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The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass *Zostera marina* (L.)

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Abstract

The herbicides Irgarol 1051 (2-(*tert*-butylamino)-4-cyclopropylamino)-6-(methylthio)-1,3,5-triazine) and Diuron (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) are commonly incorporated into antifouling paints to boost the efficacy of the compound towards algae. Previous investigations have identified environmental concentrations of these herbicides as being a threat to non-target organisms, such as seagrasses. Their individual toxicity has been assessed, but they can co-occur and interact, potentially increasing their toxicity and the threat posed to seagrass meadows. Chlorophyll fluorescence (F_v:F_m) and leaf specific biomass ratio (representing plant growth) were examined in *Zostera marina* L. after a 10-day exposure to the individual herbicides. The EC_{20} for each herbicide was determined and these then used in herbicide mixtures to assess their interactive effects. Irgarol 1051 was found to be more toxic than Diuron with lowest observable effect concentrations for F_v:F_m reduction of 0.5 and 1.0 μg/l and 10-day EC_{50} values of 1.1 and 3.2 μg/l, respectively. Plants exposed to Irgarol 1051 and Diuron showed a significant reduction in growth at concentrations of 1.0 and 5.0 μg/l, respectively. When *Z. marina* was exposed to mixtures, the herbicides commonly interacted additively or antagonistically, and no significant further reduction in photosynthetic efficiency was found at any concentration when compared to plants exposed to the individual herbicides. However, on addition of the Diuron EC_{20} to varying Irgarol 1051 concentrations and the Irgarol 1051 EC_{20} to varying Diuron concentrations, significant reductions in F_v:F_m were noted at an earlier stage. The growth of plants exposed to Diuron plus the Irgarol 1051 EC_{20} were significantly reduced when compared to plants exposed to Diuron alone, but only at the lower concentrations. Growth of plants exposed to Irgarol 1051 and the Diuron EC_{20} showed no significant reduction when compared to the growth of plants exposed to Irgarol 1051 alone. Despite the addition of the EC_{20} not eliciting a further significant reduction when compared to the herbicides acting alone for most of the mixtures, the lowest observable significant effect concentration for growth and photosynthetic efficiency decreased to 0.5 μg/l for both herbicides. Irgarol 1051 and Diuron have been shown to occur together in concentrations above 0.5 μg/l, suggesting that seagrasses may be experiencing reduced photosynthetic efficiency and growth as a result.

Keywords: Irgarol; Diuron; Interactive effects; *Zostera*; Photosynthesis; Fluorescence

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1. Introduction

Seagrasses are unique amongst flowering plants by growing and reproducing immersed in seawater. Approximately 50 species are known, occupying temperate and tropical shallow soft-sedimentary coastal waters and estuaries (Fortes, 1990), with *Zostera marina* (L.), eelgrass, the commonest species in temperate regions (Tubbs and Tubbs, 1983). Seagrasses are of great ecological importance; meadows are the most productive of all shallow, soft-sediment environments with the plants providing shelter for flora and fauna and stabilising the seabed (Barnes and Hughes, 1995). This facilitates the development of a highly diverse assemblage of organisms ranging from microscopic epiflora and fauna to large mammals, many of which have conservational or commercial importance (Burchmore et al., 1984; Larkum et al., 1989; Kemp, 2000). Previous reductions in seagrass populations have caused significant diminution in biodiversity and population numbers of the associated flora and fauna (Stauffer, 1937; Rasmussen, 1973; Pollard, 1984).

Since the ban on using tributyltin (TBT) as an antifouling agent on vessels under 25 m, paint manufacturers now often utilise the organic biocides Irgarol 1051, 2-(*tert*-butylamino)-4-cyclopropylamino)-6-(methylthio)-1,3,5-triazine, and Diuron, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, both of which are herbicides. Diuron is currently estimated to be found in 50% of all antifouling paints sold in the UK and Irgarol 1051 found in 30% (Environment Agency, 1998). Both herbicides are commonly found in coastal waters and sediments throughout the world, and often they are the most prevalent herbicides (Thomas et al., 2001b). Aqueous estuarine concentrations have been reported to reach 0.68 μg/l for Irgarol 1051 (Voulvoulis et al., 2000) and 0.7 μg/l for Diuron (Readman et al., 1993a). Concentrations in marinas and ports are usually higher and have been recorded at 1.7 μg/l for Irgarol 1051 (Readman et al., 1993b) and 6.7 μg/l for Diuron (Thomas et al., 2001a). Herbicide concentrations are even greater in sediments with Diuron concentrations up to 10 times higher than those in the overlying waters (Haynes et al., 2000b) and Irgarol 1051 up to 300 times higher (Voulvoulis et al., 2000). Both herbicides are slow to degrade, with Irgarol 1051 having a half-life of 100 days (Okamura et al., 2000), increasing the likelihood of accumulation.

Both Irgarol 1051 and Diuron have the same mode of toxicity towards plants, inhibiting photosynthesis by blocking electron transport (Hall and Rao, 1995). They bind with high affinity to the Q_B site of photosystem II (PS II), displacing the Q_B quinone and preventing electron transfer from Q_A to Q_B (Jansen et al., 1993; Hall et al., 1999). This inhibition ultimately leads to reduced carbon dioxide uptake, decreased carbohydrate production and the eventual starvation of the plant.

Of the few published reports on the effects of Irgarol 1051 and Diuron on seagrasses, Haynes et al. (2000a) found Diuron concentrations of 0.1–1.0 μg/l to significantly limit photosynthesis in *Halophila ovalis* and *Zostera capricorna* while Scarlett et al. (1999a) found Irgarol 1051 concentrations of 10 and 0.18 μg/l to significantly limit growth and photosynthetic efficiency, respectively, in *Z. marina*. These levels are below those found in the environment and thus suggest that seagrasses may be suffering from a loss of viability.

Due to an increased awareness of the environmental problems associated with antifouling paints, the UK Health and Safety Executive (HSE) have imposed a ban on the application of both Diuron and Irgarol 1051 as antifoulants as of November 2002 (Advisory Committee on Pesticides, 2000). The ban will be total for Diuron but boats over 25 m will still be allowed to use Iragrol 1051. However, the ban is not Europe-wide and the persistence of Irgarol 1051 and Diuron suggest the herbicides may still pose a threat to the marine environment.

When pollutants occur together in the environment they may interact in a synergistic, additive or antagonistic manner. Bonnemain and Dive (1990) found ziram and copper, both used in antifouling paints, to act synergistically towards a ciliate, whereas Roberts et al. (1990) found no significant interactions between Irgarol 1051 and copper to either algae or protozoa. Teisseire et al. (1999) investigated the interactive effects of Diuron and copper on duckweed, *Lemna minor*, finding additivity or non-significant antagonism to occur. Irgarol 1051 and Diuron have been reported to occur together (Ferrer and Barcelo, 1999; Thomas et al., 2000; Thomas et al., 2001a) and could therefore interact to further increase toxicity to seagrasses. To our knowledge this has not been previously reported.

2. Methodology

2.1. Collection and preparation of Zostera marina plants

Plants of *Z. marina* were collected from well established beds in the Yealm Estuary, UK (50°18.55′N, 4°3.85′W) between June and August 2001, during low water spring tides. The Yealm Estuary is a flooded ria system with low freshwater input. It has a relatively low Irgarol 1051 concentration of 0.01 μg/l (Scarlett et al., 1999a) but no data is available for Diuron. The Yealm estuary has no marinas and large vessels do not use it, but there is a yacht club with numerous small craft moorings.

Care was taken to collect only plants with no apparent disease or damage and without any visible epiphytes. Seagrasses were removed with their roots intact in an effort to ensure they remained healthy and that any influence the roots might have on the uptake kinetics of the herbicides remained. The number of plants collected was kept to a minimum to limit any ecological impact. Plants were standardised by removing all but the three youngest leaves and these were trimmed to 20 cm above the basal leaf meristem and wiped with a paper towel to remove any epiphytes. Plants were then rinsed in seawater and sediment attached to the roots was removed to avoid any influence it may have on the uptake kinetics.

The seagrasses were transferred to plastic tanks containing filtered seawater (Gelman filter +0.45 μm, Sussex, UK) and kept at 16 °C under "cool white" fluorescent tubes, giving a photon fluency rate of 35 μmol quanta m^{-2} s^{-1} photosynthetically active radiation (PAR), measured with a standard PAR sensor (Skye Instruments). They were left to acclimatise for 6 days prior to the start of the experiments.

2.2. Preparation of herbicide stock solutions

Irgarol 1051 (>97% purity) was obtained from Ciba Geigy, Basel, Switzerland and Diuron (>98% purity) from Sigma–Aldrich, Dorset, UK. Stock solutions were prepared to allow exposure concentrations to be achieved when adding 100 μl of stock solution to 1 l of seawater. Appropriate amounts of herbicide were accurately weighed and 100 ml of solvent added, ethanol (AnalaR) for Irgarol 1051 and acetone (AnalaR) for Diuron (Fisher Scientific, Leics, UK). Stock solutions were stored at 4 °C until required. Glassware was used for all experiments as the herbicides have been shown to adhere to plastic, which would affect the available concentrations (Ferrer and Barcelo, 1999). Before use glassware was cleaned using 'Decon 90', heated to 170 °C and then rinsed in acetone (AnalaR).

2.3. Individual herbicide exposure trials

To determine the interactive effects of the herbicides on photosynthetic efficiency and growth it was first necessary to assess the effects of the herbicides individually. The methods used were adapted from those described by Scarlett et al. (1999a).

To identify concentration response relationships over time, nominal individual herbicide concentrations of 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 μg/l were prepared from the stock solutions. In addition, two control solutions were prepared, one containing only seawater, equivalent to a herbicide concentration of 0.0 μg/l, and a carrier control containing 300 μl of the solvent. The control and herbicide solutions were then decanted into three replicate 1 l beakers. Three standardised plants were placed in each beaker, giving a total of nine plants per treatment. Experiments were conducted over a period of 10 days with herbicide spiked seawater changed every 48 h to ensure nutrients did not become limiting, waste metabolites were removed and the herbicides remained at the desired concentrations.

Photosynthetic efficiency was assessed using the dark adapted fluorescence induction ratio F_v:F_m. This is a useful parameter as it indicates the efficiency of the PS II photosystem, which is directly targeted by these two herbicides, and shows a high degree of correlation with the quantum yield of net photosynthesis (Hall and Rao, 1995). Measurements were taken using a plant efficiency analyser (PEA; Hansatech Instruments, UK), which was calibrated according to the conditions given by Scarlett et al. (1999a); a dark adaptation time of 20 min and a 50% light intensity (1500 μmol quanta m^{-2} s^{-1} at 650 nm peak wavelength) for 1 s. Readings were taken at the start of each experiment, and at the time of every water change. Each plant in each

replicate was analysed giving a total of 9 readings per treatment level.

Growth was assessed by measuring the ratio of new biomass to old biomass. At the start of the experiment, leaves were puncture marked with a syringe needle 1 cm above the basal leaf meristem. At the end of the 10-day exposure period plant biomass was divided into old and new growth, with leaf tissue above the scar being classified as old biomass and tissue below the scar, but above the meristem, classified as new biomass. Any new leaves without a scar were also considered as new biomass. Following separation, tissues from each replicate beaker were pooled, dried for 5 days at 90 °C and re-weighed. This provides an indication of the initial biomass before the trials and the increase in biomass during the trials, and hence growth, over the period of the experiment. As growth analyses was destructive, it was carried out only at the end of the 10-day exposure period.

2.4. Herbicide interaction trials

From the individual herbicide exposure results at day 10, dose-response curves of the F_v:F_m ratio against concentration were plotted and used to determine the herbicide concentrations that resulted in a 20% reduction in photosynthetic efficiency following the method of linear interpolation. The same method was subsequently used to determine EC_{50}s. Stock solutions of these EC_{20} concentrations were prepared following the method previously described. EC_{20} values were used in the herbicide interaction experiments in preference to EC_{50} values as they are thought to better represent chronic effects, and are also more comparable to environmental concentrations. Photosynthetic efficiency and growth responses were determined following the same procedures outlined above, with the addition of the Diuron EC_{20} to the variable Irgarol 1051 concentrations and the Irgarol 1051 EC_{20} to the variable Diuron concentrations. As the herbicides were dissolved in different solvents, the carrier control contained 100 μl/l of both ethanol and acetone.

Herbicide interactions were assessed with a widely used model; Abotts' formula (Gisi, 1996; Teisseire et al., 1999). The model compares expected and observed inhibitions where expected inhibitions expressed as percent C_{exp} can be predicted as follows:

$$C_{exp} = A + B - \left(\frac{AB}{100}\right), \quad (1)$$

where A and B are the inhibitions caused when the herbicides act alone.

The ratio of inhibition (RI) was then calculated as follows for each herbicide mixture and for both photosynthetic and growth trials:

$$\mathrm{RI} = \frac{\text{observed inhibition}}{C_{exp}}. \quad (2)$$

Interactive effects were evaluated by comparing RI with 1. RI values >1 indicated synergism; RI values equalling 1, additivity; and RI values <1, antagonism. The RI was calculated for each of the three replicates for each treatment and the mean and standard deviation determined. Only if the mean RI was greater than one standard deviation from 1 was the interactive effect assumed to be significantly different from additivity.

2.5. Statistical methods

All statistical analyses were performed using the statistical software package SPSS Version 9.0. Tests for normality and variance checks were made prior to the analyses. To allow comparisons between effects on growth and photosynthetic efficiency, statistical tests were performed on data collected at day 10, this would also allow plants to reach equilibrium, providing the most robust results. For the individual herbicide exposure trials plant growth and F_v:F_m data were analysed by one-way Analysis of Variance and to test for differences between treatments in the herbicide interaction trials plant growth and F_v:F_m data were analysed by two-way ANOVA. Differences between individual means in all tests were determined by Tukey's Honestly Significant Difference test. Differences are considered to be significant at $P = 0.05$.

3. Results

3.1. Photosynthetic efficiency

At the start of the exposure experiments, F_v:F_m values for the majority of plants were above 0.8 and were thus considered in a healthy state. All individual

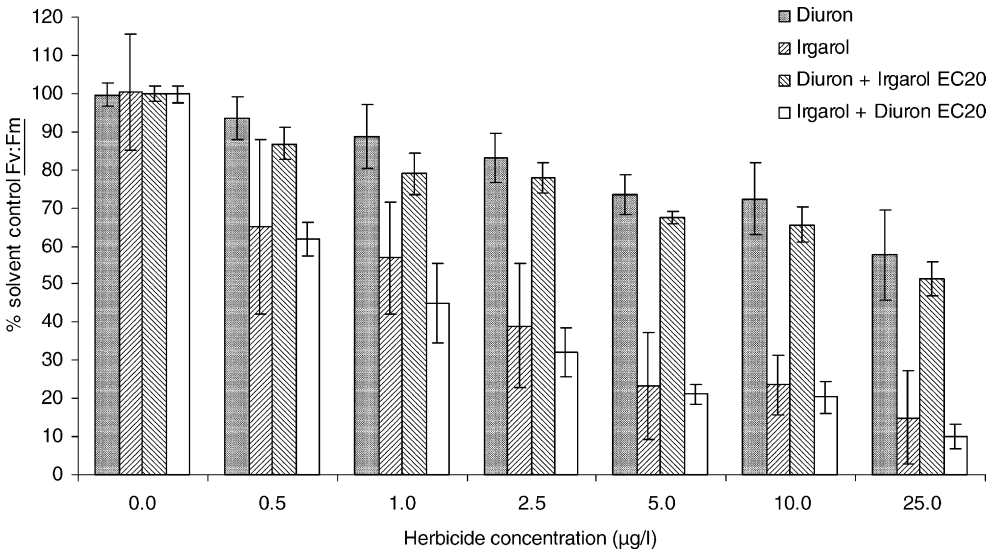

Fig. 1. Effects of Diuron, Irgarol 1051, Diuron plus the Irgarol 1051 EC_{20} and Irgarol 1051 plus the Diuron EC_{20} on the *Zostera marina* leaf F_v:F_m values (expressed as a percentage of the solvent control) after 10 days exposure. Herbicide concentration 0.0 μg/l represents the seawater control, error bars are standard deviations from the mean of three replicates.

plants maintained in the seawater and solvent controls remained at this level throughout the experimental period with very little variance.

Plants exposed to Diuron concentrations above 2.5 μg/l showed marked reductions in F_v:F_m values from day 2 onwards, whereas at lower concentrations a reduction was not observed until day 8. Plants exposed to Irgarol 1051 showed greater reductions than those exposed to the equivalent Diuron concentrations, at all time periods. After 2 days plants exposed to 25 μg/l Irgarol 1051 exhibited a decrease in F_v:F_m of 31.4% compared with a 16.6% reduction in plants exposed to Diuron.

From the day 10 results (Fig. 1) it is apparent that an increase in the concentration of the two herbicides results in a significant decrease ($P < 0.05$) in photosynthetic efficiency when compared to the solvent control. Irgarol 1051 elicited a greater decrease than Diuron at comparable concentrations ($P < 0.05$), although plants exposed to Irgarol 1051 displayed increased variation, particularly at lower concentrations. The lowest concentrations to initiate a significant decrease in F_v:F_m were 1.0 μg/l Diuron and 0.5 μg/l Irgarol 1051. The lowest values of F_v:F_m were recorded at 25.0 μg/l Diuron (40% reduction) and 25 μg/l Irgarol 1051 (80% reduction), although in the latter case the reduction did not differ significantly from that at 5 μg/l. EC_{50} values were interpolated from dose response curves to be 1.1 μg/l for Irgarol 1051 and 3.2 μg/l for Diuron, and the EC_{20} values, subsequently used in the mixed herbicide exposure experiments, estimated to be 0.23 and 0.65 μg/l for Irgarol 1051 and Diuron, respectively.

The trends identified in the individual herbicide exposures were apparent when plants were exposed to herbicide mixtures (Fig. 1). On addition of the Irgarol 1051 EC_{20} to 0.5 μg/l Diuron, values departed from controls at day 4, as opposed to day 8 in the individual Diuron exposure trials. At each time period and at all concentrations, plants exposed to Irgarol 1051 plus Diuron EC_{20} exhibited lower F_v:F_m values that plants exposed to Diuron plus Irgarol 1051 EC_{20}. For example, at day 4, exposure to 0.5 μg/l Diuron plus Irgarol 1051 EC_{20} resulted in a reduction of 2.1% compared to 9.3% for 0.5 μg/l Irgarol 1051 plus Diuron EC_{20}.

Statistical analysis of the results obtained after 10 days of exposure confirmed a significant reduction ($P < 0.05$) in photosynthetic efficiency with increasing concentrations of the principal herbicide. At all concentrations above 0.5 μg/l, plants exposed to Irgarol 1051 plus Diuron EC_{20} were found to have significantly lower ($P < 0.05$) F_v:F_m values than plants

Table 1
The interactive effect on *Z. marina* F_v:F_m values of the addition of the Diuron EC_{20} to varying Irgarol 1051 concentrations

Irgarol concentration (μg/l)	Mean RI ± S.D.	Interactive effects
0.5	0.96 ± 0.04	≈1 Additive
1.0	1.15 ± 0.13	>1 Synergistic
2.5	1.04 ± 0.04	≈1 Additive
5.0	1.01 ± 0.03	≈1 Additive
10.0	1.02 ± 0.02	≈1 Additive
25.0	1.02 ± 0.04	≈1 Additive

Table 2
The interactive effect on *Z. marina* F_v:F_m values of the addition of the Irgarol 1051 EC_{20} to varying Diuron concentrations

Diuron concentration (μg/l)	Mean RI ± S.D.	Interactive effects
0.5	0.59 ± 0.02	<1 Antagonistic
1.0	0.80 ± 0.03	<1 Antagonistic
2.5	0.72 ± 0.10	<1 Antagonistic
5.0	0.83 ± 0.03	<1 Antagonistic
10.0	0.86 ± 0.04	<1 Antagonistic
25.0	0.94 ± 0.07	≈1 Additive

exposed to equivalent values of Diuron plus Irgarol EC_{20}. The lowest concentration of Diuron in the mixture to elicit a significant reduction was 0.5 μg/l; this compares with 1.0 μg/l with Diuron alone. For plants exposed to Irgarol 1051 plus Diuron EC_{20} the lowest observed significant effect concentration of Irgarol 1051 was also 0.5 μg/l, the same as for Irgarol 1051 alone. However, since 0.5 μg/l was the lowest concentration used in this study, any further reduction would not have been apparent. The concentration of Diuron plus Irgarol EC_{20} yielding the lowest F_v:F_m values was 25.0 μg/l, with a 50% reduction in photosynthetic efficiency, although this was not statistically different from the reduction at 5 μg/l. Similarly, the lowest F_v:F_m values for Iragrol 1051 plus Diuron EC_{20} were recorded at 25.0 μg/l, causing a 90% reduction in photosynthetic efficiency, but this reduction was also not statistically different to that at 5 μg/l.

The addition of Irgarol 1051 EC_{20} to any Diuron concentration or addition of Diuron EC_{20} to any Irgarol concentration failed to result in any further significant reduction in F_v:F_m values beyond those resulting from exposure to Diuron and Irgarol alone ($P > 0.05$). Although the addition of the EC_{20} concentrations did not elicit any further significant reduction in F_v:F_m values when compared to the individual herbicides, variances were reduced.

The Diuron EC_{20} of 0.65 μg/l gave a reduction in F_v:F_m of 8.35%. When added to the varying Irgarol 1051 concentrations, the further reductions were less than 8.35% for all but 1.0 μg/l Irgarol 1051, which showed a reduction of 12.3%. The mean RIs ranged from 0.96 to 1.15 and, with the exception of 1.0 μg/l Irgarol 1051, are all within one standard deviation of an RI of 1, suggesting the interactive effects equated to additivity (Table 1). The Irgarol 1051 EC_{20} of 0.23 μg/l gave a reduction in F_v:F_m values of 17.02%. When the Irgarol 1051 EC_{20} was added to varying Diuron concentrations, the further reductions in F_v:F_m were all less than 17.02%. All RI values were less than 1, ranging from 0.59 to 0.94, and thus indicative of antagonism (Table 2).

3.2. Growth analyses

Growth was significantly reduced upon exposure to increasing concentrations of the two herbicides (Fig. 2). After 10 days exposure, an Irgarol 1051 concentration of 1.0 μg/l caused a significant ($P < 0.05$) reduction in growth with a further significant decrease between 5 and 10 μg/l. The lowest concentration of Diuron to elicit a significant ($P < 0.05$) growth reduction was 5.0 μg/l, with a further significant decrease observed at 10 μg/l. There was no significant difference ($P > 0.05$) in growth between plants exposed to Irgarol 1051 and Diuron, except at a concentration of 2.5 μg/l, where plants exposed to Irgarol 1051 experienced a 20% greater reduction.

Plant growth was significantly reduced ($P > 0.05$) by the introduction of the herbicide mixtures (Fig. 2) but there was no significant difference ($P > 0.05$) between the two herbicide mixtures at any concentration tested. For both herbicide mixtures, the lowest observed significant effect concentration was 0.5 μg/l, lower than when exposed to the individual herbicides. The addition of Irgarol 1051 EC_{20} to Diuron concentrations of less than 5 μg/l caused significantly larger reductions in the growth of *Z. marina* than upon exposure to Diuron alone ($P > 0.05$). In contrast, the addition of the Diuron EC_{20} to Irgarol 1051 had no significant additional effect on plant growth at any concentration when compared to the growth of plants

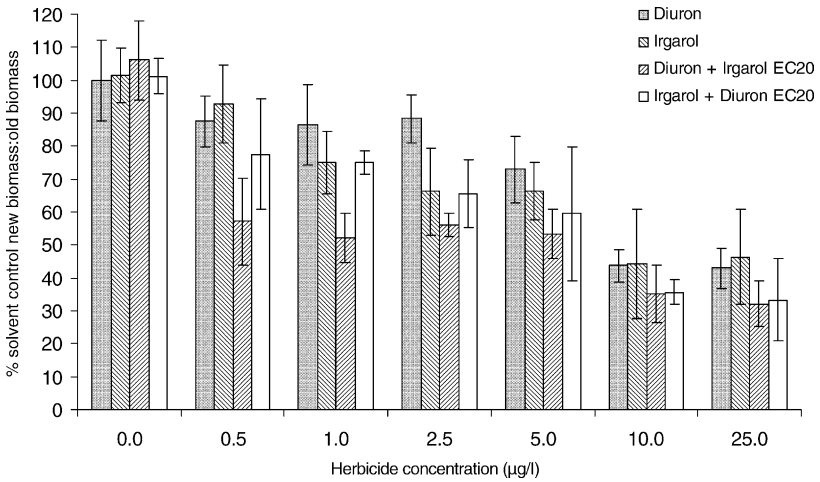

Fig. 2. Effects of Diuron, Irgarol 1051, Diuron plus the Irgarol 1051 EC_{20} and Irgarol 1051 plus the Diuron EC_{20} on the growth of *Zostera marina* plants (expressed as a percentage of the solvent control) after 10 days exposure. Herbicide concentration 0.0 μg/l represents the seawater control error bars are standard deviations from the mean of 3 replicates.

exposed to only Irgarol 1051 ($P > 0.05$). At higher concentrations (5.0, 10.0 and 25.0 μg/l), there were no significant differences in the growth of plants exposed to any of the four herbicide treatments.

From biomass ratio dose response curves, an expected growth reduction of 3.8% was interpolated for the Irgarol 1051 EC_{20} (0.23 μg/l). At all Diuron concentrations the addition of the Irgarol 1051 EC_{20} resulted in reductions greater than 3.8%, giving RIs ranging from 1.12 to 2.86, indicating that the interactive effects were synergistic (Table 3). The Diuron EC_{20} of 0.65 μg/l was found to give a reduction in plant growth of 13.5%. For Irgarol 1051 concentrations of 1.0 and 10.0 μg/l, the addition of the Diuron EC_{20} had a significantly antagonistic effect (Table 4). For all other concentrations the effect approximated additivity due to the relatively large standard deviations about the individual means.

4. Discussion

4.1. *Zostera marina* photosynthetic efficiency

Both Irgarol 1051 and Diuron have the same mode of toxic action towards plants, affecting PS II by displacing the quinone from the Q_B niche on the D1 protein and blocking Q_B reduction (Jansen et al., 1993; Hall et al., 1999). This inhibits electron transfer from Q_A to Q_B, preventing the use of absorbed light energy in photochemistry and forcing its dissipation by non-photochemical means, usually fluorescence

Table 3
The interactive effect on *Z. marina* plant growth of the addition of the Irgarol 1051 EC_{20} to varying Diuron concentrations

Diuron concentration (μg/l)	Mean RI ± S.D.	Interactive effects
0.5	2.71 ± 0.83	>1 Synergistic
1.0	2.86 ± 0.45	>1 Synergistic
2.5	2.78 ± 0.22	>1 Synergistic
5.0	1.57 ± 0.25	>1 Synergistic
10.0	1.12 ± 0.15	≈1 Additive
25.0	1.16 ± 0.12	>1 Synergistic

Table 4
The interactive effect on *Z. marina* plant growth of the addition of the Diuron EC_{20} to varying Irgarol 1051 concentrations

Irgarol concentration (μg/l)	Mean RI ± S.D.	Interactive effects
0.5	1.14 ± 0.85	≈1 Additive
1.0	0.73 ± 0.10	<1 Antagonistic
2.5	0.81 ± 0.24	≈1 Additive
5.0	0.95 ± 0.48	≈1 Additive
10.0	0.91 ± 0.05	<1 Antagonistic
25.0	0.97 ± 0.18	≈1 Additive

(Ralph, 2000). The result is a reduced F_v:F_m value proportional to the quantum yield of PS II photochemistry explaining the reduction in photosynthetic efficiency, and the overall decrease in the net photosynthesis of *Z. marina* leaves when exposed to the herbicides (Hall and Rao, 1995).

The decrease in photosynthetic efficiency was particularly apparent at low concentrations, where F_v:F_m values dropped sharply. Previous studies have also identified this trend (Scarlett et al., 1999a; Haynes et al., 2000a). Increases in the free concentration of the herbicides result in more binding to the QB niche, causing a subsequent increase in the dissipation of light energy and decrease in photosynthetic efficiency (Jansen et al., 1993). At low herbicide concentrations, a relatively high number of Q_B niches will be available for the herbicides to bind to, which could lead to the initial sharp drop in F_v:F_m values. As herbicide concentrations increase, fewer sites will be available for occupation, and the rate of binding may decrease, resulting in the levelling off in F_v:F_m values that was apparent in this investigation.

The degree of toxicity of Irgarol 1051 and Diuron shown towards *Z. marina* was similar to that observed in previous studies. Scarlett et al. (1999a) found the F_v:F_m values of plants exposed to 25.0 μg/l of Irgarol 1051 to drop by 80%, compared to the 85% found in this investigation. Despite this, the 10-day EC_{50} of 2.5 μg/l interpolated by Scarlett et al. (1999a) was higher than the 10-day EC_{50} of 1.1 μg/l obtained in this investigation. This may have resulted from natural variation in the health of the seagrasses exposed to Irgarol 1051 in the two investigations, which could have resulted in increased sensitivity of the plants to low herbicide concentrations. Plants were collected by Scarlett et al. (1999a) between summer 1997 and spring 1998, whereas plants used in this investigation were collected between June and August 2001. It is possible that the observed difference in EC_{50} values reflected the different collection periods, and a change in environmental factors may have lead to a change in the initial health status of the plants. Further trials and replicates could be used to determine if the health of seagrass plants commonly show high variance. This would have implications for their future use as a test species if high variance was common.

Although no previous studies have been conducted on the effects of Diuron on *Z. marina*, Haynes et al. (2000a) examining the effects of Diuron on three tropical species of seagrasses, found a concentration of 1.0 μg/l caused a significant reduction in photosynthetic efficiency, results comparable to those obtained in this study.

Irgarol 1051 was significantly more toxic than Diuron to *Z. marina*, with lower concentrations causing a significant reduction in photosynthetic efficiency. This greater toxicity is apparent from the difference in the 10-day EC_{20}s, with the Diuron EC_{20} being nearly three times higher than that of Irgarol 1051. The comparative toxicity of Irgarol 1051 and Diuron towards phytoplankton and periphyton have been assessed and similar results found, with Irgarol 1051 having EC_{50}s three times lower than that of Diuron (Readman et al., 2002). Ralph (2000) examined the toxicity of Diuron and atrazine, an *s*-triazine similar to Irgarol 1051, to the tropical seagrass *H. ovalis* and found the latter herbicide to be less toxic. Irgarol 1051 was developed more recently than both atrazine and Diuron and is particularly effective (Evans et al., 2000). This effectiveness is likely to stem from its high affinity for the QB niche, which may be greater than that of either atrazine or Diuron. Dahl and Blanck (1996) have shown that Irgarol 1051 is more toxic than atrazine to marine coastal plants. The greater affinity of Irgarol 1051 would mean a faster rate of binding of the molecules and in higher numbers. Toxicokinetic factors may also be responsible for the greater toxicity displayed by Irgarol 1051. If taken up more readily than Diuron it will be present at higher concentrations in the plant tissue and thus have greater toxic potential. Scarlett et al. (1999b) found seagrasses to take up Irgarol 1051 readily, documenting leaf tissue levels of 118 μg/kg, the highest plant tissue concentrations yet recorded. Haynes et al. (2000b) found seagrasses from the same region with Diuron tissue concentrations of 1.7 μg/kg. This may reflect a difference in the uptake of the two herbicides, although other factors such as herbicide water concentrations would have an influence. No previous studies have directly compared uptake rates of the two herbicides.

Although Irgarol 1051 was found to induce greater reduction in photosynthetic efficiency than Diuron, and may therefore be considered more toxic, F_v:F_m was continuing to decline at the end of the 10 day trials in plants exposed to Diuron, whereas in the Irgarol 1051 trials reduction was levelling off. This

may suggest that Diuron could be equally as toxic as Irgarol 1051, but may act more slowly.

When two toxicants having the same mode of action, as with Irgarol 1051 and Diuron, are supplied together antagonism is often found because toxicity is related to the percentage of receptor sites available for binding. The toxicants will compete for these binding sites and the compound with the greater affinity will displace the other (Walker et al., 2001). The compound with the weaker affinity will therefore exhibit a relative decrease in toxicity, as it will not bind to as many sites as it would when acting on its own, and so the overall toxicity of the mixture will be less than additive. For example, Jansen et al. (1993) found competition between Diuron and bromonitrothymol (BNT), also a PS II inhibitor, with BNT displacing Diuron, resulting in a decrease in Diuron binding. Irgarol 1051 and Diuron both seek to bind to the Q_B niche and so competition was predicted with Irgarol 1051 displacing Diuron and lowering the relative toxicity of the mixture. In most cases the herbicides were found to interact antagonistically, although large standard deviations meant this was not always significant, and at no herbicide concentration did the addition of the EC_{20} cause any further significant reduction than that caused by the herbicide acting alone. However, it is worth noting that on the addition of the EC_{20} to the primary herbicide, variation about the means decreased suggesting that the addition of the secondary herbicide was affecting the potency of the mixtures.

4.2. *Zostera marina* plant growth

The light reactions in photosynthesis, which involve PS II, are responsible for the formation of ATP (adenosine triphoshphate), NADPH (nicotinamide adenine dinucleotide phosphate) and oxygen. The inhibition of electron flow in PS II, caused by Irgarol 1051 and Diuron, impedes the formation of ATP and NADPH, leading to a decrease in carbohydrate production (Hall and Rao, 1995). Since carbohydrates are vital for plant growth exposure to Irgarol 1051 and Diuron is likely to lead to growth reduction.

The link between PS II and plant growth was responsible for the similar trends found between the reduction in plant growth and the reduction in photosynthetic activity. As herbicide concentration increased, plant growth decreased but only at 2.5 μg/l did Irgarol 1051 elicit a significantly larger reduction in growth than Diuron, in contrast to the differences seen in photosynthetic efficiency. F_v:F_m is a sensitive measure of plant health, responding rapidly to stressors (Bolhàr-Nordenkampf and Öquist, 1993). Lower F_v:F_m will result in a decrease in carbohydrate production and growth, but this is unlikely to be on the same temporal scale, since reduced photosynthetic efficiency will take longer to be reflected in growth measurements (Teisseire et al., 1999). If growth takes longer than 10 days to reach equilibrium with the F_v:F_m values, it could explain the lack of significance in the growth results between the two different herbicides.

When exposed to PS II inhibiting herbicides, higher plants have been shown to increase their chlorophyll content by the formation of shade-type chloroplasts, characterised by a higher stacking-degree of thylakoids and broader grana (Teisseire et al., 1999). This may compensate for the reduction in photosynthetic efficiency and thus plants exposed to Irgarol 1051 could maintain a growth rate comparable to plants exposed to Diuron, although this will carry an energetic cost. Changes in physiological mechanisms may also explain why, particularly at higher concentrations, there were no significant differences in the growth of plants exposed to any of the herbicide treatments, even upon the addition of the EC_{20}s. It may also be possible that the lack of difference in the growth of plants exposed to Diuron and Irgarol 1051 was due to a secondary, unspecified, toxic mechanism of Diuron, allowing Diuron to retard the growth of *Z. marina* as much as Irgarol despite having less effect on photosynthetic activity.

When the Diuron EC_{20} was added to the varying Irgarol 1051 concentrations, the interactive effects on growth were antagonistic, albeit slight and non-significant in most cases. These observations are similar to those obtained from measurements on photosynthetic efficiency. However, when the Irgarol 1051 EC_{20} was added to the varying Diuron concentrations, the interactive effects on plant growth were synergistic, particularly at low concentrations, seeming to contradict the photosynthetic efficiency trends. The reasons for this were not clear. The insensitivity of plant growth as a measurement of toxicity and the time it takes to reach equilibrium at low concentrations may have meant that the interpolation of plant

growth reduction at the Irgarol 1051 EC_{20} was underestimated. These same problems could have given rise to an overestimation of growth reduction for plants exposed to Diuron plus the Irgarol 1051 EC_{20}. This compounding of errors would lead to results that implied synergism and it may be that longer exposure periods would be more appropriate.

4.3. Risks posed to seagrasses from herbicide environmental concentrations

An Irgarol 1051 concentration of 0.5 μg/l and a Diuron concentration of 1.0 μg/l caused a significant reduction in photosynthetic efficiency whilst an Irgarol 1051 concentration of 1.0 μg/l and a Diuron concentration of 5.0 μg/l caused a significant reduction in growth. Occurring alone, these herbicide concentrations could impair the health and viability of *Z. marina*, however, when occurring together, the implications could be more severe.

Despite the addition of the secondary herbicide EC_{20}s not causing a significant reduction in F_v:F_m values when compared to the primary herbicide acting alone, a difference in the lowest observed effect concentration was noted with significant reductions in photosynthetic efficiency and growth being found at 0.5 μg/l for both herbicides. Environmental concentrations of Irgarol 1051 and Diuron exceeding 0.5 μg/l are well documented. Piedra et al. (2002) found concentrations of 0.9 μg/l of Irgarol 1051 and 0.8 μg/l of Diuron in southeast Spain, whilst Thomas et al. (2001a) found concentrations of 1.4 μg/l of Irgarol 1051 and 6.7 μg/l of Diuron in Hythe Marina, Southampton, UK.

If exposed to the concentrations documented by Thomas et al. (2001a) individually, seagrasses may experience a significant decline in photosynthetic efficiency of approximately 50% for Irgarol 1051 and 65% for Diuron. From the results of this study, the herbicide occurring at the higher concentration will have the dominant effect with the secondary herbicide eliciting a further reduction of up to 10%. Seagrasses in the vicinity of Hythe Marina could therefore suffer a reduction in photosynthetic efficiency of up to 75% and a reduction in growth of up to 60%. This potential for reduction in growth is the most pertinent parameter in assessing possible impacts upon seagrass beds as it is the most ecologically relevant. Previous studies have examined reductions of biomass in seagrass beds and been able to correlate them with subsequent loss of associated fauna (Rasmussen, 1973), as well as increased sediment suspension and light attenuation, further exacerbating seagrass bed decline (Walker and McComb, 1992).

Lower concentrations than those found by Thomas et al. (2001a) in Hythe Marina are more widespread (Boxall et al., 2000; Thomas et al., 2001b). However, as the occurrence of Irgarol 1051 and Diuron together reduces the concentrations necessary to cause a significant reduction in growth and photosynthesis, the threat towards seagrasses remains. Environmental concentrations comparable to the Irgarol 1051 10-day EC_{20} have been documented in 60% of studies reviewed for this investigation and concentrations comparable to the Diuron 10-day EC_{20} have been documented in 67% (Readman et al., 1993a; Readman et al., 1993b; Gough et al., 1994; Ferrer and Barcelo, 1999; Biselli et al., 2000; Boxall et al., 2000; Haynes et al., 2000b; Okamura et al., 2000; Piedra et al., 2002; Sargent et al., 2000; Thomas et al., 2000, 20001a; Voulvoulis et al., 2000). From the studies that have examined the environmental concentrations of both herbicides, almost half found them to occur together at levels above their EC_{20} values. If *Z. marina* were to occur in areas where EC_{20} concentrations of both Irgarol 1051 and Diuron were found, the results from this investigation suggest that plants would show a reduction in photosynthetic efficiency of over 17% and a reduction in growth of over 13%.

The highest environmental concentrations of Irgarol 1051 and Diuron are usually associated with marinas and ports, where boat activity is at its highest. Although seagrasses are found in marinas, and will therefore experience the highest herbicide concentrations occurring in the environment, their distribution is restricted and seagrasses meadows are more extensive in estuaries, outside of the marinas (Deis, 2000). Open estuaries usually exhibit lower environmental concentrations than marinas, due to increased water exchange (Thomas et al., 2001a) however, the co-occurrence of the herbicides will lower the concentrations necessary to elicit a significant reduction in photosynthesis and growth, maintaining the threat to seagrasses.

During this investigation it was noted that at the end of the 10-day exposure period, photosynthetic efficiency was continuing to show a decline, particularly

for plants exposed to Diuron. Also, as previously discussed, growth may take longer than 10 days to reach equilibrium. Both Irgarol 1051 and Diuron have half-lives in excess of 100 days, meaning seagrasses will be exposed to herbicide concentrations for far longer than they were in this laboratory study. Scarlett et al. (1999a) carried out long term Irgarol 1051 exposure trials, finding a 36-day EC_{50} of 0.2 μg/l for *Z. marina*, compared to a 10-day EC_{50} of 2.5 μg/l. This reduction in the EC_{50}, and the slow degradation rates of the herbicides, suggest that seagrass plants are likely to show even more dramatic declines in photosynthesis and growth than were discovered in this study. Longer term exposure trials may also lead to a decrease in variance and more robust results from Abotts formula. In this study, standard deviations were sometimes high, meaning antagonism or synergism were marginal.

The UK Health and Safety Executive have banned the application of Diuron as an antifoulant as of the 21st of November 2002. Irgarol was also banned in November 2002, but only to vessels under 25 m (Advisory Committee on Pesticides, 2000). Although environmental concentrations are likely to decrease over time after the ban comes into effect, the long half-lives of the herbicides will result in concentrations persisting in the environment for some time (Liu et al., 1999). The ban concerning Irgarol 1051 is similar to that concerning the application of TBT as an antifoulant, with both still being permitted on vessels over 25 m. Environmental concentrations of TBT continue to cause problems to various marine species in the UK, 14 years after its ban (Evans et al., 2000). This suggests that the risks posed to seagrasses and other non-target marine organisms from Irgarol 1051 and Diuron may still remain, and these risks may be compounded by its slow degradation. The risks may be exacerbated in ports and harbours that cater for larger vessels. Furthermore, the ban on Diuron and Irgarol 1051 relates only to the UK and not elsewhere in Europe. Non-UK boats may therefore still use the herbicides and if visiting UK waters, fresh inputs may continue to occur. In other countries, the continued application of the herbicides as antifoulants means that the risks posed to seagrasses will remain. If seagrass beds do decline as a result, a concomitent decline in the associated flora and fauna may be expected and with many of these species being of commercial or conservational importance the implications may be serious. A reduction in biomass has a cyclical effect, with an increase in sediment suspension and light attenuation causing further losses and meaning recovery and recolonisation may only take place over an 80–200-year period (Clark and Kirkman, 1989).

5. Conclusions

Irgarol 1051 and Diuron were both found to cause a decrease in the photosynthetic efficiency and growth of *Z. marina* with increases in concentration. Plants exposed to combinations of the two herbicides exhibited no significant differences in photosynthetic efficiency and growth when compared to plants exposed to the two herbicides individually. This is likely to be attributed to the antagonistic interactions frequently displayed by the herbicide mixture. Despite this antagonistic behaviour, the lowest observable significant effect concentrations were reduced by the addition of the second herbicide and this could have severe implications for seagrass populations in the environment.

Environmental concentrations of Irgarol 1051 and Diuron have been documented in the UK and elsewhere, particularly in marinas and sheltered estuaries where seagrasses can be prolific, that are above the levels found to elicit significant reductions in growth and photosynthesis. In regions where concentrations of individual herbicides are not sufficiently high to cause significant reductions, the occurrence of the herbicides together could mean the risks posed to seagrasses will remain, as the concentrations necessary to inhibit growth and photosynthesis are reduced when acting in conjunction.

Irgarol 1051 and Diuron have been banned from use in antifoulant paints in the UK as of November 2002, however, both herbicides have long half-lives. In UK waters, this could mean the threats posed to seagrasses from the herbicides will remain for several years. The ban is not Europe-wide, and in many countries the application of Irgarol 1051 and Diuron as antifoulants continues. Thus, it is likely that seagrassses will continue to experience reduced health and viability as a result of exposure to mixtures of Irgarol 1051 and Diuron.

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