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# Comparative Acute Toxicity of Herbicides to Photosynthesis of Coral Zooxanthellae

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The importance of the contribution of translocated photosynthates from endodermal symbiotic microalgae (zooxanthellae) in corals to the metabolic requirements, growth and reproduction of their hosts is well documented (Rinkevich 1989; Muscatine 1990). However, there are few published studies investigating the potential toxicity of herbicides which are known to be marine contaminants to zooxanthellae photosynthesis. The need to investigate the potential impacts of herbicides on coral photosynthesis is also important due to increasing usage of these compounds within marine antifouling paints. Of these, two herbicides have become increasingly - used : the s-triazine Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) and the phenyl urea diuron (1- (3,4 dichlorophenyl) -3,3-dimethylurea).

While it is difficult to establish global usage patterns, a recent survey (Thomas et al. 2001) reported that some 80% of antifouling paints sold in the U.K. contained either of these two herbicides. Diuron is also used extensively in the sugar cane industry in the coastal region adjacent to the Great Barrier Reef (Haynes et al. 2000). In Central America, a region adjoining significant reef communities, triazine herbicides (notably atrazine) and phenoxy acid herbicides such as 2,4 -D are important herbicide usage groups (Castillo et al.1997). These herbicides are moderately soluble, relatively persistent compounds with, for example, aqueous half lives for Irgarol 1051 of 100 to 200 days (Thomas et al. 2000) and diuron of 120 days (Haynes et al. 2000). Contamination of temperate coastal waters and sediments by atrazine, diuron, simazine (also a triazine) and Irgarol 1051 has been widely reported (Bester and Huhnerfuss 1993; Readman et al. 1993; Dahl and Blank 1996; Hall et al. 1999 and references therein; Thomas et al. 2000; 2001). Aqueous concentrations of Irgarol 1051 and diuron in marinas and coastal waters fall in the range 0.02 – 0.7μg $L^{-1}$, but with concentrations as high as 1.7μg $L^{-1}$ reported for Irgarol 1051(reviewed by Hall et al.1999) and 6.7μg $L^{-1}$ for diuron (Thomas et al. 2001). Atrazine and simazine have also been reported in coastal waters at concentrations up to 0.8μg $L^{-1}$ and 1.1μg $L^{-1}$

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respectively (Readman et al. 1993; Bester and Huhnerfuss, 1993). Little is known about the distribution of these compounds in tropical marine ecosystems. Recent investigations (Connelly et al. 2001; Owen et al. 2002) have shown Irgarol 1051 to be present in marinas and coastal waters of the Florida Keys, Bermuda and St Croix, with concentrations in the range 0.003 – 0.294μg $L^{-1}$, although higher concentrations (0.593μg $L^{-1}$) have occasionally been reported (Connelly et al. 2001). Basheer et al. (2002) have recently reported much higher Irgarol 1051 concentrations (up to 4μg $L^{-1}$) in the coastal waters of Singapore. Both Irgarol 1051 and diuron have been reported in seagrasses of the Great Barrier Reef region (Haynes et al. 2000), where diuron has also been found in coastal sediments (Haynes et al. 2000). The herbicide 2,4 -D has additionally been reported in coral tissues from Panama (Glynn et al. 1984).

Diuron and the triazines are photosystem II inhibitors, inhibiting electron transport within chloroplasts. 2,4-D acts in contrast as a plant growth regulator. We recently reported initial results (Owen et al.2002) showing Irgarol 1051 to be a potent inhibitor of zooxanthellae photosynthesis in the coral *Madracis mirabilis*. The purpose of this study was to assess, by acute *in vitro* experiments, the impact of a number of herbicides (diuron, simazine, atrazine, desethyl atrazine (a primary metabolite of atrazine) 2,4 -D and Irgarol 1051) on photosynthesis of isolated zooxanthellae from three coral species (*Diploria strigosa, Favia fragum, Madracis mirabilis*).

## MATERIALS AND METHODS

Separate experiments were undertaken in which incorporation of $H^{14}CO_3^-$ by zooxanthellae from three coral species was assessed after exposure to each of 6 herbicides at initial nominal concentrations of 2, 10, 100 and 1000μg $L^{-1}$ according to the methods outlined in Owen et al. (2002). On the basis of the results from this concentration range, additional experiments were conducted in which zooxanthellae from *Madracis mirabilis* were exposed to diuron over the concentration range 0.063 – 1μg $L^{-1}$ . For each experiment, corals (*Madracis mirabilis, Diploria strigosa, Favia fragum*) were collected by SCUBA from the coastal waters of Bermuda and maintained overnight in flowing sea water. These coral species were selected as they are common representatives both of inshore coral communities and branching, massive and small colony types. Homogeneous zooxanthellae solutions for $^{14}C$ incubations were made fresh on the day of each experiment. Zooxanthellae were isolated from corals by air brush and sequential centrifugation and resuspension steps (7mL artificial seawater (ASW)) to produce an algal pellet which was resuspended in ASW and filtered through a 15μm Nitex screen to remove residual host tissue fragments. The final, filtered zooxanthellae solution from each coral was diluted with ASW (approximately 4.5mL) to achieve a target cell density of 1 x $10^6$ cells per mL, as determined by hemocytometer. This solution was then used as a reservoir for incubations, being thoroughly vortexed prior to removal of each 100 μL aliquot for individual incubations (below). Incubations (5 per treatment / control level) were undertaken in 1.5mL

siliconized plastic flip top centrifuge vials over 6 – 8 hours under conditions of constant temperature (25.5°C) and irradiance (33 μE $m^{-2}$ $s^{-1}$). 100μL of the vortexed zooxanthellae solution above (containing 1 x $10^5$ cells) , 50μL of $^{14}C$ bicarbonate solution (41μCi $mL^{-1}$), 1μL of herbicide standard and 649μL ASW were loaded into each vial i.e. 800μL total volume. Varying dilutions of herbicide were made up in acetone to give the required exposure concentrations in the final incubation volume. 1μL of acetone was added to the control vials to account for any potential solvent effect. At each 2 hour time point each incubation vial was vortexed, 70μL drawn off and transferred to a scintillation vial. This was then acidified with 17μL of 4M acetic acid and shaken for one hour to remove unincorporated $^{14}C$ bicarbonate. 5mL of scintillation cocktail (Ultima Gold) was then added and $^{14}C$ counts determined by liquid scintillation counter (Packard). For each individual coral / herbicide exposure, pairwise comparisons between controls and each treatment group were conducted i.e. pooled / unpooled t test dependent on satisfaction of conditions of equality of variance.

## RESULTS AND DISCUSSION

Unexposed zooxanthellae exhibited linear rates of $^{14}C$ incorporation over the duration of each experiment (an example of which is shown in Figure 1). Substantial inhibition of zooxanthellae photosynthesis was exhibited at diuron and Irgarol 1051 nominal concentrations of 2 – 1000μg $L^{-1}$ in all three coral species studied. This was evident as significant reductions in $^{14}C$ incorporation after 6 hours in exposed zooxanthellae (Figure 2a and b) relative to controls. Mean reductions relative to controls at 2μg $L^{-1}$ were 33.7% (1s.d. = 9.8%) for zooxanthellae from all three coral species exposed to diuron and 19.4% (1s.d. = 7.2%) for zooxanthellae from corals exposed to Irgarol 1051. Reduction in $^{14}C$ incorporation followed a dose-dependent response with little incorporation evident at 100μg $L^{-1}$ for zooxanthellae exposed to diuron (94.2% reduction relative to controls, 1s.d. = 5.4%), or Irgarol 1051 (87.0% reduction relative to controls, 1s.d. = 7.6%). There did not appear to be any major inter species variability in response of zooxanthellae from the three coral species investigated to exposure of any one given herbicide. Baker and Rowan (1997) have shown that zooxanthellae from all three coral species investigated here are from the same clade (B) in the Caribbean region. The rather uniform response may reflect this similarity in symbiont phylotype.

A reduction in $^{14}C$ incorporation for zooxanthellae isolated from *Madracis mirabilis* was also evident at a diuron concentration of 1μg $L^{-1}$ after 8 hours incubation, but not at lower concentrations (Figure 3). This contrasted with our earlier study (Owen et al. 2002) where reduction in $^{14}C$ incorporation by isolated zooxanthellae from this species was evident after 8 hours exposure to Irgarol 1051 at concentrations as low as 0.063μg$L^{-1}$and photosynthetic inhibition of *in situ* zooxanthellae (i.e. the intact coral symbiosis) at nominal Irgarol 1051 concentrations of 0.1μg $L^{-1}$. The data suggest that these two herbicides, used extensively in tropical agricultural and marine antifouling applications, are strong

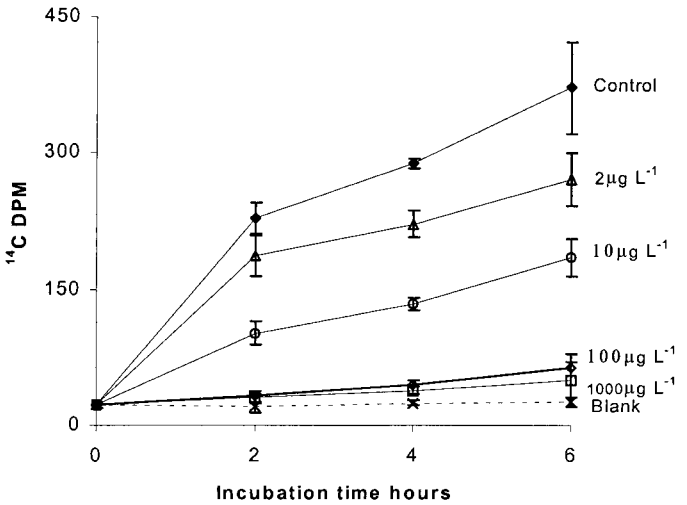


**Figure 1**. Incorporation of $^{14}C$ by isolated zooxanthellae from the coral *Favia fragum* after exposure to diuron concentrations of 2 – 1000µg L$^{-1}$. Error bars = +/- 1 s.d.

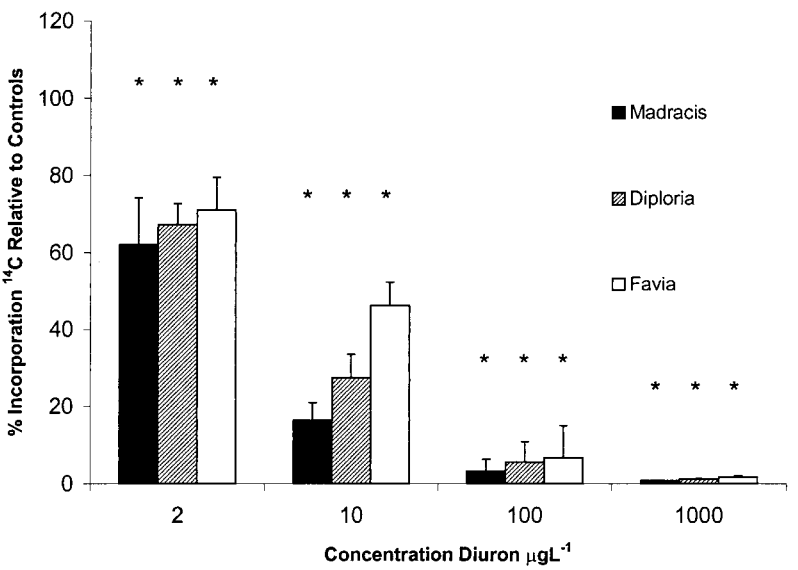


**Figure 2a**. Incorporation of $^{14}C$ by coral zooxanthellae after 6 hours exposure to diuron Error bars = +/- 1 s.d. * = significantly different from controls at p = <0.05.

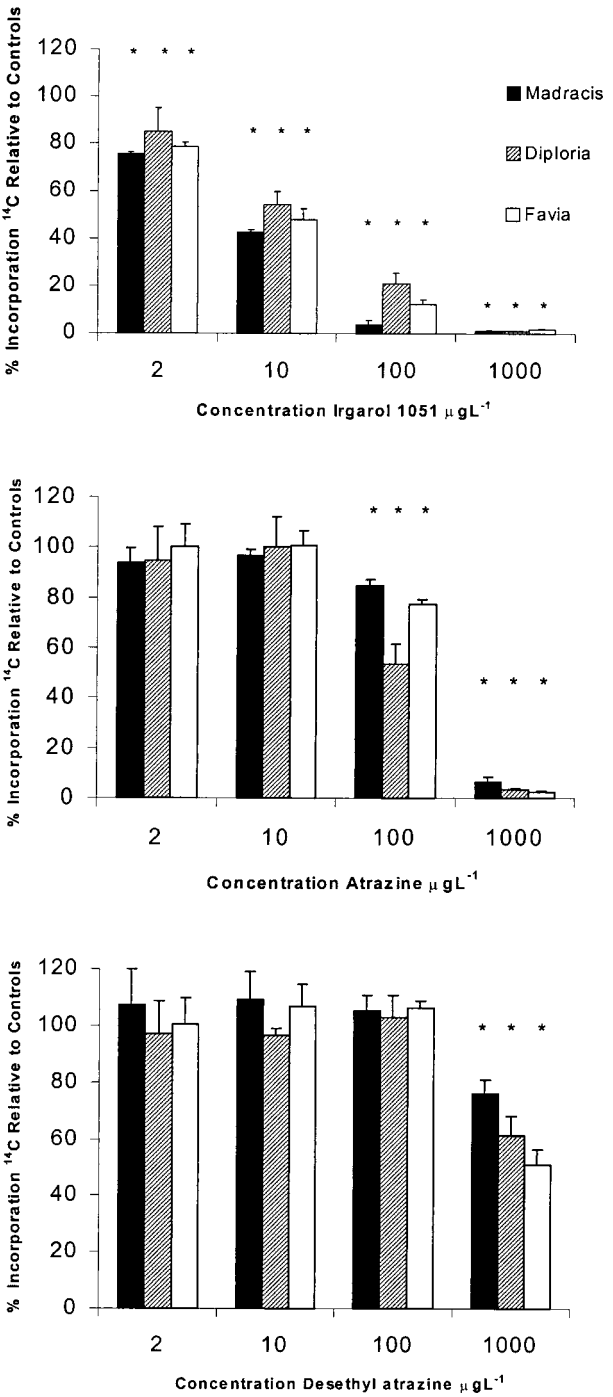


**Figure 2b-d** Incorporation of $^{14}$C by coral zooxanthellae after 6 hours exposure to Irgarol 1051, atrazine and desethyl atrazine. Error bars = +/- 1s.d. * = significantly different from controls at p = <0.05.

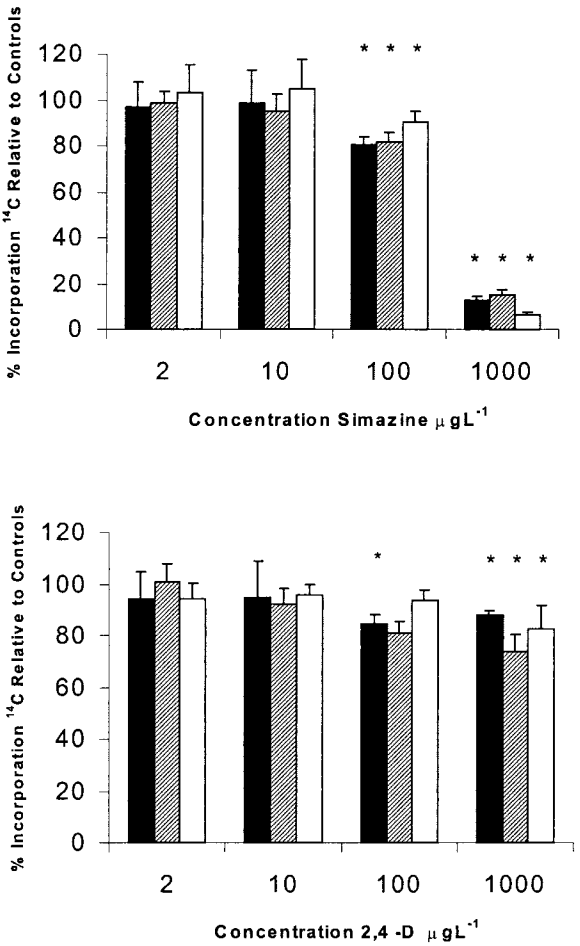


**Figure 2e-f** Incorporation of $^{14}$C by coral zooxanthellae after 6 hours exposure to simazine and 2,4-D. Error bars = +/- 1s.d. * = significantly different from controls at p = <0.05.

inhibitors of coral zooxanthellae photosynthesis at environmentally – relevant concentrations. Scarlett et al. (1999) have also reported reduction in photosynthetic efficiency in seagrasses *Zostera marina* exposed to Irgarol 1051 in the range 0.5 – 25μg L$^{-1}$, as measured by dark adapted flourescence induction ($F_v$: $F_m$), an indicator of maximum efficiency of PSII. In contrast, incorporation of $^{14}$C by zooxanthellae after 6 hours exposure to atrazine and simazine was significantly reduced only at the higher concentrations of 100μg L$^{-1}$ and 1mg L$^{-1}$, (Fig 2 c and e). Desethyl atrazine exhibited a toxic effect only at the very highest concentration (Fig 2 d), suggesting increased toxicity of atrazine to coral zooxanthellae photosynthesis in comparison with its metabolite. Small but significant reductions in $^{14}$C incorporation were evident after 6 hours exposure of isolated zooxanthellae to 2,4-D only at 1mg L$^{-1}$ (Fig 2 f). This may reflect the fact

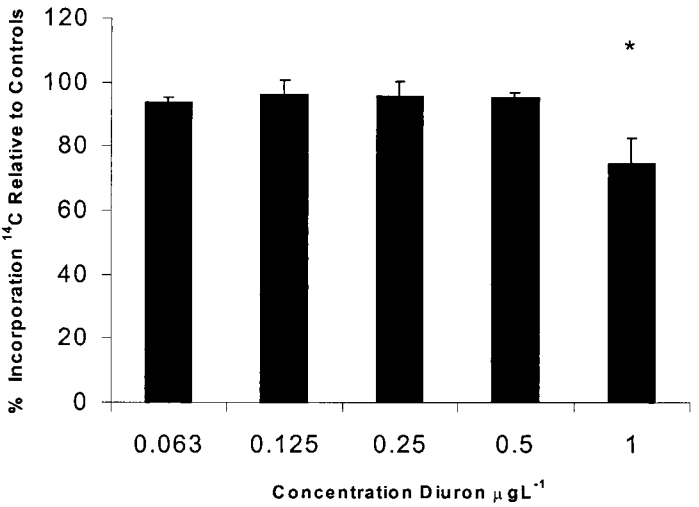


**Figure 3.** Incorporation of $^{14}$C by coral zooxanthellae isolated from *Madracis mirabilis* after 8 hours exposure to diuron. Error bars = +/- 1 s.d. * = significantly different from controls at p = <0.05.

that, unlike the other herbicides studied, the mechanism of action of 2,4-D is as a plant growth regulator rather than PSII inhibitor. The potential impacts of this widely – used herbicide on coral growth are not known. The data presented here emphasize the potent inhibitory effect of both Irgarol 1051 and diuron on zooxanthellae photosynthesis in comparison with the other herbicides. Previous studies (discussed by Dahl and Blank 1996) have shown that Irgarol 1051 is some 70 times more phytotoxic to periphyton in comparison with atrazine, consistent with the trend in zooxanthellae response for exposure to these herbicides. It should be noted that the data shown here represent acute exposures; long term experiments with periphyton and seagrasses have shown photosynthetic inhibition at far lower concentrations of Irgarol 1051 when compared to acute conditions (Dahl and Blank 1996; Scarlett et al. 1999). Additionally, many corals have been shown to ingest suspended sedimentary or particulate material (Anthony 1999). The presence of atrazine, simazine, Irgarol 1051 and diuron in coastal sediments, is well – documented (Readman et al. 1993; Haynes et al. 2000; Thomas et al. 2000) and this represents an additional potential route of exposure for corals. As such, there is a clear need to undertake seasonal distribution studies in both sediments and aqueous samples to establish the extent of contamination of reef ecosystems by these compounds. We also suggest that increased use of Irgarol 1051 and / or diuron as replacement antifouling booster biocides (e.g. when the International Maritime Organisation ban on use of organotin compounds on all shipping comes into effect in 2003) may potentially seriously impact photosynthesis of already vunerable corals.

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