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Assessing the effects of Irgarol 1051 on marine phytoplankton populations in Key Largo Harbor, Florida

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Abstract

The antifouling boosting agent Irgarol 1051 is a strong inhibitor of the photosystem II (PSII) with high efficiency/toxicity towards algae. However, because some phytoplankton species are more sensitive to Irgarol than others, its persistent release into the environment could result in adverse changes in the phytoplankton community structure at heavily impacted sites such as marinas. Continuous monitoring in the Florida Keys showed Irgarol concentrations of up to 635 ng L^{-1} in the canal system leading to Key Largo Harbor Marina (KLH) with a sharp decrease in concentration at stations offshore from the mouth of the canal. Preliminary phytoplankton community assessments from surface water samples collected in KLH between February and August 2004 showed changes in several phytoplankton species in concordance with the increase of the herbicide concentrations. Typical responses include an increase in the abundance of eukaryotes and *Cryptomonas* sp. as Irgarol concentrations increase.

Keywords: Irgarol 1051; Phytoplankton; GC/MS; Flow cytometry; South Florida

1. Introduction

The increase in use of novel biocides to prevent fouling of boats by organisms such as algae, barnacles and mussels poses a potential threat to non-target organisms. The ban of tributyltin (TBT), a compound deemed highly toxic to marine biota, has lead to the development of alternative antifouling compounds such as Sea-Nine 211, zinc pyrithione (ZPT) and Irgarol 1051 (Karlsson and Eklund, 2004).

Irgarol 1051 (2-methylthio-4-terbutylamino-6-cyclopropylamino-*s*-triazine) is a triazine-based algaecide used in marine antifouling paints. It was developed because of its high efficiency/toxicity towards cyanobacteria, thus greatly delaying the onset of fouling. Initial environmental risk assessment predicted Irgarol half-lives between 24 and 273 days in the aquatic environment, no significant bioaccumulation, and a low potential to affect non-target organisms (Giddings et al., 1998; Hall et al., 1999). The Irgarol exposure route for aquatic species is expected to be primarily through the water (Hall et al., 1999). Despite its predicted environmental fate, residues of Irgarol 1051 have been found worldwide since 1993 (Hall et al., 1999; Voulvoulis et al., 2002) and recently in Biscayne Bay, the Miami River, and the Florida Keys (Gardinali et al., 2002, 2004). The herbicide has become almost ubiquitous in large marinas worldwide.

Irgarol 1051, like other herbicides, inhibits photosystem II (Hall et al., 1999) and reduces growth and productivity of sensitive phytoplankton species such as the diatom *Navicula pelliculosa* at concentrations as low as 136 ng L^{-1} (Dahl and Blanck, 1996). Some phytoplankton species appear to be more sensitive to Irgarol 1051 than others. For instance, 23 h exposure to Irgarol (112 ng L^{-1}) decreased the abundance of some eukaryotic species to less

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than half of the controls (Readman et al., 2004). Variable sensitivity among phytoplankton species could result in changes in phytoplankton community structure at contaminated sites (Nyström et al., 2001; Dahl and Blanck, 1996). Environmentally relevant concentrations of Irgarol, as low as 126 ng L^{-1}, inhibited growth and photosynthetic activity of freshwater algae and changed phytoplankton community structure (Nyström et al., 2001). Since implications of altering the primary producers' community structure are numerous, the existence of such a response might be important. Irgarol 1051 concentrations rarely exceed 100 ng L^{-1} in estuarine/coastal zones or open circulation areas; however, enclosed marinas with low flushing rates and high density of Irgarol-treated boats present the potential to exceed concentrations at which effects have been observed (Hall et al., 1999).

Irgarol contamination of up to 182 ng L^{-1} has been documented along Key Largo Harbor Marina (KLH, Gardinali et al., 2004; Owen et al., 2002), an enclosed system formed by a series of interconnected channels with only one exit to open waters. These canals harbor a large number of marinas and boat repair facilities as well as numerous residential and commercial properties with in-water boat storage. Highest Irgarol concentrations were generally found at the inner end of the canal system, decreasing along the main navigational channel towards the entrance of the marina, where concentrations were as low as 13 ng L^{-1} (Gardinali et al., 2004).

In addition to assessing changes in phytoplankton abundance, physiological properties of cells such as pigment content and cell size can be used to assess potential shifts in community structure linked to an environmental stressor (Legendre et al., 2001). A rapid method to obtain information about these changes is flow cytometry (Readman et al., 2004). The key advantage of flow cytometry is the relative short time in which information about distribution of particle sizes, composition and fluorescence can be obtained (Jochem, 2000; Olson et al., 2002), thus providing access to water quality assessment in the time-frame required for environmental monitoring. This study aimed at comparing phytoplankton communities at different locations within the Key Largo Harbor Marina and relating observed differences to ambient Irgarol 1051 concentrations.

2. Methods

Surface water samples from 8 "impacted" locations within the KLH marina (Fig. 1), along the previously documented Irgarol gradient (Gardinali et al., 2004), were collected on February 5th, April 29th, June 14th and August 27th, 2004. These sampling sites provided two transects: stations 2, 4, 6 and 8 along the main channel; and 1, 3, 5 and 7 at the channel ends, but with increasing distance from the marina entrance.

Temperature, dissolved oxygen, pH and salinity were measured by a Hydrolab Multiprobe (Hydrolab Corp., Austin, TX). Nutrients (total inorganic nitrogen and soluble reactive phosphorous) were analyzed on a four-channel Alpkem RFA-300 (Rapid Flow Nutrient Analyzer, Alpkem Corp., Clackamas, OR). Surface water (4 L) was collected in amber glass bottles from a boat free of antifouling paint. Samples were taken to the laboratory, where they were divided for subsequent analyses.

Two liter were placed in amber glass bottles and stored at 4 °C for extraction and analysis of Irgarol. The full analytical method and its performance have been reported elsewhere (Gardinali et al., 2002). Briefly, 1 L of water was liquid–liquid extracted with methylene chloride using a separatory funnel. Blanks and laboratory spikes (LBS) using DI water (adjusted for salinity) served as quality

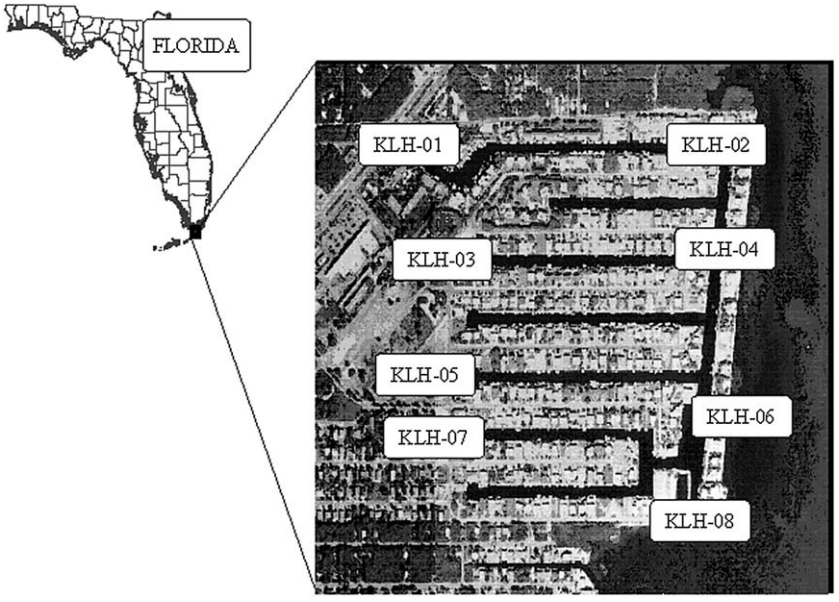

Fig. 1. Sampling stations in Key Largo Harbor Marina.

controls. Atrazine d-5 (100 μL at 1 ng μL^{-1}) was used as surrogate standard. Extractions were performed with 50 mL of pesticide-grade methylene chloride in triplicates. Organic layers were collected in 250 mL flat-bottom flasks, evaporated to almost dryness, and reconstituted in 1 mL of hexane. Upon addition of the GC recovery standard (tetrachloro-*m*-xylene, TCMX), concentrated samples were stored at 4 °C until analysis. Determination of the target analytes was conducted by GC/MS (Finnigan DSQ) using a 30 m × 0.25 mm ID and 0.25 mm film DB-5MS fused glass capillary column with helium as carrier gas (1.2 mL min^{-1}). The GC oven was programmed from 100 °C to 300 °C at a rate of 15 °C min^{-1} to achieve the separation of analytes in less then 12 min. The mass spectrometer was operated under selected ion mode (SIM) using at least three quantitation and confirmation ions. A nine-point calibration curve based on the relative responses of the analytes to the internal standard was used to determine the concentrations of Irgarol and M1, the major Irgarol metabolite.

For flow cytometric analysis of phytoplankton, 30 mL of water sample were placed in amber flasks, preserved with 1% (final conc.) of formalin and stored at 4 °C until analysis within 7 days. Samples were run on a FACSort flow cytometer (Becton-Dickinson, San José, CA) at a flow rate of 1 μL s^{-1}. Cell fluorescence, size (light scatter), and abundances were recorded on a four decades log scale and analyzed by WinMDI 2.7 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Two-parameter histograms of red fluorescence (chlorophyll *a* signal; FL3: >620 nm) versus light scatter and red fluorescence (FL3) versus orange fluorescence (phycoerythrin signal; FL2: 565–592 nm) were chosen to illustrate cellular chlorophyll versus particle size and cellular chlorophyll versus cellular phycoerythrin to differentiate phytoplankton groups. Cell abundance was calculated from measurement time based on weight calibration of the instrument's flow rate. Qualitative and quantitative comparison of plankton community parameters was assessed by multivariate analysis of cell counts and cellular fluorescence.

For the quantification of larger phytoplankton, 1 L of sample was placed in amber bottles and preserved with 1% (final concentration) of Lugol's iodine solution. Samples were placed in a 1000 mL graduated cylinder, in which they settled for one week. Thereafter, the supernatant water (900 mL) was siphoned off and the remaining 100 mL mixed and transferred into 50 mL sedimentation cylinders, where samples were left undisturbed for 24 h for analysis according to Utermöhl's inverted-microscope method (Sournia, 1978). Rare and larger cells were counted under the 20× objective using vertical "lanes" throughout the diameter of the settling chamber. Small and abundant cells were counted under the 40× objective. A number of view fields were chosen randomly depending on cell abundance.

Based on the taxonomic identification produced by flow cytometry and microscopy, a culture of *Rhodomonas salina* (5–13 μm; CCMP 1319) was incubated for 10 days in *f*/2—silica medium prepared from autoclaved 32 ppt seawater. Culture's growth was monitored until it reached a concentration of about 14×10^4 cells mL^{-1} and then diluted to 8×10^4 cells mL^{-1}. A 250 mL aliquot from the culture was placed into a 500 mL flask and spiked with a solution of Irgarol 1051 in methanol to yield final concentrations ranging from 20 to 2000 ng L^{-1}. Seven different concentrations of Irgarol 1051 were tested in triplicates. A 100 mL aliquot was withdrawn, placed in a sterilized flask and saved for chemical analysis. The remaining 150 mL were placed in a 250 mL sterilized Erlenmeyer flask. A triplicate set of cultures without Irgarol, but spiked with 100 μL of methanol, served as the control for algal growth rates. In addition, deionized water spiked with the highest Irgarol concentration was used to assess potential degradation throughout the culture experiment. Flasks were placed on a shelf in a controlled temperature room (21 ± 1 °C) under continuous light. Two cool white fluorescence bulbs provided an irradiance of 78 μmol photons m^{-2} s^{-1} photosynthetic active radiation (PAR), as measured by a Li-Cor LI 190SA photometer. A 100 mL aliquot was withdrawn from each flask at test initiation (day 0) and test termination (day 7) to assess the Irgarol concentrations. Aliquots were filtered through a 25 mm GF/B filters and saved for chemical analysis while the filters were kept in 2 mL acetone for chlorophyll *a* analysis. During the incubation period, samples were collected at daily intervals, fixed with 1% (final conc.) of formalin and analyzed by flow cytometry as described above.

3. Results and discussion

The mean hydrological parameters of the sampling sites in KLH are presented in Table 1. Temperature, salinity and

Table 1
Hydrologic parameters, represented as average ± standard deviation, for all samples within each sampling month

Parameter	February	April	June	August
Temperature (°C)	24 ± 0.7	31 ± 0.2	31 ± 0.2	32 ± 0.4
Salinity (ppt)	36.6 ± 0.2	38.2 ± 0.2	38.2 ± 0.2	36.7 ± 0.4
Dissolved O_2 (mg L^{-1})	4.8 ± 0.2	4.7 ± 0.6	4.8 ± 0.6	4.4 ± 0.8
pH	7.94 ± 0.09	7.83 ± 0.04	7.83 ± 0.04	8.02 ± 0.07

Table 2
Nutrient concentrations (total inorganic nitrogen, TIN, and soluble reactive phosphorus, SRP; μmol L^{-1}) for all stations within each month and averaged over the study period

	April		June		August		All months	
	TIN	SRP	TIN	SRP	TIN	SRP	TIN	SRP
Average	2.38	0.03	3.13	0.02	2.97	0.13	2.91	0.07
Standard deviation	0.98	0.04	1.29	0.01	1.99	0.16	1.53	0.12
Maximum	3.61	0.09	5.01	0.03	5.97	0.49	5.97	0.49
Minimum	0.98	0.01	2.12	0.02	0.60	0.03	0.60	0.01
Median	2.32	0.01	2.69	0.02	2.67	0.06	2.77	0.04

Nutrient concentrations not available for February sampling.

pH varied little between stations at any given time, and only temperature increased significantly from February to August. DO and nutrients showed large variations among stations within any given month and between stations in different months (Table 2).

Irgarol concentrations ranged from 7 to 635 ng L^{-1} throughout the study period. Concentrations were highest in February (303–635 ng L^{-1}) and lowest in April (7–96 ng L^{-1}). Within the KLH marina, Irgarol concentrations decreased from the interior to the entrance of the marina (Table 3). The gradient and concentrations of Irgarol in summer 2004 were comparable to previously reported results from KLH (Owen et al., 2002; Gardinali et al., 2004). The highest concentration exceeded concentrations reported from other locations in South Florida, i.e. as high as 99 ng L^{-1} in Key West, 70 ng L^{-1} in the Miami River, and 69 ng L^{-1} in the Coconut Grove Marina (Gardinali et al., 2004). These concentrations appear low compared to reports of <2 to 1700 ng L^{-1} in European marinas (Readman et al., 1993; Gough et al., 1994) and <3000 ng L^{-1} to 4000 ng L^{-1} in coastal waters of Singapore (Basheer et al., 2002).

Table 3
Irgarol 1051 concentrations (ng L^{-1}) in Key Largo Harbor Marina

Station	Irgarol concentration (ng L^{-1})			
	February	April	June	August
KLH-01	303		180	192
KLH-02		90	213	172
KLH-03	635	96		74
KLH-04		89	159	196
KLH-05	589	81		75
KLH-06			69	165
KLH-07	520			
KLH-08		7		106
Average	512	73	155	140
Maximum	635	96	213	196
Minimum	303	81	69	74
Median	555	89	170	165

Flow cytometric histograms distinguished clearly two populations, eukaryotes and prokaryotic phytoplankton, i.e. cyanobacteria. The abundance of eukaryotes varied little both between different stations within any given month and among stations in different months. Excluding one outlier, eukaryotic abundance at all stations from February to August ranged from 1.2×10^4 to 3.2×10^4 with a mean abundance of $2.4 \times 10^4 \pm 5.7 \times 10^3$ cells mL^{-1} (Fig. 2). The lack of eukaryotes' response to Irgarol concentrations of up to 630 ng L^{-1} is consistent with previous studies that required much higher concentrations to inhibit phytoplankton growth (Readman et al., 2004; Devilla et al., 2005).

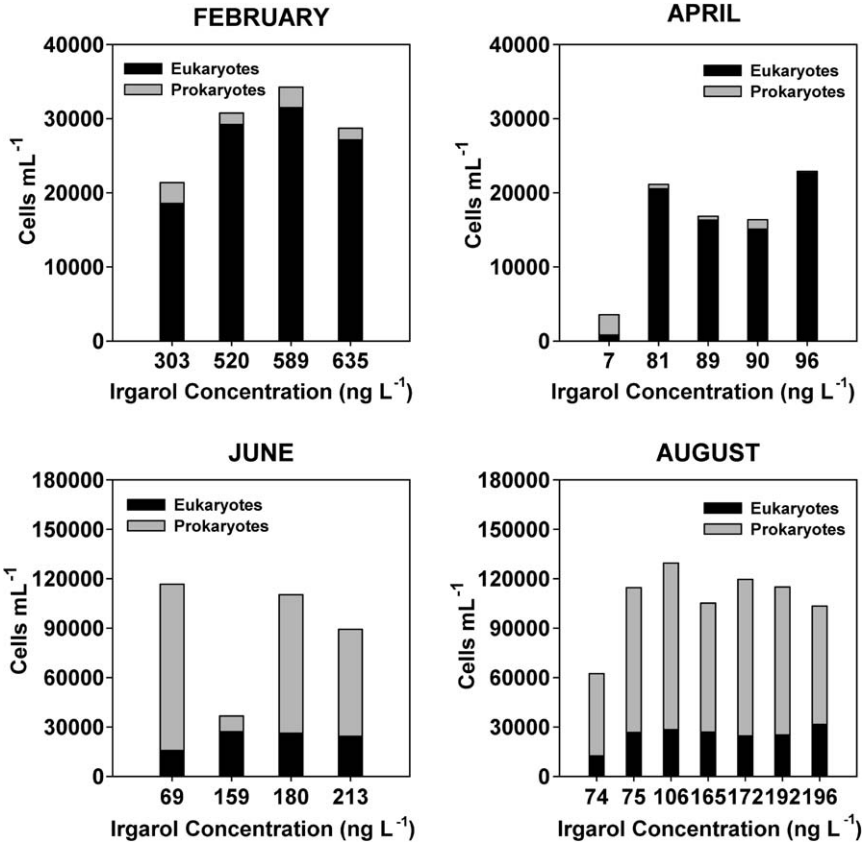

Fig. 2. Pico- and nanophytoplankton abundance in Key Largo Harbor Marina versus Irgarol 1051 concentrations (ng L^{-1}) during the months of February, April, June and August 2004.

In contrast to the small eukaryotes, prokaryotic phytoplankton showed great variation between stations in any given month and between stations in different months (Fig. 2). Prokaryotic phytoplankton abundance ranged from 1.3×10^2 to 1.0×10^5 cells mL^{-1}. At an Irgarol concentration of approximately 100 ng L^{-1}, the number of prokaryotic cells also varied from 1.3×10^2 to 1.0×10^5 cells mL^{-1}. Prokaryote abundance was not correlated with Irgarol concentrations ($r^2 = 0.160$; $P = 0.09$). Nevertheless, in contrast to the eukaryotes, the highest abundance of prokaryotes was found at lower concentrations of Irgarol. Microcosm experiments revealed a minor decrease in abundance of *Synechococcus* sp. when exposed to Irgarol concentrations as low as 100 ng L^{-1} for 29 days (Hoberg, 2004).

Seven dominant phytoplankton species were identified by microscopic analysis. Cryptophytes and *Chaetoceros* spp. were present in all samples at 1.7×10^2–2.2×10^5 and 6.8×10^2–7.7×10^5 cells mL^{-1}, respectively. Other species such as *Skeletonema costatum* and *Scrippsiella* sp. occurred at significant abundance but were confined to only one month and showed no correlation with Irgarol concentrations (Fig. 3).

Chaetoceros sp. abundance at all stations from February to August did not correlate with Irgarol concentrations ($r^2 = 0.0017$; $P = 0.86$). Correlations were also not significant for each individual month, February ($r^2 = 0.697$; $P = 0.17$), June ($r^2 = 0.778$; $P = 0.12$), April ($r^2 = 0.706$; $P = 0.16$), and August ($r^2 = 0.303$; $P = 0.20$). The variation in *Chaetoceros* sp. abundance appeared to be controlled by factors other than the concentration of the antifouling agent. However, highest abundance was restricted to Irgarol concentrations below 300 ng L^{-1} (Fig. 4).

In contrast to the diatoms (*Chaetoceros* spp., *S. costatum*) and the dinoflagellate *Scrippsiella* sp., cryptophyte abundance in KLH showed a weak but significant positive correlation with Irgarol concentrations throughout the study period ($r^2 = 0.326$; $P = 0.007$; Fig. 4). On the other hand, no consistent trend for the cryptophyte abundances with respect to Irgarol was discernible during the individual sampling events.

While the response of diatoms (*Chaetoceros* spp., *S. costatum*), dinoflagellates (*Scripsiella* sp.), and eukaryotic nanophytoplankton to changes in Irgarol concentrations remained ambiguous and potentially co-affected by other factors, such as nutrient gradients within the marina, cryptophytes appeared to exhibit the strongest tolerance against Irgarol and might, therefore, gain competitive advantage in contaminated systems. Increasing abundance of cryptophytes with increasing Irgarol contamination agrees with previously reported microcosm studies that revealed resistance of cryptophytes to Irgarol concentrations as high as 500 ng L^{-1} (Devilla et al., 2005) and 800 ng L^{-1} (Hoberg, 2004). However, since other factors, such as nutrient

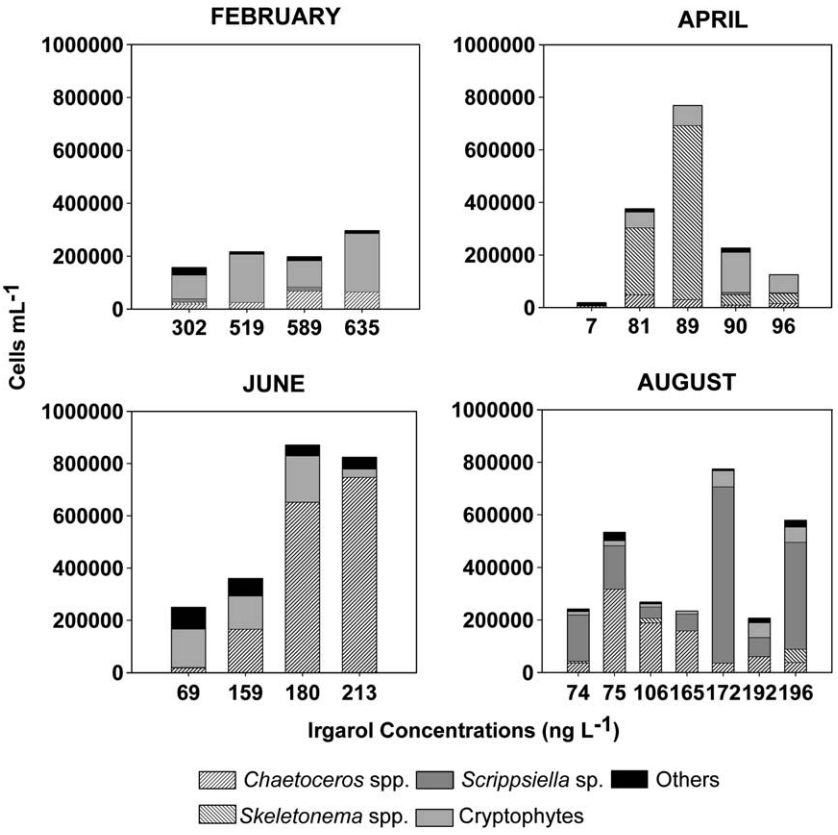

Fig. 3. Microphytoplankton abundance in Key Largo Harbor Marina versus Irgarol 1051 concentrations (ng L^{-1}) during the months of February, April, June and August 2004.

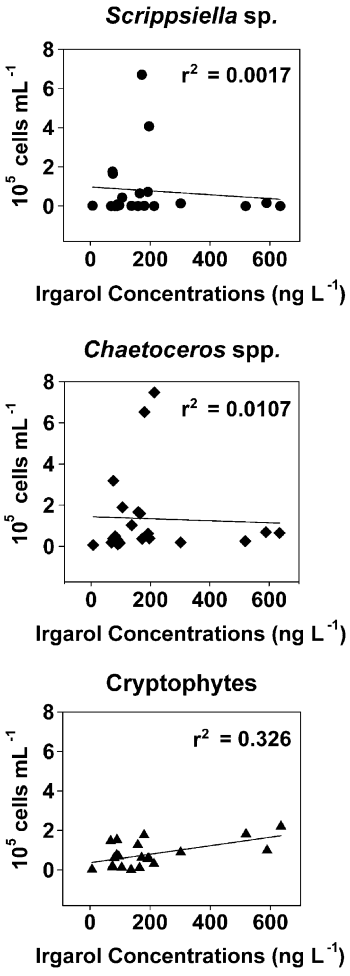

Fig. 4. Correlation between the abundance of *Chaetoceros* spp., *Scrippsiella* sp., and cryptophytes and Irgarol 1051 concentrations throughout the study period.

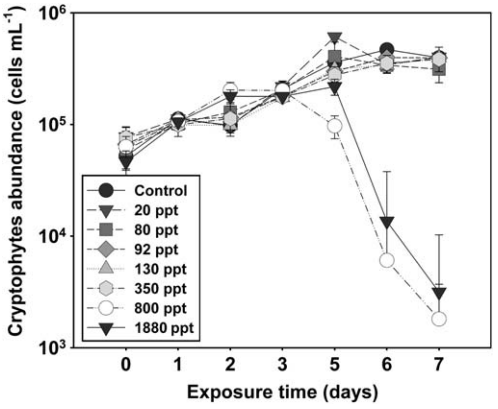

Fig. 5. Growth of the cryptophyte *Rhodomonas salina* in batch cultures as a function of Irgarol 1051 concentrations.

concentrations, might co-affect plankton composition in KLH, interpretations of high Irgarol resistance have to be made with caution. Cryptophytes are known to become dominant up to bloom formation in marine systems where nutrients are sufficient but other factors may delay diatom growth (Laanbroek et al., 1995; Jochem, 1990; Buma et al., 1992).

Due to the uncertainty of the observed behavior, the response of *R. salina* to Irgarol was assessed directly in a dose response experiment (Fig. 5). Irgarol concentrations of 800 ng $L^{-1}$and 1880 ng L^{-1} resulted in an initial population growth comparable to low concentration and control cultures but produced a severe population decline after 3 days of Irgarol exposure. *R. salina* growth at Irgarol concentrations of up to 365 ng L^{-1}did not differ from unexposed control cultures, though. Flow cytometric analyses of dose response cultures also revealed a decrease in red

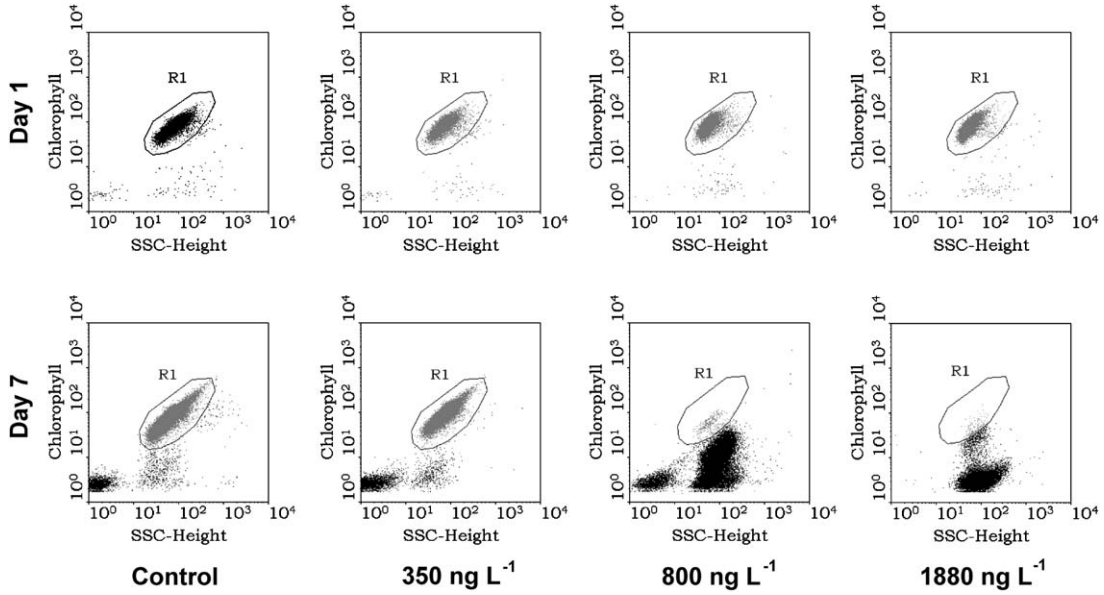

Fig. 6. Effect of Irgarol 1051 concentrations on *Rhodomonas salina* cellular chlorophyll concentrations (FL3 signal) plotted versus side-angle light scatter (SCC) as a measure of cell size. Region R1 indicates the location of the original culture population. Culture populations at days 1 and 7 of the dose response experiment for the control, 350 ng L^{-1}, 800 ng L^{-1}, and 1880 ng L^{-1} treatments.

fluorescence, a direct measure for cellular chlorophyll content (Hall and Cumming, 2003), only in cultures exposed to 800 ng L^{-1} and 1880 ng L^{-1} (Fig. 6). The dose response experiment, thus, confirmed the resistance of the cryptophyte *R. salina* towards Irgarol exposure at concentrations found in the marine environment of KLH.

The higher resistance of a photosystem inhibiting herbicide such as Irgarol in cryptophytes might be related to alternative pathways of acquiring energy in this algal group. Persistent growth of *R. salina* under severe light limitation, in conjunction with its inability to up-regulate its photosystem, has been interpreted as evidence for alternative energy acquisition, most probably through the utilization of dissolved organic carbon (Hammer et al., 2002). Phagotrophy and mixotrophy have also been discussed in cryptophytes (Roberts and Laybourn-Parry, 1999; Urabe et al., 2000; Rachiq et al., 2002). The field studies also suggest that prokaryotic phytoplankton (i.e. *Synechococcus*-like cyanobacteria) and the dinoflagellate *Scrippsiella* sp. might exhibit a higher susceptibility to Irgarol exposure. Further microcosm and dose response experiments must show if these species are, in fact, more sensitive to Irgarol or if other factors in KLH marina affected their distribution and phytoplankton community composition.

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