



# Impact of the antifouling agent Irgarol 1051 on marine phytoplankton species

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## ABSTRACT

In the present study we tested the hypothesis that environmental concentrations of the antifouling agent Irgarol 1051, as measured in coastal Western European waters, affect marine phytoplankton performance. The impact of Irgarol was investigated in the phytoplankton species *Thalassiosira weissflogii*, *Emiliania huxleyi*, *Tetraselmis* sp. and *Fibrocapsa japonica*. EC<sub>50</sub> concentrations for growth, effective quantum yield of PSII and viability were calculated from dose response relationships established during 72 h exposures to six Irgarol concentrations. Furthermore, the biological recuperation from a temporary exposure to a high Irgarol concentration (39.47 nM l<sup>-1</sup>) was monitored. Growth rates and effective quantum yield were strongly affected by Irgarol, however viability loss was never observed. EC<sub>50</sub> values differed five fold between species and ranged from 0.43 to 2.38 nM for effective quantum yield and from 0.46 to 2.44 nM for growth rate. For all species, complete biological recuperation was shown within 3–4 days after the Irgarol treatment, both for effective quantum yield and growth rate. All calculated EC<sub>50</sub> values and EC<sub>20</sub> values fall within the Irgarol concentration range measured in Western European coastal waters. We therefore conclude that present day Irgarol 1051 levels may affect the *in situ* performance of marine phytoplankton in this area.

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## 1. Introduction

Marine biofouling can be defined as the unwanted accumulation of animals, plants and microorganisms on artificial submerged surfaces in sea water (Yebra et al., 2004). Fouling is a significant problem in shipping, since even a small amount can increase the hydrodynamic drag, leading to a loss of speed and fuel efficiency. The commonly used antifouling agent tributyltin (TBT) has been shown to cause serious problems to aquatic organisms. As a result, alternative biocides with a reduced toxicity were developed. These biocides, such as Diuron, SeaNine 211, Dichlofluanid and Irgarol<sup>®</sup> 1051, also named Cybutryne, are enriched with copper oxides and are therefore called booster biocides (Boxall et al., 2000). Irgarol<sup>®</sup> 1051 (N'-tert-butyl-N-cyclopropyl-6-(methylthio)-1,3,5-triazine-2,4-diamine) further called Irgarol (Ciba<sup>®</sup> Specialty Chemicals Inc.) was registered by the United States Environmental Protection Agency (USEPA) in 1994 (Hall et al., 1999). Both previously and freshly painted boats leach Irgarol in the environment, yet recently painted boats have higher initial leaching rates (Hall et al., 1999). As a result, marinas with a limited water circulation will have elevated Irgarol concentration, as described by Lamoree et al. (2002) for Dutch marinas. Within Europe, Mediterranean and British coastal areas are found to be the most contaminated by Irgarol (up to 2.53 nM [640 ng l<sup>-1</sup>] and 5.61 nM [201–1421 ng l<sup>-1</sup>] respectively) (Tolosa et al., 1996; Boxall et al., 2000; Thomas et al., 2001; Readman, 2006).

Irgarol may affect non-target photosynthetic organisms such as phytoplankton, periphyton and aquatic macrophytes (Hall et al., 1999) when leaching into the marine environment (Dahl and Blanck, 1996). Irgarol inhibits electron transport in photosystem II (PSII) (Holt, 1993) by binding to the D1 protein (Ranke and Jastorff, 2000). However, according to the manufacturer, the photosynthetic activity is fully restored once Irgarol is removed (Ciba<sup>®</sup> Specialty Chemicals Inc. Irgarol 1051, Part I). Only a few studies have addressed the possible effect of Irgarol on marine non-target algae. The green alga *Dunaliella tertiolecta* showed a 50% decrease in growth rate during a 96 h exposure to about 3.95 nM of Irgarol while at 11.84 nM almost all (96% inhibition) cells were killed (Gatidou et al., 2003). In another study, 50% inhibition in cell number was found after 72 h of exposure to 0.63 nM Irgarol for *Synechococcus* sp., and to 0.99 nM Irgarol for *Emiliania huxleyi*, coinciding with Photosystem II (PSII) efficiency loss (Devilla et al., 2005a). Studies performed on natural phytoplankton communities showed a 50% decrease in eukaryote abundance 72 h after an addition of 0.39 nM Irgarol (Readman et al., 2004). Dahl and Blanck (1996) investigated the effect of Irgarol on periphyton colonization. This study showed a 50% decrease in the photosynthetic activity after several hours of exposure to 4.4–5.5 nM Irgarol. Prolonged (3 weeks) exposure revealed a decrease in biomass while community structure was altered in favour of the more tolerant species (Dahl and Blanck, 1996). Similarly, other studies showed shifts in phytoplankton species composition as a result of prolonged Irgarol exposure (Devilla et al., 2005b, Zamora-Ley et al. (2006).

In the present study we tested the hypothesis that present day environmental Irgarol concentrations, as measured in coastal Western

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European waters, affect marine phytoplankton performance. To this end we determined Irgarol effect concentrations of growth, effective quantum yield of PSII and viability in four temperate marine algae of different taxonomic origin (*Thalassiosira weissflogii*, *E. huxleyi*, *Tetraselmis* sp. and *Fibrocapsa japonica*). Cultures were exposed to a range of Irgarol concentrations for 72 h. Changes in growth rate and viability were determined by flow cytometry, while effective quantum yield was determined by Pulse Amplified Modulation (PAM) fluorometry. For both growth rate as well as effective quantum yield  $EC_{50}$  values were calculated using the actual Irgarol exposure concentrations, as measured in the cultures. In addition, a biological recuperation experiment was performed to test whether deleterious effects were reversible after a strong reduction in Irgarol concentration.

## 2. Materials and methods

### 2.1. Cultivation procedure

The species used were the diatom *T. weissflogii* (Gronow) (Bacillariophyceae), the coccolithophore *E. huxleyi* (Lohman) (Prymnesiophyceae), the green alga *Tetraselmis* sp. (Prasinophyceae), and the ichthyotoxic golden brown flagellate *F. japonica* (Toriumi & Takano) (Raphidophyceae) W420, all available from the culture collection of the Department of Ocean Ecosystems, University of Groningen. These species were chosen since they are all found in coastal Western European waters and belong to various algal classes. The algae were pre-cultured at 15 °C and 50–60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of PAR (Osram L, 18 W) in a 16:8 light–dark regime. This irradiance level would guarantee substantial initial growth for all species, without causing photoinhibition in the irradiance sensitive *F. japonica*. The growth medium was modified F/2 (Veldhuis and Admiraal, 1987), based on autoclaved natural seawater collected in the North Sea. The seawater was filtered on a 595 1/2 filter (Whatman®), autoclaved and stored at 4 °C. Nutrient and trace metal additions to provide F/2 strength were added just before the start of an experiment. For *F. japonica* salinity was adjusted to 25 PSU with MilliQ and for the

other three algal species 33 PSU natural seawater was used. Two batches of seawater were used (first batch coastal North Sea, second batch Central North Sea). This was done since biocide analyses revealed the presence of Diuron 0.18–0.89 nM in the first seawater batch that was used for the toxicity experiments, while the second batch revealed undetectable Diuron levels. All species were acclimated to the experimental conditions for at least 2 weeks, prior to the Irgarol exposure experiments. Cultures were diluted regularly in order to prevent nutrient limitation.

### 2.2. Toxicity experiments

At the start of the experiment the cultures were diluted five times in F/2 medium to guarantee nutrient replete growth during experimentation. The diluted cultures were distributed over 12 × 250 ml sterile glass bottles to obtain duplicate 150 ml samples per Irgarol condition. A 500 ppm (1.97 mM) Irgarol stock in HPLC grade methanol was obtained from the RIKZ, Haren (Ref. nr. PP1036) and stored in a brown glass bottle at 4 °C. This stock was diluted with MilliQ in two steps (500 ppm → 10 ppm → 1 ppm) in to a final 1 ppm working solution just prior to the experiment. From this working solution different aliquots were added to the cultures to provide a range of Irgarol concentrations (calculated as 0, 0.51, 2.64, 3.94, 5.25, 52.61 nM). The effect of carrier (methanol) concentration in the cultures was assumed to be negligible: the highest methanol concentration was 0.006% (v/v), which is more than 10 times below the maximum methanol concentration recommended for algal bioassay tests (0.78%, Abou-Waly, 2000). Then the cultures were placed back at their original culture conditions. Subsequently, after 2, 24, 48 and 72 h samples for PAM and flow cytometry were taken.

### 2.3. Algal recuperation experiment

Algae were diluted 4 times with F/2 medium in sterile 250 ml glass bottles, giving approximately 150 ml of culture per bottle. After 4 days (day 4), the algal concentration was determined by flow cytometry and PSII effective quantum yield by a water–PAM. Then the cultures

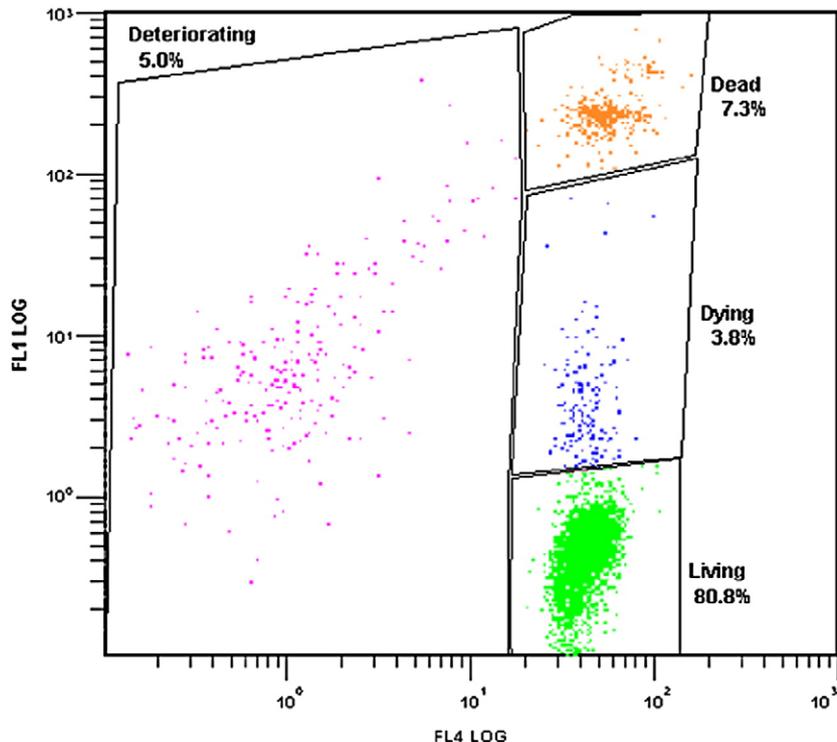


Fig. 1. Example of a flow cytometric FL1:FL4 (Sytox specific green fluorescence (FRL1) versus red (FL4) fluorescence) plot which was used to determine the percentages living, dying, dead and deteriorated algae.

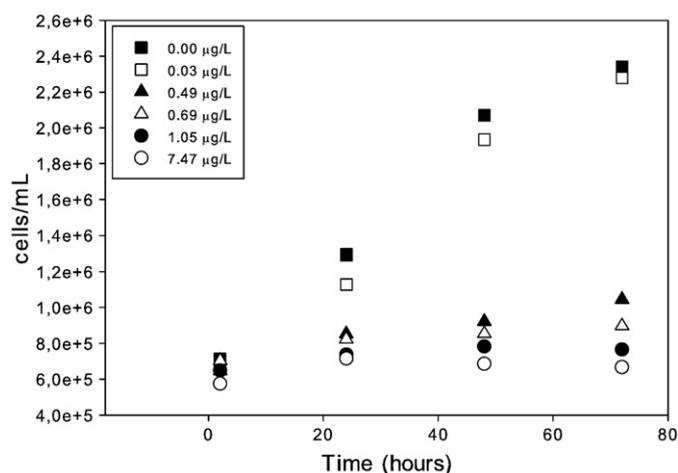


Fig. 2. Example of the time course of cell densities of *E. huxleyi*, during exposure to different Irgarol levels.

were split into two portions in sterile 60 ml serum bottles, after which 39.47 nM Irgarol (final, measured concentration) was added followed by incubation under the experimental irradiance and temperature conditions. The remainder of the culture was also incubated and served as the control (no Irgarol treatment). After 3 days of Irgarol treatment (day 4–day 7) cell numbers and effective quantum yield were measured followed by a 50× dilution step in F/2, reducing the Irgarol concentration to 0.79 nM. Effective quantum yield and growth rates were subsequently monitored until day 11. The biological recuperation experiment was performed twice for each species, using both seawater batches. *F. japonica* was not included in the recovery experiments, since this species could not handle the 50× dilution step.

#### 2.4. PSII measurements

Effective PSII quantum yield data were obtained by Pulse Amplified Modulation (PAM) fluorometry (PAM2000 and water-PAM; Walz, Germany). The water-PAM consisted of a control unit with a flow-through water-PAM chlorophyll fluorometer featuring blue (450 nm) LEDs. For the PAM 2000, several ml of sample was filtered onto a 12 mm GF/F filter (Whatman®). Caution was taken not to dry out the filter. Samples were incubated for 5 min at 15 °C in the dark prior to the measurements. Since this acclimation time does not allow for complete relaxation of NPQ, the measured parameter was interpreted as  $F_v'/F_m'$  (effective quantum yield), not  $F_v/F_m$  (max quantum yield).

#### 2.5. Viability measurements

A flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Miami, FL, USA), with 5-mW argon ion laser with excitation at 488 nm, was used for cell counts to determine growth rate as well as viability. To determine cell viability, 10 µl Green Nucleic Acid Stain Sytox® (5 mM solution in DMSO, 1:100 diluted in MilliQ, Molecular Probes) was added to 1 ml of culture, shaken gently and incubated for 30 min at room temperature. Cells were analyzed following Van de Poll et al. (2007). When more than 200 cells/s (viable – dead) were detected, samples were diluted 10× in 0.2 µ filtered seawater. The number of algae was obtained from the Forward Scatter (FS) histogram, while for cell viability only the FS gated cluster was analyzed. This was done to guarantee exclusion of non-algal particles in the calculations. Four standardized (for each species) regions were distinguished: living, dying, dead and deteriorated (Example for *E. huxleyi* in Fig. 1). The first three regions were characterized by a similar chlorophyll autofluorescence (FL4) (x-axis) and an increasing Sytox specific green fluorescence (FL1) (y-axis: viable–dying–dead). The deteriorated cells were characterized by elevated Sytox specific FL1, with simultaneously

reduced FL4 (Fig. 1). To calibrate flow cytometric viability analysis, aliquots of each species were diluted in Milli-Q and spiked with Sytox, after which viability loss was followed over a time course up to 30 min. Then identical culture volumes were analysed, clustered and gated such that all cells were retrieved. This was done for each species since species-specific differences in the position and quantitative importance of the four clusters were found. Data analysis was performed using EXPO32 ADC XL4 Color analytical software.

#### 2.6. Irgarol analysis

The analytical procedure used in trend monitoring of the marine environment, which is validated and tested by QUASIMEME round-robin tests twice a year, was applied to these experiments (RIKZ, 2006a). Samples were preserved with Hydrochloric acid at pH=2 and stored at 4 °C until extraction (RIKZ, 2006b; Barceló and Alpendurada, 1996; Jeannot, 1994). Before extraction, the sample was buffered to pH=5 with Sodiumacetate. Two surrogate standards (13C3-Atrazine and 13C6-Metolachlor) were added to check the analytical recovery of each sample. Then solid phase extraction was performed with 200 mg Styrene divinylbenzene columns (Baker, Deventer) after filtration of the sample over a Ø 25 mm GF/F filter (Whatman®). The target compounds were eluted with methanol and analyzed with Liquid Chromatography Mass Spectrometry/Mass Spectrometry (API2000 LC-MS/MS, Applied Biosystems) after concentration to 1 ml and the addition of an internal standard (D6-Diuron). This procedure was adjusted slightly for the actual tests by performing the filtration directly after the toxicity experiments. The regular quality assurance tests indicated that this had no influence on the method performance. Blank and direct Irgarol measurements in untreated samples were also performed for each series. The detection limit of Irgarol using the method described was 0.002 nM and the analytical uncertainty  $U$ , based on long term reproducibility measurements, was  $-14 < U < 21\%$  (RIKZ, 2006a).

It is advised to perform biocide analyses in dose–effect studies, since uncontrolled variability in anticipated concentrations may occur (Klamer et al., 1997; Brown et al., 2001). Indeed, a substantial difference between dilution-expected and measured Irgarol concentrations (between 10 and 80% recovery), including the procedural reference samples (between 30 and 60% recovery), was found. These stochastic uncontrolled losses could be due to phenomena related with Irgarol solubility and wall adsorption. The maximum solubility of Irgarol in water is ~10 ppm only. This may cause uncontrolled aliquot dilution to the second dilution step, while solubility also depends on salinity and pH (Ciba® Irgarol® 1051, coating effect segment). Second, despite the short contact time (seconds to minutes) of the Irgarol stocks with plastic material (tubes for dilution, pipette tips), some wall adsorption may have occurred during dilution, since an Irgarol loss of 20% was shown after one day storage in plastic tubes when diluted in MilliQ (results not shown). Although other possibilities for the loss of Irgarol, such as photodegradation, bioaccumulation by the algae or minor adsorption to glass fibre filters cannot be ruled out, these possible factors would only apply to the algal filtrates and not to the reference samples (showing between 30 and 60% recovery). The high reproducibility of the dose response curves for the duplicate experimental

Table 1

The  $p$  values resulting from the K/S test for the four 'viability groups' living, dying, dead and deteriorated

	Living	Dying	Dead	Deteriorated
<i>T. weissflogii</i>	0.657	1.000	0.771 <sup>a</sup>	
<i>E. huxleyi</i>	0.100	0.100	1.000	0.100
<i>Tetraselmis</i> sp.	0.771	0.229	0.771	0.771
<i>F. japonica</i>	0.229	0.229	0.429	0.229

$p > \alpha =$  no significant difference ( $\alpha = 0.05$ ).

<sup>a</sup> for *T. weissflogii*, dead and deteriorated were one group.

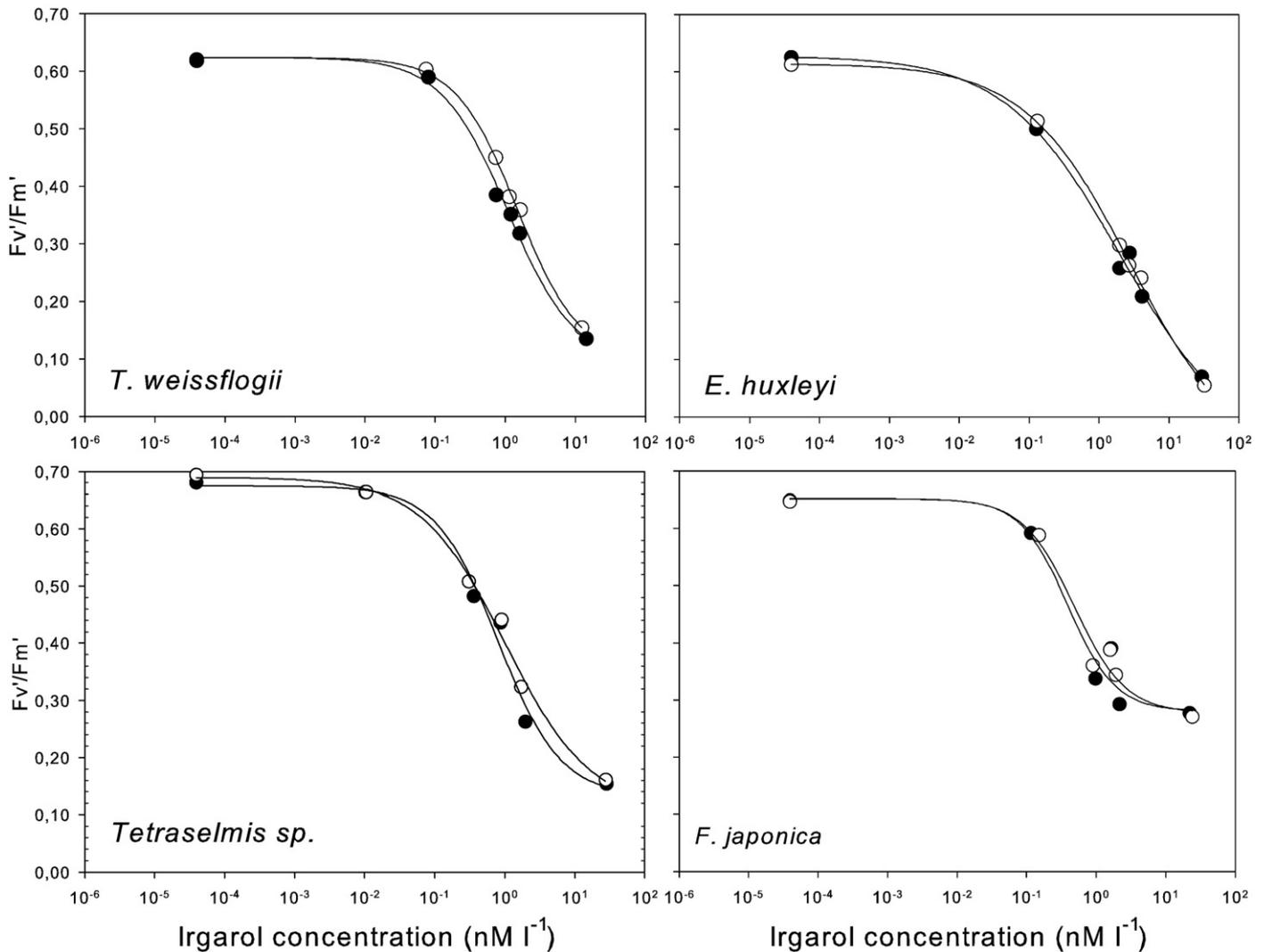


Fig. 3. Irgarol effects on effective quantum yield ( $F_v'/F_m'$ ) and derived  $EC_{50}$  curves for four marine phytoplankton species. Black and white dots represent the duplicate series.  $R^2$  values are shown in Table 3.

series (Figs. 3 and 4) underline the necessity of using actual exposure concentrations instead of dilution derived concentrations.

Early contaminant analyses also demonstrated the presence of Diuron, in the seawater batch that was used for the  $EC_{50}$  experiments. It is known that the co-occurrence of different antifouling agents can have an antagonistic or synergistic effect. For example, Irgarol and Diuron were found to act synergistically in the microalga *S. capricornotum* (Fernández-Alba et al., 2002). In our experiments however, there was no significant difference between the biological recuperation experiment using the first or second seawater batch (Table 3). This indicates that the detected Diuron levels did not, or were too low to influence the test results. This is also in accordance with the reported 7 fold lower toxicity of Diuron and the low levels compared to the experimental Irgarol levels.

### 2.7. Calculation of $EC_{50}$ values

Effective concentration values ( $EC_{50}$ ) were obtained for 50% growth rate reduction and PSII efficiency for all species. Calculations were done using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California, USA) or SigmaPlot 10.0. Besides the  $EC_{50}$  values,  $EC_{10}$  and  $EC_{20}$  values were also calculated using:

$$EC_x = \left( \frac{x}{100-x} \right)^{1/H} \cdot EC_{50}$$

where  $x$  is, 10 or 20 and  $H$  is the slope as calculated for the  $EC_{50}$  curve.

### 2.8. Statistical analysis

All statistical analyses were performed with SPSS 14.0. To test whether there was a significant difference between the four 'viability groups' of cultures with (highest concentration) and without Irgarol a Two-sample Kolmogorov–Smirnov (K–S) test was done. This same K–S test was done to investigate if there was a significant difference between the recovery experiment with the first and second seawater batch. For all statistical analysis  $\alpha=0.05$  and when  $p>\alpha$ , results were considered non significant.

## 3. Results

### 3.1. Toxicity experiments

The four species showed similar responses towards Irgarol. At the lower Irgarol concentrations cell numbers increased significantly over time, while numbers only slightly increased, if at all, at higher Irgarol concentrations (example *E. huxleyi* in Fig. 2). The viability measurements showed no difference between the Living, Dying, Dead and Deteriorated clusters of the blank samples and the ones treated with

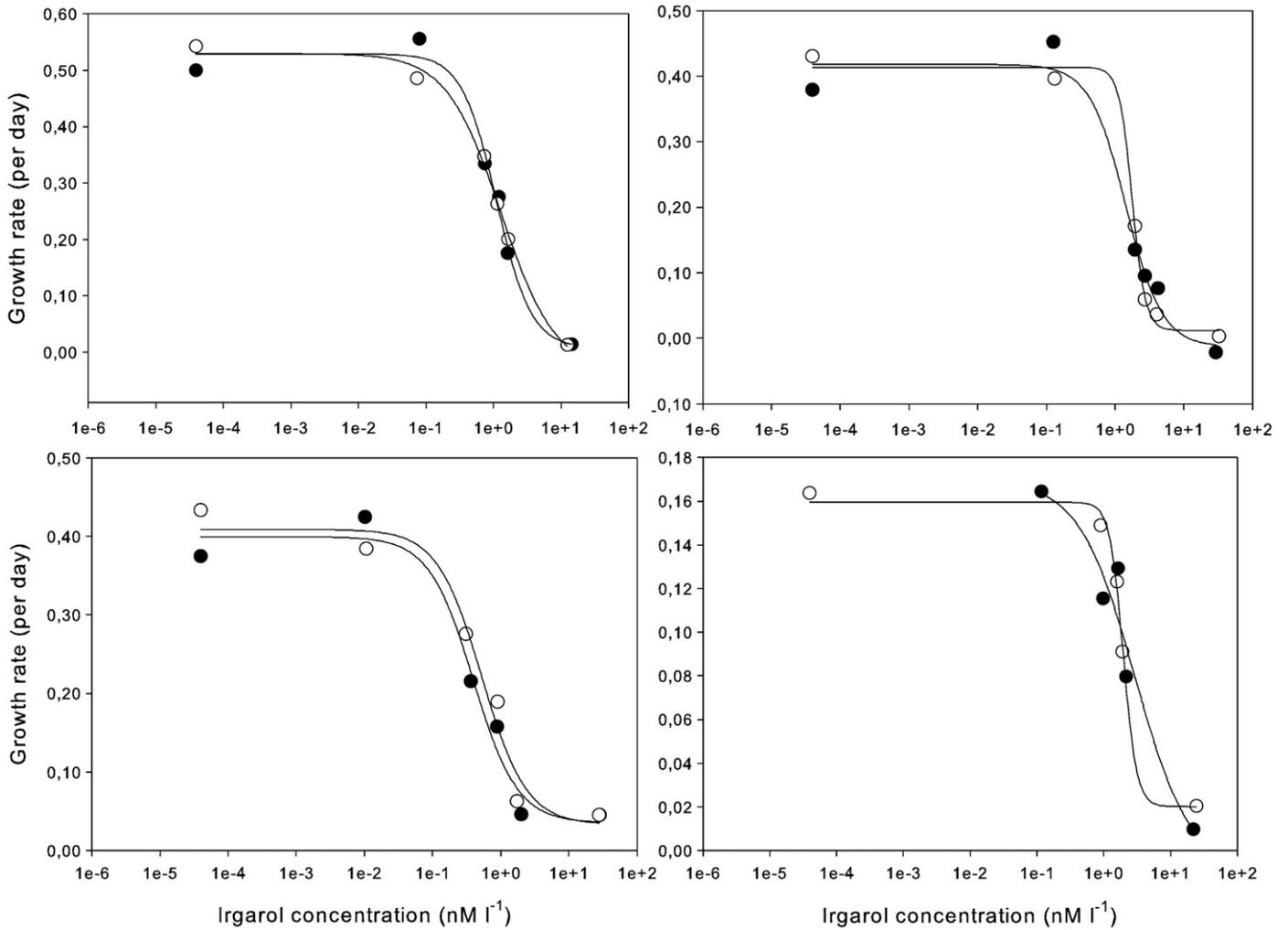


Fig. 4. Irgarol effects on growth rate and derived EC<sub>50</sub> curves for four marine phytoplankton species. Black and white dots represent the duplicate series. R<sup>2</sup> values are shown in Table 3.

the highest Irgarol concentration. Yet, *E. huxleyi* had a slightly higher percentage of deteriorated cells, while the amount of living algae slightly decreased during Irgarol exposure. However, statistical testing of the four clusters with (= highest Irgarol concentration) and without Irgarol revealed no significant ( $\alpha=0.05$ ) difference for any of the algae (Table 1). Therefore, any increase in the relative amount of dead cells as a result

of Irgarol exposure could not be demonstrated for the duration of the experiments. All species showed loss of colour during exposure to the highest Irgarol concentrations (results not shown). In addition, for all species a trend was shown in decreased red autofluorescence (FL4) at the highest Irgarol concentrations. *F. japonica* also showed decreased FL4 fluorescence values at lower Irgarol concentrations (results not shown).

Table 2

EC<sub>50</sub> values ( $\mu\text{g l}^{-1}$ ; nM) as determined for effective quantum yield and growth rate for all tested species

	EC <sub>50</sub> ( $\mu\text{g l}^{-1}$ ; nM) Fv'/Fm'	R <sup>2</sup>	Average $\pm$ S.D.	EC <sub>50</sub> ( $\mu\text{g l}^{-1}$ ; nM) Growth rate	R <sup>2</sup>	Average $\pm$ S.D.
<i>T. weissflogii</i>	0.275	0.997	0.327 $\pm$ 0.073	0.280	0.985	0.303 $\pm$ 0.032
	1.09			1.11		
	0.378	0.998		0.325	0.995	1.20 $\pm$ 0.13
	1.49			1.28		
<i>E. huxleyi</i>	0.505	0.992	0.604 $\pm$ 0.140	0.363	0.976	0.406 $\pm$ 0.060
	1.99			1.43		
	0.703	0.999		0.448	0.995	1.60 $\pm$ 0.24
	2.77			1.77		
<i>Tetraselmis</i> sp.	0.204	0.985	0.230 $\pm$ 0.038	0.100	0.976	0.116 $\pm$ 0.024
	0.81			0.39		
	0.257	0.993		0.133	0.972	0.46 $\pm$ 0.09
	1.01			0.52		
<i>F. japonica</i>	0.098	0.960	0.110 $\pm$ 0.016	0.740	0.939	0.618 $\pm$ 0.172
	0.39			2.92		
	0.121	0.972		0.479	0.994	2.44 $\pm$ 0.68
	0.48			1.89		

The duplicate measurements were calculated separately and used to calculate the average  $\pm$  S.D. R<sup>2</sup> represent the fit of the EC<sub>50</sub> curves as shown in Figs. 4 and 5.

During Irgarol exposure all species showed a decrease in effective quantum yield (Fig. 3A–D) as well as in growth rate (Fig. 4A–D) at increasing Irgarol concentrations. The effect on effective quantum yield was shown already 2 h after the Irgarol addition for all algal species. Since the duplicate measurements contained different Irgarol concentrations, these measurements were treated as different samples. For all species and for both effective quantum yield and growth rate the EC<sub>50</sub> curves showed a good fit (lowest R<sup>2</sup> of 0.94). However the fit for effective quantum yield was better than for growth rate, especially in the case of *F. japonica* (Table 2). Besides the EC<sub>50</sub> value for these four species, EC<sub>10</sub> and EC<sub>20</sub> values for reduction in PSII efficiency were calculated. They revealed values of 0.126, 0.047, 0.067,

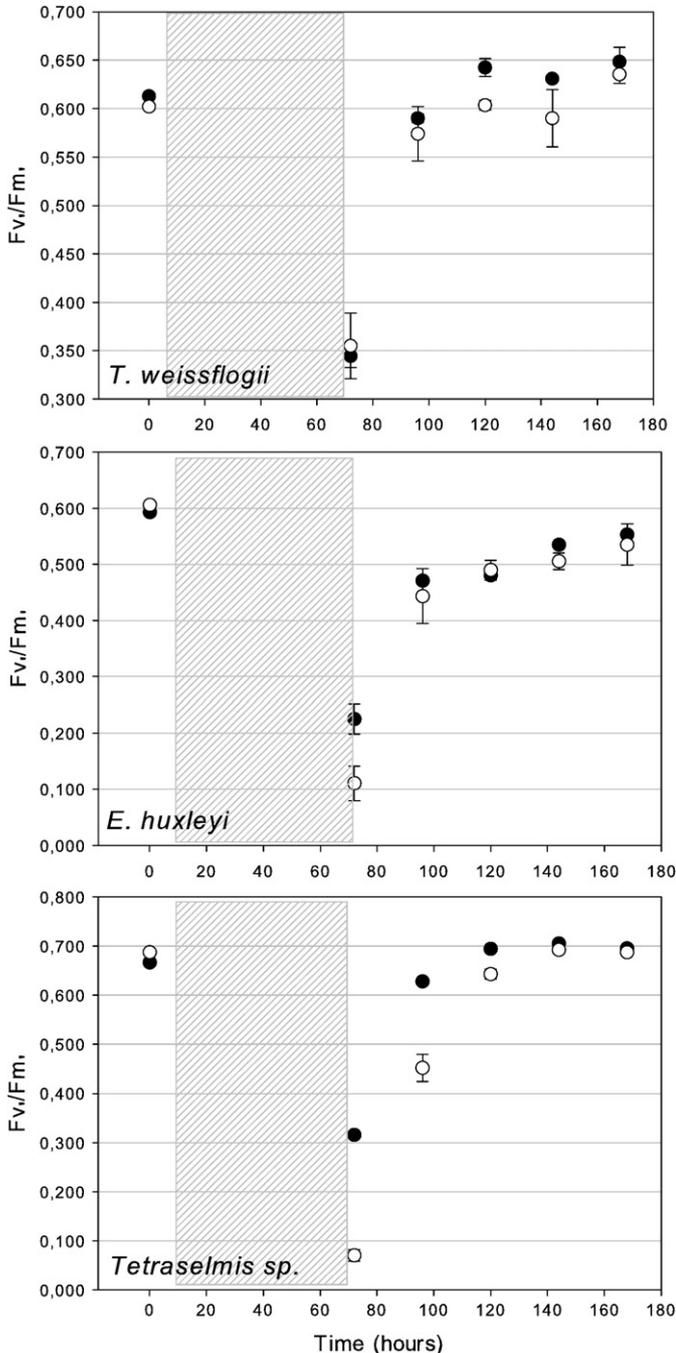


Fig. 5. Reversibility of Irgarol effects on three phytoplankton species based on the recovery of the  $F_v/F_m$ . The grey area indicates the period of Irgarol exposure. Filled circles: first seawater batch; Open circles: second seawater batch. Error bars represent mean  $\pm$  S.D.

Table 3

Growth rates during the recuperation experiment

	Original growth rate	After 3 days (day 7)	After 4 days (day 8)
<i>T. weissflogii</i> <sup>a</sup>	0.522	0.568	0.568
<i>T. weissflogii</i> <sup>b</sup>	0.332	0.447	0.482
<i>E. huxleyi</i> <sup>a</sup>	0.404	0.912	0.914
<i>E. huxleyi</i> <sup>b</sup>	0.723	0.966	0.893
<i>Tetraselmis</i> sp. <sup>a</sup>	0.407	0.377	0.407
<i>Tetraselmis</i> sp. <sup>b</sup>	0.451	0.247	0.424

The original (blank) growth rate as well as the growth rate 3 and 4 days after Irgarol was removed are represented (measured at days 7 and 8 respectively).

<sup>a</sup> first seawater batch.

<sup>b</sup> second seawater batch.

0.071 nM Irgarol (EC<sub>10</sub>) and 0.300, 0.197, 0.170 and 0.138 nM Irgarol (EC<sub>20</sub>) for *T. weissflogii*, *E. huxleyi*, *Tetraselmis* sp. and *F. japonica* respectively.

### 3.2. Biological recuperation experiment

The adverse effects of 39.47 nM Irgarol were shown to be reversible for the tested species *T. weissflogii*, *E. huxleyi* and *Tetraselmis* sp. (Fig. 5A–C). *T. weissflogii* returned to its original level 2 days after the Irgarol was removed when considering effective quantum yield, while *Tetraselmis* sp. reached this level after 3 days. *E. huxleyi* did not recover completely, although effective quantum yield increased rapidly after the Irgarol was removed. The effect of Irgarol on growth rate was also shown to be reversible. Within 3 days after the Irgarol was removed growth rates of *T. weissflogii* and *E. huxleyi* were back at their original value, while for *Tetraselmis* sp this took 4 days (Table 3). No significant difference ( $\alpha=0.05$ , K–S test) between the two seawater batches was found (*T. weissflogii*, *E. huxleyi* and *Tetraselmis* sp.,  $p=0.474$ ,  $p=1.000$  and  $p=0.416$  respectively). Finally, in agreement with the toxicity experiments, all cultures lost colour during Irgarol exposure, however, for all species the colour was back to normal 3–4 days after the Irgarol was removed.

## 4. Discussion

Our data did not demonstrate any Irgarol mediated viability loss in the four tested species within the experimental time frame, even when growth was completely halted. This would contradict the study of Gatidou et al. (2003) who suggested that Irgarol had a lethal effect on *D. tertiolecta*. However, in their study viability loss was not directly assessed, but it was assumed that a growth inhibition of 96% would result from substantial viability loss. The lethal effect of Irgarol was furthermore described in the ACE (2002) final report in which flow cytometric analyses revealed that approximately half of the phytoplankton was killed at concentrations as low as 0.39 nM (100 ng l<sup>-1</sup>). In addition to the finding that Irgarol exposure was not lethal, fast recovery from a high Irgarol dose occurred, when considering both effective quantum yield and growth rate. It must be noted that Irgarol was not removed completely during the recovery experiment, since the 39.47 nM (10  $\mu$ g l<sup>-1</sup>) was 1:50 diluted. However, the earlier executed toxicity experiments had shown that an Irgarol concentration of 0.79 nM did not have a measurable effect on the algae, although this concentration would slightly affect *E. huxleyi*, as confirmed by the recovery experiments.

Based on effective quantum yield, *F. japonica* was shown to be the most Irgarol sensitive species, followed by *Tetraselmis* sp., *T. weissflogii* and *E. huxleyi*, with an average EC<sub>50</sub> value of 0.43, 0.91, 1.29 and 2.38 nM (0.110, 0.230, 0.327 and 0.604  $\mu$ g l<sup>-1</sup>) respectively. However, the EC<sub>50</sub> values for growth rate gave a different order, namely *Tetraselmis* sp., *T. weissflogii*, *E. huxleyi* and *F. japonica* (respectively 0.46, 1.20, 1.60, 2.44 nM or 0.116, 0.303, 0.406 and 0.618  $\mu$ g l<sup>-1</sup>). The reason for the observed differences in sensitivity of *F. japonica* could largely be explained by the fit of the EC<sub>50</sub> curve, which was much better for effective quantum

yield. *F. japonica* was very carefully homogenized prior to the flow cytometric measurements, given the risk of cell clump formation and mucus thread ejection when handled too roughly. This could have given more variable cell counts than obtained for the other species. For the other species,  $EC_x$  values for growth rate and PSII inhibition were highly similar. The  $EC_{50}$  value of *E. huxleyi* was not in accordance with the  $EC_{50}$  value of 0.99 nM (0.250  $\mu\text{g l}^{-1}$ ) as calculated by Devilla et al. (2005a). However, this  $EC_{50}$  value was based on 50% inhibition in cell number and not on growth. In addition, comparison of  $EC_{50}$  values between studies is restricted by experimental constraints, such as the irradiance levels applied. It must be stressed that the irradiance level applied in our study could deviate from *in situ* irradiances experienced by phytoplankton cells in (turbid) coastal waters. Higher *in situ* irradiances than applied here might increase Irgarol mediated oxidative stress, caused by uncontrolled radical formation when D1 is blocked. Thus, estimated  $EC_{50}$  values could have been lower when higher (experimental) irradiance levels had been used. Due to differences in response slopes  $EC_{20}$  and  $EC_{10}$  values revealed a different ranking, as compared with the  $EC_{50}$  values, especially for *E. huxleyi*. Generally, however, a 20% loss was found for all species in a relatively narrow concentration range, namely between 0.14 and 0.30 nM (35–76  $\text{ng l}^{-1}$ ).

No clear explanation for the observed species specific Irgarol responses was found when comparing cell characteristics such as the presence of cell coverings, average cell size of surface–volume ratio. For example, the species with the highest surface–volume ratio, i.e. *E. huxleyi*, was shown to exhibit the highest  $EC_{50}$  level. Note that a higher vulnerability for the species with the highest surface–volume ratio would only be revealed in the beginning of the experiment since an equilibrium between external and intracellular Irgarol would be installed more rapidly than for the other species. Yet, within the first 2 h of Irgarol exposure, effects on effective quantum yield were already established for all four species tested. Therefore no physical barrier or features related with surface/volume ratio was shown to restrict Irgarol uptake within this time frame.

When the calculated  $EC_{50}$  values were compared with maximum Irgarol concentrations in the Dutch Wadden Sea and Eems–Dollard (0.36 nM [90  $\text{ng l}^{-1}$ ], Bellert and van de Ven, 2003), the tested algae would not be significantly affected *in situ* (lowest  $EC_{50}$  value 0.43 nM [0.110  $\mu\text{g l}^{-1}$ ]). The  $EC_{10}$  and  $EC_{20}$  values, however, were lower than ambient. Therefore, the competitiveness and fitness of algae could very well be affected at environmental Irgarol levels, reducing growth as well as affecting species composition, given the species specific, 2–3 fold differences in  $EC_{10}$  and  $EC_{20}$  values. The Dutch National Monitoring Program data show a slight increase over the years for a number of locations, which may well substantiate after the banning of TBT by the European Union as off 2008. Furthermore, Irgarol concentrations in Dutch coastal waters are relatively low, as compared with other nearby regions in Western Europe, such as those found in British coastal waters (0.79–5.61 nM [201–1421  $\text{ng l}^{-1}$ ], Boxall et al., 2000; Thomas et al., 2001) and the Baltic (1.58–1.74 nM [400–440  $\text{ng l}^{-1}$ ], Dahl and Blanck, 1996, Biselli et al., 2000). Finally, Irgarol concentrations in the environment are known to vary with season, due to its seasonal application. While monitoring data are temporal as well as geographical snap-shots, the actual Irgarol concentrations could give rise to considerable temporary effects. Given the lowest effective concentration of 0.04 nM [11.3  $\text{ng l}^{-1}$ ] ( $EC_{10}$  of *E. huxleyi*) and the highest effective concentration of 2.38 nM [604  $\text{ng l}^{-1}$ ] ( $EC_{50}$  *E. huxleyi*), it is therefore concluded that present or future Irgarol levels may exhibit a significant impact on marine phytoplankton growth and community structure in coastal Western Europe.

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