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Impact of antifouling booster biocides on single microalgal species and on a natural marine phytoplankton community

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ABSTRACT: Phytoplankton were exposed to 4 antifouling booster biocides (Sea-Nine 211®, Irgarol 1051®, diuron and zinc pyrithione) to investigate toxicological responses. Initially, single species/single biocide exposure experiments revealed changes in pigment ratios under all biocide exposures for the prymnesiophyte *Emiliania huxleyi*, but not for the cyanophyte *Synechococcus* sp. Growth inhibition results following 72 h exposures indicated that *Synechococcus* sp. was more tolerant to zinc pyrithione (NOEC of 1.0 µg l^{-1}) and Sea-Nine 211® (NOEC of 0.9 µg l^{-1}) than *E. huxleyi* (EC_{50} of 0.54 and EC_{50} of 0.35 µg l^{-1}, respectively). In contrast, *Synechococcus* sp. was more sensitive to diuron (EC_{50} of 0.55 µg l^{-1}) than *E. huxleyi* (EC_{50} of 2.26 µg l^{-1}), whereas exposure to Irgarol 1051® similarly impacted both species (EC_{50} of 0.16 and 0.25 µg l^{-1}, respectively). In addition, the impact on photosynthesis and on pigment chemotaxonomy was investigated through a laboratory exposure experiment using a natural phytoplankton community. Pigment signatures were measured by High Performance Liquid Chromatography (HPLC) and densities of size-classified phytoplankton groups were monitored using Analytical Flow Cytometry (AFC). Group-specific sensitivity of the natural phytoplankton community was detected through pigment composition after 72 h exposure to 5 µg l^{-1} zinc pyrithione and 10 µg l^{-1} Sea-Nine 211®. Zeaxanthin increased proportionally, indicating a relative increase in Cyanophyceae. This result was corroborated using AFC. Primary production, estimated by $^{14}C\text{-}HCO_3^-$ uptake, was compared to maximum quantum yield of Photosystem II (F_V/F_M), which was quantified by Fast Repetition Rate Fluorimetry (FRRF). The 2 techniques were in good agreement ($R^2 = 0.89$, $p = 0.0001$), both primary production and F_V/F_M being impaired by exposure to all biocides tested. These results are discussed in the context of the potential environmental impact of biocides on phytoplankton communities and the ecological implications of any modifications in species composition.

KEY WORDS: Marine phytoplankton · Antifouling booster biocides · *Emiliania huxleyi* · *Synechococcus* sp. · Analytical Flow Cytometry · Pigments · Fast Repetition Rate Fluorescence · $^{14}C\text{-}HCO_3^-$ uptake

INTRODUCTION

The application of antifouling paints to the hulls of ships or boats is to prevent attachment and growth of fouling biota, but these chemical applications pose a threat to non-target organisms (Readman et al. 1993). Most contain organic biocides, which are used singly or as an additional component to copper-based formulations (Health and Safety Executive, HSE 2002). Such organic biocides enhance the efficacy of the formulation and are commonly referred to as 'booster' biocides. Since tri-*n*-butyltin (TBT) restrictions were intro-

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duced in the late 1990s (HSE 2002), the use of booster biocides has become more widespread and, worldwide, around 18 compounds have been used (Thomas 2001). Among them, Irgarol 1051® and diuron were, until recently, the most commonly used, with levels up to 1.42 and 6.74 µg l^{-1} respectively being reported in UK marinas (Thomas et al. 2001).

Phytoplankton are highly susceptible to herbicidal antifouling compounds due to the latters' damaging effects on photosynthesis. Due to their short generation times and differential responses and susceptibilities to biocides, algae can be affected over time scales from hours to days, leading to changes in community structure. Therefore, toxicological studies on phytoplankton are particularly relevant when safe environmental regulations are to be established. These are typically based on unialgal tests, but toxic responses of natural phytoplankton communities are more environmentally relevant, yet such studies are scarce (e.g. Dahl & Blanck 1996, Nyström et al. 2002, Readman et al. 2004).

The aims of this study were to investigate changes in biochemical and physiological function of selected microalgal species and in the structure of a marine phytoplankton community, induced by exposure to 4 antifouling biocides, two of which have been reported in Plymouth waters (diuron and Irgarol 1051®) and two of which are in common usage (Sea-Nine 211® and zinc pyrithione). Diuron and Irgarol 1051® are PSII inhibitors and impair photosynthesis by displacing a plastoquinone (Q_B) from its binding site in the D1 protein of photosystem II (Cremlyn 1991). Sea-Nine 211® has a broad spectrum activity (e.g. against fungi, algae, bacteria and marine invertebrates) and reacts with thiol-containing enzymes (Collier et al. 1990). Zinc pyrithione inhibits bacterial ATP synthesis and membrane transport (Dinning et al. 1998) and generates hydroxyl radicals when photolysed with visible or UV light (Aveline et al. 1996). Since the detection of sensitive toxicological endpoints is crucial for accurately determining toxic effects on communities, we also assessed the suitability of several biomarkers, growth, photosynthesis, pigment:chlorophyll *a* (chl *a*) ratios and chemotaxonomy.

MATERIAL AND METHODS

Preparation of biocide solutions and chemical reagents. Authentic analytical standards were acquired: Irgarol 1051® (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine; Ciba-Geigy), diuron Pestanal® ([3-(3,4-Dichlorophenyl)-1,1 dimethyl-urea]; 99% purity; Riedel-de Haën), Sea-Nine 211® (4,5-dichloro-2-n-octyl-4-isothiazolin-3-one; Institute for Environmental Science, The Netherlands), and zinc pyrithione (1-Hydroxypyridine-2-thione (pyrithione) zinc salt; 95% purity; Sigma Chemical). Stock solutions of Irgarol 1051®, diuron Pestanal® and Sea-Nine 211® were dissolved in methanol and their working solutions prepared with sterile seawater. The zinc pyrithione stock and working solutions were prepared by dilutions into sterile seawater at the start of the experiment.

Algal cultures and single-species experiments. The prymnesiophyte *Emiliania huxleyi* (PCC 92D) and the cyanophyte *Synechococcus* sp. (PCC 543) were obtained from the Plymouth Culture Collection, UK. Species selection was based on previous studies (Readman et al. 2004) that showed *E. huxleyi* to be highly sensitive to, and *Synechococcus* sp. to be comparatively resistant to, Irgarol 1051® exposure.

Non-axenic cultures were maintained in f/2 culture medium (Guillard 1975), without Trizma, and prepared with filtered (0.45 µm) seawater (salinity of 33‰; initial pH of 8.3 to 8.4). Ideal growth rates (~1 division d^{-1}) were achieved by varying culture conditions in both species. *Emiliania huxleyi* was grown in a 12:12 h light:dark cycle at 15.5 ± 2.5°C under an average irradiance of 160 µmol photons m^{-2} s^{-1} photosynthetic active radiation (PAR), provided by cool white light bulbs (Sylvania; 70 W); *Synechococcus* sp. was grown on a 12:12 h light:dark cycle at 20.5 ± 1.5°C under an average irradiance of 80 µmol photons m^{-2} s^{-1} PAR. Cultures were grown for at least 4 d to ensure cell acclimatisation prior to experimentation. Sterile techniques were used for all culture work in an attempt to minimize bacterial growth.

Experiments were conducted using 2 l borosilicate bottles containing 1 l of f/2 medium spiked with aliquots of biocide working solutions. *Emiliania huxleyi* was exposed to the nominal concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 µg l^{-1} zinc pyrithione; 0.4, 0.8, 1.5 µg l^{-1} Sea-Nine 211®; 0.1, 0.2, 0.5 µg l^{-1} Irgarol 1051®; and 0.2, 0.5, 5.0, 10, 50 µg l^{-1} diuron. *Synechococcus* sp. was exposed to 0.4, 0.6, 1 µg l^{-1} zinc pyrithione; 0.2, 0.4, 0.9 µg l^{-1} Sea-Nine 211®; 0.2, 0.5, 1 µg l^{-1} Irgarol 1051®; 0.2, 0.4, 2.2 and 3.3 µg l^{-1} diuron. Exponentially growing cells were inoculated into bottles to obtain ~1×10^4 cells ml^{-1}. Controls and carrier controls were included in all experiments. Final concentrations of methanol for each treatment did not exceed 0.00036% (v/v). Experiments were static and carried out in triplicate over 72 h. Samples for pigment analyses and biocide determinations were taken at the beginning and end of the experiments, while samples for Analytical Flow Cytometry (AFC) were taken daily.

Experiment with a natural phytoplankton community. Coastal water was collected from the well characterised station L4 (http://www.pml.ac.uk/L4), Plymouth, UK (50°15′N, 04°13′W) in August 2001. The

in situ temperature was 15°C and salinity was 33‰. Phytoplankton composition on the day of sampling, as characterised by cell abundance, was 15.6% flagellates (≤5 µm), 3.5% diatoms, 2.2% colourless Dinophyceae, 0.2% Dinophyceae, 0.05% coccolithophorids, and 78.5% picoplankton. Highest carbon biomass was attributed to Dinophyceae (35%), flagellates (24%), colourless Dinophyceae (16%), diatoms (18%), and picoplankton (6%) (http://www.pml.ac.uk/L4).

Seawater containing phytoplankton (<63 µm) was transferred into 2 l borosilicate bottles and spiked with the biocide working solutions, in triplicate. The concentrations tested were selected from a previous scaling experiment and were: 0.03 and 0.05 µg l^{-1} Irgarol 1051®, 0.5 and 5 µg l^{-1} zinc pyrithione, 1 and 10 µg l^{-1} diuron, and 1 and 10 µg l^{-1} Sea-Nine 211®, including controls and carrier controls (methanol). Bottles were incubated for 72 h under a 12:12 h light:dark cycle, an average temperature of 17°C and irradiance of 230 µmol photons m^{-2} s^{-1} PAR. Daily sampling was carried out for AFC and FRRF determinations. After 72 h, samples were filtered (0.5 to 1.5 l; 0.7 µm GF/F) for pigment analyses. Samples for biocide determination were taken at the sampling station and at the beginning and end of the experiment. A set of 60 ml transparent polycarbonate bottles containing the phytoplankton community under the same exposure concentrations was also incubated to determine carbon dioxide fixation by ^{14}C-HCO_3^- uptake.

Determination of biological parameters. The rate of carbon fixation was estimated from the incorporation of ^{14}C-HCO_3^- (Joint et al. 2002). Aliquots of ^{14}C-HCO_3^- were added to each 60 ml bottle, giving a final activity of 5 µCi ^{14}C-HCO_3^- per bottle. After 24 h, incubations were terminated by filtration through Nuclepore polycarbonate membrane filters (0.2 µm) and samples were counted in a liquid scintillation counter (LSC) LKB Wallac 1219 Rackbeta.

In vivo fluorescence measurements were determined by a FAST^{tracka} fluorometer (FRRF) (Chelsea Instruments) operated as a benchtop unit (Suggett et al. 2001). Samples (25 ml) were dark-adapted (30 min) and data were analysed by FRRF software FRS 1.8 to provide values for the maximum quantum yield of PSII (F_V/F_M). The FRRF was configured with only the dark chamber activated, with auto-ranging mode gain and 20 discrete acquisitions.

Pigment composition was determined by filtering seawater samples (1 to 2 l) onto Whatman glass fiber filters (0.7 µm GF/F) and frozen in liquid nitrogen (–196°C). Pigments were extracted with 90% acetone (containing internal standard) by ultrasonication. Extracts were clarified by centrifugation and supernatants were mixed with 1 M ammonium acetate (50%:50% v/v) before injection into the HPLC. Analyses of pigments were conducted using the reverse phase HPLC procedure outlined by Barlow et al. (1997) using a 3 µm Hypersil MOS2 C-8 column (100 mm × 4.6 mm; Alltech Associates) on a Shimadzu HPLC system coupled to a photo-diode array detector (UV6000). Calibration was performed with standards purchased from DHI Water and Environment and Sigma-Aldrich.

Samples for AFC were fixed with 1 to 2% glutaraldehyde (final concentration) (microscopy grade; Merck). Samples were fast-frozen in liquid nitrogen and stored at –80°C in the dark to preserve their fluorescence. *In vivo* samples derived from the unialgal cultures were analysed immediately after sample collection and therefore did not require fixation. Phytoplankton samples were analysed for enumeration and size classification using a Becton Dickinson FACSort™ flow cytometer as described in Readman et al. (2004).

Antifouling booster biocides analyses. Seawater samples were collected from sub-surface (0.5 m depth) at the sampling station (2.7 l) and from the experiments (1 to 2 l), spiked with an internal standard (Ametryn or Atrazine-d_5), and were stored at 4°C awaiting extraction. Extractions and analyses of Irgarol 1051® and Sea-Nine 211® were based on the methods described in Zhou et al. (1996) (for solid-phase extraction) and Sargent et al. (2000) (for liquid-liquid extraction). Extracts (1 to 2 µl) were injected into a GC-MS (6890 N Network GC System) coupled to a 5973 Mass Selective Detector (Agilent Technologies) and operated in selective ion monitoring (SIM) mode using m/z values of 182 for Irgarol 1051®, 169 for Sea-Nine 211®, and 227 for Ametryn or 205 for Atrazine-d_5. Spiked recoveries were 85% (±3%; n = 5) for Sea-Nine 211® and 81% (±6%; n = 5) for Irgarol 1051®. Samples for diuron determination were analysed using an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Sension). Zinc pyrithione is notoriously difficult to analyse, and for this reason, nominal concentrations were used.

Data analyses. For the unialgal experiments, the effective concentration which reduces growth by 50% (EC_{50}) was calculated by graphical interpolation (Walsh 1988). The lowest observable effective concentration (LOEC) and the non-observable effective concentration (NOEC) after 72 h exposure to biocides were determined by one-way ANOVA, followed by Tukey honest significant difference test (HSD; α = 0.05), in which LOEC represented $p < 0.05$ and NOEC represented $p > 0.05$. Growth rates (μ) were estimated from the slope of linear fitting curves between $\log_2$ of cell number versus time and were expressed as divisions d^{-1}. Cell number, μ and pigment ratio data were analysed by 1-way ANOVA followed by the Tukey HSD test (α = 0.05). All data were tested for homoscedasticity (Cochran's test) and normality (χ^2 test) and 'rank' values transformed when necessary to meet the

assumptions for parametric tests. All tests were carried out using the statistical package Statistica for Windows v 5.1 (StatSoft).

For the experiment with natural phytoplankton, a distance matrix (Bray Curtis similarity) was generated using fourth root transformed pigment data and submitted to analysis of similarities (ANOSIM analysis). The contribution of individual pigments to each biocide exposure was determined by SIMPER analysis (Similarity Percentages–Species Contributions) using the statistical software PRIMER for Windows v 5.2.9.

Estimates of biomass, as chl *a*, for each phytoplankton group were obtained using the matrix factorisation program CHEMTAX (Mackey et al. 1996). The pigment:chl *a* ratios used to create the initial matrix were derived from published literature (Mackey et al. 1996, Schlüter et al. 2000) as well as from the unialgal culture data obtained in this study. The pigment data were run under 3 different pigment:chl *a* ratios for Prymnesiophytes, obtained from the *Emiliania huxleyi* cultures exposed to biocides (see Table 2). For Cyanophyceae, zeaxanthin:chl *a* ratios were constant, as determined for *Synechococcus* sp. throughout biocide exposures (see Table 2). The pigments β-carotene, diadinoxanthin and chlorophyll c_1c_2 were excluded from the matrix as recommended by Mackey et al. (1996). Algal classes were selected according to the phytoplankton composition on the day of sampling (see section above 'Experiment with a natural phytoplankton community').

RESULTS

Unialgal experiments

Growth inhibition

The EC_{50} (72 h) values for cell numbers of *Emiliania huxleyi* indicated differences in the sensitivity to the 4 biocides. EC_{50} values were 2.26 µg l^{-1} for diuron, 0.54 µg l^{-1} for zinc pyrithione, 0.35 µg l^{-1} for Sea-Nine 211® and of 0.25 µg l^{-1} for Irgarol 1051® (Table 1). At concentrations of 0.5 µg l^{-1} diuron and 0.2 µg l^{-1} zinc pyrithione, no effect was observed at 72 h (NOEC) ($p > 0.05$) (Table 1). The LOEC (cell numbers) was 0.4 µg l^{-1} for zinc pyrithione and 0.1 µg l^{-1} for Irgarol 1051® ($p < 0.05$).

Under the selected experimental conditions, *Synechococcus* sp. was not inhibited by the concentrations of zinc pyrithione and Sea-Nine 211® tested ($p > 0.05$), whereas diuron (EC_{50} of 0.55 µg l^{-1}) and Irgarol 1051® (EC_{50} of 0.16 µg l^{-1}) significantly affected cell density ($p < 0.05$) (Tables 1 & 2). The NOEC for diuron was 0.21 µg l^{-1}, 1.0 µg l^{-1} for zinc pyrithione, and 0.9 µg l^{-1} for Sea-Nine 211® ($p > 0.05$) (Table 1). Results indicated that the carrier solvent did not significantly influence growth compared to the controls ($p > 0.05$).

Pigment composition

For *Emiliania huxleyi*, all marker pigment to chl *a* ratios changed significantly when exposed to diuron and zinc pyrithione ($p < 0.05$); whereas chlorophyll c_3 (chl c_3):chl *a*, fuco:chl *a* (fucoxanthin:chl *a*) and 19′-hex:chl *a* (19′-hexanoyloxyfucoxanthin:chl *a*) varied with Sea-Nine 211® concentrations, and only the fuco:chl *a* ratio varied with Irgarol 1051® concentrations ($p < 0.05$) (Table 2). A pattern of reduction in pigment to chl *a* ratio was observed with increasing exposure to biocides, except for the 19′-hex:chl *a*, diadino:chl *a* and chl c_3:chl *a* ratios, which increased at intermediate concentrations of biocides, followed by a decline at the highest concentrations (≥5 µg l^{-1} diuron) (Table 2). However, when these ratios were normalised to the number of cells, a general pattern of decreased pigment per cell with increasing toxicant concentrations was apparent.

For *Synechococcus* sp., there was no significant difference in marker pigment to chl *a* ratios within the concentration range of the biocides tested ($p > 0.05$) except for Irgarol 1051®, where significant differences in β-carotene:chl *a* and also in chl *a*:cell were recorded ($p < 0.05$) (Table 2).

Natural phytoplankton community experiment

Toxic effects on pigment composition

Biocide treatments significantly affected the pigment composition ($R = 0.477$; $p = 0.001$; ANOSIM analysis). Largest differences in pigment proportions were found at concentrations of 5 µg l^{-1} zinc pyrithione and 10 µg l^{-1}

Table 1. EC_{50}, NOEC, and LOEC (µg l^{-1}) 72 h as cell number inhibition of *Emiliania huxleyi* and *Synechococcus* sp. exposed to the 4 biocides tested. Numbers in parentheses indicate the percentage of inhibition at the given concentration. nd: not determined due to insufficient data

	Emiliania huxleyi			*Synechococcus* sp.		
	EC_{50}	NOEC	LOEC	EC_{50}	NOEC	LOEC
Zinc pyrithione	0.54	0.2	0.4 (21%)	nd	1.0	nd
Diuron	2.26	0.54	nd	0.55	0.21	0.43 (45%)
Irgarol 1051®	0.25	nd	0.1 (21%)	0.16	nd	nd
Sea-Nine 211®	0.35	nd	nd	1.25	0.9	nd

Table 2. Growth rate (μ, divisions d^{-1}), cell numbers (cell ml^{-1}), chlorophyll *a* to cell (pg $cell^{-1}$) and marker pigment to chlorophyll *a* ratios for the prymnesiophyte *Emiliania huxleyi* and the cyanophyte *Synechococcus* sp. exposed to different type and concentrations of antifouling biocides. Data represent means of 3 replicated cultures. *Significant differences in pigment ratios and growth rates due to different concentrations of biocides for each marked column ($p < 0.05$)

Species	Biocide type	Biocide (μg l^{-1})	μ[a] (div d^{-1})	Cell ml^{-1} ($\times 10^4$)	Chl *a*: cell	Chlc_1c_2: chl *a*	Chlc_3: chl *a*	Fuc: chl *a*	19′-Hex: chl *a*	Diad: chl *a*	β-Car: chl *a*	Zea: chl *a*
Emiliania huxleyi (Prymnesiophyceae)	Diuron	0	1.12	7.23	0.40	0.28	0.20	0.60	0.19	0.17	0.032	
		MEOH	1.11	7.48	0.40	0.27	0.20	0.57	0.18	0.17	0.032	
		0.2	1.05	6.38	0.37	0.26	0.22	0.50	0.25	0.16	0.030	
		0.5	1.02	6.54	0.26	0.25	0.22	0.48	0.27	0.17	0.031	
		5	0.51	2.23	0.25	0.24	0.21	0.48	0.27	0.17	0.029	
		12	0.42	1.74	0.23	0.23	0.21	0.50	0.24	0.18	0.031	
		50	0.38	1.70	0.16	0.16	0.19	0.45	0.23	0.19	0.025	
			*	*	*	*	*	*	*	*	*	
	Zinc pyrithione	0	1.15	5.86	0.46	0.24	0.11	0.42	0.19	0.19	0.022	
		0.2	1.13	5.63	0.52	0.23	0.11	0.39	0.20	0.18	0.024	
		0.4	1.06	4.55	0.23	0.24	0.12	0.37	0.31	0.34	0.022	
		0.6	0.84	2.54	0.16	0.19	0.17	0.23	0.35	0.38	0.026	
		0.8	0.18	6.88	0.11	1.37	0.61	1.98	3.27	0.94	0.004	
		1	−0.62	1.23	0.12	4.70	1.25	7.05	10.37	2.09	0.000	
			*	*	*	*	*	*	*	*	*	
	SeaNine 211®	0	1.17	3.02	0.81	0.25	0.17	0.51	0.15	0.11	0.024	
		MEOH	1.13	2.91	0.80	0.23	0.12	0.58	0.19	0.14	0.019	
		0.4	0.74	1.24	0.69	0.26	0.14	0.45	0.22	0.12	0.023	
		0.8	−1.33	0.02	0.76	0.19	0.07	0.43	0.35	0.16	0.011	
			*	*			*	*	*			
	Irgarol 1051®	0	1.17	3.02	0.81	0.25	0.17	0.50	0.15	0.11	0.024	
		MEOH	1.13	2.91	0.80	0.23	0.12	0.58	0.19	0.14	0.019	
		0.1	1.02	2.33	0.37	0.82	0.29	2.15	0.83	0.28	0.009	
		0.2	0.86	1.61	0.46	0.21	0.17	0.39	0.23	0.13	0.022	
		0.5	0.52	0.80	0.40	0.31	0.16	0.72	0.35	0.24	0.015	
			*	*	*			*				
Synechococcus sp. (Nostocophyceae)	Diuron	0	1.23	12.85	0.0033						0.12	0.61
		MEOH	1.26	11.89	0.0034						0.12	0.64
		0.2	1.03	8.76	0.0037						0.14	0.64
		0.4	1.07	7.82	0.0043						0.12	0.56
		2.2	0.53	2.96	0.0025						0.18	0.64
		3.3	0.29	1.80	0.0035						0.13	0.65
			*	*								
	Zinc pyrithione	0	1.18	5.37	0.0049						0.11	0.71
		0.4	1.18	5.15	0.0042						0.13	0.64
		0.6	1.15	7.10	0.0046						0.11	0.66
		1	1.02	4.76	0.0054						0.11	0.64
	SeaNine 211®	0	1.18	5.37	0.0049						0.11	0.71
		MEOH	1.19	5.15	0.0045						0.11	0.68
		0.2	1.23	5.60	0.0042						0.12	0.69
		0.4	1.18	6.70	0.0040						0.12	0.67
		0.9	0.91	3.61	0.0046						0.11	0.71
	Irgarol 1051®	0	1.13	8.39	0.0036						0.14	0.74
		MEOH	0.97	6.11	0.0050						0.13	0.83
		0.2	0.58	3.07	0.0022						0.21	0.65
		0.5	0.08	1.08	0.0037						0.12	0.71
		1	−0.10	0.77	0.0031						0.14	0.81
			*	*	*						*	

[a] μ was estimated by taking into account the 3 days of the experiment

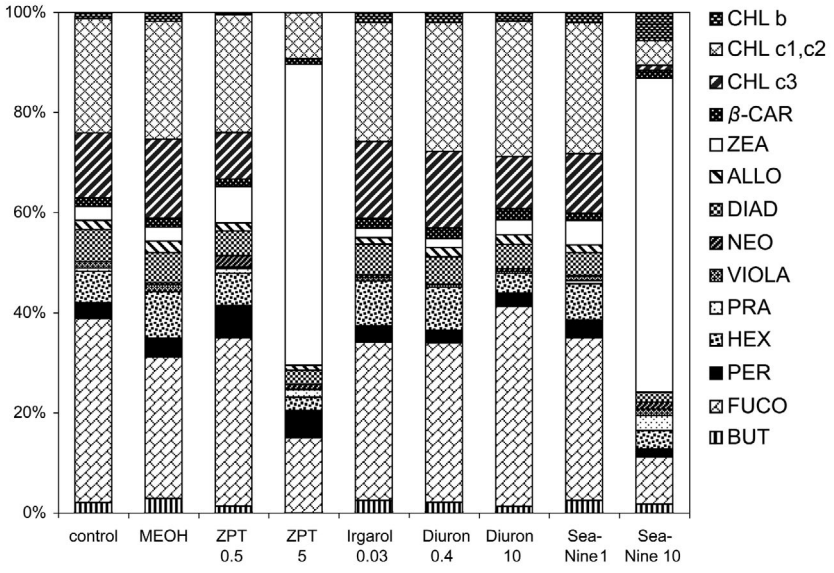

Fig. 1. Pigment composition (% of total) of phytoplankton after 72 h exposure to concentrations (µg l^{-1}) of zinc pyrithione (ZPT), Irgarol 1051® (Irgarol), diuron, Sea-Nine 211® (SeaNine), including control (no biocide addition) and the carrier control (MEOH). Data are mean values (n = 3). Samples from the 0.05 µg l^{-1} Irgarol 1051® were lost during analyses. CHL b: chlorophyll *b*; CHL c_1,c_2: chlorophyll c_1,c_2; CHL c_3: chorophyll c_3; β-CAR: β-carotene; ZEA: zeaxanthin; ALLO: alloxanthin; DIAD: diadinoxanthin; NEO: neoxanthin; VIOLA: violaxanthin; PRA: prasinoxanthin; HEX: 19′-hexanoyloxyfucoxanthin; PER: peridinin; FUCO: fucoxanthin; BUT: 19′-butanoyloxyfucoxanthin

Sea-Nine 211® (Fig. 1). Whilst the contribution of chl *a* to total pigments was reduced drastically, zeaxanthin levels either increased (Sea-Nine 211® exposure) or remained unchanged (zinc pyrithione exposure) resulting in an increase in its relative contribution (Fig. 1). There was a trend towards dissimilarity (>40%) between untreated samples and those treated with 5 µg l^{-1} zinc pyrithione and 10 µg l^{-1} Sea-Nine 211® (SIMPER analysis). The pigments chl c_3, fucoxanthin, 19′-butanoyloxyfucoxanthin and 19′-hexanoyloxyfucoxanthin contributed to 54% of the dissimilarity between 5 µg l^{-1} zinc pyrithione and controls; whilst fucoxanthin, chl c_3, alloxanthin, peridinin and 19′-hexanoyloxyfucoxanthin contributed to 54% of the dissimilarity between 10 µg l^{-1} Sea-Nine 211® and the controls. Dissimilarities amongst treatments (>30%) were observed for concentrations of (1) 5 µg l^{-1} zinc pyrithione with diuron (0.4 and 10 µg l^{-1}), Irgarol 1051® (0.03 µg l^{-1}), Sea-Nine 211® (1 µg l^{-1}) and zinc pyrithione (0.5 µg l^{-1}); and (2) 10 µg l^{-1} Sea-Nine 211® with zinc pyrithione (0.5 µg l^{-1}), Irgarol 1051® (0.03 µg l^{-1}), diuron (0.4 and 10 µg l^{-1}) and Sea-Nine 211® (1 µg l^{-1}). All other comparisons showed dissimilarities lower than 30%.

Estimation of the community structure using CHEMTAX identified diatoms, dinoflagellates, prymnesiophytes and cyanophytes as the main contributors to the total chl *a* biomass of the phytoplankton community. Cyanophytes contributed 74 and 79% to the total biomass (as chl *a*), respectively, at the highest concentrations of zinc pyrithione and Sea-Nine 211® (Fig. 2). The contribution of dinoflagellates to biomass increased at 0.5 and 5 µg l^{-1} zinc pyrithione (11 and 15%, respectively) when compared to the controls (8%), although it remained almost unchanged under the other biocide treatments. At 10 µg l^{-1} diuron and 0.5 µg l^{-1} zinc pyrithione, a reduction in prymnesiophytes (15 and 40%, respectively) and an increase in diatoms (21 and 38%, respectively), relative to controls, were observed. At 1 µg l^{-1} Sea-Nine 211®, a reduction in diatoms and an increase in prymnesiophytes were observed.

Toxic effect on phytoplankton growth

AFC results demonstrated a reduction in total cell numbers compared to the controls from 7 to 95% under all biocide concentrations after 72 h ($p < 0.05$), except for 0.03 µg l^{-1} Irgarol 1051® and 0.4 µg l^{-1} diuron ($p > 0.05$; 1-way ANOVA, Least Significant Difference post hoc test) (Fig. 3). Compared to controls, results indicated a decrease in nanoeukaryotes following exposures to zinc pyrithione, Sea-Nine 211® and 10 µg l^{-1} diuron ($p < 0.05$), a reduction in picoeukaryote numbers in all biocide exposures ($p < 0.05$) except for 1 µg l^{-1} Sea-Nine 211®, and no changes in Cyanophyte abundances in all treatments ($p > 0.05$), except for 10 µg l^{-1} diuron (data not shown). The relative contributions of cyanophyte to the population increased at the highest levels of zinc pyrithione and Sea-Nine 211®; picoeukaryotes appeared to contribute similarly (5 µg l^{-1} zinc pyrithione) or more (10 µg l^{-1} Sea-Nine 211®) compared to the controls, whilst the contribution of nanoeukaryotes decreased (Fig. 4).

Toxic effect on photosynthesis

The development of the phytoplankton community was followed daily using *in vivo* chlorophyll fluorescence. All 4 antifouling biocides impaired photosynthesis at the concentrations tested (Fig. 5). After 72 h exposure, reductions between 20 and 90% in F_V/F_M

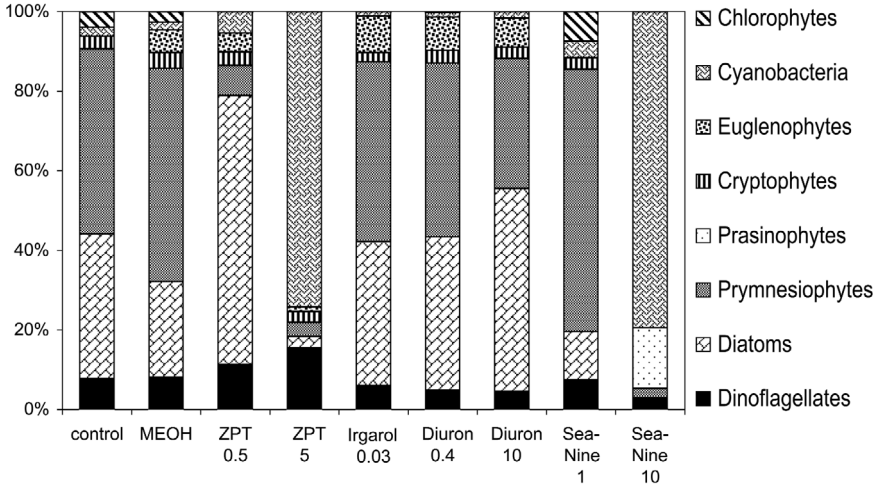

Fig. 2. Contribution of different algal groups, as determined from pigment: chlorophyll *a* ratios using CHEMTAX, after 72 h exposure to concentrations (µg l^{-1}) of zinc pyrithione (ZPT), Irgarol 1051® (Irgarol), diuron, Sea-Nine 211® (Sea-Nine), including the control (no biocide addition) and the carrier control (MEOH)

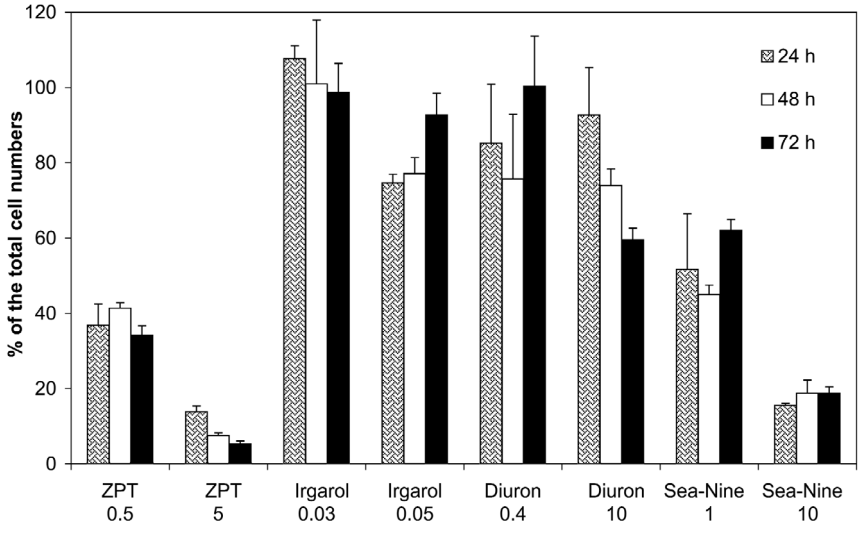

Fig. 3. Percentage of total cell numbers (measured by Analytical Flow Cytometry) related to carrier controls (MEOH) after 24 h, 48 h and 72 h exposure to concentrations (µg l^{-1}) of zinc pyrithione (ZPT), Irgarol 1051® (Irgarol), diuron, and Sea-Nine 211® (Sea-Nine). Data are mean values ± SD (n = 3)

were apparent depending on the concentration of the biocide. For zinc pyrithione, inhibition was more pronounced at 24 h exposure, contrasting with Sea-Nine 211®, where inhibition appeared to decrease with time of exposure (Fig. 5).

In vivo inhibition of fluorescence measured using FRRF was compared with inhibition of $^{14}C\text{-}HCO_3^-$ incorporation after a 24 h incubation period (Fig. 6). Linear regression indicates a significant correlation between them ($R^2 = 0.89$; $p = 0.0001$).

Changes in antifouling biocide concentrations with time

In the single species experiments, measured concentrations of Irgarol 1051® and diuron were within ±3% of the nominal concentration at time zero, and did not change substantially after 72 h (±3% of the measured initial values). Measured Sea-Nine 211® concentrations were lower than the nominal concentrations and probably relate to the rapid degradation of Sea-Nine 211® (half-life ≤ 24 h). Indeed, after 72 h no Sea-Nine 211® was detected in the samples. Therefore, nominal concentrations were used for the calculations of toxicological endpoints.

No Irgarol 1051® was detected at the sampling site (n = 3) or in the control treatments (n = 3). For the natural phytoplankton, concentrations of Irgarol 1051® in the carrier controls on Day 1 of the experiment were close to the detection limit. Initial Irgarol 1051® concentrations were within ±5% of the nominal concentrations (n = 2). Diuron concentrations on Day 1 were 0.4 and 9.7 µg l^{-1} and after 72 h were 0.4 and 6.0 µg l^{-1}, respectively.

DISCUSSION

Unialgal experiments

The intensity of toxic effects on growth and pigmentation differed according to the type of biocide and the species tested. For *Emiliania huxleyi*, Irgarol 1051® was the most toxic (EC_{50} = 0.25 µg l^{-1}) with respect to growth and diuron the least toxic (EC_{50} = 2.26 µg l^{-1}). Although Sea-Nine 211® is readily degraded (Jacobson & Willingham 2000), and is therefore suggested to be less harmful to non-target species, it impaired *E. huxleyi* growth to a similar extent to that of Irgarol 1051® (EC_{50} = 0.35 µg l^{-1}). Values of NOEC for Sea-Nine 211® and zinc pyrithione in *Synechococcus* sp. were higher than the EC_{50}s calculated for *E. huxleyi*, which suggests that *Synechococcus* sp. is more tolerant to these 2 biocides. Comparing the EC_{50}s of the 2 species, *Synechococcus* sp. was more sensitive to diuron than *E. huxleyi*, whereas both showed similar sensitivities to Irgarol

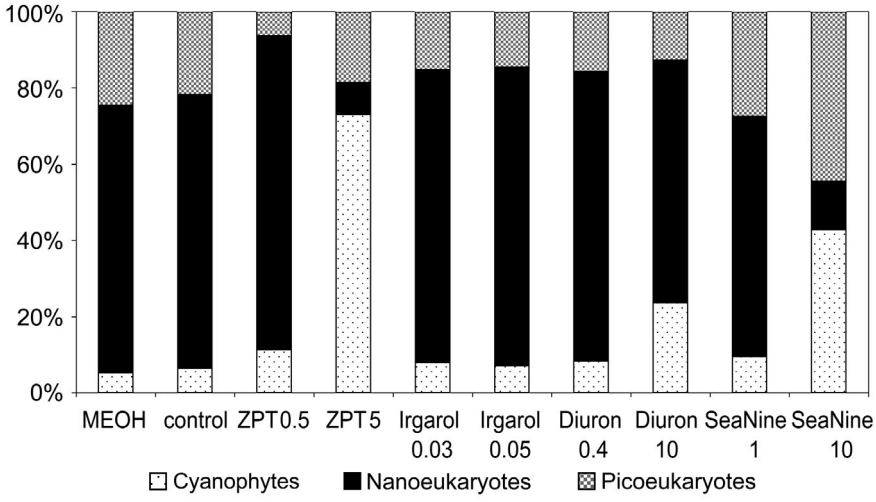

Fig. 4. Cell number of picoeukaryotes, nanoeukaryotes and cyanophytes measured by Analytical Flow Cytometry after 72 h exposure to concentrations (µg l^{-1}) of zinc pyrithione (ZPT), Irgarol 1051® (Irgarol), diuron, Sea-Nine 211® (Sea-Nine), including the control (no biocide addition) and the methanol control (MEOH). Data are mean values (n = 3)

1051®. Our data indicate higher sensitivities in the species tested than those reported for the freshwater alga *Selenastrum capricornutum* exposed to Sea-Nine 211® (EC_{50} = 3 µg l^{-1}), diuron (EC_{50} = 45 µg l^{-1}), and Irgarol 1051® (EC_{50} = 11 µg l^{-1}) (Fernández-Alba et al. 2002). Differences in sensitivities observed in this study and compared to the literature are probably related to the species studied, culture conditions and the measures of toxic response, and are further discussed.

In cultures of *Emiliania huxleyi*, changes in pigment: chl *a* ratios showed increase in 19′-hexanoyloxyfucoxanthin, diadinoxanthin and chl c_3 per chl *a* with increasing concentrations of biocides, and resulted from a relative reduction of chl *a*. Increases in 19′-hex:chl *a* and diadino:chl *a* ratios have also been reported by Schlüter et al. (2000) for *Phaeocystis* sp. and *E. huxleyi* in response to increasing light levels. The relative increase of 19′-hexanoyloxyfucoxanthin might be related to hydroxylation of fucoxanthin to 19′-hexanoyloxyfucoxanthin, causing a reduced efficiency of energy supply by the light harvesting complex (Vanleeuwe & Stefels 1998, Schlüter et al. 2000). This coincides with a decrease in fucoxanthin: chl *a* and an increase in 19′-hex:chl *a* ratios under diuron exposures in this study.

In contrast to *Emiliania huxleyi*, *Synechococcus* sp. showed no overall differences in pigment ratios at the concentrations of biocides tested, owing mostly to the lack of inhibitory response of growth (zinc pyrithione and Sea-Nine 211® exposures). For Irgarol 1051®, however, where growth inhibition was more pronounced, the changes in β-carotene:chl *a* and chl *a*:cell ratios might have been related to protective mechanisms. When exposed to PSII inhibitors (such

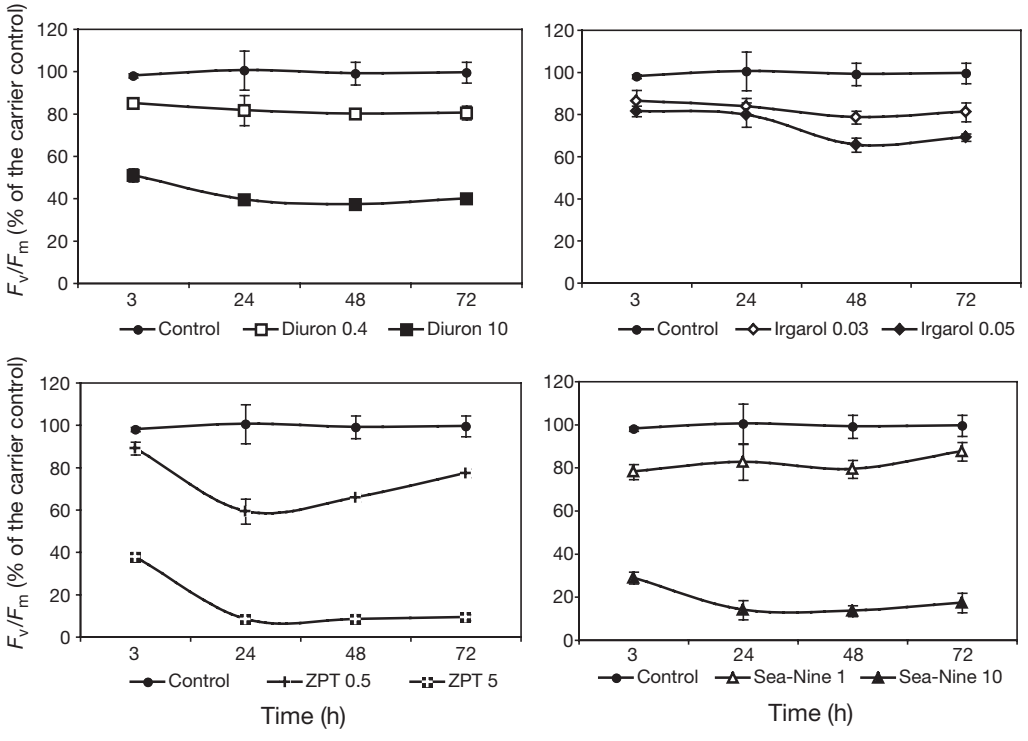

Fig. 5. Photosynthetic efficiency (F_V/F_M) responses of phytoplankton exposed to concentrations (µg l^{-1}) of zinc pyrithione (ZPT), Irgarol 1051® (Irgarol), diuron and Sea-Nine 211® (Sea-Nine) for the period of the experiment (72 h). Data are mean values ± SD (n = 3)

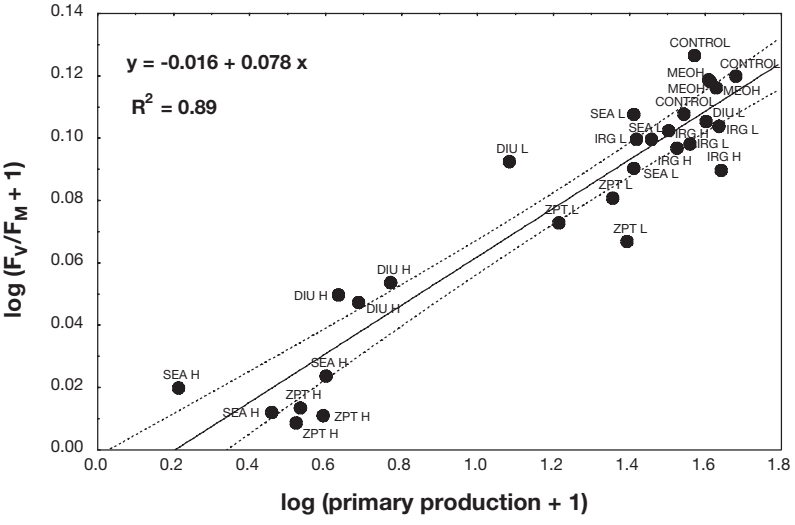

Fig. 6. A linear model relating primary production and F_V/F_M data after 24 h exposure of natural phytoplankton to Irgarol 1051®, zinc pyrithione, Sea-Nine 211® and diuron (p = 0.0001). The confidence intervals (95%) correspond to the outer bounds on the graph. Controls, carrier controls (MEOH), diuron 0.4 µg l^{-1} (DIU L) and 10 µg l^{-1} (DIU H), Irgarol 1051® 0.03 µg l^{-1} (IRG L) and 0.05 µg l^{-1} (IRG H), zinc pyrithione 0.5 µg l^{-1} (ZPT L) and 5 µg l^{-1} (ZPT H), Sea-Nine 211® 1 µg l^{-1} (SEA L) and 10 µg l^{-1} (SEA H)

as Irgarol 1051® and diuron), algae decrease their electron transport capacity and increase the production of oxy-radicals, giving rise to destructive effects when the protective mechanisms are overwhelmed (Dahl & Blanck 1996). In contrast to algae, cyanobacteria lack the zeaxanthin cycle-dependent antenna quenching, and so other mechanisms of energy dissipation, such as D1 exchange might be involved in cell protection (Sane et al. 2002). The unialgal culture studies reported here indicated that, under the selected experimental conditions, *Synechococcus* was more sensitive to PSII inhibitors than *E. huxleyi*. Elucidating why the biochemical mechanisms appear more susceptible to PSII inhibitors in *Synechococcus* sp. requires further investigation.

The values of pigment to chl *a* ratios obtained in controls for *Emiliania huxleyi* and *Synechococcus* sp. are in close agreement with those reported in the literature (Schlüter et al. 2000, Henriksen et al. 2002). While it is well documented that changes in natural environmental conditions (e.g. nutrient-limitation, irradiance and cell growth phase) can alter pigment to chl *a* ratios (Schlüter et al. 2000, Henriksen et al. 2002), our results indicate that anthropogenically derived substances such as biocides may also influence pigment to chl *a* ratios and therefore must be taken into account when estimating phytoplankton composition from group-specific pigment ratios used in programmes such as CHEMTAX.

Natural phytoplankton community experiment

Toxic impairment on phytoplankton composition

Estimation of phytoplankton composition using CHEMTAX provides an indication of the structural changes following exposures to biocides. Our results showed a selective toxicity to the natural phytoplankton assemblage, especially following exposure to the highest concentrations of zinc pyrithione and Sea-Nine 211® tested. A relative increase in peridinin indicated an increase of dinoflagellates and a relative increase in zeaxanthin denoting a greater proportion of Cyanophyceae present in the community under exposure to zinc pyrithione. Similar structural changes to these were also noted at 10 µg l^{-1} Sea-Nine 211®, although the relative proportion of peridinin in this case remained almost unchanged. Such tolerance to both biocides (zinc pyrithione and Sea-Nine 211®) is corroborated by the lack of sensitivity found in *Synechococcus* sp. toxicity experiments.

Under almost all biocide exposures, cyanophycean cell numbers remained unchanged compared to controls, even though marked reductions in other phytoplankton groups occurred. For instance, picoeukaryotes were sensitive to almost all biocide treatments, possibly as a result of their large surface:volume ratio, absorbing more chemical per body mass. The selective reduction of other groups resulted in a relative increase in Cyanophyceae abundance mainly at zinc pyrithione and Sea-Nine 211® exposures. Moreover, AFC results and CHEMTAX estimates were in good agreement in detecting these changes in Cyanophyceae. Such relative lack of sensitivity of Cyanophyceae in comparison to other groups could possibly lead to development of potentially toxic Cyanophyceae species with environmental consequences.

Cyanophycean cell numbers increased under exposure to 10 µg l^{-1} diuron; this was not detected from the pigment results, due probably to them being masked by major algal groups. *Synechococcus* sp. has been reported to be capable of exchanging the D1:1 and D1:2 proteins, triggered by diuron treatments (Campbell et al. 1996), therefore conferring resistance to PSII inhibitors. Cells containing the D1:2 form generally have a higher quantum yield of oxygen evolution and lower non-photochemical quenching than cells

containing only D1:1 protein (Campbell et al. 1996). The transient exchange of the D1:1 protein, which appears to be the initial response, may thus provide the time required for full cellular acclimation to an increased excitation pressure by modification in protein and lipid composition. This mechanism might have aided cyanophytes to outcompete other algal groups in the community, when exposed to high diuron concentrations.

Although the concentrations of Irgarol 1051® tested were too low to induce any detectable impact under the selected experimental conditions, it is likely that environmental concentrations (of up to 1.42 µg l^{-1}; Thomas et al. 2001) would modify phytoplankton composition (Readman et al. 2004). Other studies have demonstrated that, depending on the concentrations, Irgarol 1051® (0.07 to 2.5 µg l^{-1}) can modify algal community structure, by reduction in prymnesiophytes and development of pennate diatoms, cryptophytes, chrysophytes and cyanophytes (Bérard et al. 2003, Readman et al. 2004). A similar pattern was observed in this study for 0.5 µg l^{-1} zinc pyrithione and 10 µg l^{-1} diuron, where the relative contribution of diatoms increased, while prymnesiophytes (indicated by 19′-hexanoyloxyfucoxanthin) were reduced. Reported environmental concentrations of diuron (up to 6.74 µg l^{-1}; Thomas et al. 2001) also pose a threat to phytoplankton, as indicated in this study. Very little data is available on environmental concentrations of Sea-Nine 211® and zinc pyrithione (www.pml.ac.uk/ace).

Phytoplankton were more sensitive to antifoulants than other planktonic groups, such as bacteria and crustaceans (Fernández-Alba et al. 2002). However, phytoplankton toxic responses such as compositional changes and low productivity might have an indirect impact on grazers by reducing their food resources. These compositional changes also affect nutrient cycling through the microbial food web and subsequent flow to higher trophic levels (Goldman et al. 1987). Bioaccumulation and consequent trophic transfer of antifoulants is unlikely to be of major significance.

Toxic effect on photosynthesis

The quantum efficiency of PSII (F_V/F_M) was diminished by all concentrations of all biocides tested on the natural phytoplankton community. Diminished F_V/F_M can indicate a large proportion of inactive PSII reaction centres due to oxidation, degradation of D1 proteins (Anderson et al. 1997) or severely reduced pigment concentrations (Marwood et al. 2001). Based on F_V/F_M measurements, the phytotoxicity of the biocides for the natural phytoplankton community can be ranked: Irgarol 1051® > zinc pyrithione ~ Sea-Nine 211® > diuron. Variations in this toxicity ranking of biocides might differ for other communities depending on species composition or physiological state.

Irgarol 1051® affected F_V/F_M at very low (0.03 µg l^{-1}) and environmentally relevant concentrations. Even though a higher concentration of diuron was required to impair photosynthesis (10 µg l^{-1}), similar levels (up to 6.74 µg l^{-1}) have been detected in the environment (Thomas et al. 2001). Degradation products of diuron can also pose a toxic threat to the environment (Tixier et al. 2001). However, since Irgarol 1051® and diuron are not easily degraded, exposures and toxicity were not significantly reduced over time during the experiments in this study. Zinc pyrithione and Sea-Nine 211® are, however, transformed more rapidly than diuron and Irgarol 1051® (Thomas 2001) reducing exposure concentrations and toxic response. Reversibility of the F_V/F_M impairment was faster for zinc pyrithione than for Sea-Nine 211®, which was observed at the lower concentrations tested, most likely due to the fact that these compounds undergo degradation within hours. Biological processes appear to be more important than abiotic processes for the transformation of the active substance of Sea-Nine 211® (DCOI), with half-lives varying from <1 to 24 h according to the matrices and temperature investigated (Jacobson & Willingham 2000). In contrast to Sea-Nine 211®, zinc pyrithione is rapidly transformed by photolysis with a half-life of <1 h in natural seawater. Toxicity of metabolites is lower than zinc pyrithione and this may have contributed to the F_V/F_M recovery observed after 24 h exposure (Turley et al. 2000).

FRRF versus ^{14}C-HCO_3^- uptake

Photosynthesis-related endpoints are sensitive measures for assessing short-term responses to antifouling biocides. Although F_V/F_M and ^{14}C-HCO_3^- uptake measure different phases of the photosynthetic process, a good correlation between both methods was found (R^2 = 0.89). Analysis of chlorophyll fluorescence is a sensitive and early indicator of damage to the photosynthetic apparatus (Krause & Weis 1991), whilst ^{14}C-HCO_3^- uptake measures the end result of the photosynthetic process, i.e. carbon assimilation. A 1:1 relationship between ^{14}C-HCO_3^- measurements and FRRF estimates of carbon fixation has been reported by the originators of the technique (Kolber & Falkowski 1993). Good comparability between a pulse-amplitude modulated fluorometer and ^{14}C-HCO_3^--based primary production has also been reported by Dorigo & Leboulanger (2001) for freshwater periphyton exposed to herbicides and for natural phytoplankton exposed to Irgarol 1051® (Nyström et al. 2002).

CONCLUSIONS

The differential toxicity of the tested biocides to algae induced modifications to the community structure of the natural phytoplankton under investigation, which resulted in changes to the taxonomic composition of natural seawater. The cyanophyte *Synechococcus* sp. was more resistant than the prymnesiophyte *Emiliania huxleyi* to zinc pyrithione and Sea-Nine 211® in culture experiments. In contrast, *Synechococcus* sp. was more sensitive than *E. huxleyi* to diuron, while exposure to Irgarol 1051® showed a similar impact on both species. Irgarol 1051® toxicity was up to twice that of zinc pyrithione and Sea-Nine 211®, whilst diuron was the least toxic of the biocides studied.

The biocides impact on the selected natural phytoplankton community and the differences in group-specific sensitivity were detected through pigment composition. Following exposure to zinc pyrithione and Sea-Nine 211®, the pigment zeaxanthin proportionally increased indicating a relative increase in Cyanophyceae, which was corroborated with AFC results. Cyanophyceae is a group of particular concern because of toxic blooms. Consequences of biocide pollution on marine diversity during conditions which promote toxic blooms require further investigation in mesocosm-type studies.

However, further investigations should be conducted on inter-species variations in pigment to chl *a* ratios under the influence of toxicants. Biocidal effects were detected by both $^{14}C\text{-}HCO_3^-$ uptake and F_V/F_M measurements at very low concentrations. F_V/F_M is considered to be a sensitive and rapid endpoint to assess the impact of antifouling booster biocides.

Concentrations of antifouling booster biocides in the environment are still a matter for concern. In the UK, the use of Irgarol 1051® was revoked by legislation in July 2003. While both Sea-Nine 211® and zinc pyrithione are readily degradable, our results suggest that the consequences of continuous use, providing persistent contamination, could be environmentally damaging. The effects of short-term pulses needs to be more researched.

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