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Extreme irgarol tolerance in an *Ulva lactuca* L. population on the Swedish west coast

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ABSTRACT

The herbicide irgarol 1051 is commonly used on ship hulls to prevent growth of algae, but as a component of self-eroding paints it can also spread in the surrounding waters and affect non-target organisms. The effect of irgarol on settlement and growth of zoospores from the marine macro algae *Ulva lactuca* from the Gullmar fjord on the Swedish west coast was investigated in the present study. The zoospores were allowed to settle and grow in the presence of irgarol, but neither settlement – nor growth inhibition was observed at concentrations of up to 2000 nmol l^{-1}. This is between 10 and 100 times higher than effect concentrations reported earlier for algae. Irgarol also induced the greening effect (4-fold increase in chlorophyll *a* content) in the settled zoospore/germling population, typical for photosystem II inhibitors like irgarol. This study support previous findings that irgarol constitutes a selection pressure in the marine environment.

1. Introduction

The herbicide irgarol 1051 (2-(tert-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine), is used in antifouling paints to prevent algae from growing on ship hulls and other underwater structures. Irgarol is also known under the trade name Cybutryne, and belongs to the chemical group s-triazines (Thomas and Brooks, 2010). In the US, the first irgarol-containing paint was registered for use in 1998, and in Europe irgarol has been in use since the mid 80's (Hall et al., 1999). However, in more recent years its use has been restricted in countries such as the UK, Denmark and Sweden (Thomas and Brooks, 2002). The mode of action of irgarol is the inhibition of photosynthesis (Dahl and Blanck, 1996) by binding to the plastoquinone binding-niche at the D1 protein in photosystem II (PSII) (Tietjen et al., 1991). This blocks the electron transfer and inhibits the D1 protein turnover (Jansen et al., 1993). It is also known that PSII inhibiting herbicides cause oxidative stress through production of reactive oxygen species, and it is this production of radicals, rather than starvation following the blockage of the photosystem, that causes cell death in exposed organisms (Rutherford and Krieger-Liszkay, 2001; Fufezan et al., 2002). Photosynthetic organisms exposed to triazine herbicides at sub-lethal concentrations are known to increase their chlorophyll and accessory pigment content. This so-called greening effect is assumed to be a compensation mechanism for the loss of photosynthetic efficiency (Hatfield et al., 1989; Koenig, 1990; Boura-Halfon et al., 1997).

Antifouling paints are designed to release biocides into the surrounding water and thereby create a hostile environment at the painted surface. As a consequence, antifouling biocides also end up in the environment where they can affect non-target organisms. Irgarol was first discovered as contaminant in the Mediterranean by Readman et al. (1993), and it has since then been found in marine environments worldwide, with the highest reported concentration of 17 nmol l^{-1} found in Singapore (see the review by Konstantinou and Albanis, 2004). Along the Swedish west coast, irgarol was first detected in 1993 in the range of 0.24 to 1.2 nmol l^{-1} by Dahl and Blanck (1996), and was subsequently found regularly over a period of 10 years (Blanck et al., 2009). During this 10 year period the periphyton communities, mainly consisting of diatoms and cyanobacteria, developed a community tolerance for irgarol. The most tolerant community had an EC50 value for photosynthesis inhibition of <40 nmol l^{-1}, which was from a location with irgarol exposure levels of 0.29 nmol l^{-1} (Blanck et al., 2009). Hence, irgarol acted as a selection pressure in the area. The most probable tolerance mechanism to irgarol in periphyton communities is an increased turnover of the D1 protein (Eriksson et al., 2009). In terrestrial plants, tolerance mechanisms to PSII inhibitors have been shown to include point mutations leading to amino-acid substitution ($serine_{264}$ substituted with glycine) in the D1 protein (e.g. Bettini et al., 1987; Oettmeier, 1999;

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Devine and Shukla, 2000; Kumata et al., 2001), and an increased detoxification of the herbicide via glutathione conjugation (Anderson and Gronwald, 1991; Gray et al., 1996).

In this study we investigated the effects of irgarol on settlement and growth of the marine macro algae *Ulva lactuca* from the same geographical area where tolerance development was observed in periphyton communities by Blanck et al. (2009).

U. lactuca, also known as sea lettuce, is a macroalga species commonly found in the littoral zone in temperate waters (Loughnane et al., 2008; Hofmann et al., 2010). One of the reasons for its wide distribution is the high capacity to colonize new surfaces. Species within the *Ulva* genus are capable of spreading to new areas both through the dispersal of the two types of reproductive bodies, asexual zoospores and sexual gametes, and through the dispersal of detached pieces of thalli that remains capable of producing spores/gametes (Callow and Callow, 2000). *U. lactuca* has been extensively used in laboratory studies (e.g. de Nys et al., 1996; Turner et al., 2009), and grows readily when cultured.

2. Materials and methods

Thalli of *U. lactuca* were collected from two different locations (58°14′65″N 11°25′85″E and 8°15′10″N 11°26′20″E) in the mouth of the Gullmar fjord on the Swedish west coast between 2009 and 2010. Sporophytes were kept in the laboratory in a flow-through system with temperature-controlled natural seawater (salinity 32, temperature 19 °C). The light–dark regime was 16 h of light and eight hours of darkness, and average photon flux density of photosynthetically active radiation (PAR) of 100 μmol photons m^{-2} s^{-1}. Phosphate and nitrate were added to the water when growth stimulation of the thalli was required. Zoospore production was induced by exposing the sporophytes to additional light (250 μmol photons m^{-2} s^{-1} PAR). Pieces of thalli containing spores were collected and placed in Petri dishes with filtered natural seawater (0.2 μm membrane filter, Pall Corporation, VWR International, Stockholm, Sweden) with a salinity of 32. Spore release was stimulated by increasing the concentration of phosphate and nitrate with 0.07 mmol l^{-1} and 0.7 mmol l^{-1} respectively. Spores were then collected using a plastic Pasteur pipette. To prevent settling prior to the exposure to irgarol the spores were put on ice until the start of the experiment. Stock solutions of irgarol were prepared in dimethyl sulfoxide (DMSO) and were stored in darkness at −18 °C for a maximum of four weeks. Test solutions for the experiments were prepared at maximum one hour prior to start by adding aliquots of the stock solutions to filtered seawater. To avoid nutrient limitation macronutrients were added to the filtered seawater, increasing the nutritional concentrations with 40 μmol l^{-1} NO_3 and 4 μmol l^{-1} PO_4, in agreement with the previous study by Mercado et al. (2006).

Test solutions and spores were added to 20-ml scintillation glass vials to a final volume of 10 ml. The vials were incubated on a rotary shaker, giving the water a gentle stirring. Transparent Plexiglas covers enabled light to penetrate but prevented evaporation. All treatments were run in five replicates together with 10 controls with co-solvent (DMSO 100 μl l^{-1}, 0.2 μm filtered seawater) and 10 controls without co-solvent (0.2 μm filtered seawater). The temperature was set to 15 °C to mimic typical conditions on the Swedish west coast during the pleasure craft season. The day-night regime was 16 h light (photon flux density 40–60 μmol photons m^{-2} s^{-1}, PAR) and eight hours darkness and the incubation time was 72 h with a daily change of solutions. The chlorophyll *a* content of the initial zoospore population was between 310 and 790 μg l^{-1}. Unattached spores were washed away at the first change of solutions 24 h after the start, and inhibition was always related to controls containing co-solvent from the same experimental run, in order to allow comparisons between experiments. The chlorophyll *a* content was used as an indirect measure of the attached biomass, including both settled zoospores as well as germlings. Chlorophyll *a* was extracted with ethanol (96%) (Jespersen and Christoffersen, 1987) for 24 h on a shaking table in darkness. The samples were measured using a 10-AU Turner Fluorometer, Sunnyvale California USA. The experiment was repeated independently four times with thalli collected on three occasions during a total period of one and a half years.

The irgarol stock solutions in DMSO were diluted into dichloromethane for analysis by gas chromatography-time of flight-mass spectrometry (GCT-Premier, Micromass, Sweden). The analysis was performed in electron impact mode (70 eV) using full scan (m/z 50–800 mD) and a source temperature of 180 °C. The accurate masses used for compound determination were 253.136 + 182.053 for irgarol. Gas chromatographic separation was carried out using a DB-5MS column (60 m × 0.25 mm × 0.25 μm) (J&W Scientific). The temperature program was as follows: 70 °C for 3 min, 20 °C/min to 170 °C, 2 °C/min to 185 °C held for 2 min, and 10 °C/min to 300 °C held for 2 min. A splitless mode injection at 250 °C was used. The analytical concentrations show only minor deviations from the nominal concentrations, and hence all toxicity data for irgarol could be based on nominal concentrations.

No statistically significant differences were observed between the settlement and growth of organisms in pure filtered seawater and of organisms exposed to filtered seawater and DMSO (100 μl l^{-1}) (two tailed t-test, $\alpha = 0.05$). The DMSO-treated samples were hence designated as controls for statistical calculations. One-factor analysis of variance (ANOVA) was applied for testing for significant differences between treatments and the Student–Newman-Keuls (SNK) test was used for *a posteriori* comparisons.

3. Result and discussion

No inhibition of zoospore settlement and growth occurred in irgarol treatments up to 2000 nmol l^{-1} (Fig. 1). Instead, irgarol exposures consistently led to increase in the overall chlorophyll *a* content of the settled population in a concentration-dependent fashion. Visual inspection of the incubation vessels confirmed germling growth even at high irgarol concentrations. Increased chlorophyll *a* content was found in all concentrations above 16 nmol l^{-1} (one-factor ANOVA, $\alpha = 0.05$) with a maximum increase to almost 400% of the control values at an irgarol concentration of 100 nmol l^{-1} (Fig. 1D). The concentration-dependent increase in chlorophyll *a* values were observed in all four independent experiments, using thalli collected at three different occasions at two different collection sites (Fig. 1). These findings are in sharp contrast to previously published data. The distribution of sensitivity reported as EC/LC50 among aquatic plants an algae, including both micro- and macroalgae, for irgarol range between 0.54 nmol l^{-1} and 32 nmol l^{-1} (Hall and Gardinali, 2004; Hall et al., 2009). This is more than 60 times lower than the highest tested concentration in the present study, at which *U. lactuca* grows unaffected. For settlement- and growth inhibition specifically in marine macro algae, irgarol effect concentrations are reported in a range between 10 and 100 times lower than the highest tested concentration in the present study (Table 1). Scarlett et al. (1997) found a complete inhibition of zoospore settling of *Ulva intestinalis* at an irgarol concentration of 39 nmol l^{-1}, and no zoospores could survive a concentration of 200 nmol l^{-1}, which is an order of magnitude less than the highest concentration tested in this study. Settling of *U. lactuca* gametes was completely inhibited at a surface concentration of 1 μg cm^{-2} (de Nys et al., 1996), and growth of adult thalli of *Ulva intestinalis* is reported to be reduced in concentrations above 49 nmol l^{-1} (Lewis et al.,