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Microalgal sensitivity varies between a diuron-resistant strain and two wild strains when exposed to diuron and irgarol, alone and in mixtures

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HIGHLIGHTS

- Two microalgal species were exposed to irgarol, diuron, and mixtures of both.
- At 0.5 μg L^{-1}, irgarol was more toxic than diuron, for both species.
- A mutation was found in the *psbA* gene coding sequence of the diuron-resistant strain.
- The mutation induced no resistance to irgarol in the diuron-resistant strain.
- Mixture (D5+I0.5) induced stronger effects than I0.5 in the diuron-resistant strain.

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ABSTRACT

A wild strain of *Chaetoceros calcitrans* and wild and diuron-resistant strains of *Tetraselmis suecica*, were exposed to the PSII inhibitor herbicides diuron and irgarol, individually and in mixtures. The effects of three concentrations of diuron and irgarol and four binary mixtures were evaluated on doubling time, relative reactive oxygen species and lipid content by flow cytometry, and on photosynthetic efficiency by pulse amplitude modulated fluorescence.

In both wild strains, significant effects were observed for each molecule at the highest concentration tested: at irgarol 0.5 μg L^{-1}, *C. calcitrans* was shown to be more sensitive than *T. suecica* (+52% and +19% in doubling time, respectively), whereas at diuron 5 μg L^{-1}, *T. suecica* was more affected (+125% in doubling time) than *C. calcitrans* (+21%). Overall, irgarol had a higher toxicity at a lower concentration than diuron (no effect at diuron 0.5 μg L^{-1}) for both wild strains. The strongest mixture (irgarol 0.5 μg L^{-1} + diuron 5 μg L^{-1}) increased doubling time by 356% for *T. suecica*, thus showing amplified effects when the two compounds were mixed.

Sequencing of the diuron-resistant strain demonstrated a single mutation in the *psbA* gene coding sequence. Although resistance of this strain to diuron was confirmed with no effect at the highest diuron concentration, no resistance to irgarol was shown. In addition, the mutant strain exposed to the strongest mixture showed a 3.5-fold increase in doubling time compared with irgarol alone, thereby supporting the hypothesis of a biochemical interaction between these two compounds.

1. Introduction

Irgarol (2-methylthio-4-tertbutylamino-6-cyclopropylamino-s-triazine) and diuron (1-(3,4 dichlorophenyl)-3,3 dimethyl urea) are two biocides commonly used in copper-based antifouling paints to replace TBT (Tributyltin) (Manzo et al., 2006). Diuron has also been

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used as an herbicide in agriculture. The use of diuron as a biocide and herbicide was prohibited in France in 2008 (Directive biocide 98/8/CE and Arrêté du 21/08/2008). However, its persistence in the environment means that it is still found in rivers and coastal waters. In the Water Framework Directive (2000/60/EC), diuron, and later irgarol (Directive 2013/39/UE), were included in the list of "48 priority pollutants to be monitored in European waters", which will lead to their progressive prohibition over the next 20 years. While diuron is no longer used in most European countries, it is still of great concern in other countries, such as in Australia where it is known to be harmful to the Great Barrier Reef (Lewis et al., 2009; Holmes, 2012). In contrast, irgarol is still widely used in antifouling paints all around the world despite reports of high toxicity in some studies from the U.K. (Thomas et al., 2001; Chesworth et al., 2004), where its use in antifouling paints has been prohibited. Along the French coasts, average irgarol concentrations from 10 to 40 ng L^{-1} were found in Arcachon Bay, while concentrations up to 0.1 μg L^{-1} were reported in Arcachon port (Auby and Maurer, 2004). More recently, irgarol concentrations up to 0.186 μg L^{-1} were reported in Vilaine Bay (Caquet et al., 2013). In Singaporean coastal waters, irgarol concentrations in the range of 3–4 μg L^{-1} have been reported (Basheer et al., 2002). As for diuron, concentrations from 11 to 33 ng L^{-1} were reported in Mediterranean coastal waters (Munaron et al., 2012), and 0.268 μg L^{-1} in Vilaine Bay (Caquet et al., 2013). The highest concentrations reported in European rivers and ground waters have been 0.279 μg L^{-1} and 0.864 μg L^{-1}, respectively (Loos et al., 2009, 2010). In addition, diuron and irgarol have been measured at maximal concentrations of 2.583 and 0.824 μg L^{-1}, respectively, in careening areas of several ports (Cozic and Durand, 2013).

Irgarol, a triazine, and diuron, a phenylurea, both act as photosystem II (PSII) inhibitors: their binding action on the D1 protein in PSII prevents electron transfer between quinones Q_A and Q_B, impeding Hill's reaction (Nimbal et al., 1996; Jones and Kerswell, 2003). As PSII structure is very well conserved among plants and microalgae, numerous non-target organisms could suffer deleterious effects if environmental pollution occurs (Readman et al., 1993).

Effects on phytoplankton have been recorded in a number of studies. Koutsaftis and Aoyama (2006) reported 72 h IC_{50} values of 1.1 μg L^{-1} and 36 μg L^{-1} for irgarol and diuron respectively, on the growth of the microalga *Chaetoceros gracilis*. Nyström et al. (2002) established that irgarol concentrations ranging from 441 to 647 ng L^{-1} were responsible for 50% photosynthesis inhibition in Lake Geneva phytoplankton. Larras et al. (2013) assessed the sensitivity of benthic diatoms to diuron and irgarol under both planktonic and benthic conditions. They established EC_{50} values of 4.27 and 10.07 μg L^{-1} for planktonic conditions and 9.50 and 0.070 μg L^{-1} for benthic conditions, for diuron and irgarol, respectively, based on the 96 h growth rate of the population. Devilla et al. (2005) established EC_{50} values based on 72 h cell number inhibition of 2.26 and 0.25 μg L^{-1} for diuron and irgarol, respectively, on the microalga *Emiliania huxleyi*. For diuron, tropical estuarine microalgae species *Navicula sp*. and *Navicula pyriformis* showed EC_{50} values of 7.8 and 8 μg L^{-1}, respectively, based on 72 h growth rate (Magnusson et al., 2008). In another study, Magnusson et al. (2010) found diuron IC_{50} values of 2.6, 2.01, 2.71 and 4.4 μg L^{-1} for *Navicula sp*., *N. pyriformis, Phaeodactylum tricornutum* and *Cylindrotheca closterium,* respectively, based on photosynthetic efficiency.

In the environment, organisms are exposed to cocktails of chemicals, it is thus of interest to study the effects induced by mixtures of contaminants. Fernández-Alba et al. (2002) showed that a mixture of irgarol and diuron resulted in a synergistic interaction impacting three different organisms, including a microalga. Gatidou and Thomaidis (2007) showed that the harmful effects of interactions between irgarol and its metabolites were additive on phytoplankton, while the interaction between diuron and its metabolites was shown to be synergistic. Recently, Cedergreen (2014) reviewed the main interactions resulting from different types of pollutants: metals, pesticides and antifouling agents, revealing that synergistic interaction often occurred with antifouling mixtures.

Following chronic exposure to many different chemicals, genetic variants resistant to certain types of molecules might arise in some species. It was demonstrated that PSII inhibitor resistance was mainly due to a mutation in the gene sequence coding for the D1 protein (Erickson et al., 1989; Oettmeier, 1999). However, according to the literature available, such mutations were not involved in resistance to irgarol (Eriksson et al., 2009). Cells resistant to contaminants arise randomly by rare spontaneous pre-selective mutation during replication (Costas et al., 2001; López-Rodas et al., 2001). In the case of environmental pollution, such mutants would allow a population to become resistant (López-Rodas et al., 2009; Carrera-Martinez et al., 2011; Romero-Lopez et al., 2012). In the particular case of diuron resistance, it has been demonstrated that diuron itself was not responsible for the first appearance of resistant cells (López-Rodas et al., 2001).

The microalgae used in this study were the chlorophyte *Tetraselmis suecica* and the diatom *Chaetoceros calcitrans*. In addition to their use in aquaculture, both of these species are encountered in the temperate coastal waters of the East Atlantic. The testing of species from two different phyla enabled us to cover a broader range of potential responses to pesticide exposure. Furthermore, two different strains of *T. suecica* were used in this study: (i) a "wild" strain and (ii) a diuron-resistant strain (Stachowski-Haberkorn et al., 2013).

In order to understand to what extent environmental contamination with herbicides can affect microalgal populations, this study aimed:

1. To evaluate the toxicity of diuron and irgarol separately and to explore the effects of binary mixtures, on four physiological endpoints, using two species of microalgae.
2. To identify the mutation responsible for diuron resistance in the mutant strain of *T. suecica.*
3. To investigate the effects on the mutant strain of irgarol and of binary mixtures of both herbicides.

To answer these questions, the impacts of irgarol and diuron (individually and in mixtures) were assessed on three strains of two marine phytoplankton species. The genetic basis of the resistance to diuron was investigated and effects of the herbicides were measured on four parameters. Growth, measured by doubling time (T_D), is a parameter obviously related to the survival process in microalgae. Two other parameters related to the physiological status of the strains are expected to vary because of photosynthesis inhibition caused by diuron and irgarol: the photosynthetic efficiency (ϕ'_M) and the relative reactive oxygen species (ROS; $FL1_{ROS}$) content. Since the two phytoplankton species are commonly used in aquaculture, the relative lipid content ($FL1_{Lipids}$) was also measured, as it is related to the nutritive quality of the cells.

One major interest of the present study is that, to our knowledge, no ecotoxicological studies have yet established the effects of herbicide mixtures toward both wild and resistant strains of the same phytoplankton species.

2. Materials and methods

2.1. Chemical/toxicant preparation

Irgarol (Irgarol Pestanal® ≥ 98.4%) and diuron (>98%) were purchased from Sigma Aldrich. Stock solutions of irgarol