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Combined Effects of Tri-*n*-butyl Tin (TBT) and Diuron on Marine Periphyton Communities Detected as Pollution-Induced Community Tolerance

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Abstract. The effects of combined toxicity were studied, using marine periphyton communities exposed to mixtures of tri-*n*-butyl tin (TBT) and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU) in indoor aquaria during four weeks. The experimental design of the study followed a central composite design (CCD) and utilized dose-response surface methodology for evaluation of the results. The detection of pollution-induced community tolerance (PICT) was accomplished by short-term (1 h) tests on inhibition of photosynthesis. Both single-toxicant and two-toxicant short-term tests were used. Two tentative measures of tolerance are proposed to achieve convenient comparisons of the tolerances from the two-toxicant tests. With the detection of PICT, effects of the long-term exposure were recorded on diatom species richness, chlorophyll *a* accumulation and copepod abundance. The decrease of diatom species richness was accompanied by an increased tolerance (PICT), which was detectable by all tolerance measures used. Primary effects on microalgae were recorded as a decrease in chlorophyll *a* at higher toxicant concentrations. At lower concentrations, primary effects on copepods were found, which resulted in reduced grazing and increased chlorophyll *a* content.

Ecotoxicological testing aiming at predictions of effects in the field still mostly employs single species/single substance tests, despite the considerable difficulty in extrapolating results from simple test system to effects of complex effluents in receiving ecosystems (Blanck 1984; Cairns and Pratt 1989; Okkerman *et al.* 1991). Tests with more than one toxicant have been performed (see reviews by Sprague 1970; Alabaster and Lloyd 1982; Vouk *et al.* 1987) but only very few have been done with more than three components (Könemann 1981; Hermens and Leeuwangh 1982; Deneer *et al.* 1988), unless effluents have been tested. Almost all testing for joint toxicity are performed with one species test systems. A very limited number of studies have employed complex biological test systems, at the community level, and with more than one test substance (Cairns *et al.* 1990).

Pollution-induced community tolerance (PICT) has been used to detect effects on microalgal communities and its validity has been examined (Blanck *et al.* 1988; Blanck and Wängberg 1988a; Molander and Blanck 1991; Wängberg *et al.* 1991). The PICT-concept is based on the idea that toxicants act as selection pressures (Luoma 1977; Pitelka 1988 and references therein; Klerks and Levinton 1989 and references therein), when applied at high enough concentrations over long enough time. When the toxicant selection pressure is acting on a community with species or individuals showing a variation in sensitivity, the community is restructured, and due to the exclusion of the sensitive components the community tolerance increases (*i.e.*, PICT appears). This increase is detectable with a series of shortterm toxicity tests (giving a measure of the community tolerance), and claimed to reflect the primary effect of the toxicant on the community. Thus, PICT is indicated by the gradual increase of the community tolerance, as a response to an increasing long-term exposure. So far the PICT-methodology has not been examined in a situation where communities have been exposed to several concomitant toxicants.

The aim of this study was to examine the detection of PICT in a situation of combined toxicity, with short-term tests using both individual toxicants and mixtures, and to relate PICT to other effects of the long-term exposure, such as effects on biomass (chlorophyll *a*) and diatom species richness.

In this work, where marine periphyton communities were used, we studied the combined toxicity of two, mechanistically unrelated and highly toxic biocides: 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, diuron) and tri-*n*-butyl tin (TBT). Diuron is a specific inhibitor of the photosynthetic electron transport, with the target site at the photosystem II (PS II) acceptor side (Izawa 1977; Renger 1986; Hansson and Wydrzynski 1990). The light-dependent toxicity is exerted by decreasing the electron transport capacity through PS II, thus causing both a decreased CO_2-incorporation and damage to the chloroplast membrane system. TBT is an organotin compound, used as an active component in antifouling paints, and shown to inhibit ATP-synthesis in both chloroplasts (Watling-Payne and Selwyn 1974) and mitochondria (Aldridge *et al.* 1977).

To give a good description of the combined toxicity, efficient strategies for the experimental settings are required, since the number of treatments increase exponentially with the number of toxicants. However, experimental schemes efficient in order to modelling complicated phenomena are well established, and

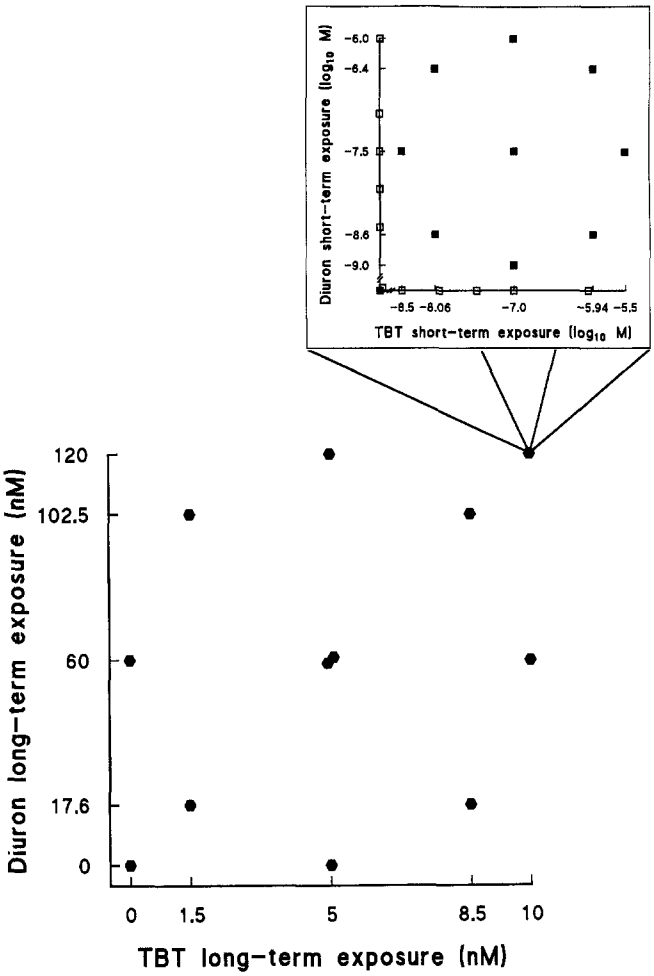

Fig. 1. Illustration of the central composite designs (CCD's) used in (i) the long-term exposure of marine periphyton communities to TBT and diuron and (ii) the short-term experiments for detection of pollution-induced community tolerance (PICT). Open squares in the short-term design refers to concentrations used in single-toxicant short-term tests

statistical experimental designs such as factorial, fractional factorial and central composite designs (CCDs), are well known tools for generating balanced conditions for statistical tests (Bayne and Rubin 1986; Box *et al.* 1978). Here, the chosen experimental outline was a central composite design. 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 are kept to a minimum.

Materials and Methods

Experimental Design

Central composite designs (CCD) were used for both the long-term exposure (selection) and the short-term (detection) exposure (Figure 1). In the long-term experiment five concentration levels of each of the toxicants were used, giving 11 different combined treatments, with the single constituents ranging from 0 to 10 nM TBT and 0 to 120 nM diuron. In addition to the usual star-shaped CCD a control-treatment, and a treatment of 10 nM TBT and 120 nM diuron were used. The center treatment (5 nM TBT + 60 nM diuron) in the design was duplicated. Former experience with the toxicants acting alone were used to choose long-term exposure levels ranging from just detectable to strong effects on the periphyton communities as measured by diatom species richness, biomass (chlorophyll *a* content) and PICT. Tolerance was quantified by a short-term test of photosynthesis inhibition using mixtures of the toxicants. The short-term experiments consisted of six concentration levels of each toxicant, which gave 10 different combined treatments. The concentrations of the single constituents ranged from 0 to 3.2 μM for TBT and from 0 to 1.0 μM for diuron. There were four replicates of each treatment, except the center point which was run with 12 replicates.

Short-term tests with the single toxicants were also performed using geometrical series of five concentrations ranging from 3.2 nM to 1 μM for both TBT and diuron. There were four replicates of each treatment while the control was run with six replicates (Figure 1).

Marine Microcosms

A marine microcosm system at Kristineberg Marine Biological Station (KMBS) by the Gullmar fjord on the Swedish west coast was used for periphyton colonization and long-term exposure to toxicants. The experiment ran from 900511 to 900607. Water from the fjord, with its indigenous microbiota, was pumped from about 3 m depth into the laboratory by an air-driven teflon-membrane pump (Dominator Maskin AB, Sweden). A continuous water-flow through twelve 22-L glass aquaria was maintained by a flow distributor according to Granmo and Kollberg (1972) but modified to a radial symmetry. The mean flow rate was about 200 ml/min giving a mean residence time of about 120 min. Water solutions of diuron (99% purity for analytical purposes, Pestanal-quality, Riedel-de-Haën, Germany) and TBT (tri-*n*-butyltin chloride >97% purity, Fluka AG, Germany) were prepared from acetone stock solutions by diluting with water purified by reversed osmosis giving an acetone concentration of 1 ml/L. The acetone solutions were stored cold and in the dark. The water solutions of diuron and TBT were prepared and kept, for a maximum of 7 days, at room temperature (18–22°C) and covered from the light of the aquaria, to minimize the risk of photodegradation. The water solutions were delivered to the aquaria by means of a peristaltic pump (Ismatech IPN 26, Ismatech AG, Switzerland) and subject to a 450-fold dilution in the aquaria giving an acetone concentration of 2.2 μl/L. Flow rates of diluting sea water and water solutions of toxicants were checked daily and adjusted when deviating from desired values. The accuracy of the dosing was checked by analysis of the toxicants in the aquaria during earlier experiments and found to be between 65% and 100% of nominal concentrations for diuron and from 85% to 155% for TBT. Each aquarium was equipped with a stirring device to maintain a thorough mixing and to minimize the boundary layer between bulk water and periphyton. The aquaria was illuminated from above by two fluorescent tubes (Osram Lumilux Daylight L 18W/12) that gave a photon flux density of about 125μE m^{-2} s^{-1} at the water surface. Periphyton communities colonized cleaned glass discs (area = 1.5 cm^2, 170 per aquaria) mounted on polyethylene holders (Blanck and Wängberg 1988a). The glass discs were cleaned in hot concentrated HNO_3 and rinsed in purified water. The cleaning was completed by rinsing in 70% ethanol immediately before submersing in the aquaria.

Chlorophyll and Taxonomic Analysis

Periphyton biomass was estimated as the chlorophyll *a* content. Chlorophyll absorption was measured spectrophotometrically after extraction in dimethyl sulfoxide (DMSO) (60°C, 30 min) (Hiscox and Israelstam 1978) and addition of an equal volume 90% distilled acetone

(Shoaf and Lium 1976). The amounts of chlorophyll *a* were calculated by the appropriate equation (chlorophyll $a + c_1 + c_2$, since the communities were almost free from chlorophyll *b*) of Jeffrey and Humphrey (1975). Qualitative taxonomic analyses were made on fresh material using one to three glass discs.

Copepod Abundance

As a means to estimate the grazing on periphyton the copepod populations of the aquaria were counted. Sampling occurred once at the end of the experiment by adding 10 ml formaldehyde solution (37%) to each aquarium to immobilize all copepods. After 30 min, the aquaria were sampled after thorough mixing. After sedimentation of the coarser material (including copepods), the sample volume was reduced to 46–63 ml, from which four or five 10 ml samples were counted.

Quantification of Short-Term Community Tolerance

The periphyton short-term tolerance was quantified by a short-term test of photosynthesis inhibition. The activity was estimated as incorporation of ^{14}C-carbon after incubation in sea water with ^{14}C-labeled bicarbonate added. Each periphyton disc was incubated in a glass scintillation vial containing 2 ml of test solution and placed in a thermostated water bath. The periphyton discs were taken from the aquarium and stored in toxicant-free sea water during the handling time required to sort out atypical samples (= samples with abnormal biomass, causing large and heterogenous variation in activity). The test solutions were prepared from filtered (Whatman GF/F, Whatman Paper Ltd, England) sea water and from acetone stock solutions of TBT and diuron, giving a final acetone concentration of 100 µl/L. The concentrations used are given above under "Experimental Design." Samples prepared for determination of abiotic ^{14}C-fixation were treated with 0.1 ml 37% formaldehyde solution ($n = 2$) and run with the test concentration series. The temperature in the incubator was set to the aquarium temperature (15°C). The samples were gently shaken during the 60 min of incubation. The light from fluorescent tubes (Osram Lumilux Daylight L18W/11) in the incubator was filtered through 20 cm water and regulated to give a photon flux density of 125 µE m^{-2} s^{-1}. After 30 min preincubation in light, 50 µl aliquots of ^{14}C-bicarbonate were added to each of the scintillation vials. The ^{14}C-bicarbonate solutions were prepared by a 1+49 (v/v) dilution of Amersham CFA2 stock solution (2 mCi/ml, 55 mCi/mmol, Amersham Laboratories, England) in GF/F-filtered sea water (ca. 1.9 mM total inorganic carbon) giving an activity of 2 µCi (74 kBq) per vial. The incorporation of ^{14}C-carbon was terminated after 30 min by adding 0.1 ml of concentrated formaldehyde solution. The test solution was then removed with a suction pump and the samples were acidified by adding 1 ml of concentrated acetic acid. Remaining inorganic carbon was driven off when drying the acidified samples. The release of incorporated ^{14}C-carbon from the periphyton was enhanced by addition of 1 ml of DMSO (Filbin and Hough 1984) before addition of scintillation cocktail (Ready Safe, Beckman Inc.). The DMSO addition gave the same yield of detectable radioactivity as a standard tissue solubilizer (Soluene 350, Packard Instrument Co., Inc.). The samples were thoroughly mixed both after the DMSO and the cocktail addition. A liquid scintillation counter (LS 3801, Beckman Inc., USA) was used to determine the amount of incorporated ^{14}C. The activities, as disintegrations per minute (dpm), were calculated from the counts per minute (cpm) data, using an external standard technique and the appropriate correction factors for the sample quench characteristics and machine efficiency. The counting efficiency was about 90%.

Data Analysis

The tolerance levels were estimated from dose-response surfaces, each comprising activity data from 50 periphyton discs (two-toxicant tests) or from dose response-curves comprising data from 26 discs (single toxicant tests). The response surface modelling and the statistical analysis of the dose-response surfaces were done with the MODDE-software (Umetri AB, Umeå, Sweden). The central composite design made it possible to approximate the present dose-response data with a semi-cubic polynomial model by multiple linear regression:

$$\ln Y = k_1 + k_2*[T] + k_3*[D] + k_4*[T]^2 + k_5*[T]*[D] + k_6*[D]^2 + k_7*[T]^2*[D] + k_8*[T]*[D]^2 + e \quad (1)$$

where ln Y is the natural logarithm of the response (photosynthesis activity as % of control) and [T] and [D] are the logarithms (base 10) of the TBT and diuron concentrations, respectively. The polynomial coefficients k_1–k_8 thus contain the information about the shapes of the dose-response curves and the interactions between the two toxicants as recognized by the analysis. The use of a relatively complicated model is justified by the known sigmoid-shape of dose-response curves which calls for a model which can approximate this curvature. The logarithmic transformation of the response data was necessary to obtain homogeneous variance.

The fitted models were checked for goodness-of-fit (i) by inspecting the residuals (e) plotted versus predicted values, (ii) by inspecting the normal distribution plots of residuals, (iii) by an F-test of the $MS_{residuals}$ over $MS_{model\ coefficients}$, and (iv) by an F-test on the partitioning of the residual MS into $MS_{pure\ error}$ and $MS_{lack\text{-}of\text{-}fit}$. The statistical significance of the separate coefficients of the polynomials were also calculated. For a detailed description of experimental design and the above diagnostic tests see for example Box *et al.* (1978) or Bayne and Rubin (1986).

From the dose-response surfaces two different measures of tolerance were obtained, both giving a single number (analogous to the EC- or LC-values from conventional dose-response curves) aiming at a simple comparison of the tolerances from the different long-term experiments, although excluding information about the shapes of the dose-response surfaces. The first measure is called max-EC_{50} and is defined by the length of a vector from the origin (= 3.2 nM TBT and 1 nM diuron, since the origin is not defined on a log scale) to the point farthest away from the origin on the 50%-inhibition isobole (the curve connecting the points of equal effect) of the contour plots of the dose-response surfaces. The value was transformed to a linear scale as

$$\text{max-}EC_{50} = \sqrt{(10^T)^2 + (10^D)^2} \quad (2)$$

where T and D are the number of log-scale units on the TBT and diuron axes respectively. The second measure is called surface-EC_{50} and consists of the area under the 50%-inhibition isobole. The surface was transformed to linear scale as

$$\text{surface-}EC_{50} = (10^{\sqrt{A}})^2 \quad (3)$$

where A is the area in log-scale units. The transformation approximates the surface area as a square.

The data from the single toxicant tests were approximated by a nonlinear regression procedure with the Fit.P-software (Biosoft, Cambridge, England) using a logistic model, modified from Clarke & Green (1988)

$$Y = A + (C - A)/(1 + e^{-l}*X^{-k}) \quad (4)$$

where Y is the carbon incorporation activity (in dpm), X is the toxicant concentration, C the carbon incorporation activity of the control samples and A a constant referring to the measured abiotic carbon incorporation, k and l are the parameters controlling the slope and position of the curve, e is the base of the natural logarithms. Weighting was performed in the nonlinear regression analysis and was obtained after adjusting the weighting function to give residuals evenly distributed around the fitted line.

Table 1. EC_{50}-values for periphyton carbon incorporation from single toxicant tests with TBT and diuron (EC_{50}-TBT and EC_{50}-diuron) together with tentative measures of tolerance from two-toxicant short-term tests

Long-term exposure (nM TBT/nM diuron)	EC_{50}-TBT (nM)	EC_{50}-diuron (nM)	max-EC_{50}[b] (nM)	surface EC_{50}[b] (arbt. units)
0/0	12	10	4.2	19.8
1.5/17.6	120	52	32	628
5/0	530	44	41	1060
1.5/102.5	680	61	570	40700
0/60	350	43	58	1390
5/60	955	82	767	9260
5/60	1340[a]	84	251	42500
5/120	1400[a]	68	1050	61000
8.5/17.6	800	62	269	42900
8.5/102.5	36400[a]	93	1190	154000
10/60	1000	64	378	23900
10/120	1870[a]	143	2960	127000

[a] EC-value extrapolated beyond highest test concentration (= 1000 nM)
[b] See "Materials and Methods" for definitions. Values calculated from short-term test with 46 to 48 samples, except 1.5/17.6 where $n = 42$

Results

Short-Term Effects on Photosynthesis in Unexposed Communities

The EC_{50}-values from the single toxicant tests on periphyton photosynthesis (Table 1) were 12 nM for TBT and 10 nM for diuron in the control aquarium of the long-term experiment. These values were in good agreement with earlier results obtained with unexposed periphyton communities (Dahl and Blanck 1990; Molander and Blanck 1991).

The dose-response surface from the unexposed long-term control showed a slightly convex curvature (Figure 2, top, Figure 2, bottom left), indicating an interaction between TBT and diuron in the short-term experiment. This was also reflected by the value of the k_5-coefficient (Table 2) of the interaction term in the model polynomial.

Detection of Induced Tolerance and Cotolerance in Exposed Communities

Both single toxicant and two-toxicant short-term experiments were run on preexposed communities to detect PICT, and four different measures of tolerance were calculated; the EC_{50} for (i) diuron and (ii) TBT respectively (from the single toxicant tests) and the tentative measures (iii) max-EC_{50} and (iv) surface-EC_{50} (from the two-toxicant tests, see Materials and Methods for definitions).

The results from the short-term two-toxicant experiments (Figure 3) showed a gradual increase in tolerance, reflected by the gradual shift of the 50% isobole to higher concentrations. Also, in the lowest range of long-term exposures, (5 nM TBT/0 nM diuron, 0nM TBT/60 nM diuron and 1.5 n M TBT/17.6 nM diuron) an increased tolerance was evident. The statistical significance of the fitted models was high (Table 2) with coefficients of determination (R^2) ranging from 0.85 to 0.96. The tentative measures of tolerance derived from the two-toxicant short-term tests and the EC_{50}-values from the single toxicant short-term tests were all able to detect the increased tolerance as the combined long-term exposures increased (Figure 4, Table 1).

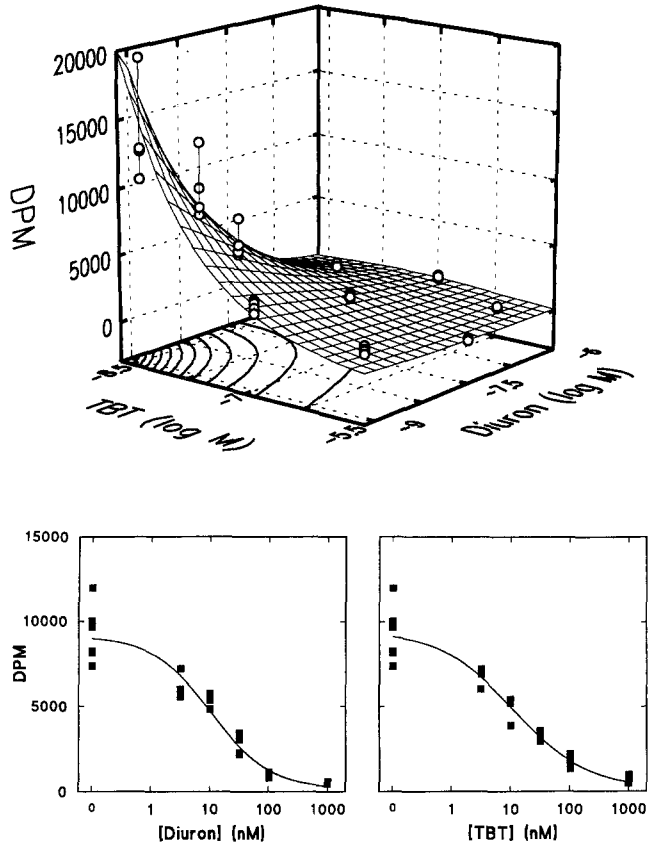

Fig. 2. Inhibition of photosynthesis in single-toxicant and two-toxicant short-term tests with TBT and diuron for marine periphyton communities from the long-term control aquarium. The photosynthetic activity is given as dpm/sample

Table 2. Model coefficients, according to Eq. (1), and statistics of the polynomials approximating the short term tests of carbon incorporation inhibition on periphyton communities exposed to combinations of TBT and diuron. Data were normalized to % of the short-term tests control to obtain compatibility. The coefficients of each model are given together with their calculated significance (p)

TBT	DCMU	n	k_1	p	k_2	p	k_3	p	k_4	p	k_5	p	k_6	p	k_7	p	k_8	p	$R^{2\,a}$	$p_{mo}{}^{b}$	$p_{lof}{}^{c}$
0	0	46	2.4198	.000	−1.318	.000	−.8732	.000	−.5436	.000	.5914	.000	−.2061	.082	−.5190	.029	.2528	.262	.9599	.0000	.009
1.5	17.6	42	3.3979	.000	−1.026	.000	−1.711	.000	−.8102	.000	.6319	.000	−1.142	.000	.1771	.589	.1874	.446	.9563	.0000	.068
5	0	48	3.5621	.000	−.9552	.000	−1.573	.000	−.8645	.000	.7628	.000	−1.039	.000	−.0061	.982	.4389	.103	.9447	.0000	.007
1.5	102.5	46	4.3021	.000	−.2803	.006	−.9489	.000	−.3129	.010	.2741	.036	−.7195	.000	.2639	.272	.0542	.811	.8494	.0000	.012
0	60	47	3.7234	.000	−.4511	.000	−1.272	.000	−.3056	.024	.4208	.005	−1.022	.000	.2911	.272	−.1845	.472	.911	.0000	.051
5	60	46	4.4489	.000	−.3917	.000	−1.535	.000	−.2865	.018	.4337	.001	−1.307	.000	.4694	.045	.2549	.253	.9428	.0000	.253
5	60	46	4.1009	.000	−.4585	.000	−1.374	.000	−.4372	.001	.4881	.001	−1.160	.000	.0471	.846	.3883	.104	.9323	.0000	.000
5	120	47	4.5030	.000	−.1962	.055	−1.205	.000	−.3598	.004	.3753	.006	−1.044	.000	.728	.004	−.2188	.350	.8898	.0000	.000
8.5	17.6	46	4.6938	.000	−.7816	.000	−1.282	.000	−.8039	.000	.3922	.003	−1.279	.000	−.1031	.659	.6319	.006	.9395	.0000	.052
8.5	102.5	46	4.5225	.000	−.3015	.000	−.9979	.000	−.2335	.008	.3791	.000	−.8627	.000	.3288	.054	.2607	.112	.9301	.0000	.090
10	60	47	4.3912	.000	−.2280	.075	−1.152	.000	−.6698	.000	.4720	.010	−1.096	.000	.0067	.983	−.0536	.856	.8688	.0000	.128
10	120	47	4.6356	.000	−.1322	.166	−1.019	.000	−.4274	.000	.5402	.000	−.9530	.000	.7363	.002	−.2022	.360	.8704	.0000	.019

[a] The coefficient of determination for the entire model

[b] The significance of the F-test of $MS_{residuals}$ over $MS_{model\ terms}$

[c] The significance of the F-test for lack-of-fit ($MS_{lack\text{-}of\text{-}fit}$ over $MS_{pure\ error}$)

The single toxicant EC_{50}-values from the long-term exposure of 5 nM TBT and 0 nM diuron were 530 nM for TBT and 44 nM (4.4 × control) for diuron (Table 1, Figure 5). This result shows the expected TBT-tolerance increase, but also an unexpected diuron-tolerance increase, indicating a cotolerance since the communities gained tolerance also for diuron while exposed only to TBT (5nM). The results from the long-term exposure of 0 nM TBT and 60 nM diuron; EC_{50}-TBT = 350 nM (29 × control) and EC_{50}-diuron = 43 nM, showed a corresponding cotolerance to TBT after diuron exposure.

Other Long-Term Effects in Exposed Communities

Other long-term effects were found on the diatom species richness, the chlorophyll *a* accumulation, and on copepods grazing on the periphyton. The diatom species richness showed a decrease from a mean of 20 species per disc in the control to 10 at a long-term exposure of 8.5 nM TBT and 102.5 nM diuron (Figure 6). This indicates the exclusion of species sensitive to the toxicants.

The effects of the long-term exposures on the mean chlorophyll *a* content of the periphyton communities ranged from 0.19 μg/disc to 0.54 μg/disc, and showed a clear peak at the combined concentrations of 5 nM TBT and 60 nM diuron (Figure 6). This response might be a compound effect, caused by a primary effect on the algae at high concentrations, besides a secondary effect caused by reduced copepod abundance already at lower concentrations (Figure 6).

Discussion

The detection of PICT by the short-term tests of periphyton photosynthesis inhibition was clear, and an increase of the periphyton tolerance was reflected in all tolerance measures (Figure 4 and 5, Table 1) as well as directly from the short-term dose-response surfaces of the two-toxicant short-term tests (Figure 3). This is the first evidence that PICT is detectable also in a situation with combined toxicity when two concurrent selection pressures are acting. Former studies of PICT under controlled exposures (Blanck and Wängberg 1988a and b; Molander et al. 1990; Molander and Blanck 1991) have detected PICT under single toxicant selection pressures.

It is, however, not only a question of detecting PICT but also a question of its validity, *i.e.*, whether it correctly indicates the disturbance of the exposed community. A comparison of PICT with other long-term responses shows a consistent picture in the sense that they all respond in the same concentration range (Figures 4 and 5 compared to Figure 6). The selection of more tolerant species was indicated by a decreasing diatom species richness, and this effect was followed by a decrease of chlorophyll *a* (in the highest exposures) which we interpret as the primary toxicant effect on the algae. The increase in tolerance was several-hundred-fold while the decrease in diatom species richness and copeped abundance was about 50% (Figure 6). Thus PICT appears to be a valid tool for description of the disturbed communities. The use of chlorophyll *a* as an indicator of long-term impact is clearly not valid as judged from its peak response.

We have earlier suggested (Blanck *et al.* 1988) that PICT has the potential to differentiate between primary and secondary effects of a toxicant. Our interpretation of the chlorophyll *a* peak would thus be that in the control and low concentrations the copepods (or other grazers) were actively grazing and reducing the periphyton biomass. The decrease in chlorophyll *a* at high concentrations was caused by primary toxicant effects on the algae, while the peak in chlorophyll *a* at intermediate concentrations, was a secondary effect caused by decreased grazing due to primary toxicant effects on copepods. Similar effects have been observed in an experiment with trichloroguaiacol (TCG) (Molander *et al.* 1990) where the population of grazing polychaetes was more sensitive than the algal community, giving an increased chlorophyll *a* content, together with low tolerances for TCG, until a shift with lower chlorophyll *a* and higher tolerance was found at a higher long-term exposure concentration. Both these observations show the ability of PICT to differentiate between primary and secondary toxicant effects.

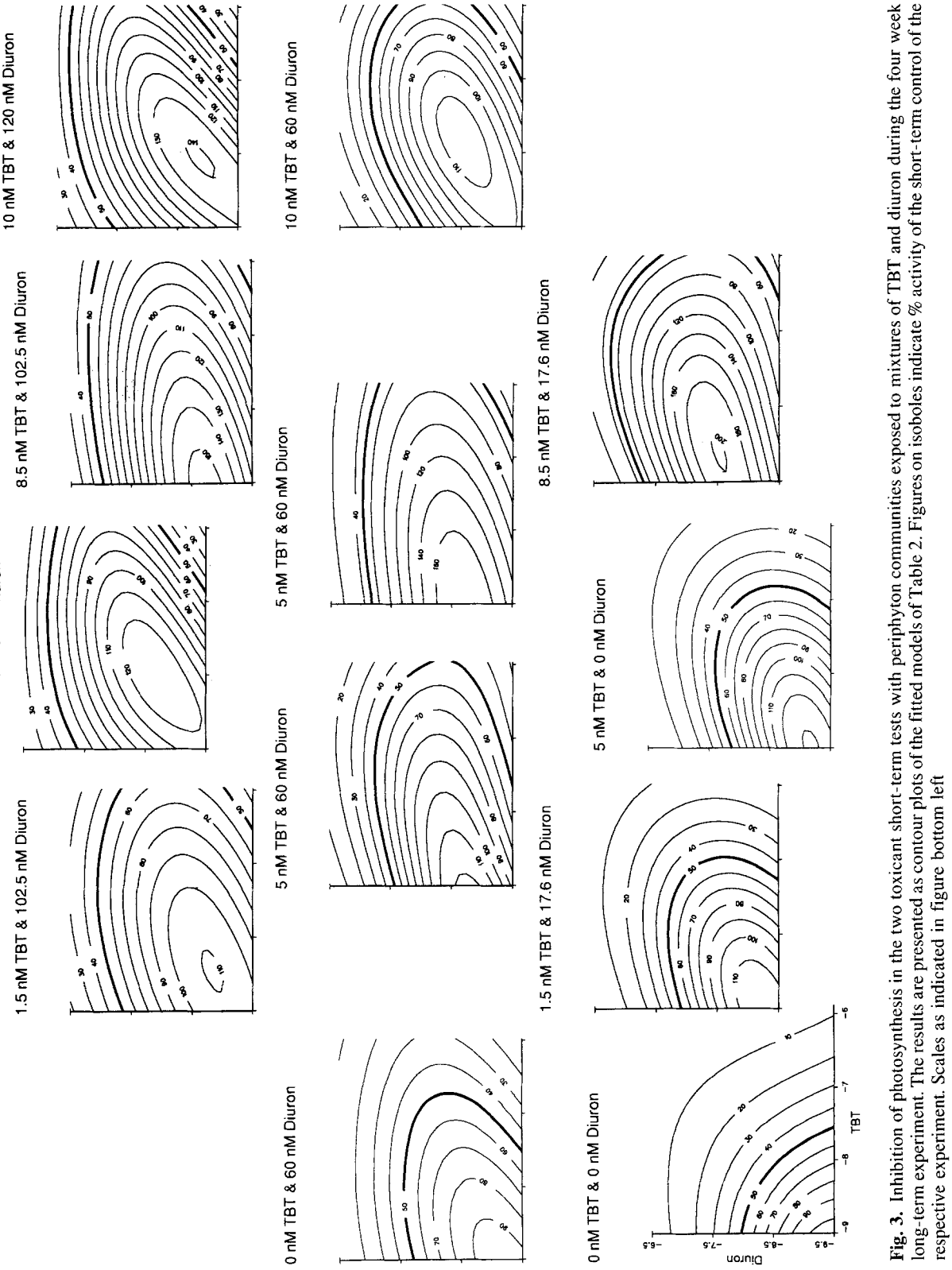

Fig. 3. Inhibition of photosynthesis in the two toxicant short-term tests with periphyton communities exposed to mixtures of TBT and diuron during the four week long-term experiment. The results are presented as contour plots of the fitted models of Table 2. Figures on isoboles indicate % activity of the short-term control of the respective experiment. Scales as indicated in figure bottom left

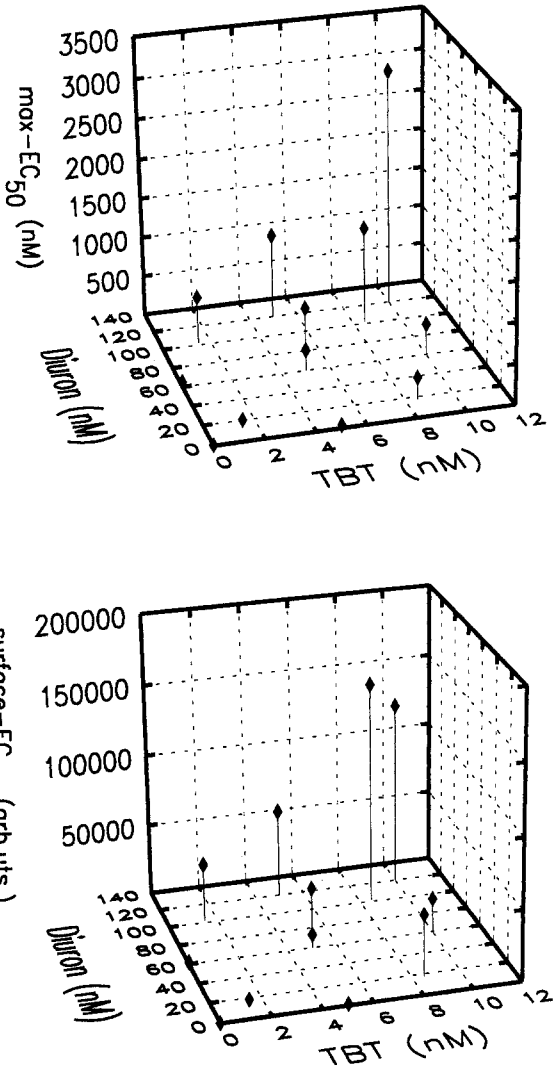

Fig. 4. Pollution-induced community tolerance (PICT) of marine periphyton communities exposed to mixtures of TBT and diuron during the four week long-term experiment. The tolerances are given for the tentative tolerance measures - max-EC_{50} and surface-EC_{50} - calculated from the two-toxicant short-term tests

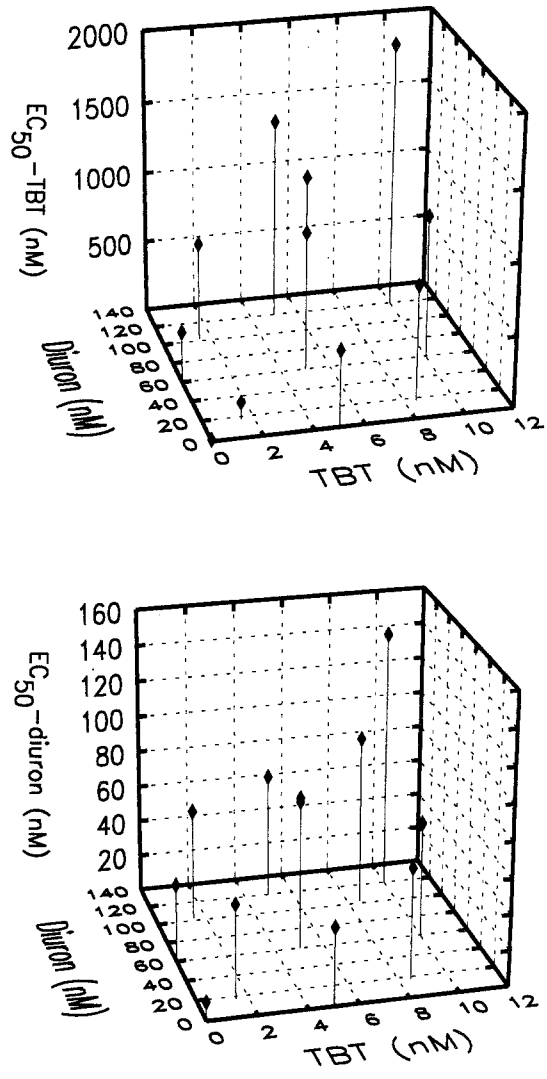

Fig. 5. Pollution-induced community tolerance (PICT) of marine periphyton communities exposed to mixtures of TBT and diuron during the four week long-term experiment. The tolerances are given as the EC_{50}-TBT and EC_{50}-diuron derived from the single-toxicant short-term tests

Ben-Shlomo and Nevo (1988) have proposed that a combination of two toxicants can act as a unique, third, selection pressure. They found different allel frequencies in populations of *Palaemon*-shrimps after exposure to cadmium and mercury singly or in combination, and concluded that "the interactive contamination of cadmium and mercury acts as a specific contaminant." In our experiment the tolerance increase was about a factor 10 for diuron, while the tolerance increase for TBT was about a factor of 100 (Table 1) when using single-toxicant tests for tolerance detection. The observed effect difference between the two toxicants is clearly visible also in the dose-response surfaces from two-toxicant tests (Figure 3) where the tolerance increase along the TBT-axis is larger than along the diuron-axis. A comparison between max-EC_{50} and EC_{50}-diuron also showed a large difference in magnitude, while the differences in magnitude between max-EC_{50} and EC_{50}-TBT were small (Table 1). Thus the magnitude of the max-EC_{50} seems more influenced by TBT than by diuron which is in accordance with the single-toxicant results. It appears that the selection pressure from the combined toxicants in this experiment still reflects the properties of each of the two toxicants and does not act as a third unique selection pressure.

Increased tolerance for a compound which has not exerted any selection pressure on the community is termed a cotolerance. This is a problem when using single-toxicant tests for detection of PICT in the field where the exposure histories of the communities are unknown, and when the occurrence of PICT is used to infer a causal relationship between the induced tolerance and exposure to a certain compound (Blanck *et al.* 1988; Dahl and Blanck 1990). Cotolerance can be expected for compounds closely related, either chemically or in their mode of action (Blanck et al. 1988). In this experiment, two toxicants with differences in chemical structure, chemical properties and mode of action were used. Despite this a cotolerance was de-

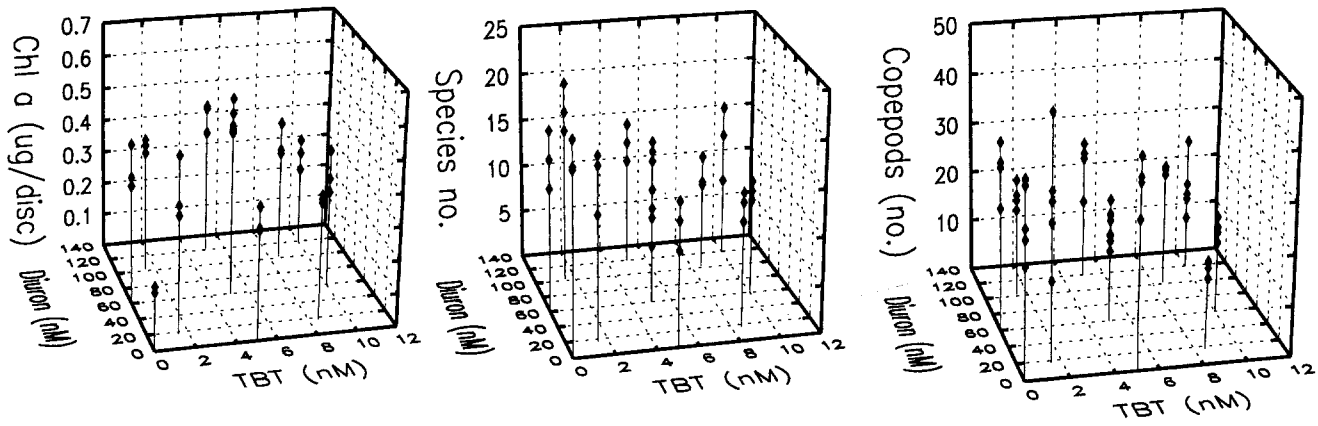

Fig. 6. Effects on diatom species richness, chlorophyll *a* content and copepod abundance in aquaria microcosms exposed to mixtures of TBT and diuron during the long-term experiment

tected, using single-toxicant tests. This is in contrast to the findings of Blanck and Wängberg (1991) who in periphyton exposed to arsenate found a large cotolerance for thiophosphate, a compound with a mode of action similar to arsenate, while the cotolerances of other tested compounds (diuron among others) were insignificant. The observation of cotolerance between TBT and diuron, two compounds unrelated both chemically and in their mode of action, imply the occurrence of a common tolerance mechanism functional for both toxicants and utilizing a presently unconsidered property of the chemicals. Such a tolerance mechanism might be related to the uptake, storage, metabolization or excretion but not to specific modifications of the target sites (Brattsten *et al.* 1986 and references therein).

It is concluded that PICT was detectable, by the short-term tests of photosynthesis inhibition and response surface methodology, also in a situation of combined toxicity and that the response was a valid estimator of other effects in the community. PICT was also able to discriminate between primary and secondary effects of the toxicants on the algae, while *e.g.*, biomass was clearly not valid since it was confounded by secondary effects due to grazing. In this experiment the mixture of toxicants did not act as a unique, third, selection pressure (*sensu* Ben-Shlomo and Nevo 1988). The unexpected occurrence of cotolerance merits further investigations since this question is crucial for the specificity of PICT when single-toxicant tests are used for detection.

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