional under selection of irgarol, since they make up the majority of all PEST sequences (Fig. 2C lower panel). Thus, the PEST region of D1 is a putative irgarol tolerance regulating region in marine periphyton. It should be stressed that this represents a novel discovery of a tolerance mechanism functional under environmental(-like) conditions, *i.e.* in a multi-species system with continuous immigration from the natural environment, ecological interactions like competition and predation present, and naturally fluctuating nutrient and light history conditions. This is in contrast to many studies where tolerance to PS II inhibitors has been studied in artificial systems using site-directed mutants of standard laboratory species. It is also important to point out that most of the irgarol selected PEST sequence types were already present in the background community. This is consistent with the observations that there was irgarol contamination (Table 1) and irgarol selection (detected as a comparatively high EC50 value) in the experimentally untreated microcosm (Fig. 1).

D1 is naturally a high-turnover protein (32, 65), and its turnover is involved in regulating electron flow and activating/inactivating PS II as a mechanism of light adaptation, *e.g.* during photoinhibition (18, 83, 93, 94). Importantly, PS II inhibitors have been shown to block D1 turnover (32, 47, 54, 56, 63, 76). This occurs when the inhibitor binds to D1 and either blocks its proteolytic site or induces a conformational change which reduces the accessibility of this site (25, 65, 103). This means that there are at least three toxic effects of PS II inhibitors: (i) inhibition of photosynthetic electron transport, leading to reduced ATP and NADPH production; (ii) increased oxidative stress due to accumulation of ROS at PS II, and importantly; (iii) blockage of D1 turnover. Since D1 turnover is vital for continued photosynthesis, it is evident that its impairment will be an ecologically relevant effect of PS II inhibitors. In fact, the amino acid substitutions Ser_{264} or Val_{219}, thought to confer tolerance through a reduced affinity of the herbicide to the Q_B-binding niche, also alter the turnover rate of D1. Both increases (82, 99) and decreases (22, 95) in turnover have been described and in the former cases the tolerance could also originate from a less affected D1 turnover rate during exposure to PS II inhibitors.

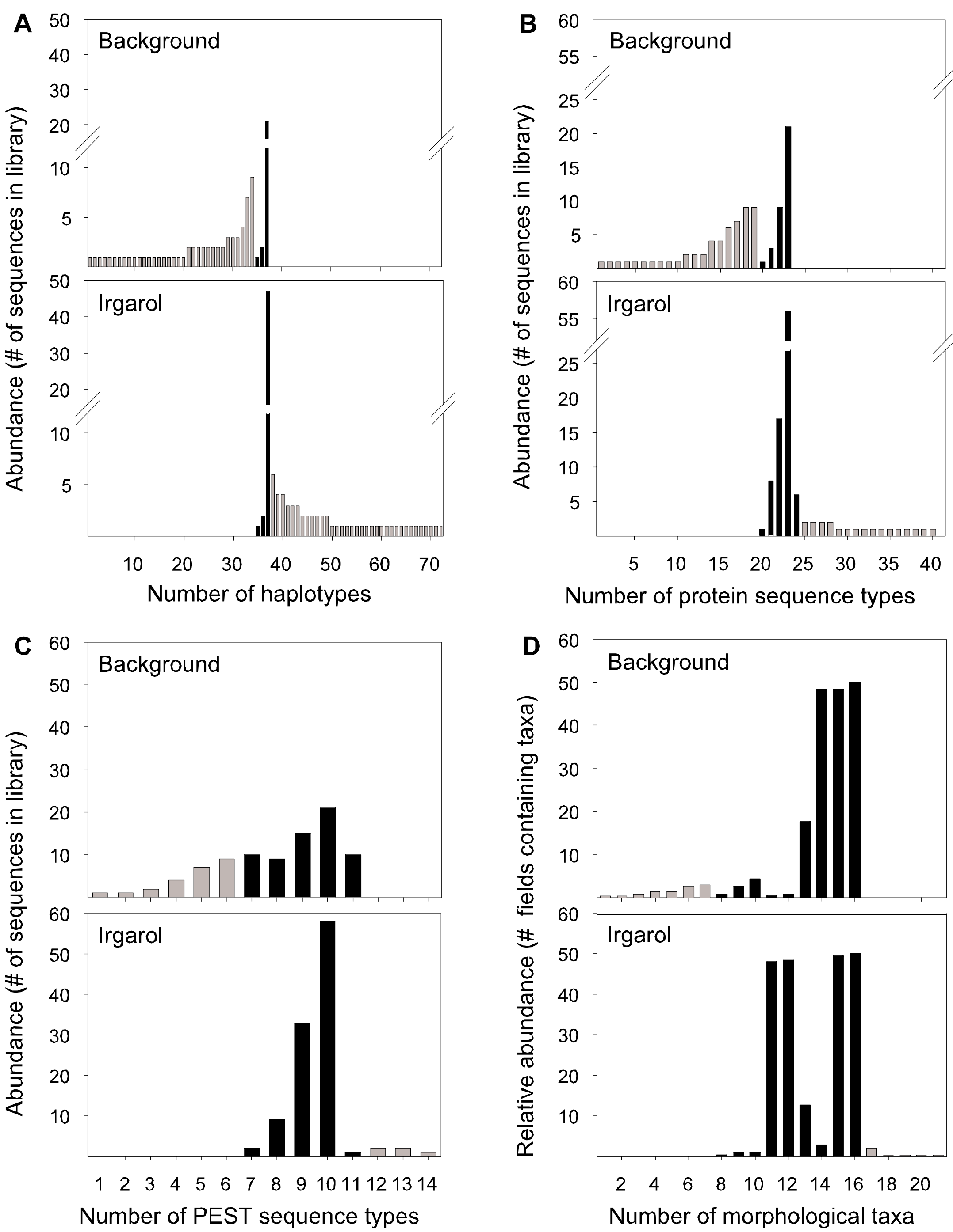

Figure 2. A1 and A2 represent distributions of different psbA haplotypes in background and irgarol communities respectively. B1 and B2 represent distributions of different protein sequences, i.e. all the unique amino acid sequences produced when translating the psbA haplotypes, in background and irgarol communities respectively. Analogously, C1 and C2 represent the distributions of different PEST sequences in background and irgarol communities respectively. In the same way, D1 and D2 represent the distributions of all morphologically

distinct taxa detected in background and irgarol communities respectively. In all graphs, the x-axis shows the number of unique types of the different variables (A; haplotypes, B; protein sequences, C; PEST sequences and D; morphologically distinct taxa) and y-axis shows the abundance of each unique type for the different variables. Black bars indicate types or taxa common to both the background and the control community whereas shaded bars indicate types or taxa present in either one of the background or the control community. The level of exposure, 0.02 nM in control and 9.75 nM in exposed microcosms, is indicated in each graph.

There are three conditions that must be met for the PEST region to be regulative for irgarol tolerance. Firstly, the amino acid sequence of the PEST region must be able to modulate the degradation rate of D1. Support for this comes from studies showing point mutations in the PEST region results in both higher (75) and lower (105) D1 degradation rates. Since several studies have shown that deletion mutants with no PEST domain retain the ability to degrade D1 (6, 74, 75, 78) the complete PEST signal does not seem to be necessary for D1 degradation. Although the PEST signal was removed in these mutants the regulatory role of this part of D1 persisted since the mutants gave either higher (74, 75, 78) or lower (6) turnover rates. The mechanism by which the PEST region regulates D1 degradation could be via interaction with other PS II components. It has been proposed that when amino acids in the PEST region of D1 are oxidised (*e.g.* by ROS) intermolecular cross-links to the D2 protein are formed, and this conformational change can act as a signal for D1 protein degradation (69). One should also keep in mind that the rate of D1 degradation is dependent on light intensity, and that a mutation giving a higher turnover rate at low light (40 µmol photons m^{-2} s^{-1}) can produce a lower turnover rate in high light (1500 µmol photons m^{-2} s^{-1}) (71). This finding is important in an ecological context since the environmental light regime is often well in excess of 40 µmol photons m^{-2} s^{-1} and is fluctuating constantly.

The second condition is that D1 degradation must be rate limiting for its overall turnover. This is again a premise supported by many studies that have shown that the turnover of D1 is indeed limited by its degradation (63, 66, 77, 98). The final condition is that increased or optimised turnover of D1 should result in increased tolerance. Pace *et al*. (84) provide experimental support to the hypothesis of increased D1 turnover as a tolerance mechanism to triazine PS II inhibitors (atrazine) in a moderately tolerant species (pea). Furthermore, Trebst (102, 103) and Draber (25) have argued that the inhibition of D1 turnover by PS II-inhibiting herbicides, and thereby the interference of key regulatory processes in photosynthesis, might be much more devastating than mere inhibition of electron transport. Consequently, any mechanism maintaining the rate of D1 turnover rate closer to that in non-exposed situation would give tolerance. Also

in a study by Kless *et al.* (52) moderate tolerance was detected in PEST deletion mutants of *Synechocystis* sp. PCC 6803. Even though this study does not directly couple the tolerance to increased turnover of D1, it shows that alterations in the PEST region affect tolerance to PS II inhibitors. Interestingly, these moderately tolerant mutants showed no reduction in photosynthesis (as measured as O_2 evolution) or in growth rate. These authors also point out that the PEST region may be the rate limiting factor of D1 degradation under conditions of stress.

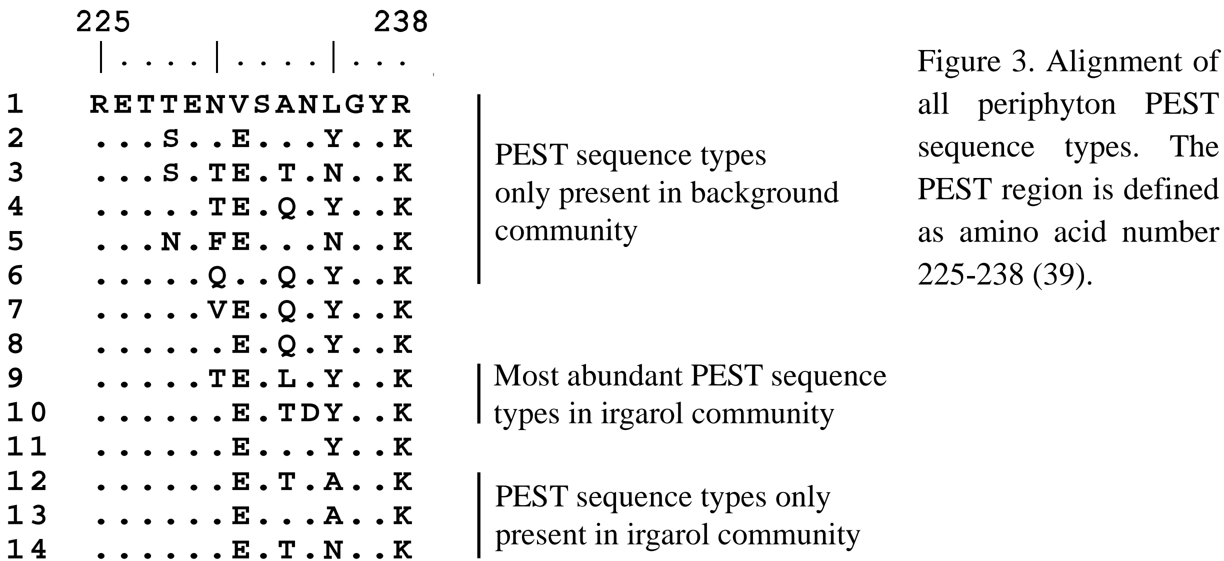

Figure 3. Alignment of all periphyton PEST sequence types. The PEST region is defined as amino acid number 225-238 (39).

The toxic effects of acute irgarol exposure (*e.g.* impaired electron transport, increased production of ROS and blockage of D1 turnover) are interrelated, producing complex cause-effect-relationships. Proteins damaged by increased ROS production, or by UV/light radiation, need to be replaced. However, replacement of D1 has to be preceded by its degradation (2, 4, 5, 113) and since irgarol blocks the degradation of D1 this repair mechanism is prevented, resulting in entire PS II complexes becoming non-functional. This situation is similar although more severe to that of photoinhibition, in which the rate of photodamage to the D1 protein by high irradiation exceeds the rate of repair. Interestingly, it has been shown that in *Synechocystis* mutants with amino acid substitutions in the PEST region, photoinhibition that is reversible in the wild type is irreversible, dependent on *de novo* D1 synthesis (59). Also, Mulo et al. (74) showed that the reversible and irreversible components of photoinhibition in the same cyanobacterium were affected differently by deletions in the PEST region. Collectively, this also implies that the amino acid sequence in the PEST region is one factor influencing the light regulation of photosynthesis, its susceptibility to

high excitation pressures as occurs during photoinhibition, and its tolerance to PS II inhibitors.

Some studies have also described increased D1 turnover as a physiological stress-compensating mechanism. Increased D1 turnover occurs in response to fumigation with ozone (36) or with mixtures of air pollutants (O_3, NO_2 and SO_2) (58), as well as during potassium deficiency (58), cadmium exposure (30, 34) and drought (35). It has been proposed that D1 turnover acts as a general adaptive response to environmental extremes (35), which gives further support to our hypothesis of increased D1 turnover as an irgarol tolerance mechanism.

When the occurrence of PEST sequence types in background and irgarol communities were analysed (Fig. 2C), all non-PEST amino acids were excluded from the sequences. This analysis does not reveal whether PEST sequence types, selected for by irgarol, occur also in background D1 proteins. We therefore analysed the distribution of the two most abundant irgarol-selected PEST sequences (type 9 and 10; Fig. 2C) in the D1 protein sequence types. PEST type 9 and 10 were found in 14 out of the 40 D1 protein types. Most interesting, the occurrence pattern of the two PEST types are different (Fig. 4). PEST type 10 was present only in one type of D1 protein in the background community, namely protein type 1 (Fig. 4, upper panel). In the irgarol community, not only was protein type 1 more abundant, but the PEST type number 10 was also present in two additional D1 protein sequence types exclusively found in the irgarol community, namely protein sequence type 2 and 3 (Fig. 4, lower panel). Thus, PEST type 10 is irgarol-favoured both in terms of its abundance in the community and its occurrence in different types of irgarol-selected D1 proteins. PEST type 9 was also more abundant in the irgarol than in the background community (compare upper and lower panel in Fig. 2C) but, in contrast, this PEST type occurred in some protein types which were exclusively found in the background community. This is shown as the presence of protein type 11-14 in the upper panel of Figure 4. Consequently, PEST sequence type 9 is less likely than type 10 to contribute to tolerance in the irgarol community.

In spite of the evolutionary conservation of *psbA*, and the fact that only a small part of the gene was sequenced, we found as many as 72 unique haplotypes within periphyton from both microcosms. This number of periphyton haplotypes can be compared to the number found in marine picophytoplankton communities, which were 3, 12 and 28 from the Mediterranean Sea, the Red Sea and the Pacific ocean around Hawaii, respectively (112). Even though there was biased sampling in this study, excluding cells >3 µm, it seems like the phototrophic part of periphyton communities are very diverse. The number of periphyton haplotypes can also be compared to the 21 morphologically distinct species found in these microcosms (Fig. 2D, Table 2). The corresponding value at

the protein level is 40, which still is almost twice the number of morphologically distinct species. This is not so surprising since identification of a species by microscopy does not necessarily reflect the genetic identity of that species. Moreover, microscopic visual inspection cannot reliably detect very small photosynthetic species, even at the highest magnifications used. Since the identities of the morphologically distinct species are uncoupled from the identities of the *psbA* haplotypes a phylogenetic analysis of the haplotypes has been performed and will soon be submitted for publication elsewhere.

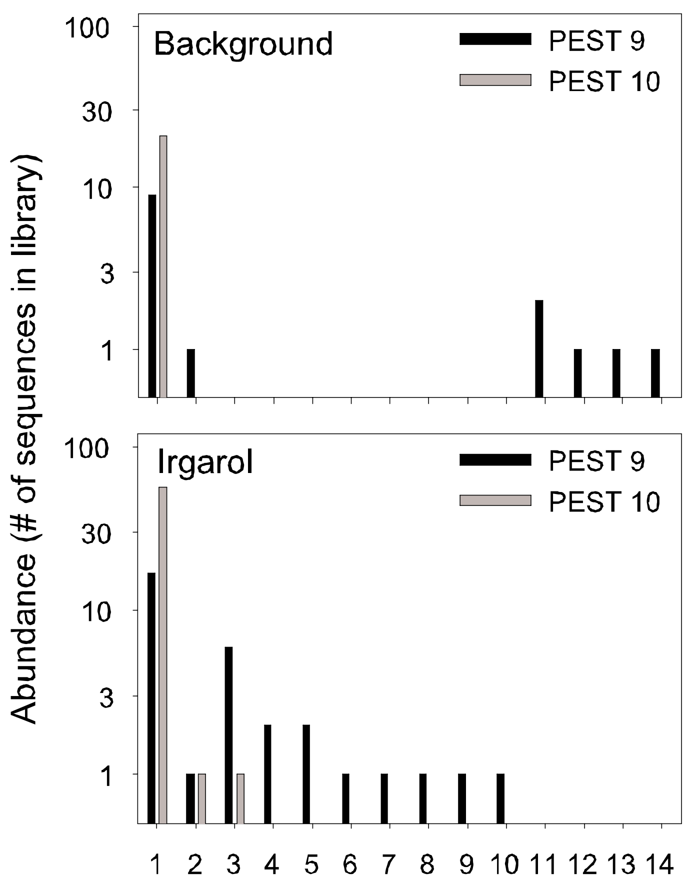

Figure 4. Distribution of D1 protein sequence types containing PEST region 9 (black bars) and 10 (grey bars) in background (upper panel) and irgarol (lower panel) communities. Note the logarithmic scale of the y-axis of both panels. Note also the PEST region 9 containing protein sequence types 11-14 in the background community (black bars to the right in the upper panel).

The different outcome in terms of PICT induction from this study and that of Dahl and Blanck (21) is probably due to the environmental contamination of irgarol between the years 1994 and 2001. This is further supported by the study of Blanck et al. (11) which shows that PICT to irgarol has slowly developed in environmental periphyton communities in this area between 1994 and 2004. The spatial and temporal PICT pattern reported in that study also shows that community tolerance in waters nearby the microcosm inlet is relatively high

during mid-summer when this study was performed. This is again consistent with the elevated EC50 value in the background exposure microcosm and means that the indigenous community in the fjord, continuously sampled in this study, already contained irgarol-tolerant components. The experimental exposure then further selected for increased tolerance. This is also consistent with the fact that the PEST sequences selected for in the irgarol treatment was already present in the background exposure microcosm. Thus, there is a three-step selection process behind the observed PICT response, namely a long-term selection over the years, a selection over the season and the experimental selection in the microcosm. The first two steps are documented in the studies by Dahl and Blanck (21) and Blanck et al. (11), while the higher community tolerance observed here also involves an experimental selection step and thus is dependent on all three steps.

In conclusion, this study shows that PICT to irgarol can now be clearly induced in periphyton communities. The mechanism of tolerance is not the previously identified mutations in the *psbA* gene giving amino acid substitutions at Ser_{264} or Val_{219}, which are known to convey tolerance to PSII inhibitors. Instead it seems to be related to the degradation of D1 as a result of non-neutral amino acid differences in the PEST region of D1 being more prevalent in the microcosm with elevated irgarol exposure. This is consistent with findings in biochemical studies of photosynthesis over the last two decades and we can now add that it is only modifications in the PEST region that are functional under environmental(-like) conditions in the irgarol-contaminated marine periphyton habitat on the Swedish west coast.

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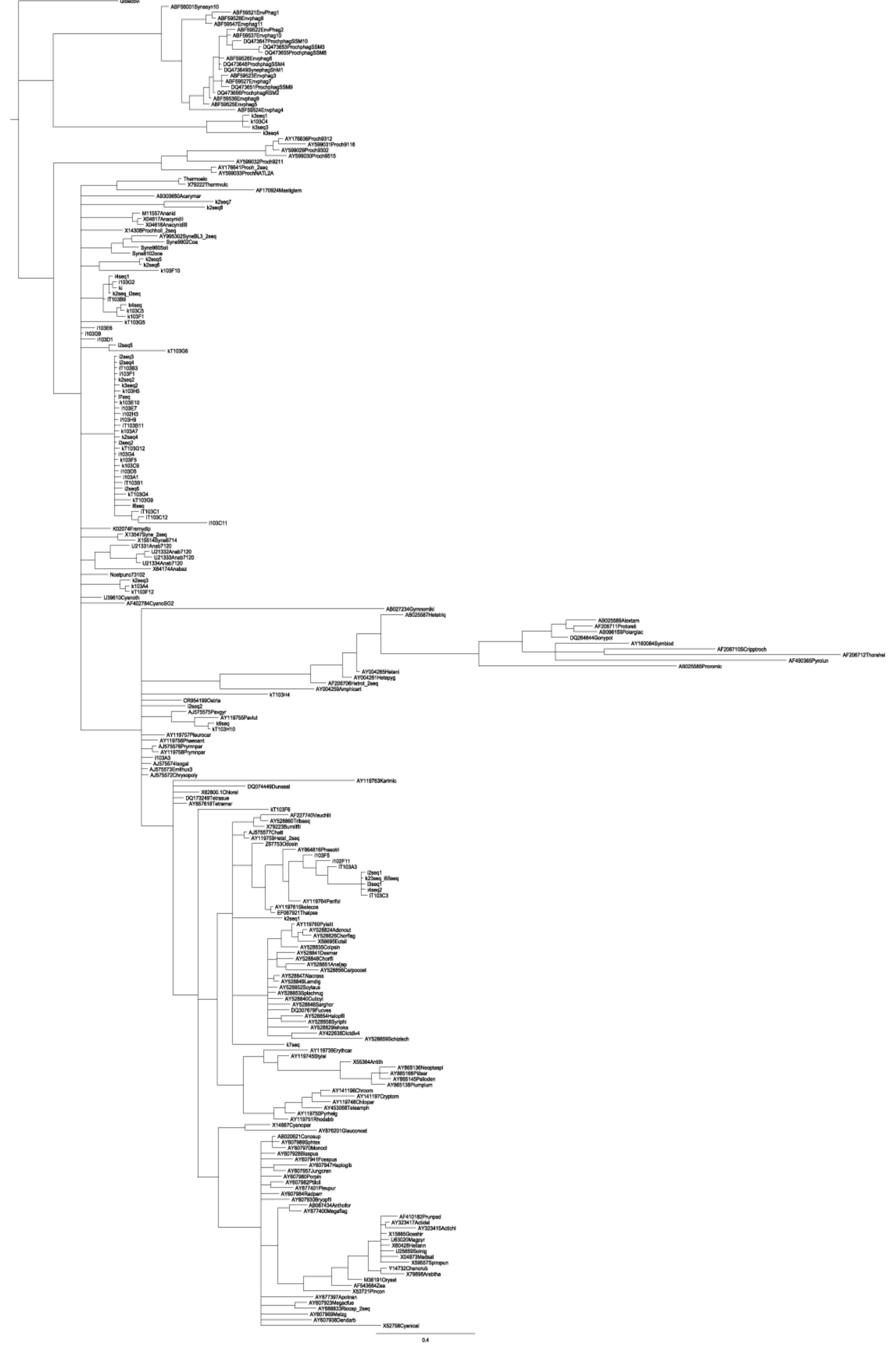
Paper IV



A phylogenetic approach to detect selection on the target site of the antifouling compound irgarol in tolerant periphyton communities

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Summary

Using DNA sequence data for phylogenetic assessment of toxicant targets is a new and promising approach to study toxicant-induced selection in communities. Irgarol 1051 is a photosystem (PS) II inhibitor used in antifouling paint. It inhibits photosynthesis through binding to the D1 protein in PS II, which is encoded by the *psbA* gene found in genomes of chloroplasts, cyanobacteria and cyanophages. *psbA* mutations that alter the target protein can confer tolerance to PS II inhibitors. We have previously shown that irgarol induces community tolerance in natural marine periphyton communities and suggested a novel tolerance mechanism, involving the amino acid sequence of a turnover-regulating domain of D1, as contributive to this tolerance. Here we use a large number of *psbA* sequences of known identity to assess the taxonomic affinities of *psbA* sequences from these differentially tolerant communities, by performing phylogenetic analysis. We show that periphyton communities have high *psbA* diversity and that this diversity is adversely affected by irgarol. Moreover, we demonstrate that within tolerant periphyton the suggested tolerance mechanism is used by diatoms only, whereas some groups of irgarol-tolerant cyanobacteria seem to use other tolerance mechanisms. However, identifying periphyton *psbA* haplotypes to species or genus level proved difficult, which indicates that the periphyton genomic pool is poorly studied and represented in international sequence databases.

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Introduction

Dramatic advances have been made in exploring microbial diversity using recently developed molecular techniques. DNA sequencing of clone libraries of specific genes, and later of the metagenome of natural communities has

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revealed a hitherto unknown microbial diversity of massive proportions. *Metagenomics* is a descriptive term for studies that in these ways address DNA sequences of all individuals in a community as a common genomic pool (Kowalchuk *et al.*, 2007). The metagenomic approaches have so far had limited use in the study of periphyton communities. These communities consist of organisms growing on surfaces of submerged objects in aquatic environments (Wetzel, 1975). They are very diverse and are composed of different groups of organisms, *e.g.* bacteria, cyanobacteria, eukaryotic microalgae and microfauna. Although poorly described in a metagenomic context, periphyton communities have been extensively used in water quality assessments and in ecotoxicological research (*e.g.* see Niederlehner and Cairns, 1990; Patrick *et al.*, 2007; Sabater *et al.*, 2007). Since the sensitivity to a toxicant differs between species (Blanck *et al.*, 1984; Vaal *et al.*, 1997), exposure will result in a Toxicant-Induced Succession (TIS) where sensitive genotypes, strains or species are replaced by more tolerant ones. As a consequence, the tolerance of the whole community to the specific toxicant will increase, according to the concept of Pollution-Induced Community Tolerance (PICT) (Blanck *et al.*, 1988). Thus, a pollution-tolerant community will have a different genotype, strain and/or species composition compared to a sensitive community. PICT in combination with community structure change is an integrating endpoint for estimating ecologically relevant effects of toxicants (Blanck, 2002; Boivin *et al.*, 2002).

Only few metagenomic studies have dealt with effects of toxicants on periphyton communities. Dorigo *et al.* (2002) compared rDNA composition in eukaryotic organisms from river periphyton with different exposure concentrations of atrazine and isoproturon, and Brummer *et al.* (2003) studied rDNA composition in the β-proteobacterial part of river periphyton with different exposure to various organic and inorganic pollutants. The present study experimentally estimates the effects of a single toxicant and is therefore not confounded by other environmental variables. Moreover, it estimates effects on the composition of the entire oxygenic and phototrophic part of the communities including both pro- and eukaryotes. We studied the impact of the antifouling compound irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine) on the phylogenetic composition of the *psbA* gene in marine periphyton communities. The *psbA* gene encodes the D1 protein which is one of the components of photosystem (PS) II. Irgarol is used in antifouling paint to prevent settling of aquatic organisms on underwater surfaces (*e.g.* boat hulls and oil rigs). Since the first reports of irgarol contamination in 1992 (Readman *et al.*, 1993) it has been found in numerous aquatic environments around the globe (reviewed by Konstantinou and Albanis, 2004 and later detected by Hall *et al.*, 2005; Harino *et al.*, 2005; Carbery *et al.*, 2006; Gatidou *et al.*, 2007; Blanck *et al.*, 2008). This is consistent with observations that irgarol is quite persistent in the environment (Callow and Willingham, 1996; Hall *et al.*, 1999) and suggests that wherever irgarol is used

there is a considerable potential for contamination. Irgarol is a PS II inhibitor that binds to the D1 protein and thereby blocks photosynthetic electron transport in cyanobacteria, algae and higher plants (Moreland, 1980; Pfister *et al.*, 1981; Fedtke, 1982; Vermaas *et al.*, 1983). Therefore, the amino acid sequence of D1, and thus the DNA sequence of the *psbA* gene, is of great importance for irgarol binding and thus irgarol tolerance. Moreover, since *psbA* is a functional gene that encodes the target protein for the studied toxicant, there is a direct link between the mechanism of action of the toxicant and the community composition of the *psbA* gene pool.

psbA is found in genomes of cyanobacteria, cyanophages and chloroplasts, and it is evolutionary very conserved (Zurawski *et al.*, 1982; Svensson *et al.*, 1991; Trivedi *et al.*, 1994). Therefore, sequences from as distantly related organisms as the early diverging cyanobacteria *Gloeobacter violaceus* and the conifer *Pinus contorta* can be aligned and compared in a satisfactory way. Genomes of cyanobacteria can contain 2–5 *psbA* genes: *psbA1* – *psbA5*. However, these genes only encode two forms of the D1 protein (Curtis and Haselkorn, 1984; Mulligan *et al.*, 1984; Vrba and Curtis, 1990). These forms impart different functional characteristics to PS II and interchange of them is a mean for light adaptation in cyanobacteria (Campbell *et al.*, 1996). In most eukaryotic organisms there is only one *psbA* gene in each chloroplast genome and it typically lacks introns (Palmer, 1985; Dwivedi and Bhardwaj, 1995). However, there are exceptions with regard to copy number, *e.g.* the presence of two copies in the chloroplast genome of *Pinus contorta*, *P. banksiana* (Lidholm *et al.*, 1991) and *Chlamydomonas reinhardtii* (Erickson *et al.*, 1984). There are also exceptions in gene organisation, such as the presence of two introns in *Chlamydomonas moewusii* (Turmel *et al.*, 1989) and four in *Chlamydomonas reinhardtii* (Erickson *et al.*, 1984). Although there are generally only one or two *psbA* copies in each chloroplast genome, there can be up to hundreds of copies of the genome within a single chloroplast and, furthermore, several chloroplasts in one cell (Lee and Haughn, 1980; Bendich, 1987; Birky and Walsh, 1992). These genome copies may be polymorphic (Frey *et al.*, 1999b), resulting in a set of different haplotypes within the genomes. During selection, frequencies of such haplotypes can change among the chloroplast genomes and within the cell. This additional level of intra-organelle and intra-individual selection can induce changes in gene frequencies within the life-span of an individual and provide a mechanism for very rapid evolution, which has been demonstrated as increases in the point mutation conferring tolerance to triazine herbicides in *Senecio vulgaris* (Frey, 1999a). Perhaps the most interesting example of chloroplast genome organisation is that of many dinoflagellate species. Their chloroplast genomes have been shown to be substantially reduced down to 16 functional genes, including psbA, and divided into so-called minicircles, containing only one to three genes each (Zhang et al., 1999; Hackett et al., 2004; Barbrook et al., 2006).

Similarly to the copy number of chloroplast chromosomes in other species, the copy number of minicircles is variable at different growth stages (Koumandou and Howe, 2007), further demonstrating the variable nature of chloroplast genes. This variability in *psbA* gene organisation and copy number among organisms suggests that in diverse periphyton communities i) irgarol selection could have different rates in different taxa and ii) there may be only a weak correlation between the number of cells and the number of *psbA* genes. In addition to these biological reasons there are also methodological limitations, such as the inability to detect small species in the microscope, or primer mismatch in the polymerase chain reaction (PCR) producing the clone libraries. A strict correlation between clone libraries and morphologically distinct species should therefore not be expected. Instead, these two approaches should be viewed as complementary through which we can examine different aspects of the microbial community.

We have previously shown that richness in *psbA* haplotypes was very high in periphyton communities, and that irgarol-induced community tolerance was accompanied by a dramatic change in the composition of the *psbA* gene pool and of the predicted D1 protein types within the community (Eriksson *et al.*, in press). These changes indicate that the sequences of the D1 protein and *psbA* play important roles in organisms exposed to irgarol. In this study, we put the *psbA* haplotypes from two periphyton communities, one with low and one with elevated irgarol tolerance, in a phylogenetic context. The aims are to use phylogeny to (i) identify *psbA* haplotypes to lower taxonomic levels, *e.g.* genus or species, (ii) test whether phylogenetic affiliations of *psbA* are indicative of sensitivity or tolerance and (iii) describe the diversity of periphyton species in a broad taxonomic context.

Results and Discussion

We used natural marine surface water in an experimental flow-through system, and since irgarol is used as an antifouling agent in Sweden, it was not possible to achieve a true control community completely unexposed to irgarol. Therefore, this community was exposed to the current background concentration of irgarol. (For overview of irgarol field contamination and periphyton community tolerance levels in the same area, see Blanck *et al.* 2008.) In the following we therefore use the terms "background" and "irgarol" to couple sequences, species or communities to exposure to either background or experimentally elevated irgarol concentrations. The background concentration was 0.02 nM while the experimentally elevated concentration was 10 nM (Eriksson *et al.*, in press). The 10 nM exposure equals the upper range of detected irgarol concentrations in the environment (Konstantinou and Albanis, 2004; Lam et al., 2005; Carbery et al., 2006).

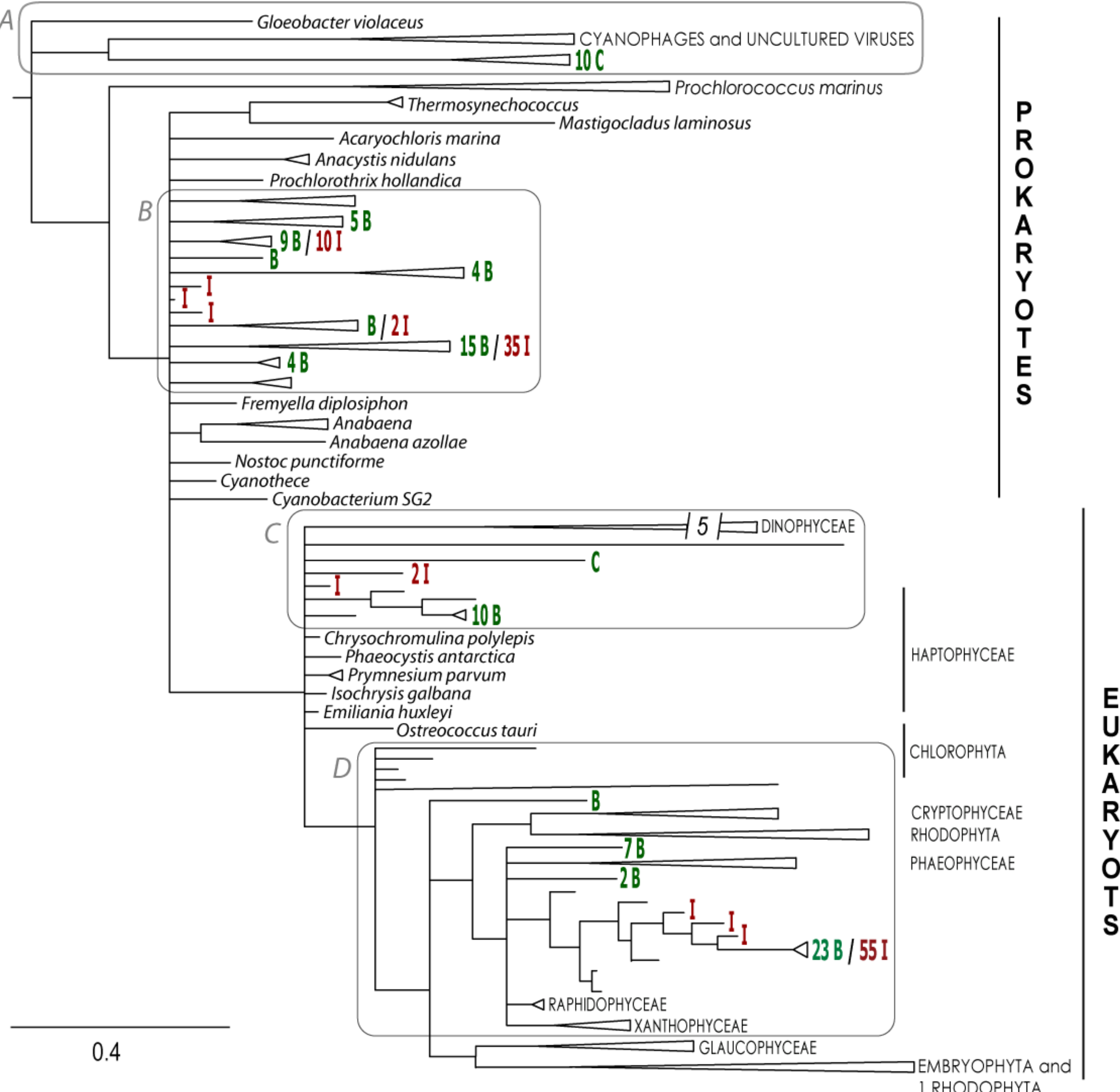

Figure 1. Simplified tree showing the phylogenetic relationships of *psbA* haplotypes. Evolutionary distances were determined from an alignment of 285 nucleotides. The tree is a 50 % majority-rule consensus of 32,000 trees from a Bayesian inference of phylogeny. *psbA* sequences amplified from the microcosms are indicated using the green letter B for sequences from microcosm with background exposure and the red letter I for sequences from microcosm with elevated irgarol exposure. The numbers before these letters indicate the total number of sequences found in the respective microcosm. The parts of the tree indicated with A, B, C and D are enlarged in Figure 2. The scale bar represents the expected changes per site. The length of the Dinophyceae clade break is five scale bars.

Since several authors have demonstrated that artefacts can be produced in PCR-produced clone libraries (e.g. see Liesack et al., 1991; e.g. see Polz and Cavanaugh, 1998; Acinas et al., 2005), we developed a strategy in order to minimize these problems (see Experimental Procedures). This resulted in exclusion of 23 haplotypes from further analyses and the establishment of libraries with very low probability of containing erroneous sequences. Within these revised libraries, 72 unique *psbA* haplotypes were found in the background and irgarol communities, but they were distributed very differently among the two groups. Only three haplotypes were common to both communities while 34 were found only in the background community and 35 only in the irgarol community. BLAST searches (Altschul *et al.*, 1997) were performed against the nucleotide collection (nr/nt) in GenBank (Benson *et al.*, 2008) in order to identify the haplotypes. Since there were no satisfactory BLAST matches to any known sequences, we used phylogeny to infer the periphyton *psbA* haplotypes to taxonomic groups.

We aligned periphyton *psbA* haplotypes to a large number of *psbA* sequences of known identity and determined their phylogenetic relationships. Some bacteriophage sequences of unknown identity were also included in order to increase taxon sampling in this group (Table S1). After a first phylogenetic analysis some taxa from Embryophyta and Phaeophyceae were excluded since they were identified as less relevant for assigning periphyton taxa to known taxonomic groups. Even though we used a fairly short segment of *psbA* (285 bp.) the resulting tree identifies eukaryotes as a lineage separate from the prokaryotes (Fig. 1). The prokaryote part of the communities is very diverse, containing 56 haplotypes. Unfortunately, this part of the tree was largely unresolved and almost no periphyton haplotypes could be assigned to taxonomic groups (Fig. 1 and 2B). This demonstrates that these organisms are poorly studied and poorly represented in the nucleotide databases. However, one periphytic prokaryote clade (clade 1; Fig. 2A), composed of 10 taxa from the background community, seems related to viral cyanophage sequences. The puzzling discovery that bacteriophages infecting *Synechococcus* and *Prochlorococcus* species have incorporated *psbA* in their genomes was made by Mann *et al.* in 2003, and it has been studied by several authors since then (e.g. Lindell et al., 2004; Zeidner et al., 2005; Sharon et al., 2007). Although the cyanophage and periphyton prokaryote clade is only moderately supported (0.72 Bayesian posterior probability; Clade 1 in Fig. 2A), the fact that only haplotypes from the background community are found in this clade is in agreement with earlier findings in virology. These studies showed that viral phage production depends on host photosynthesis during infection and that factors inhibiting photosynthesis, like irgarol does, also have the effect of decreasing phage production (*e.g.* see Sherman and Haselkorn, 1971; Allen and Hutchison, 1976; Sherman, 1976).

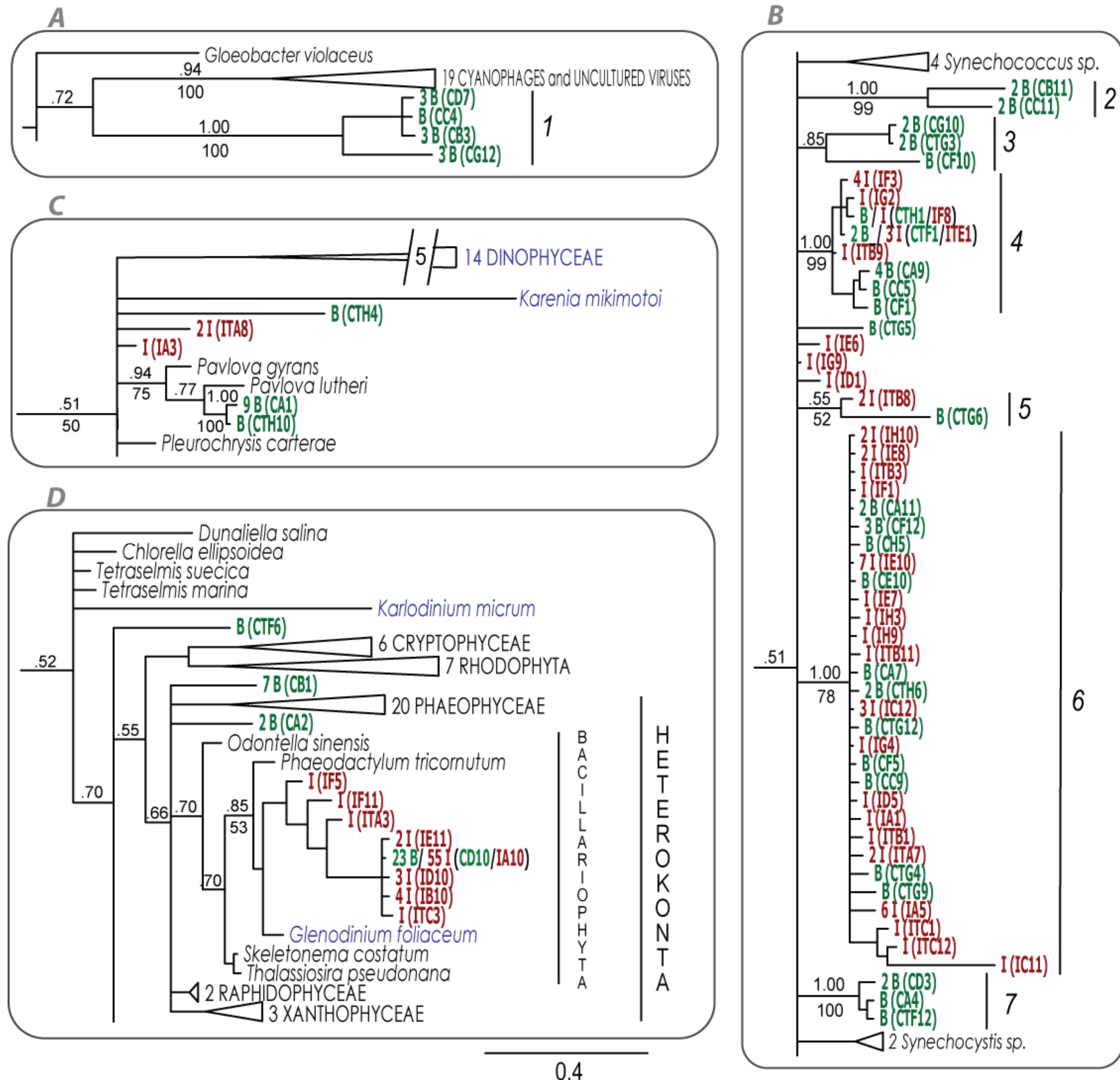

Figure 2. Enlarged parts (A-D) of the phylogenetic tree shown in Figure 1. The labels of the periphyton sequences are as in Fig. 1, but here the numbers indicate the total number of the specific haplotype found in the respective microcosm. Taxon names in blue indicate species belonging to Dinophyceae. The unidentified prokaryote periphyton clades in box A and B are numbered 1-7 to facilitate references in the text. Numbers above and below branches show posterior probability and jackknife support values, respectively, as applicable. The scale bar represents the expected changes per site. Branches of Glaucophyceae and Embryophyta were excluded from box D since they did not contribute to the identification of periphyton haplotypes.

Many studies of microbial communities have revealed high sequence microheterogeneity, meaning that even though many of the sequences are

unique, a large proportion show high similarity to each other (e.g. see Ferris and Ward, 1997; e.g. see Casamayor et al., 2002; Ferris et al., 2003). Similarly to what Field *et al.* (1997), Acinas *et al.* (2004) and Klepac-Ceraj *et al.* (2004) found for 16S rDNA sequences, we detected clades composed of many closely related prokaryote *psbA* haplotypes (*e.g.* clades 4 and 6–7 in Fig. 2B) in spite of substantial corrections for PCR artefacts. This pattern can partly originate from multiple paralogous copies of these genes. We evaluated this possibility by comparing sequence similarities of the periphyton prokaryote taxa to those of paralogous *psbA* copies within sequenced cyanobacterial genomes (Table S2) and to those of orthologous genes in non-periphytic cyanobacterial taxa through pairwise comparisons of the studied fragment. This analysis revealed that non-identical paralogous *psbA* fragments within prokaryote genomes have an average similarity of 90.6%, but also showed that it can be as low as 73.3% (*Gloeobacter violaceus* [PCC 7421]). The sequence similarities of the orthologous *psbA-1* (in *Anabena sp.* [PCC 7120], *Synechocystis sp.* [PCC 6714] and *Thermosynechococcus vulcanus*); and *psbA-2* (in Cyanobacterium SG2, *Anabaena sp*. [PCC 7120], *Synechocystis sp.* [PCC 6803], *Prochlorothrix hollandica, Synechococcus sp.* [BL3] and *Anacystis nidulans* [R2]) gene fragments are in average 82.5% and 82.9%, respectively. This indicates that in spite of the different functions of the paralogous genes (Campbell *et al.*, 1996), there is a higher degree of *psbA* divergence among species than among functions in one species. Moreover, even though the similarity of paralogous sequences on average is higher than that of orthologous sequences, sometimes these similarities overlap. For the periphyton prokaryote clades 4 and 6–7, the sequence similarities were 95.7%, 86.3% and 97.8%, respectively. Thus, from this analysis and the topology of the tree we conclude that these clades are likely to contain paralogous *psbA* copies but may also harbour orthologous genes, especially clade 4 since that grouping contains several subclades.

The eukaryote part of the tree contains only 16 periphyton haplotypes but is more resolved than the prokaryote part. Still, identification of periphyton haplotypes to species or genus level was not particularly successful. The majority of the periphyton eukaryote haplotypes belong to the Bacillariophyta clade (Fig. 2D). This is consistent with data on relative abundance of species based on morphological characters in these communities (Eriksson *et al.*, in press). The Bacillariophyta clade also harbours the dinophycean species *Glenodinium foliaceum* (formerly named *Peridinium foliaceum*), which at first would appear to be an erroneous placement (Fig. 2D). However, the grouping of *G. foliaceum* in the diatom clade is consistent with the fact that this taxon previously has been shown to have acquired a diatom endosymbiont via tertiary endosymbiosis (Chesnick et al., 1996; Chesnick et al., 1997; Horiguchi and Takano, 2006). Such endosymbiotic events, where a dinoflagellate has engulfed an eukaryote algal cell (secondary endosymbiosis) or where the secondarily acquired chloroplast has been replaced with yet another eukaryote

plastid (tertiary endosymbiosis), have actually occurred many times within this group, with an exceptionally complex chloroplast phylogeny as a result (Scherer et al., 1991; Moestrup and Daugbjerg, 2007). Our analysis is also consistent with previous dinoflagellate phylogenies in that the species with fucoxanthin-pigmented chloroplasts, *Karenia mikimotoi* (formerly named *Gymnodinium mikimotoi*) and *Karlodinium micrum* (synonymous names: *Karlodinium veneficum* and *Gymnodinium micrum*), fall outside the clade composed of species with peridinin-pigmented chloroplasts, *i.e.* the Dinophyceae (Fig. 2C–D). The reason is that the two fucoxanthin-pigmented species most likely have acquired a tertiary haptophyte chloroplast (Tengs et al., 2000; Ishida and Green, 2002; Patron et al., 2006). Although our analysis supports the different phylogenetic affiliations of fucoxanthin and peridinin pigmented dinoflagellates, (*i.e.* the placement of *K. mikimotoi* and *K. micrum* outside the dinophycean clade in Fig. 2C–D), it does not support the view of them as sister groups, as suggested by Takashita *et al.* (1999) and Yoon *et al.* (2002). However, the question whether fucoxanthin-pigmented plastids are more closely related to peridinin-pigmented plastids or to haptophyte plastids has proven especially difficult to resolve (Inagaki *et al.*, 2004; Shalchian-Tabrizi *et al.*, 2006).

One of the most striking features of the trees shown in Figure 1 and 2C–D is the extremely long branches of dinoflagellate taxa. The total branch length of the dinophycean clade consisting of 14 species (Fig. 1 and Fig. 2C) range from 1.20 to 3.33 expected substitutions per site, with an average of 2.07, and that of *K. mikimotoi* (Fig. 2C) and *K. micrum* (Fig. 2D) correspond to 1.48 and 1.36 expected substitutions per site, respectively. This can be compared to land plants, which in this tree have a range of total branch lengths from 1.04 to 1.61 with an average of 1.28. Several studies (*e.g.*, Holder and Lewis, 2003) have shown that Bayesian methods are less sensitive to long-branch attraction (Felsenstein, 1978) than parsimony methods, which suggests that this is a true result rather than an artefact of phylogenetic reconstruction. Moreover, these results are in agreement with previous studies (Cavalier-Smith, 1999; Zhang et al., 2000; Bachvaroff et al., 2006), which have also shown that dinoflagellates have long branches and thus elevated evolutionary rates as compared to other organisms. The complex dinoflagellate plastid phylogeny makes it difficult to determine whether some of the periphyton haplotypes are of dinoflagellate origin. Marine periphyton attached to glass surfaces have previously been shown to harbour dinoflagellates (Webster *et al.*, 2006). Even though our analysis of morphologically different taxa only detected a very low abundance of the dinoflagellate *Prorocentrum lima* in the irgarol microcosm, it is still possible that some of the unidentified haplotypes with long branches, *e.g.* CTH4, CTF6 or ITA8, are of dinoflagellate origin.

There is a general taxonomic correspondence between morphologically distinct taxa and haplotypes in the microcosms. The general composition of

the communities is the same in both data sets, *i.e.* the dominance of cyanobacteria and diatoms. There are also some examples of correspondence at lower taxonomic levels. There is one case of a likely identification to the level of genus in this analysis, namely the two background haplotypes (CA1 and CTH10) that group with two species of genus of *Pavlova* (Fig. 2C). These may come from the irgarol-sensitive "green-like" algal cells detected in the analysis of morphologically distinct taxa. There were also sprouts of brown algae in the data set of morphologically distinct taxa, which could be represented by the CB1 or CA2 haplotypes within the broader taxonomic group of Heterokonta (Fig. 2D). There are also discrepancies between the data sets of morphologically distinct taxa and haplotypes. Obviously, the methodological limitations of the two approaches can give rise to such discrepancies. For example, cyanophages cannot be detected with a microscope but their DNA can readily be extracted, amplified and cloned. However, such discrepancies can also come from the inherent differences of the two levels of biological organisation. Measuring gene and species frequency is simply not the same thing, for instance due to differences in number of gene copies among different species.

Even though it is not entirely straightforward, our phylogeny of periphyton *psbA* can be compared to that made for marine phytoplankton *psbA* (Zeidner *et al.*, 2003). These authors amplified a larger fragment of *psbA*, which theoretically should result in detection of greater haplotype richness. On the other hand they specifically selected for the picoplankton-sized (<3 µm) cells during cell fractionation, which in turn should lower the detected haplotype richness. In periphyton we found 72 haplotypes, belonging to diverse taxonomic groups (Fig. 1 and 2), whereas Zeidner *et al.* (2003) found 3, 12 and 28 haplotypes from the Mediterranean Sea, the Red Sea and the central North Pacific Ocean, respectively. The sampled picophytoplankton community was dominated by *Prochlorococcus* and *Synechococcus* haplotypes, even though some green algae, stramenopiles, haptophytes and one cryptophyte were found. Obvious differences between these phytoplankton communities and the periphyton communities described in this study are the dominance of diatoms and the absence of *Prochlorochoccus* in periphyton. Although there are differences in sampling and in sequence length, it is noteworthy that higher richness was found when we sampled periphyton from two microcosms, at one occasion and one geographical area, as compared to phytoplankton sampled at three different depths and at three locations around the world during three different years. We thus have to conclude that periphyton communities harbour a great diversity but also that the high haplotype richness from the two microcosms is a consequence of the experimental exposure to irgarol, since this altered the composition of the *psbA* gene pool.

The topology of the tree (Figs. 1–2) shows that phylogenetic affiliations of periphyton haplotypes in some cases, but not in all, are correlated with sensitivity or tolerance to irgarol. Examples of sensitive taxonomic groups are prokaryote clade 1–3 and 7, the eukaryotic *Pavlova* clade and some single unidentified taxa (Fig. 2A–D). One larger phylogenetic group is unquestionably selected for by irgarol, namely the clade within Bacillariophyta (Fig. 2D). Although this clade harbours the most abundant haplotype in the background community, its abundance increased almost three-fold (from 23 to 68 sequences) in the irgarol community. Moreover, the diversity in this grouping increased due to irgarol exposure (Fig. 2D). In contrast, the prokaryote clades 4–6 harbour both background and irgarol haplotypes. This suggests that the studied part of D1, corresponding to the sequenced fragment of *psbA*, is less important for the ecological performance during irgarol exposure in the prokaryote clades than in the Bacillariophyta clade. Interestingly, the two different patterns of sensitivity/tolerance to irgarol in the *psbA* phylogeny are consistent with the proposal of two distinct tolerance mechanisms to irgarol in periphyton (Eriksson *et al.*, in press). That study showed that the only region of D1 that differed between the background and irgarol community was the PEST domain. Irgarol induced a change in distribution of PEST sequence types, and since this domain is suggested to regulate degradation of proteins (1986; Rechsteiner and Rogers, 1996), it implies that the tolerance mechanism involves increased degradation and turnover of the D1 protein, which has also been suggested previously (Trebst et al., 1988; Draber et al., 1991; Trebst, 1996). However, the two most irgarol-selected PEST sequence types differed in their occurrence pattern. The most abundant PEST sequence type in the irgarol community was present only in haplotypes that occurred in the irgarol community, which makes this PEST sequence type a likely irgarol tolerance-conferring trait. Importantly, all *Bacillariophyta* taxa have this PEST sequence type. In contrast, the second most abundant PEST sequence type in the irgarol community was present in haplotypes that occurred in both the irgarol and the background communities. Thus, the second most abundant PEST sequence type is less likely to contribute to irgarol tolerance in the irgarol community. According to our phylogenetic analysis, this PEST sequence type was present in haplotypes in prokaryote clade 6 and in the prokaryote single-sequence clades CTG5, IG9, IE6 and ID1 (Fig. 2B), which also occur both in background and irgarol communities. Thus the phylogenetic analysis detected irgarol selection in *Bacillariophyta* because their tolerance mechanism is likely to involve the PEST domain in D1, and thus depends on the sequence of *psbA*. In contrast, irgarol selection for prokaryote taxa was not detected by the phylogenetic analysis since their tolerance mechanism most likely is directed towards some other trait. Even though no tolerance mechanism has been described in phytoplankton communities, the irgarol-induced selection for diatoms and

cyanobacteria reported here is consistent with effects of irgarol in phytoplankton communities (Readman *et al.*, 2004; Devilla *et al.*, 2005).

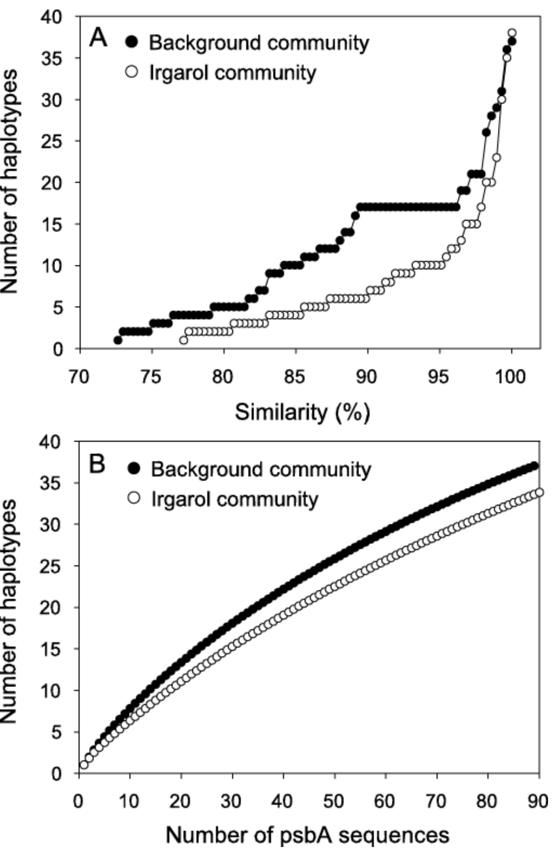

Figure 3. Compositional pattern of periphyton *psbA* sequences from background and irgarol communities. A) Relation between number of haplotypes and sequence similarity calculated with the UPGMA algorithm. B) Rarefaction curves describing the number of haplotypes as a function of the number of *psbA* genes sampled.

Irgarol selection caused decreased diversity that was detected as the absence of some taxonomic groups in the irgarol community (Fig. 2A–D) and as lowered *psbA* sequence diversity from 0.216 (SE 0.040) for the background community to 0.184 (SE 0.043) in the irgarol community. As discussed above for the prokaryote part of the communities, a large fraction of the haplotypes are closely related, which can be seen in Figure 3A as an almost exponential increase in the number of *psbA* clusters when similarity of sequences increases. The lower diversity in the irgarol community is also shown as a higher degree of sequence similarity in the restructured irgarol-tolerant community (Fig. 3 A). When we analysed molecular selection by calculating non-synonymous and synonymous mutations (Hill and Hastie, 1987; Hughes and Nei, 1988) for the periphyton pool of *psbA* genes, no significant positive selection from irgarol could be detected. This is likely due to the high degree of conservation of this fragment of *psbA*. When we expanded the analysis to all taxa in the alignment, positive selection was detected only within the group of phage sequences (data not shown). Similar to most metagenomic studies, the number of detected genes accounted for only a small fraction of the total diversity,

indicated by the steadily increasing rarefaction curves for both background and irgarol periphyton (Fig. 3B).

In conclusion, the *psbA* gene seems promising for community studies in a taxonomic framework, since even such a short fragment as 285 base pairs gave high phylogenetic resolution. The haplotype diversity of *psbA* in periphyton communities is very high and its composition at least partly correlated to traditional measures of species diversity. Consistent with the ecotoxicological community-tolerance response, specific haplotypes of *psbA* were shown to be irgarol-sensitive and the overall diversity of periphyton decreased after irgarol exposure. Phylogenetic affiliations are indicative of irgarol sensitivity/tolerance for some taxa, but not for others. This is probably due to one tolerance mechanism being regulated by the PEST domain of D1, and thus encoded by *psbA*, while one (or several) tolerance mechanism(s) are being encoded elsewhere. Thus, clone libraries and phylogenetic analysis are high-precision tools well suited to trace selection on targets of toxicants.

Experimental procedures

Microcosm experiment

The flow-through microcosm experiment was performed indoors at Sven Lovén Centre for Marine Sciences, Kristineberg on the west coast of Sweden during July and August in 2001 as described by Eriksson *et al.* (in press).

Accession numbers

The periphyton *psbA* sequences have Genbank accession numbers from AM933675 to AM933749. The accession numbers of the non-periphytic *psbA* sequences used in the phylogenetic analysis are given in Table S1.

Sequence and phylogenetic analysis

A strategy was developed to minimize PCR artefacts within our libraries. We removed sequencing errors by excluding clones with non-complementary forward and reverse sequences from further analysis. To avoid artefacts from polymerase errors we excluded sequences that fulfilled the two criteria of occurring only once in each library and differing by only one nucleotide from any other sequence within the library. Moreover, we manually identified possible recombination points among the sequences in each library, and

checked whether any sequence could originate from other sequences, *i.e.* be of chimeric origin.

psbA sequences of known species, and some bacteriophage sequences with unknown identity, was retrieved from the EMBL-EBI database, and 161 sequences were aligned to the 72 periphyton *psbA* sequences. Sequences were aligned using Mafft 5.64 (Katoh et al., 2002; Katoh et al., 2005) with the settings: FFT-NS-i (Slow; iterative refinement method), the gap opening penalty 1.53 and the offset value 0.123. Similarity and rarefaction calculations of periphyton *psbA* haplotypes were made with UPGMA in the Clusterer application (version 1) (Klepac-Ceraj *et al.*, 2006). Sequence diversity within the communities was calculated using pairwise analysis of sequences and the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Standard error estimates were obtained by a bootstrap procedure with 1000 replicates. Positive molecular selection was tested with a codon-based Z-test under the test hypothesis of positive selection, using the Nei-Gojobori method (Nei and Gojobori, 1986). The Z-variance of the difference was computed using a bootstrap of 1000 replicates. For these analysis the software MEGA4 (build number 4028) (Tamura *et al.*, 2007) was used. When comparing similarities of paralogous and orthologous *psbA* sequences from non-periphytic cyanobacterial taxa and periphyton prokaryote *psbA* sequences, BioEdit 7.0.5.3 (Hall, 1999) was used.

For the phylogenetic analysis, both Bayesian and maximum parsimony methods were used as implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) and PAUP* 4.0b10 (Swofford, 2002), respectively. We used *Gloeobacter violaceus* as outgroup since this cyanobacterium is a member of an early branching lineage (Nelissen et al., 1995; Turner et al., 1999; Cavalier-Smith, 2007). The evolutionary model suggested by the Akaike Information Criterion as implemented in MrModelTest 2.2 (Nylander, 2004) was incorporated in the input file of MrBayes. For each run, 8 parallel Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were performed, running 20 million generations, sampling every 1000 generations and saving branch lengths. The software Tracer 1.4 (Rambaut and Drummond, 2006) was used to determine when the tree sampling stabilized, and a burn-in value of 4000 was subsequently used when computing the results. The analysis gave a 50% majority-rule consensus tree from 32,000 sampled trees. Jackknife support values (Farris *et al.*, 1996) were then estimated in PAUP* by running 10000 replicates with 37% deletion, 20 random addition sequence replicates, TBR branch swapping and saving up to 10 trees per replicate.

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Supplementary information

Table S1. Organism names and accession numbers of the non-periphytic sequences in the phylogenetic analysis.

Organism	Accession number
Gloeobacter violaceus PCC 7421	BAC91085
Thermosynecococcus elongatus BP-1	BA000039
Thermosynechococcus vulcanus	X79222
Nostoc punctiforme PCC 73102	NC_010628
Synechococcus sp. CC9902	NC_007513
Synechococcus sp. CC9605	NC_007516
Synechococcus sp. WH 8102	NC_005070
Mastigocladus laminosus	AF170924
Synechococcus sp. BL3	AY995302
Anacystis nidulans	M11557
Anacystis nidulans R2	X04617
Anacystis nidulans R2	X04618
Synechocystis sp. PCC 6803	X13547
Synechocystis sp. PCC 6714	X15514
Anabaena sp. PCC 7120	U21331
Anabaena sp. PCC 7120	U21332
Anabaena sp. PCC 7120	U21333
Anabaena sp. PCC 7120	U21334
Anabaena azollae	X64174
Cyanothece sp. ATCC51142	U39610
Prochlorothrix hollandica	X14308
Acaryochloris marina MBIC11017	AB303650
Fremyella diplosiphon	K02074
Trichodesmium sp.	AF107784
Cyanobacterium SG2	AF402784
Prochlorococcus marinus MIT 9312	AY176636
Prochlorococcus marinus NATL1MIT	AY176641
Prochlorococcus marinus MIT 9302	AY599029
Prochlorococcus marinus MIT 9515	AY599030
Prochlorococcus marinus MIT 9116	AY599031
Prochlorococcus marinus MIT9211	AY599032
Prochlorococcus marinus NATL2A	AY599033
Synechococcus cyanophage syn10	ABF56001
Synechococcus cyanophage S-ShM1	DQ473649
Prochlorococcus cyanophage P-SSM9	DQ473651
Prochlorococcus cyanophage P-SSM4	DQ473648

Prochlorococcus cyanophage P-RSM2	DQ473656
Prochlorococcus cyanophage P-SSM10	DQ473647
Prochlorococcus cyanophage P-SSM3	DQ473653
Prochlorococcus cyanophage P-SSM8	DQ473655
Uncultured virus, environmental samples	ABF59521
Uncultured virus, environmental samples	ABF59522
Uncultured virus, environmental samples	ABF59523
Uncultured virus, environmental samples	ABF59524
Uncultured virus, environmental samples	ABF59525
Uncultured virus, environmental samples	ABF59526
Uncultured virus, environmental samples	ABF59527
Uncultured virus, environmental samples	ABF59528
Uncultured virus, environmental samples	ABF59536
Uncultured virus, environmental samples	ABF59537
Uncultured virus, environmental samples	ABF59547
Alexandrium tamarense	AB025589
Gonyaulax polyedra	DQ264844
Amphidinium carterae	AY004259
Heterocapsa triquetra	AB025587
Heterocapsa rotundata	AF206706
Heterocapsa pygmaea	AY004261
Heterocapsa niei	AY004265
Protoceratium reticulatum	AF206711
Prorocentrum micans	AB025585
Symbiodinium sp. Tm	AY160084
Protoceratium reticulatum	AF206711
Polarella glacialis	AB096159
Scrippsiella trochoidea	AF206710
Pyrocystis lunula	AF490365
Karlodinium micrum	AY119763
Karenia mikimotoi	AB027234
Glenodinium foliaceum	AY119764
Emiliania huxleyi	AJ575573
Chrysochromulina polylepis	AJ575572
Isochrysis galbana	AJ575574
Pavlova gyrans	AJ575575
Pavlova lutheri	AY119755
Pleurochrysis carterae	AY119757
Phaeocystis antarctica	AY119756
Prymnesium parvum	AJ575576
Prymnesium parvum	AY119758
Tetraselmis marina	AY857618
Tetraselmis suecica	DQ173249

Dunaliella salina	DQ074449
Chlorella ellipsoidea	X62800.1
Ostreococcus tauri	CR954199
Chattonella sp. KST-2003	AJ575577
Heterosigma akashiwo	AY119759
Bumilleriopsis filiformis	X79223
Vaucheria litorea	AF227740
Tribonema aequale	AY528860
Phaeodactylum tricornutum	AY864816
Skeletonema costatum	AY119761
Odontella sinensis	CAA91657
Thalassiosira pseudonana	ABK20797
Pylaiella littoralis	AY119760
Adenocystis utricularis	AY528824
Chordaria flagelliformis	AY528826
Ectocarpus siliculosus	X56695
Colpomenia sinuosa	AY528835
Desmarestia sp. SMB-2004	AY528841
Alaria crassifolia	AY528847
Laminaria digitata	AY528849
Scytothamnus australis	AY528852
Splachnidium rugosum	AY528853
Chorda filum	AY528848
Cutleria cylindrica	AY528840
Analipus japonicus	AY528851
Carpomitra costata	AY528856
Sargassum horneri	AY528846
Fucus vesiculosus	DQ307679
Halopteris filicina	AY528854
Syringoderma phinney	AY528858
Ishige okamurae	AY528829
Dictyopteris divaricata	AY422638
Schizocladia ischiensis	AY528859
Chilomonas paramecium	AY119748
Pyrenomonas helgolandii	AY119750
Rhodomonas abbreviata	AY119751
Cryptomonas sp. M1094	AY141197
Chroomonas sp.	AY141196
Teleaulax amphioxeia	AY453068
Glaucocystis nostochinearum	AY876201
Cyanophora paradoxa	X14667
Cyanidium caldarium	X52758
Antithamnion sp.	X55364

Stylonema alsidii	AY119745
Erythrotrichia carnea	AY119739
Neoptilota asplenioides	AY865136
Ptilota serrata	AY865166
Plumaria plumosa	AY865138
Psilothallia dentata	AY865145
Conocephalum supradecompositum	AB020621
Sphaerocarpos texanus	AY607989
Monoclea sp.	AY607970
Blasia pusilla	AY607928
Fossombronia pusilla	AY607941
Haplomitrium gibbsiae	AY607947
Jungermannia crenuliformis	AY607957
Porella pinnata	AY607980
Ptilidium ciliare	AY607982
Radula perrottetii	AY607984
Pleurozia purpurea	AY877401
Bryopteris filicina	AY607930
Anthoceros formosae	AB087434
Megaceros flagellaris	AY877400
Apotreubia nana	AY877397
Megaceros cf. fuegiensis	AY607923
Riccardia capillacea	AY688833
Metzgeria sp.	AY607969
Prunus padus	AF410182
Actinidia deliciosa	AY323417
Gossypium hirsutum	X15885
Magnolia pyramidata	U63020
Helianthus annuus	X60428
Solanum nigrum	U25659
Medicago sativa	X04973
Chenopodium rubrum	Y14732
Arabidopsis thaliana	X79898
Landoltia punctata	X59557
Zea mays	AF543684
Oryza sativa	M36191
Actinidia chinensis	AY323415
Pinus contorta	X53721
Dendrohypopterygium arbuscula	AY607938

Table S2. Paralogous *psbA* sequences from prokaryote species.

Organism	Gene copy number [a]	Accession	ID	Protein ID
Acaryochloris marina	*psbA2*	CP000828	CP000828_4292	ABW27180
Acaryochloris marina	*psbA3*	CP000828	CP000828_5729	ABW27888
Anabaena variabilis ATCC 29413	*psbA1*	CP000117	CP000117_3407	ABA21206
Anabaena variabilis ATCC 29413	*psbA2*	CP000117	CP000117_3435	ABA21220
Anabaena variabilis ATCC 29413	*psbA3*	CP000117	CP000117_4595	ABA21758
Anabaena variabilis ATCC 29413	*psbA4*	CP000117	CP000117_5290	ABA22075
Cyanothece sp. ATCC 51142	*psbA1*	CP000806	CP000806_7085	ACB52849
Cyanothece sp. ATCC 51142	*psbA2*	CP000806	CP000806_6905	ACB52759
Cyanothece sp. ATCC 51142	*psbA3*	CP000806	CP000806_542	ACB49618
Anabaena sp. PCC7120	*psbA1*	BA000019	BA000019_4919	BAB76565
Anabaena sp. PCC7120	*psbA2*	BA000019	BA000019_3767	BAB75426
Anabaena sp. PCC7120	*psbA3*	BA000019	BA000019_4641	BAB76291
Anabaena sp. PCC7120	*psbA4*	BA000019	BA000019_3611	BAB75271
Gloeobacter violaceus PCC 7421	n.d.	BA000045	BA000045_788	BAC88720
Gloeobacter violaceus PCC 7421	n.d.	BA000045	BA000045_1729	BAC89647
Gloeobacter violaceus PCC 7421	n.d.	BA000045	BA000045_2350	BAC90263
Gloeobacter violaceus PCC 7421	n.d.	BA000045	BA000045_2690	BAC90597
Microcystis aeruginosa	*psbA1*	AP009552	AP009552_1024	BAG00844
Microcystis aeruginosa	*psbA2*	AP009552	AP009552_1040	BAG00860
Microcystis aeruginosa	*psbA3*	AP009552	AP009552_1053	BAG00873
Microcystis aeruginosa	*psbA5*	AP009552	AP009552_5863	BAG05636
Synechococcus elongatus BP-1	*psbA1*	BA000039	BA000039_1875	BAC09395
Synechococcus elongatus BP-1	*psbA2*	BA000039	BA000039_1876	BAC09396
Synechococcus elongatus BP-1	*psbA3*	BA000039	BA000039_1503	BAC09029
Synechococcus elongatus PCC7942	*psbA1*	CP000100	CP000100_919	ABB56456
Synechococcus elongatus PCC7942	*psbA2*	CP000100	CP000100_1932	ABB56923
Synechococcus elongatus PCC7942	*psbA3*	CP000100	CP000100_3009	ABB57419
Synechococcus sp. ATCC 27264	n.d.	CP000951	CP000951_2836	ACA99409
Synechococcus sp. ATCC 27264	*psbA2*	CP000951	CP000951_314	ACA98171
Synechococcus sp. ATCC 27264	n.d.	CP000951	CP000951_4328	ACB00145
Synechococcus sp. CC9311	*psbA1*	CP000435	CP000435_737	ABI46731
Synechococcus sp. CC9311	*psbA3*	CP000435	CP000435_3717	ABI46741
Synechococcus sp. CC9605	*psbA1*	CP000110	CP000110_664	ABB34083
Synechococcus sp. CC9605	*psbA3*	CP000110	CP000110_2245	ABB34800
Synechococcus sp. CC9902	*psbA1*	CP000097	CP000097_1899	ABB25909
Synechococcus sp. CC9902	*psbA2*	CP000097	CP000097_3677	ABB26771
Synechococcus sp. RCC307	*psbA1*	CT978603	CT978603_1470	CAK28343

Synechococcus sp. RCC307	*psbA2*	CT978603	CT978603_1471	CAK28344
Synechococcus sp. WH7803	*psbA1*	CT971583	CT971583_375	CAK22792
Synechococcus sp. WH7803	*psbA2*	CT971583	CT971583_808	CAK23210
Synechococcus sp. WH8102	*psbA1*	BX569693	BX569693_68	CAE07985
Synechococcus sp. WH8102	*psbA2*	BX569691	BX569691_381	CAE07498
Synechococcus sp. WH8102	*psbA3*	BX569694	BX569694_461	CAE08666
Synechococcus sp. WH8102	*psbA4*	BX569694	BX569694_158	CAE08434
Synechocystis sp.	*psbA1*	Y00885	Y00885_6	CAA68778
Synechocystis sp.	*psbA2*	X13547	X13547_2	CAA31899
Trichodesmium erythraeum	*psbA1*	CP000393	CP000393_337	ABG49673
Trichodesmium erythraeum	*psbA3*	CP000393	CP000393_9439	ABG53717

[a] n.d. indicates that the paralogous identity is not described in the database.