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Detection, Validity and Specificity of
Pollution-Induced Community Tolerance (PICT)

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Sverker Molander

Akademisk avhandling

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GÖTEBORG 1991

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DETECTION, VALIDITY AND SPECIFICITY OF POLLUTION-INDUCED
COMMUNITY TOLERANCE (PICT)

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DISSERTATION ABSTRACT: Pollution-induced community tolerance (PICT) has been proposed by Blanck et al. 1988 as an ecotoxicological tool for use in advanced hazard assessment of chemicals. PICT is based on the apprehension that toxicants act as selection pressures on biota, affecting the community by excluding sensitive individuals and species. These effects are detectable as an increased tolerance at the community level by a short-term metabolic test. A model for induced tolerance in communities is presented and discussed together with results from long-term (3-5 weeks) experiments using marine periphyton communities colonizing artificial substratum in a microcosm system under controlled exposure to three different toxicants: a herbicide (diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU), a biocide and constituent of anti-fouling paints (tri-*n*-butyltin, TBT), and a chlorophenolic compound found in kraft mill bleachery effluents (4,5,6-trichloroguaiacol, 4,5,6-TCG). Biomass, microalgal species richness, and functional parameters was recorded in parallel to the detection of PICT, which was achieved by short-term tests of photosynthesis inhibition. The results show a general agreement between the PICT response and the other measures of toxicant impact. PICT was able to separate primary from secondary toxicant effects as shown from results where grazers were more sensitive to toxicants than periphyton. In these cases a good agreement between PICT and decreases in species richness and biomass was found at high concentrations. At lower concentrations the tolerance and microalgal species richness was unaffected while biomass increased. The specificity of PICT was studied by analysis of cotolerance patterns for periphyton established under exposure to a specific inhibitor of photosynthetic electron transport; diuron. The patterns found revealed a similarity to patterns found for microalgae and chloroplasts with alterations in the herbicide target site at the D1 protein of photosystem-II. Effects of the combined selection pressures of TBT and diuron was also investigated in a long-term experiment with mixtures of the toxicants. PICT was also detectable in this situation, using both single-toxicant and two-toxicant short-term tests. The results presented indicate the general applicability of the PICT methodology for studies of toxicant impact on communities.

Keywords: aufwuchs, central composite design, cotolerance, combined toxicity, cross-resistance, diatoms, ecotoxicological test system, herbicides, microcosm, periphyton, photosynthesis, toxicity testing

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To Whom
it may concern

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(PICT)

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This dissertation is based on the following papers,
which are referred to in the text by their respective
Roman numerals:

- I. Molander S, Blanck H, Söderström M, 1990.
Toxicity assessment by pollution-induced community tolerance (PICT), and identification of metabolites in periphyton communities after exposure to 4,5,6-trichloroguaiacol. *Aquatic Toxicology* 18:115-136.
- II. Molander S, Blanck H, 1991.
Detection of pollution-induced community tolerance (PICT) in marine periphyton communities established under diuron exposure. Accepted for publication in *Aquatic Toxicology*.
- III. Blanck H, Molander S, 1991.
Cotolerance pattern of periphyton communities restructured by the herbicide diuron. (Manuscript)
- IV. Molander S, Dahl B, Blanck H, Jonsson J, Sjöström M, 1991.
Combined effects of tri-*n*-butyl tin (TBT) and diuron (DCMU) on marine periphyton communities detected as pollution-induced community tolerance. (Submitted manuscript)

CONTENTS

PREFACE	1
SELECTION	3
Toxicants.....	4
DETECTION	5
Sketch to a model of community tolerance.....	5
The choice of test parameter for short-term tests.....	8
Variation in base-line tolerance.....	9
Relations between short-term effects and long-term effects	10
Conclusions	12
VALIDITY	13
Parameters	13
Comparisons and discussion	14
Conclusions	16
SPECIFICITY.....	17
Primary and secondary effects.....	17
Cotolerance.....	18
Interactions	19
DIRECTIONS.....	23
ACKNOWLEDGMENTS	24
REFERENCES	26

When the Lord created the world and people to live in it - an enterprise which, according to modern science, took a very long time - I could well imagine that He reasoned with Himself as follows: "If I make everything predictable, these human beings, whom I have endowed with pretty good brains, will undoubtedly learn to predict everything, and they will thereupon have no motive to do anything at all, because they will recognize that the future is totally determined and cannot be influenced by any human action. On the other hand, if I make everything unpredictable, they will gradually discover that there is no rational basis for any decision whatsoever and, as in the first case, they will thereupon have no motive to do anything at all. Neither scheme would make sense. I must therefore create a mixture of the two. Let some things be predictable and let others be unpredictable. They will then, amongst many other things, have the very important task of finding out which is which."

E.F. Schumacher
from "Small is beautiful"

PREFACE

..now I know in part...

I. Corinthians XIII:12

Sustainable functions of ecosystems are the key prerequisite of all life forms, including mankind. No other species on earth is presently able to threaten the functions of ecosystems to such an extent as man is, due to his technical and social skills. As a consequence of technical development a vast amount of chemicals is produced and used by man. These chemicals will finally appear in the ecosystems, in one form or another. In the ecosystems biota will be *exposed* to the chemicals and some chemicals will reach concentrations high enough to give *effects*. To avoid undesired effects of chemical stress in the ecosystems, information about the fate and effect of pollutants is required. This is the concern of ecotoxicology.

The scientific analysis of fate and potential effects of a chemical have been called a "hazard assessment", which often is approached with a multi-level and sequential strategy (Cairns 1982, Landner 1987, Anon. 1989, Landner 1989). Ecotoxicological test systems have been developed to generate the necessary experimental information for a hazard assessment.

Pollution-induced community tolerance (PICT) has been proposed by Blanck et al. (1988) as an ecotoxicological tool for work at the level of advanced hazard assessment (Landner et al. 1989). PICT is based on the apprehension that a toxicant exerts a selection pressure (Luoma 1977, Pitelka 1988, Klerks and Levinton 1989) when the exposure reaches a certain level for a sufficient period of time. Since organisms vary in their tolerance to toxicants, the chemical selection pressure will exclude those not withstanding the toxicant. The result is a community with an increased tolerance due to the exclusion of the sensitive components. The difference in tolerance between the unselected and the selected communities can be detected by the comparison of results from short-term tests of metabolic inhibition performed with the respective community.

All studies regarding the PICT-response include two steps - the selection step and the detection step. The selection step requires a long-term exposure to a toxicant under controlled conditions in a micro- or mesocosm system or in a pollution gradient in the field, while the detection step is the employment of short-term metabolic tests for quantification of the tolerance levels. The comparison of tolerances among different communities might then reveal a PICT.

PICT can be used in micro- or mesocosm studies to predict the environmental hazard of chemicals as well as to detect the impact of chemicals retrospectively in polluted ecosystems. In predictive studies the use of PICT might give the

possibility to determine the No-Effect Concentration (NEC) which is relevant for the community under study and in retrospective studies PICT might give the possibility to trace the significant toxicant stress (Blanck et al. 1988, Landner et al. 1989b).

The PICT methodology has been used for studies of arsenate tolerance (Blanck and Wängberg 1988a, 1988b, Wängberg and Blanck 1990) and cotolerance (Blanck and Wängberg 1991) using marine periphyton and freshwater phytoplankton and periphyton in limnocorrals (Wängberg et al. 1991). These arsenate studies were summarized by Wängberg 1989. Freshwater phytoplankton in microcosms have also been used for studies of PICT for copper and atrazine (Gustavsson and Wängberg 1991). The PICT methodology has also been used in field studies of chemical impact by an industrial effluent (Molander et al. 1986), tri-*n*-butyltin (TBT; Dahl and Blanck 1990) and copper and arsenate (Wängberg 1991).

The aim of this dissertation is to give some background to the PICT methodology and to present a summary of results of relevance for the detection, validity and specificity of PICT. The first two chapters deal with the principles for the selection step and the detection step together with some results from paper I-IV in relation to other PICT-studies. The two next chapters deal with the validity of the PICT response, compared to other long-term effects, and with the specificity of PICT.

SELECTION

Selection can be the result of an environmental stress. Those organisms that cannot withstand the environmental stress are excluded. From the viewpoint of the organisms there is no difference between anthropogenic, xenobiotic, naturally occurring chemicals or other environmental stresses. To survive the organisms have to cope with them all, thus the exposure to detrimental concentrations of chemicals can be regarded as an environmental stress (Luoma 1977). The concept of stress in biological systems (Selye 1973) is used both in (eco)physiology (Levitt 1980) and evolutionary biology (Pitelka 1988, Bradshaw and Hardwick 1989, Holloway et al. 1990), and can be defined, following Grime (1989), as *external constraints limiting the rates of resource acquisition, growth or reproduction of organisms*. This definition focuses upon the coupling between stress and fitness of the individual organism. In the case of PICT the attention however is not on the fitness of an individual, but on the response of the entire community.

Variation is the fundamental prerequisite for selection. In the context of PICT our main concern is variation among individuals or species in their tolerance to toxicants. Tolerance to chemicals is the ability to withstand the exposure of chemicals (for discussions of the tolerance concept see LeBaron and Gressel 1982, Kelly and Harwell 1989). Tolerance is relative and subject to changes due to genetic and phenotypic alterations. The cause of variation in tolerance is differences in uptake, metabolization, excretion or other mechanisms related to the mode of action or to the ability to circumvent the effects of toxicants.

The implementation of the PICT methodology for studies of specificity and validity of PICT demands a controlled exposure of the test community. Periphyton communities, colonizing artificial substrata in an indoor aquaria system were used following Blanck and Wängberg (1988a). (See also "Material and methods" in paper I-IV). Here "periphyton" is operationally defined as the microbial community found on submerged substrata (Wetzel 1975, see Weitzel 1979 for a discussion of periphyton definitions and nomenclature). This definition includes bacteria, fungi, protozoa, and microalgae found on the substratum, but exclude benthic meiofauna despite their obvious influence as grazers on the periphyton community (Round 1971 and references therein, Connell and Slatyer 1977, paper I, II and IV).

Colonization of periphyton have been described in terms of a succession (Characklis 1981, Bakus et al. 1986, Wahl 1989 and references therein) starting with a biochemical conditioning of the surface and bacterial colonization. Both processes are rapid. Then unicellular and multicellular eucaryotes adhere to the

surface. In marine systems the diatoms are dominating among the unicellular organisms (Wahl 1989). In our experiments the colonization started when the cleaned glass discs were submerged into the flow-through aquaria which received fjord-water with its indigenous biota.

The rate of colonization on the glass discs is highly relevant for the structure of the resulting community, and found to be a sensitive effect parameter in toxicity tests using freshwater protozoa (Cairns et al. 1980). The colonization time, 3 to 5 weeks, is relatively short, and mature communities, in the sense of communities at a successional equilibrium, are not established under this period of time. The rate of colonization and growth is dependent on water temperature, nutrient availability, grazing and other factors changing with season in addition to the toxicant effects. The duration of the experiments is a compromise in order to obtain a sufficient amount of metabolically active biomass (for easy measurement of activities in the short-term tests and other analyses) and to avoid nutrient shortage which might cause sloughing, i.e. a sudden loss of biomass from the community giving highly variable samples.

Toxicants

Three different toxicants have been used as selection pressures in the long-term experiments. 4,5,6-trichloroguaiacol (4,5,6-TCG, paper I) is a chlorinated phenolic compound found in kraft mill bleachery effluents (Neilson et al. 1989, paper I). Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU, paper II-IV) is a herbicide inhibiting photosynthesis, at a well-known and specific target site on a 32 kD protein of photosystem-II (PSII; Moreland and Hilton 1976, Hansson and Wydrzynski 1990). The third toxicant used was tri-*n*-butyltin (TBT, paper IV), which is a biocide used in, e.g. anti-fouling paints. TBT inhibits the ATP-synthesis in both mitochondria (Aldridge et al. 1977) and chloroplasts (Watling-Payne and Selwyn 1974) at low concentrations.

All of the used toxicants needed cosolvents to achieve the high concentrations required in the stock solutions. In one case (4,5,6-TCG), ethanol under alkaline conditions was used. The dosing of sodium hydroxide into the aquaria caused the sea-water pH to increase, which was counteracted by addition of an equimolar amount of HCl. For the two other toxicants, acetone was used as cosolvent. Ethanol caused an enhancement of bacterial growth in the periphyton communities which got a "slimy" appearance (paper I) despite the low levels of ethanol in the aquaria. The use of acetone was not followed by such effects.

DETECTION

The detection of PICT involves the comparison of tolerances between communities, exposed and unexposed to toxicant selection pressures. The quantification of tolerance is by a short-term metabolic test (Blanck et al. 1988, Blanck and Wängberg 1988a) giving an EC-value, the concentration of a toxicant that inhibits the metabolic activity to a certain extent, as a measure of tolerance. The basis for tolerance detection by tests of metabolic inhibition is outlined in a proposed model for community tolerance which begins this chapter.

Sketch to a model of community tolerance

All measures of tolerance are relative, which means that all use of tolerance measures must be related to the tolerance of another organism or community - reference. For the detection of PICT a comparison of tolerances between toxicant-selected, and -unselected communities is necessary. Because toxic effects are perpetuated from lower levels (biochemical, physiological) of organization they may also be detected at these lower levels. The following sketch to a model of community tolerance is intended to give the motives for using short-term metabolic tests for detection of PICT and to give some concepts for discussion of factors affecting PICT.

The dose-response curve (Figure 1a) for a metabolic process may be apprehended as a cumulated probability density function (Sokal and Rohlf 1981) for the *tolerance* versus exposure (= dose or toxicant concentration; Figure 1b). When exposure is increased an increasing proportion of the area (representing total metabolic activity) under the distribution curve is inhibited (Figure 1b). This proportion can be subtracted from the initial value of unity, giving Figure 1c, the normalized dose-response curve.

The distribution of sensitivity of a community (Figure 1b) has three features that are of importance for the PICT methodology.

The first is the position along the exposure axis. The position determines the *relative tolerance* (or sensitivity) of the community when compared to other communities. The median of the probability density distribution corresponds to the EC₅₀-value as determined from the normalized dose-response curve (Figure 1c).

The second is the area under the curve, i.e. the total activity of the community, which is controlled by the number of organisms, their biomass and activity.

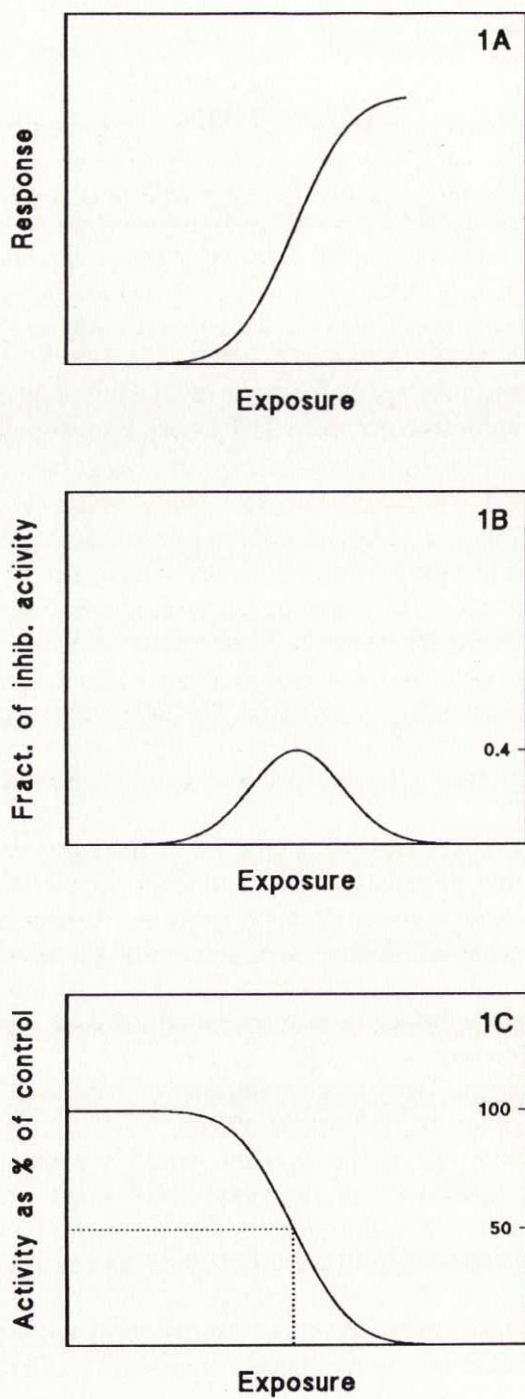


Figure 1. See text for discussion

The third feature is the range spanned by the distribution. This range marks the ends of the dose-response curve (Figure 1c), and depends on the community tested as well as on the short-term test parameter and the test compound used. Therefore differences in the slope of the dose-response curves between different test parameters and between different toxicants should be expected. This range may be called a *range of sensitivities*.

The distribution of sensitivities within the community can be partitioned into distributions for each of the species found in the community. All these distributions have their own characteristic position, area and range of sensitivities. Together they form the distribution of sensitivities giving the dose-response curve of the community when cumulated and subtracted from the initial activity.

The selection process can be viewed from this perspective. "Adaptation" of a species can e.g. be described as an expansion of the range of sensitivities *or* as skewing of the distribution towards higher concentrations, *or* as a transition of the entire distribution to higher concentrations. The composition of the community will, in a similar way, be reflected by the position and shape of the dose-response curve. The absence of species will for instance not necessarily influence the range of sensitivities. The exclusion of species in either extreme will however appear as a diminished range of sensitivity. When this exclusion is accomplished by the selection pressure of a toxicant and sensitive individuals are excluded the dose-response curve will shift towards higher exposure levels - this is PICT.

Possible explanations for different shapes of dose-response curves for communities can also be derived from the model. A community with its metabolic activity mainly from one or a few dominating species, dispersed along the exposure axis, may give dose-response curves with one or more plateaus. With this and the proposed model above as background it is clear that the detection of PICT by comparisons of dose-response curves is a statistical challenge.

The range of sensitivities is in most cases larger in a community, containing many species, compared with a species or a community with few components. The species dependent variation in sensitivity to chemicals can span three (Blanck et al. 1984), or in extreme cases up to six orders (Blanck and Björnsäter 1988) of magnitude among different species of microalgae. The larger range of sensitivities in communities is important for, at least, two reasons. The detection of an increased tolerance is facilitated by a large difference in tolerance - the larger the range of sensitivities the higher is the probability for a large, and easily detected, tolerance increase (Blanck et al. 1988). Secondly, the potential tolerance increase, which we may call the response range of PICT, is larger in a situation with a large range of sensitivities.

The artificial substratum as well as the environment in the aquaria system and all treatments of the periphyton samples represent "selection pressures" on the community that give structural deviations from epilithic and epiphytic periphyton communities found in the source ecosystem. Of the approximately 425 benthic diatom species in the Kattegatt/Skagerrak area about 110 have been found on glass discs, in the field or in the aquaria system (M.Kuylenstierna pers.comm.). At any occasion in the aquaria system no more than about 40 species have been found. The use of this subset in PICT-studies is however of minor importance as long as the selection pressures caused by handling and laboratory environment do not exclude species systematically in either tail of the sensitivity distribution. The number of species in the community and their variation in tolerance does however influence the *shape* of the dose-response curves and might restrict the use of regression techniques for estimation of EC-values.

The presence or absence of species in a community is claimed to be a sensitive response to stress (Gray 1989 and references therein). The problem with presence/absence data is however to link them to a toxicant since fluctuations in other environmental factors can cause shifts in species composition. If the shift in species composition can be linked to an increased tolerance (PICT) this may imply a toxicant impact on the community (Blanck and Wängberg 1988). Such close coupling between species composition and PICT have been reported by Blanck and Wängberg 1988a and b, Wängberg et al. 1990, paper I and IV. Thus PICT tends to bridge the gap between ecotoxicological measures based on the structure of communities and measures based on the function.

The choice of test parameter for short-term tests

The response of the test parameter is related to both the mode of action of the toxicant and to the tolerance mechanism of the organisms/community. When choosing between short-term test parameters the parameter giving as large response range as possible should be preferred. Such a parameter will improve the detection of PICT. The potential response range could be judged from the slope of the dose-response curves.

It is self-evident that the precision of the short-term test parameter will influence the detection limit of PICT. Test parameters that offer high precision and reproducibility is thus mandatory. A high test capacity is also required since a large number of samples is needed to accurately establish dose-response curves for several communities in a short-time. In our studies 20 to 48 samples of periphyton have been used to achieve sufficient descriptions of the dose-response curves.

In principle, any metabolic process that can be measured on the community level and that has a sufficiently large range of sensitivities can be used as a

short-term test parameter. The short-term test must also be relatively quick since no selection during the test can be allowed. The test parameter of choice should also give a good reflection of the structure of the community. Metabolic processes that are performed only by a small fraction of the community should generally be avoided, unless the mode of action is known and a highly specific short-term test is desirable. For many toxicants both mode of action and tolerance mechanisms are less well known and in such cases a more general approach has to be used.

In paper I-IV the inhibition of photosynthesis, estimated as incorporation of inorganic ^{14}C -carbon (ΣCO_2), was used as test parameter in the short-term experiments. The method is relatively easy, giving the high test capacity needed.

Incorporation of ΣCO_2 in periphyton communities includes however both photosynthesis of microalgae and cyanobacteria, and light-independent processes in a range of microorganisms (Overbeck 1979).

Photosynthesis is a highly integrated process giving the possibility of detecting disturbances in many different metabolic subsystems while the light-independent incorporation is an anapleurotic reaction of the TCA cycle aiming at regeneration of metabolic intermediates (Overbeck 1979). In a community both processes occur concomitantly in light. Photosynthesis is however dominating under normal conditions; the light-independent CO_2 -incorporation amounts only to a few per cent of the total incorporation (page II:10, Figure 8). Both photosynthesis and light-independent CO_2 -incorporation are processes with many possible sites for inhibition as well as for regulation and adaptation. Differences between individuals and species in these systems give differences in tolerance, these differences are the sources of PICT.

Variation in base-line tolerance

The detection of PICT involves a comparison of tolerances between a selected, tolerant, community and an unselected community with the original tolerance; the base-line. This base-line tolerance is not absolute but differs among communities and fluctuates with season due to variations in species composition and phenotypical adaptations caused by other environmental factors than toxicants, such as nutrients and light. As an example there was a strong influence of nutrient regime on arsenate tolerance (Wångberg and Blanck 1990, Blanck et al. 1989). High cell content of phosphorus caused a large (3 orders of magnitude) increase in tolerance, which is understandable since arsenate works as a phosphate analogue. Arsenate seems to be an extreme since the base-line tolerance for diuron was found to vary within a factor 2-3 (EC_{50} 31 nM, SD 15 nM, $n=56$) in periphyton communities sampled from various localities in the Gullmars

fjord archipelago, at the west coast of Sweden, during the period April 1988 to October 1991 (B.Dahl pers.comm.).

The potential for variability in base-line tolerance appears to be highly dependent on the toxicant and related to its mode of action of the toxicant and the nature of the tolerance mechanisms.

The implications of variable base-line tolerances include the proper choice of unexposed control communities and the determination of the tolerance for these. Knowledge of the base-line tolerance variation is also important to distinguish PICT from normal variations in base-line tolerance when control communities from different localities are used. When microcosms are used, and not run for extended periods, this variation in base-line tolerance is no serious problem since the variation seems to be more related to factors varying at larger time and room scales, which means that variation in base-line tolerance occur between experiments and not within them.

Relations between short-term effects and long-term effects

The model sketched above implies a close connection between inhibitory concentrations of a toxicant in a short-term test of an unselected community and the concentrations that affect the community in long-term experiments. For three of the so far investigated toxicants, arsenate (Blanck and Wängberg 1988b), 4,5,6-TCG (paper I), and TBT (page IV:9 Figure 2, and page IV:13 Figure 4, Dahl and Blanck 1990), there is a close relationship between concentrations causing inhibition in short-term tests and concentrations affecting periphyton communities in long-term experiments. Effects detectable on a low level of organisation does, however, not automatically give rise to effects on higher levels due to the possibility of physiological adjustments (metabolic regulation, phenotypic plasticity, adaptation etc.) which may counteract the direct effect. Diuron is such an exception (paper II).

The short-term test with diuron was more sensitive (at least a factor 4) than all other effect parameters including PICT (page II:12, table II, and page II:14, Figure 9), which suggests compensating mechanisms not manifested in the short-term tests, such as e.g. synthesis of chloroplast membrane proteins, or that the photosynthetic capacity originally has a large redundancy which can allow for a certain inhibition without affecting net production. Thus, the assumption of a simple relation between the long-term and the short-term effects, i.e. the same concentration of diuron giving effects in both short-term and long-term tests, is not valid in this case.

A possible explanation to these observations is related to the mode of action of the herbicide. Diuron is a specific inhibitor of the photosynthetic electron transport at the binding site of oxidized plastoquinone (PQ) on the D1 protein of

PSII (Pfister et al. 1981, Wolber and Steinback 1984). The photosynthetic electron transport system is however able to cope with variations in both light-quantity and light-quality due to different regulatory mechanisms (Powles 1984, Anderson 1986, Raven 1989, Smith et al. 1990). Cyanobacteria, treated with sublethal doses of PSII inhibiting herbicides, have shown responses similar to an adaptation to low light (Koenig 1987, 1990, Hatfield et al. 1989). This is logical since evaluated at a position in the electron transport down-stream the D1 protein, low concentrations of diuron is comparable to low light conditions, both giving a reduced electron flow. This might trigger an adaptive response, restoring the capacity of the electron transport system. Regulatory mechanisms giving this result have been proposed by Melis et al. (1985) and Fujita et al. (1987). Hatfield et al. (1989) have characterized diuron adapted *Anacystis nidulans* and found increased chlorophyll *a* and lipid contents but no effects on electron transport or *in-vivo* fluorescence kinetics. Such a mechanism could contribute to the observations of increased chlorophyll *a* content between 4 and 40 nM (page II:9, Figure 4), provided that the chlorophyll *a* increase was not caused by reduced grazing. This seems however improbable since the EC₅₀ for freshwater crustaceans is at least 15 times higher than the concentrations giving increased chlorophyll *a* content in our experiments (Mayer and Ellersieck 1986).

The chlorophyll *a* increase (page II:9, Figure 4) implies that low concentrations of diuron can be overcome by mechanisms of homeostasis, and accordingly, effects of low diuron concentrations might be detected with the short-term test, but not in the long-term test, depending on the time for the regulating systems to react and compensate for the diuron exposure. Thus, the inherent possibility of regulation at different points in the photosynthetic electron transport system might be reflected in the relatively high concentrations needed to give long-term effects compared to the concentration that causes the first effects in the short-term test.

A prerequisite for this tentative explanation is that diuron does not give permanent damages to the photosynthetic apparatus. Böger and Schlue (1976) found, in a study with diuron on *Bumilleriopsis*, that the photosynthetic activity of the alga, cultured at concentrations reducing the growth to 20-30% of control, was restored when transferred to herbicide-free medium. These observations are consistent with our finding that no detrimental effect of diuron was detectable on the chlorophyll *a* specific photosynthetic rate (P_{stc} ; page II:11, Figure 7), i.e. the chlorophyll *a* specific CO₂-incorporation was unaffected or increased when the diuron exposure was released (diluted) in the concentrations of the short-term tests that were lower than the respective long-term experiment concentration.

Conclusions

I conclude that it was possible to use short-term tests of photosynthesis, as measured with ^{14}C -incorporation to detect PICT, and that in three cases of four there were good agreements between concentrations giving inhibition in short-term tests and those concentrations that gave effects on communities in long-term experiments. In the diuron case the short-term test gave a false positive response (at least a factor 4 too sensitive), for which we have a tentative explanation. The short-term test may thus under certain circumstances be an unreliable estimator of effects (PICT or others) since long-term exposure may trigger off mechanisms of homeostasis that might give unanticipated effects in the short-term tests. Thus, the short-term test cannot *per se* uncritically be used for the purpose of an ecotoxicological evaluation. Despite this shortcoming the short-term test can be used to detect PICT and in that respect be used for evaluating the long-term effects.

VALIDITY

The validation discussed in this chapter is a mere comparison between PICT and other test parameters. PICT is thus said to be a valid estimator when there is an agreement between PICT and other test parameters involved in the comparison. The first main question, from an ecotoxicological point of view, is whether PICT can estimate other long-term effects on the periphyton community. The comparison is thus between the concentrations in the long-term experiment that give PICT and those which cause other effects on the periphyton community.

The validity of conclusions based on the PICT response is of obvious interest for the use of PICT as an ecotoxicological tool. The rather narrow perspective employed in this work differs much from the views expressed by Cairns and Smith (1989), who points out the more general criteria for validation of test systems as predictive tools. The validation undertaken in this work is based on dependent data sets and not intended for an evaluation of PICT-based predictions into real ecosystem situations, so in the context of Cairns and Smith (1989) this validation can be considered as the first step in a more rigorous validation of predictive PICT-studies.

For the purpose of validation, toxicant effects on both structural and functional parameters, other than community tolerance, were recorded.

Parameters

In all papers the content of **chlorophyll *a*** has been used as an indicator of periphyton microalgal and cyanobacterial biomass. Chlorophyll *a* is in most cases a valid approximation for biomass in comparisons between the long-term exposures, since it is found that for most species of microalgae the chlorophyll *a* content is within 0.5-2% of dry-weight (1-4% of ash-free dry-weight; Reynolds 1984). It is however known that algal responses to some PSII inhibitors resemble shade adaptation and result in changed pigment composition of the algae (Anderson 1986, paper II and references therein). In paper I **total carbon** and **total nitrogen** were used in parallel to chlorophyll *a* as indicators of periphyton biomass.

Microalgal species richness (number of species) has also been used as a measure of toxicant impact in the long-term experiments. Changes in microalgal, or mostly diatom, species richness were used as a measure of toxicant effects on community structure, instead of various available quantitative indices (Green 1979).

In paper II two **functional measures** of toxicant impact were used beside the detection of PICT. Both the chlorophyll *a* specific photosynthetic rate (P_{stc}) and the fraction of light-independent ^{14}C -incorporation (CI_{li}/CI_{stc}) were derived from the short-term tests.

To estimate the impact on periphyton biomass of **grazing** two different types of grazers were studied at two occasions. In paper I we report the effects of 4,5,6-TCG on Nereid abundance and in paper IV the abundance of copepods as influenced by mixtures of TBT and diuron. The composition of grazers found in the aquaria is highly dependent on the present situation in the fjord, from which the aquaria water is taken, where natural variations between seasons and years occur. For both nereids and copepods effects on the grazers gave effects on periphyton biomass.

Comparisons and discussion

In the experiment with 4,5,6-TCG (paper I) we found PICT concomitantly with a drop in diatom species richness and chlorophyll *a* content at the highest test concentration (page I:10, Figure 3, and page I:12 table IV). Between 1 and 12 μM of 4,5,6-TCG was required to affect the periphyton photosynthesis as judged from the short-term tests on previously unexposed periphyton communities. When the exposure in the aquaria exceeded this range primary toxicant effects on the algae were recorded as decreased species richness, decreased chlorophyll *a* content and PICT. Thus, a good agreement was found between short-term inhibition of ^{14}C -incorporation, PICT, species richness, and decreased chlorophyll *a* content.

Similar agreements between PICT, inhibition of short-term ^{14}C -incorporation (unexposed communities) and decreasing species richness and chlorophyll *a* content have been found for arsenate (Blanck and Wängberg 1988a and b) and TBT (Dahl and Blanck 1990).

Different relations between the test parameters appeared however in the study of diuron impact on periphyton communities (paper II). The results are summarized in Figure 9, page II:14. Also in this case, PICT was found in the same concentration range (>40 nM) as a decrease in both microalgal species richness and chlorophyll *a* content. At lower concentrations both species richness and chlorophyll *a* content was however slightly increased compared to control values.

Increases in chlorophyll *a* content at low concentrations of atrazine have also been found in studies with another PSII inhibiting herbicide, atrazine, in artificial ponds (Larsen et al. 1986), and microcosms (Larsen et al. 1986, Pratt et al. 1988). It is plausible that mechanisms similar to those described in the literature

and cited above ("Detection", page 11) have caused these effects since the modes of action are similar for atrazine and diuron.

The increase in diatom species richness found in the concentration range 8-40 nM (page II:10, and page II:12, Table II) is also paralleled by observations from microcosms studies with atrazine at low to intermediate concentrations (3.2 - 32 µg/l, 15 - 150 nM; Pratt et al. 1988). Gray et al. (1990), in a study of marine benthos in a pollution gradient from an oil platform, found increased species number at intermediate distances from the platform. The interpretation, following Connell (1975), was "that disturbance gives an advantage to some species which increase abundance, and leads to eradication of some rare species, whereby different species are able to colonise in low numbers." In a similar way low concentrations of PSII inhibitors may give advantages to certain species, expelling other species and giving room for newcomers. Evidence for this is given in paper II, Figure 5, page II:10, and table II, page II:12, where the exclusion of the Rhodophycean-algae at concentrations higher than 8 nM was followed by an increase in the diatom species richness in a concentration range between no-effect and clearly deleterious effects. The increase in species richness together with the increase in chlorophyll *a* may thus be considered as the initial responses to stress, followed by decreasing chlorophyll *a* content and microalgal species richness at higher concentrations. For some presently unknown reason PICT failed to detect this slight response, and is thus not valid in this case.

It is presently unknown if PSII inhibitors are exceptional cases or if the response scheme with increased species richness and biomass can be found for other toxicants. A necessary methodological prerequisite for the detection of the intermediate increase in species richness is a sufficiently close spacing of concentrations in controlled exposures. The paucity of data for toxicants may be related to the common practice of running only a few treatments in micro- or mesocosms experiments isolated from surrounding ecosystems. Thus, the detection of increased species richness at intermediate exposures is impossible since colonization is a necessary prerequisite.

In similarity to the studies with 4,5,6-TCG and arsenate the study of combined toxicity with TBT and diuron (paper IV) reported an agreement between PICT and a decrease in species richness, while the decrease in chlorophyll *a* appeared in concentrations giving very high tolerances. The comparison between the short-term test and PICT is more complicated in this case since two toxicants are involved, the relationship seems however to be influenced by the diuron exposure in the same way as discussed above for single-toxicant exposures.

Conclusions

We may conclude that PICT is a valid estimator of effects on the algal community as reflected by decreased species richness. This implies that exclusion of species is the main reason for tolerance increase, while adaptations on a physiological level is of less importance. The initial increase in species richness found in the diuron studies (paper II) is not reflected by PICT. The reasons for this is not presently understood, but might be related to the detection limit of PICT.

PICT is also a valid estimator of effects on algal biomass or chlorophyll *a* content when effects on biomass is not confounded by grazing.

SPECIFICITY

The specificity of PICT is influenced by the short-term test parameter used for detection of PICT, the mode of action(s) of the toxicant(s) under study and the tolerance mechanism(s). Since this "equation" contains at least three variables the outcome might be immensely complex. The following treatment of the specificity of PICT focuses on three different aspects.

The first concern is the possibility to separate primary from secondary toxicant effects using PICT.

The second is the degree of specificity of PICT in a situation when cotolerance occurs between chemicals.

The third concern is the ability of PICT to detect effects in a situation where two toxicants exert their selection pressures concomitantly.

Primary and secondary effects

The ability to distinguish between primary and secondary effects of toxicants is a key problem in ecotoxicology for understanding the complicated cause-effect relationships in ecological systems. An obvious situation appears when the periphyton community structure is controlled by grazing and the grazers are more sensitive to the toxicant than the periphyton is. Two such cases were reported in paper I and IV respectively. In both cases the periphyton biomass, estimated as chlorophyll *a* content, increased with initially increasing toxicant exposures as a consequence of reduced grazing (page I:10, Figure 3, and page IV:15-16, Figure 6). The PICT response, however, followed the decrease only in species number and chlorophyll *a* content at even higher concentrations. Thus PICT was able to distinguish the primary effects, decrease in species richness and chlorophyll *a* content, from a secondary effect, the increased chlorophyll *a* content at lower exposure levels.

In the study of paper II a more complicated situation appeared when the first primary effects seemed to be an increased chlorophyll *a* content, due to physiological adaptations, and an increase in species richness (paper II, Figures 4, 5 and 9). These slight effects were not mirrored by an increased tolerance. At higher concentrations the consistency between PICT, and decreases in species richness and chlorophyll *a* content was found.

PICT is not a valid estimator of effects on the grazers, since their tolerance is not assayed in the short-term tests.

Cotolerance

Cotolerance (cross-resistance) arises when an organism or community is exposed to a selection pressure and gains increased tolerance for the compound exerting the selection pressure together with tolerance for other compound(s) which not occurred as selection pressure(s). Cotolerance can be expected to occur between toxicants which share some property related to chemical structure or mode of action (Blanck et al. 1988 and references therein). Two classes of mechanisms on the biochemical level are generally claimed to cause cotolerance. The first class is tolerance mechanisms related to uptake, translocation or metabolization/excretion of the toxicant, while the second class is tolerance mechanisms related to modifications of the target site or bypass reactions. The combination of these two mechanisms may also occur. In multicellular organisms the situation is further complicated on a physiological level due to differences between tissues and organs in their sensitivity to toxicants (Brattsten et al. 1986).

In a community cotolerance may occur as a response to toxicant selection pressure, due to the mechanisms outlined above, but also as a stochastic event, i.e. caused by chance. The reason for this is that species originally vary in their tolerance to different chemicals, they have different "tolerance patterns". Thus, among the remaining species in a selected community, some species might originally have been more tolerant for certain chemicals than the excluded species. In such a case cotolerance will be detected without any strict mechanistic connection. An analogy is bacterial plasmid systems, which in some cases are found to convey cotolerance between antibiotics and heavy metals (Silver and Misra 1988).

In paper III we examine the potential of PICT to distinguish between tolerance mechanisms on the biochemical level giving a high potential specificity. The approach was to establish a tolerance for the herbicide diuron and then detect the cotolerance pattern with a set of toxicants with modes of action more or less related to the mode of action of diuron. A high specificity of cotolerance patterns for herbicide tolerance conveyed by mutations in the active site of the photosystem-II D1 protein have been found (Mets and Thiel 1989). The specificity is occasionally so high in these biochemical systems that cotolerance does not appear even for toxicants attacking the same target site (Brusslan and Haselkorn 1988, Mets and Thiel 1989).

The found cotolerance pattern (page III:7, table 2, and page III:8, Figure 2) was similar, but not identical, to any of those reported for chloroplasts and microalgae (Mets and Thiel 1989) tolerant to diuron. Our interpretation was that PICT was able to distinguish between the major groups of herbicides (urea/triazine-type and phenol-type) included in the study, as well as to sort out an uncoupler of photophosphorylation. The very high specificity found in

studies of cotolerance patterns with chloroplasts and single microalgal species was however not reached. The reason for this might be that many different tolerance mechanisms act concomitantly in the community, giving an indistinct cotolerance pattern.

In the study reported in paper IV and also in other studies with TBT (Dahl and Molander unpubl.) a cotolerance between TBT and diuron appeared. Since TBT and diuron are known to have different modes of action this cotolerance must be caused by another mechanism than by differences in the target site. Several mechanisms conferring cotolerance between chemically unrelated toxicants are known from the literature, e.g. for cancer drugs (Endicott and Ling 1989), and for heavy metals (Silver and Misra 1988, Hogstrand 1991).

The consequences of cotolerance in PICT-studies regards the specificity of PICT, especially when PICT is used in field studies to imply a close cause-effect connection between an affected community and a certain toxicant. At present, when the knowledge of cotolerance patterns still is mostly lacking, the interpretation of PICT in situations with unknown exposure(s) must be made with caution. The increased tolerance, indicating an affected community, is undisputable if distinguishable from base-line tolerance, the cause is however to be found within a group of chemicals.

Interactions

In situations with more than one toxicant interactions between toxicants may be important. In the ecotoxicological context this is of interest since many toxicants are found together in wastewaters and recipients (Vouk et al. 1987 and references therein). Interactions are by definition results deviating from the expected, the question is however "what is expected?". According to Morse (1978) two different "reference models" can be distinguished, to assess whether the measured effects deviates from the expectations, showing interaction or not (see also reviews by Hatzios and Penner 1985 and Bödeker et al. 1990). The reference models are called the "additive dose model" (ADM) and the "multiplicative survival model" (MSM). The ADM assumes a qualitative similarity in the mode of action (simple similar action *sensu* Bliss 1939) of the toxicants while the MSM assumes toxicants with completely different modes of action (independent action *sensu* Bliss 1939). However, Drury (1980) states that "by choosing an algebraic model of a function, $f(x,y)$ one by that very fact also chooses the interaction". Moreover, as pointed out by Unkelbach and Pösch (1988), when the dose-response curves of the toxicants under study have slopes with a certain difference, the expected model is ADM in one dose range and MSM in another. An additional argument against the adoption of one or the other of these models, stemming from the assumptions regarding modes of action underlying the models, is that

both models can be considered as extreme points on a scale where many combinations of chemicals must be regarded as falling between these models. It is for instance well known that chemicals with a very specific mechanism at low concentrations can act much more unspecifically at higher concentrations, violating the idea of completely different mechanisms of the MSM. On the other side, many pesticides have completely different mechanisms of toxicity and can not be expected to follow the ADM, when mixed.

Since none of the proposed models is generally applicable a mere description of the response-surfaces seems appropriate. This can be achieved using central composite designs (CCD). The use of a CCD gives a good description of the dose-response surface and does not dispense the possibility of detecting "interactions" or nonlinearities while the number of experiments is kept to a minimum. This is in contrast to the application of special, and laborious methods to detect deviations from the two reference models (Flint et al. 1988, Altenburger et al. 1990). In paper IV two-toxicant short-term tests following a CCD was used for description of short-term inhibition of carbon incorporation together with the conventional single toxicant short-term tests.

The result of the modelling using a CCD is summarized on page IV:11, Figure 3, and page IV:12, Table II. From these models two measures of tolerance, max-

Table 1. Coefficients of fitted models and statistics of the polynomials approximating the long-term effects on various tolerance measures from short-term tests of periphyton communities exposed to combinations of TBT and diuron. The coefficients of each model are given together with their calculated significance (p), the coefficient of determination for the model (R^2), and the significance of the F-test of $MS_{residuals}$ over MS_{model} coefficients and the F-test for lack-of-fit ($MS_{pure\ error}$ over $MS_{lack\ of\ fit}$). The numbering of the coefficients refers to "Materials and methods" of paper IV.

EFFECT	n	k_1	p	k_2	p	k_3	p	k_5	p	R^2	$P_{(m)}$	$P_{(lof)}$
EC ₅₀ -TBT	11	870.1	.0000	447.23	.0043	459.7	.0037	80.1	.6156	.8728	.022	.577
EC ₅₀ -diuron	12	65.4	.0000	22.65	.0231	23.39	.0201	11.4	.3477	.7601	.0532	.057
Max-EC ₅₀	12	512.5	.003	416.36	.0426	694.0	.0039	711.7	.0196	.8347	.0279	.603
Surface-EC ₅₀	12	37153	.0031	30356	.0424	38930	.0148	29542	.1355	.7624	.0523	.544

EC_{50} and surface- EC_{50} (for definitions see page IV:7-8), were derived for evaluation of PICT, together with conventional EC_{50} -values derived from single-toxicant tests. As shown in Figure 4 and 5, page IV:13-14, the occurrence of PICT was clear regardless of the measure used. PICT was thus detectable also in a situation with two concomitant selection pressures.

The results of modelling the tolerance-data from the long-term experiment with mixtures of TBT and diuron are given in Table 1 and Figure 2. The same techniques were used as for the short-term tests (see page IV:7-8).

The contour-plots of all four tolerance measures (Figure 2) show more or less bending. This indicates a slight deviation from the ADM, which should be straight lines connecting points of equal response on the two axes. Whether this result is a synergism (=deviation from expected ADM) or not is an open question, despite high statistical significances (Table 1), since a tolerance-increase surface like Figure 1 might be the expected outcome, if the tolerance-increase curves of TBT and diuron have different slopes (Unkelbach and Pöch 1988).

Ben-Shlomo and Nevo (1988) studied the differential survivorship of allozyme genotypes in populations of *Palaemon*-shrimps exposed to cadmium and mercury singly or in combination, and proposed that the combined selection pressure of the two toxicants acted as a third, unique, selection pressure giving a specific result in terms of the surviving population. In our investigation of the combined toxicity of TBT and diuron (paper IV) we arrive at another conclusion, based on arguments of the relative magnitudes of the tolerance measures. Since all tolerance measures, including the single-toxicant based EC_{50} -values, indicated PICT, and since the max- EC_{50} reflects the properties of the two single toxicants we conclude that the mixture of the toxicants does not act as a third, unique selection pressure.

The specificity of PICT can thus be regarded as sufficient for detection of the combined primary effects of TBT and diuron on the periphyton community, as well as to recognize the contributions from the two toxicants in the combined effect.

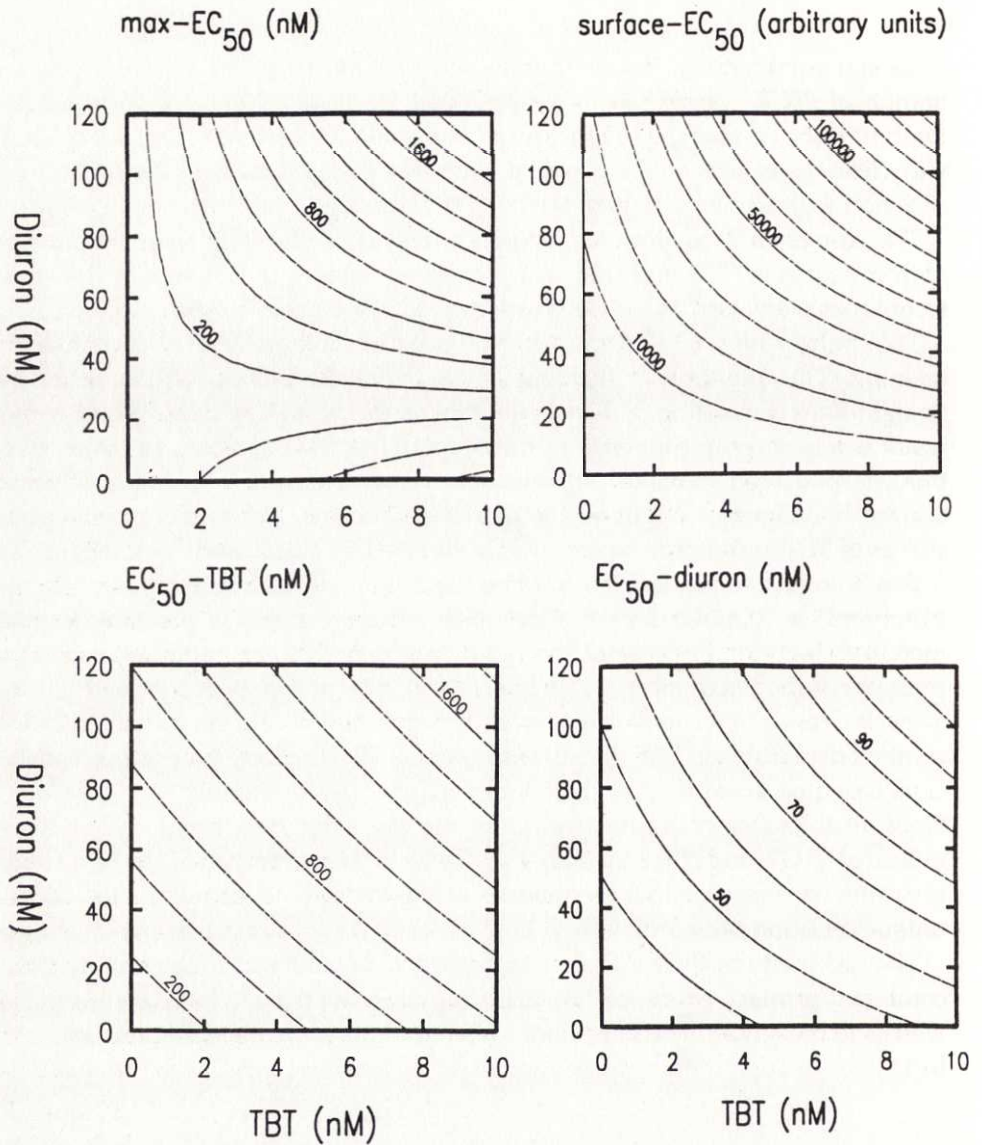


Figure 2. Induced community tolerance of periphyton communities exposed to mixtures of TBT and diuron. The contour plots show models fitted to the responses of the four tolerance measures; max- EC_{50} , surface- EC_{50} , EC_{50} -TBT and EC_{50} -diuron (see also Table 1).

DIRECTIONS

Future research prospects for the PICT methodology are mainly related to two problems - the detection of the induced tolerance and the mechanisms of cotolerance.

The first can be approached by studying PICT with other communities and on different trophic levels using different short-term test parameters suitable for these communities and with regard to the biological process of concern. This would widen the use of PICT to other communities than periphyton and phytoplankton.

The central questions with cotolerance are between which chemicals cotolerance will occur and which chemical and/or biological properties of the toxicant/organism system that are the determinants of cotolerance. A challenge is also to find and define groups into which chemicals can be classed, since with this knowledge predictions of cotolerance patterns might be possible. Such a knowledge together with detection of PICT using mixtures (paper IV) might be useful for assessing impacts, in field situations, of toxicants not identified.

With these tools available, experimental work might give the information necessary for better predictions of toxicant impact on biota than the presently available methods attain.

The PICT methodology is linked to a fundamental biological feature - the possibility of functional adaptation within organisms, communities and ecosystems. A consequence of this adaptability of biota is that stress will cause change, sooner or later giving an ecosystem with biota adapted to the situation, regardless of the causes of stress; manmade or natural.

The question of how much the ecosystem might sustain of chemical stress is thus closely connected to the question of the change of the ecosystem. Sketches to scientific criteria for "healthy" ecosystems have been formulated focusing on different aspects; e.g. sustained production and yield, sustained species diversity and sustained self-regulation of the ecosystems (Odum 1985, Rapport et al. 1985, Schindler 1987, Cairns 1991).

The question is however not only a scientific one but also an ethical - "Which changes in the ecosystems are acceptable, or desirable?" The development of science and technology in the western societies have forced us to put the question since man now is able to change also the course of natural history (Idso 1989). The answer to the ethical question can however hardly be found from within the reductionist system of science and technology. - Anyhow, humility is part of the answer.

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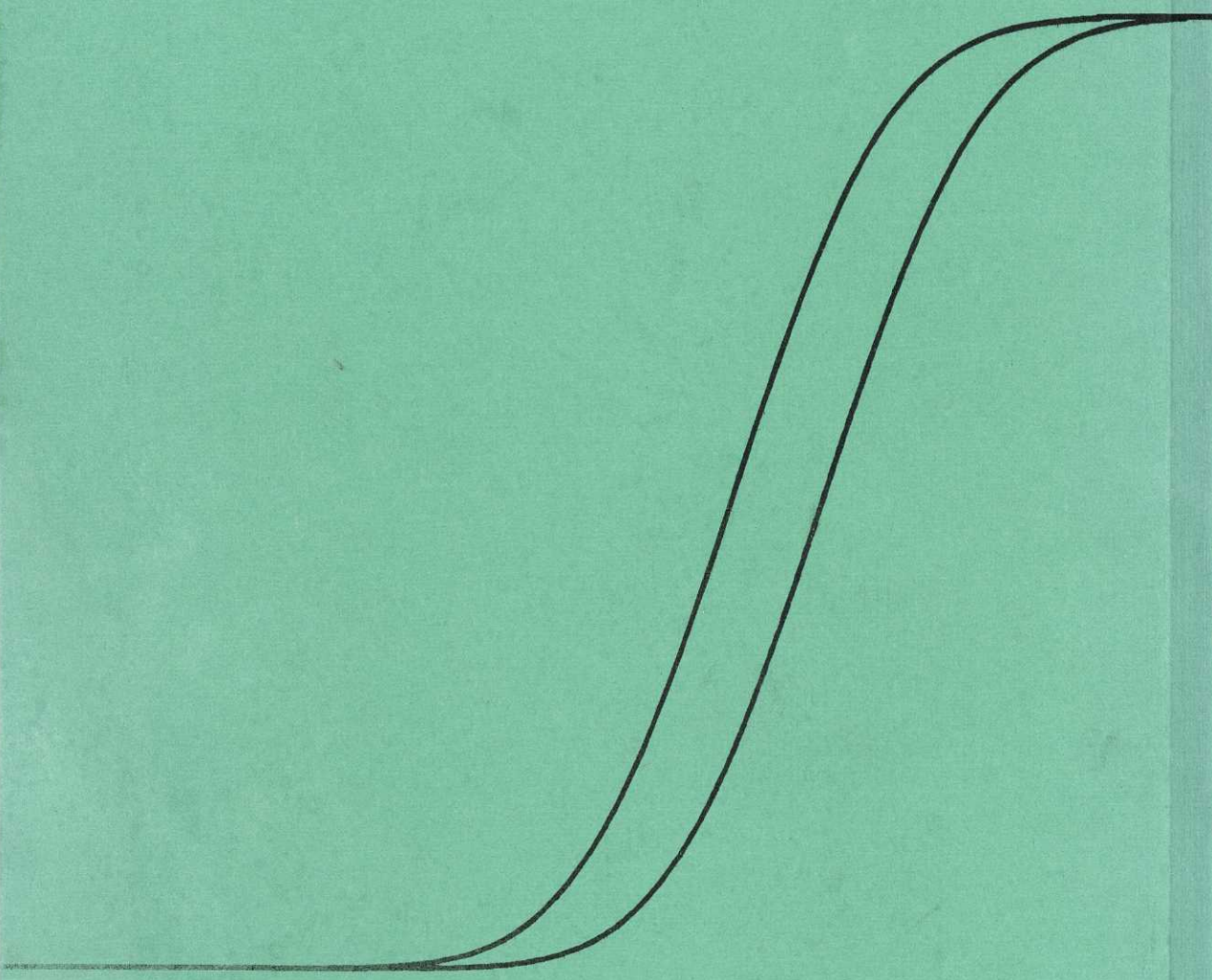
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