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Bioaccumulation of the Antifouling Paint Booster Biocide Irgarol 1051 by the Green Alga *Tetraselmis suecica*

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Following a ban on the use of tributyltin based antifouling paints during the 1980s on vessels less than 25 m in length, alternative booster biocides were added to improve the efficacy of copper based paints (Voulvoulis et al. 1999). The developed paints have been, and are, extensively used on small boats that frequent marinas and other areas of low water exchange. Under these conditions, Irgarol 1051 and diuron were found to be present at elevated concentrations (Scarlett et al. 1999, Thomas et al. 2001); a direct result of the compounds' environmental persistence in surface waters and sediments (Scarlett et al.1999; Thomas et al. 2002, 2003). Following an assessment of the risk posed by antifouling paint biocides (European Commission's Biocidal Products Directive (98/8/EC)), the UK Health and Safety Executive (HSE) restricted the use of both Irgarol 1051 and diuron in antifouling paints (Thomas et al. 2002). However, although the use of Irgarol 1051 is restricted in the UK, Denmark and Sweden, following the reports of damage to microalgal communities in coastal waters (Dahl and Blanck 1996), it is still used on vessels in other regions of Europe as well as the United States of America (Hall et al. 2005).

Irgarol 1051 is an effective antifouling biocide that disrupts the photosystem II process and reduces the photosynthetic efficiency of algae. As a result Irgarol 1051 is much more toxic to plants than animals (Hall et al. 1999; Okamura et al. 2000). In the laboratory these effects have been demonstrated on the eelgrass, *Zostera marina*, where growth rate and photosynthetic efficiency were both reduced (Scarlett et al. 1999). Similarly, in the green macroalga *Ulva intestinalis* Irgarol 1051 was seen to significantly reduce the growth of *U. intestinalis* germlings and to reduce photosynthetic efficiency with an EC50 (72 h) of 2.5 µg L^{-1} (Scarlett et al. 1997). Recently published work also suggests that Irgarol 1051, and its principal metabolite GS26575, also present a hazard to freshwater macrophytes (*Apium nodiflorum, Chara vulgaris,* and *Myriophyllum spicatum*) with suppression of the relative growth rate observed at low ng L^{-1} concentrations (Lambert et al. 2006). As well as GS26575, two other metabolites have been identified for Irgarol 1051. These are M2 (Lam et al. 2004) and M3 (Lam et al. 2005). As yet, few data are available regarding the environmental persistence and toxicities of these compounds.

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The hazard posed by any substance deliberately released into the environment is often classified by its persistence, bioaccumulation and toxicity (e.g. OSPAR 2005). From the studies presented above it is clear that Irgarol 1051 is persistent and toxic to specifically to algal species, however few data are available on its biological uptake. Scarlett et al. (1997, 1999) reported bio-concentration factors (BCFs) of up to 25,000 in *Z. marina* while Tóth et al. (1996) has reported BCFs of 30,000 in freshwater macrophytes. However, we are unaware of any work having been conducted on the bioconcentration of Irgarol 1051 by phytoplankton. Bioconcentration in phytoplankton has obvious importance to pelagic food webs due to their small individual size and large surface area. Uptake by phytoplankton is also important since it offers an additional transport mechanism (Ferard et al. 1983).

Irgarol 1051 has a log K_{OW} of 3.95 which suggests that the potential to bioaccumulate is low (Thomas 2000). Using a predictive approach developed for polychlorinated biphenyls (PCBs) to assess the uptake of Irgarol 1051 by phytoplankton it is possible to estimate a BCF of 55,000 once equilibrium is reached (Mailhot 1987). This compares with the BCFs of 25,000 to 30,000 measured for macrophytes (Tóth et al. 1996; Scarlett et al. 1997, 1999).

The purpose of the study described herein was to establish whether at sub-lethal concentrations, Irgarol 1051, a photosystem II inhibitor, uptake occurs in marine phytoplankton and whether a predictive model developed for PCBs was suitable for predicting the bioconcentration of Irgarol 1051 in *Tetraselmis suecica*.

MATERIALS AND METHODS

HPLC-grade methanol, acetonitrile, acetone and water were obtained from Rathburns (Walkeburn, UK). Chlorotoluron (dimethyl-d_6), was obtained from Promochem (Wewyn Garden City, UK), and Irgarol 1051 and GS26575, from Ciba Geigy (Basel, Switzerland). Octadecyl silane (C18) Isolute SPE (1g/6mL and 100 mg/3mL) columns were obtained from Jones Chromotography (Hengoed, Mid Glamorgan, UK).

Prior to exposure the marine microalga *T. suecica* was cultured in large polyethylene containers (4 x 400 L) over two-weeks. The algae were exposed to constant light (approximately 3000 lux). An additional container contained 400 L of filtered (0.2 µm) seawater (FSW) and no algae. All containers were aerated constantly from the base to prevent the algae settling. The temperature, dissolved oxygen concentration, salinity and pH were measured and recorded daily using a WTW Multiline P4 handheld probe and remained between the following parameters, temperature: 14.2-18.2°C, dissolved oxygen: 90-100%, salinity: 25.5-27.4‰, pH: 6.44-8.32. Cell densities were determined on days 0, 1, 3, 7, 14 and 21 of the experiment in order to calculate growth rates and establish the photosynthetic health of the algae.

Irgarol 1051 was introduced to the 3 culture containers containing algae and the

one containing only filtered seawater. At the start of the experiment the concentration of Irgarol 1051 in each container was 1 µg L^{-1}. On day 7, the three bags containing algae were spiked with a further 1 µg L^{-1}. After the addition of Irgarol 1051 the bags were left for 1 hour before sampling occurred to allow adequate mixing of the Irgarol 1051.

Samples were collected on days 0, 1, 3, 7, 14 and 21. On these days water/algae samples (1 L) were collected for the determination of Irgarol 1051 concentrations in both water and algae. Samples were taken in triplicate from each of the vessels containing algae (control and spiked). These samples were filtered (Whatman GF/D) on pre-weighed filter papers. The algae retained on the filter paper were then dried and retained at -20°C prior to chemical analysis. The filtrate, along with 1 mL of dimethyl-chlorotoluron-d_6 (internal standard; 20 µg mL^{-1}) was extracted by solid phase extraction (SPE; Isolute C18 solid phase extraction cartridges, Jones Chromatography) pre-conditioned with methanol and water.

Three 100 mL samples were also taken from each bag (control, spiked and FSW bags). Each of these samples had 1 mL of the chlorotoluron internal standard added. These samples were then solid phase extracted, again using Isolute C18 cartridges, pre conditioned with methanol and water. All solid phase extraction cartridges were stored at -20 °C and retained for chemical analysis.

In this experiment the photosynthetic health of the algae was monitored through measuring the photosynthetic efficiency of the algae. When algae absorb light, the energy can be dissipated in three ways. Firstly, it can be used in photosynthesis. Any excess energy above the requirements for photosynthesis will be released as fluorescence (re-emission of light at a slightly longer wavelength) or will be lost as heat. When algae have been stored in the dark for a period of 10 minutes, all residual energy is processed and the amount of fluorescence is small (minimum fluorescence, F_o). When the algae is flashed with a bright light the fluorescence will increase to a maximum (F_m) as not all the energy is required for photosynthesis. The proportion of the maximum possible fluorescence used for photosynthesis, normally around 80%, is then expressed as the variable fluorescence (F_v) (which is the difference between F_m and by F_o) divided by F_m (F_v/F_m). Photosynthetic Efficiency (F_v/F_m) measurements were determined using a Hansatech Handy PEA (Photosynthetic Efficiency Analyser). F_v/F_m measurements were taken on sampling days 0, 1, 3, 7, 14 and 21. Samples were taken, in triplicate, from each container. For each measurement, 4 mL of algae was dark adapted for 10 min before exposure to light at 100% saturation, 3000 µM m^{-2} sec^{-1}.

On day 0, an image analyser (Image Pro Plus version 4.0) was used to count algal cells in each of the containers. In addition to this a fluorescence plate reader was used to calculate cell numbers counted via image analysis. On all other sampling days cell counts were determined using only the plate reader method.

Solid phase extraction columns were eluted using methanol (2 x 5 mL). Methanol

Table 1. *Tetraselmis suecica* 14-day bio-concentration factors for Irgarol 1051.

	Mean Irgarol 1051 Concentration		Irgarol 1051 bio-concentration factor
	Water ($\mu g\ L^{-1}$)	Algae ($\mu g\ Kg^{-1}$)	(± standard deviation)
Replicate 1	0.27	22,900	84,822 ± 32,394
Replicate 2	1.2	18,640	15,538 ± 8,457
Replicate 3	0.44	108,590	47,780 ± 26,429

was added to each dry column and allowed to soak into the column, under vacuum, for 5 min. After this period the solvent was drained at a rate of approximately 10 mL min^{-1} and collected. The column was allowed to dry before this process was repeated twice more. For each column 15 mL of solvent was blown down under nitrogen to 1 mL using a Turbovap and stored at -20°C prior to analysis.

Dried filter papers were extracted using 20 mL of a 50:50 mix of methanol and dichloromethane. 1 mL of the chlorotoluron internal standard solution was added to each sample. This mixture was shaken mechanically (20 min.), sonicated in an ultrasonic bath (10 min.) and then centrifuged (4000 rpm, 10 min.). The solvent was removed and the process repeated. For each filter paper, the 40 mL of solvent was blown down to 1 mL using a Turbovap and analysed using LC-MS.

Analysis was performed by high performance liquid chromatography-coupled to atmospheric pressure chemical ionisation mass spectrometry (HPLC-APCIMS) operated in the single ion-monitoring (SIM) mode (Thomas et al. 2000, 2003). Recoveries for authentic reference compounds using these methods were 99 ± 6 % and the limits of detection in water were 0.01 $\mu g\ L^{-1}$ and in algae 0.01 $\mu g\ g^{-1}$ Quality assurance was maintained by using a blank with each series of samples, and a response factor standard that was repeated after every three samples.

RESULTS AND DISCUSSION

The measured concentration of Irgarol 1051 in the algal culture containers ranged from 0.14 to 2.3 $\mu g\ L^{-1}$ (Table 1). The results show that Irgarol 1051 is readily taken up by *T. suecica* (Table 2, Figure 1), whilst GS26575 was taken up at a much slower rate (data not shown). Exposure of *T. suecica* over a period of 21-days shows a rapid uptake of Irgarol 1051 over days 1-7, followed by a period of equilibration. Following equilibrium, bio-concentration factors (BCF) of between 15,000 and 80,000 were measured (Figure 1). Comparison of the mean 14 day BCFs obtained for the different replicates shows that there is a significant difference between the BCFs determined in replicates 1 and 2 with replicate 3 (5% significance level, determined by comparison of means). There is high variability in the data; however this reflects the high variability in the accumulation of

Table 2. Mean concentration (µg L^{-1}) of Irgarol 1051 in the test containers.

Day	Mean Irgarol 1051 Concentration (µg L^{-1})†				
	Control (No Irgarol 1051)	Replicate 1	Replicate 2	Replicate 3	Control (No algae)
0	< LOD	0.2	0.2	0.9	1.6
1	< LOD	0.2	0.1	0.1	2.2
3	< LOD	0.1	0.1	0.1	1.7
7	< LOD	0.3	0.3	0.3	2.1
14	< LOD	0.2	1.2	0.4	1.9
21	< LOD	0.0	1.2	0.1	1.3

† n=3, LOD = 0.01 µg L^{-1}.

Table 3. Mean concentration (µg L^{-1}) of Irgarol 1051 in the algae.

Day	Mean Irgarol 1051 Concentration (µg Kg^{-1})†			
	Control (No Irgarol 1051)	Replicate 1	Replicate 2	Replicate 3
0	< LOD	769	1893	1,112
1	< LOD	5,254	1825	1,464
3	< LOD	3,761	5,752	4,917
7	< LOD	21,643	28,756	37,486
14	< LOD	22,900	18,640	108,590
21	< LOD	22,158	493	15,860

† n=3, LOD = 0.01 µg Kg^{-1}.

photosystem II inhibitors in healthy alga. The reduced BCFs measured in replicate 3 after 7 days are due to the failure of the algae to grow.

The photosynthetic health and growth rate of the algae can be seen in Figures 2 and 3. Figure 2 shows that photosynthetic health of *T. suecica* is reduced by 50% with Irgarol 1051 present at concentrations of between 0.14 and 1.39 µg L^{-1}. Since Irgarol 1051 is a photosystem II inhibitor, this reduction in photosynthetic efficiency would be expected to occur at relatively high, but not environmentally irrelevant, concentrations. Even though the photosynthetic efficiency of the algae has been reduced, the rate of *T. suecica* growth remains good with 1 to 3 doublings per day in cell density recorded. The *T. suecica* growth rate is reduced following the addition for further Irgarol 1051 into the system on day 7, and in the case of replicate 3, the concentration of Irgarol 1051 in the system is sufficient to prevent the further growth of the algae.

Based upon a log K_{OW} of 3.95, and using the PCB bioaccumulation model (Mailhot 1987), a BCF of 55,000 was predicted for Irgarol 1051. The BCFs measured in this study for *T. suecica* are within the same range of the predicted values and those reported for marine macrophytes (Scarlett et al.1997, 1999). This

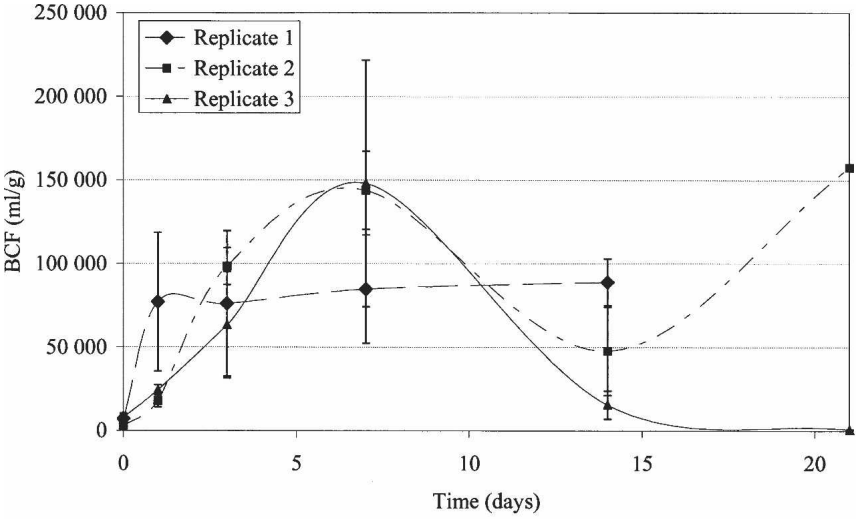

Figure 1. Uptake of Irgarol 1051 in *Tetraselmis suecica* over 21 days.

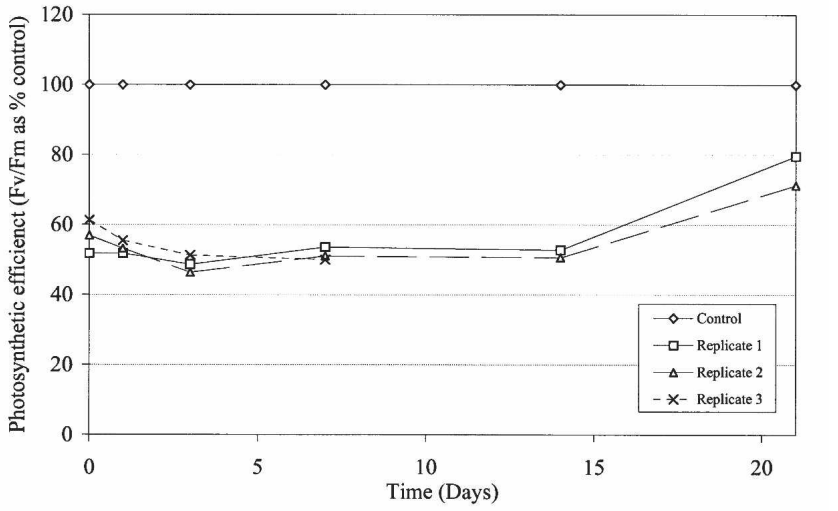

Figure 2. *Tetraselmis suecica* photosynthetic efficiency over 21 days exposure to Irgarol 1051.

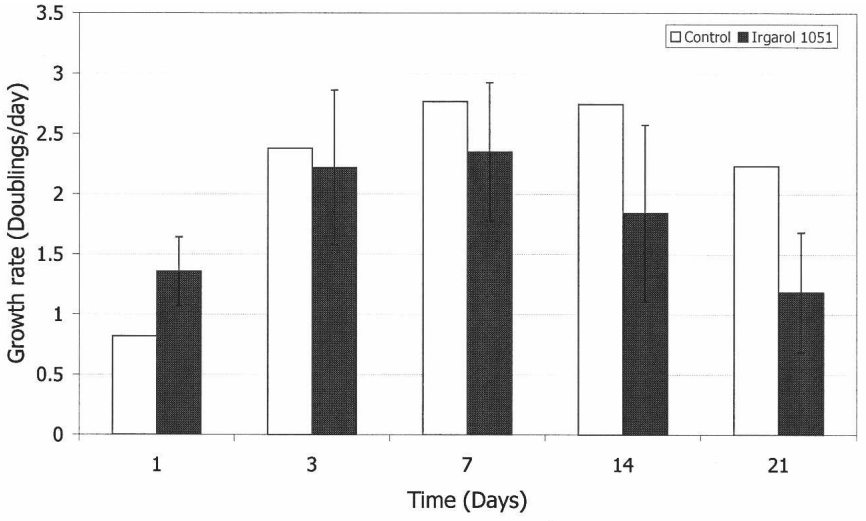

Figure 3. *Tetraselmis suecica* growth over 21 days exposure to Irgarol 1051.

suggests that the model described by Mailhot (1987) is a good predictor of Irgarol 1051 bioaccumulation in *T. suecica.*

Overall the data suggest that Irgarol 1051 can be bio-concentrated by phytoplankton at concentrations that are sufficiently high to reduce the algae's rate of photosynthesis but not sufficiently high to prevent the algae from growing. This is important since previous studies have focused on the ability of Irgarol 1051 to reduce photosynthesis and subsequently algal growth. These data show for the first time that when Irgarol 1051 is present at sub-lethal concentrations, bioaccumulation occurs. This also suggests that this may be an important pathway for the uptake of Irgarol 1051 into marine food webs. The large volume culturing system used within this study is commensurate for the production of high volumes of algae that would be required to further assess the food web transfer of Irgarol 1051 or any other environmental contaminant. In addition to impacts on marine food chains, the bioaccumulation of algae may serve as a mechanism for transporting persistent biocides from one location to another, for example, from areas of intense boating activity to the open ocean.

The risks associated with Irgarol 1051 use are presented in the scientific press as uncertain. The UK has restricted its use, whilst reports from the US suggest that it is likely that adverse effects will only been seen in areas of high boat density (Hall et al. 2005). The data presented here serve to further inform the risk assessment process and provide additional information on how Irgarol 1051 behaves in the environment and the possible effects it may have on marine food webs.

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