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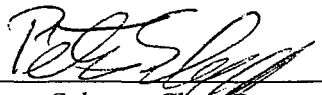
Title: The effect of the biocide Irgarol® 1051, and Roundup® Ready, on planulae of the brooding coral, *Leptastrea purpurea* (Cnidaria: Scleractinia).

Approved: 

Dr. Peter Schupp, Chair Person, Thesis Committee

The objectives of this study were to establish the acute toxicity of the antifouling agent Irgarol®1051, and the herbicide, Roundup® Ready, to larvae of the brooding coral, *Leptastrea purpurea* (Cnideria: Scleractinia), and to determine the minimum concentration of each chemical required to adversely effect larval settlement on their preferred substrate, crustose coralline algae (CCA). The major components of Roundup® Ready, i.e., glyphosate, the active ingredient, and polyoxylene amine (POEA), a surfactant added to enhance the effect of glyphosate, were similarly tested.

The 24-h LC_{50} values for Roundup® Ready, POEA and glyphosate were 9.6 mg (active ingredient)/L, 1.5 mg/L and 9.3 mg/L respectively. Acute toxicity threshold concentrations for each chemical were 6.2 mg a.i./L, 1.1 mg/L and 5.7 mg/L respectively. Irgarol®1051 was not acutely toxic up to concentrations of 100 mg/L, the maximum level tested.

Settlement bioassays ran for 24 h and employed two experimental treatments. In the first treatment, settlement was determined with larvae previously exposed to the test chemicals for 24 h. The second treatment monitored larval settlement on previously exposed CCA. Irgarol®1051, at 100 mg/L, reduced settlement in both treatments but was not effective at any of the lower concentrations tested. The minimum experimental concentrations of Roundup® Ready, POEA and glyphosate

that significantly reduced larval settlement were 1 mg a.i./L, 10 mg/L and 3.6 mg/L respectively in the first treatment. No significant reduction in larval settlement was observed at these concentrations in the second treatment. However, settlement was inhibited when CCA were exposed to 10 mg a.i./L and 100 mg/L of Roundup® Ready and glyphosate respectively, while POEA had no effect at a maximum test concentration of 36.6 mg/L. The significance of the findings is discussed with respect to levels of Irgarol®1051 and Roundup® encountered in aquatic environments elsewhere in the world.

Effects of the biocide, Irgarol® 1051 and Roundup® Ready on planulae of the brooding coral *Leptastrea pupurea* (Cnidaria: Scleractinia)

BY

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INTRODUCTION

Coral reefs are home to more than 25 % of all marine life and are among the most diverse and productive communities on earth (Field *et al.*, 2001). Coral reefs occur in tropical and sub-tropical regions of the world and are important for small tropical islands like Guam because they provide shoreline protection from breaking waves, especially during rough weather conditions. Reefs also provide a vital habitat for fish, an important food source for millions of people, and have considerable commercial and recreational importance, particularly with regard to tourism. Many small tropical islands are heavily dependent on healthy coral reefs to maintain their fragile economies. A decline in reef health, then, may result in serious economic losses.

Despite the known value of coral reefs, they are being damaged at an alarming rate by pollution, habitat destruction, overfishing, deforestation, soil erosion, and other anthropogenic disturbances (Richmond, 1993). Biocides that migrate outside their target areas can also cause a potential disturbance to coral reefs if they reach ecologically relevant concentrations. Such occurrences have been shown to cause reproduction and recruitment failure in benthic invertebrates and other marine organisms (Moore and Waring, 1998).

Corals use chemical cues to synchronize spawning among individuals, to help sperm locate eggs, and to induce larval settlement and metamorphosis (Richmond, 1993). Therefore, water quality is important for the successful reproduction and recruitment of these organisms. Corals have a narrow range of tolerance for many environmental factors and are sensitive indicators of water quality (Richmond, 1993). The larval stage is especially sensitive to physical and chemical changes in water quality. Bioassays to

determine the effect of contaminants on coral larvae settlement and metamorphosis have been performed with chlorpyrifos (Leota, 2000), copper (Reichelt-Brushett and Harrison, 2000; Victor, 2002), nickel (Goh, 1991), petroleum (Te, 1991), nutrients (Koop *et al.*, 2001) and sediments (Hodgson, 1990; Babcock and Davies, 1991). Thus far, most studies have shown a significant reduction of larval settlement after exposure to environmentally realistic concentrations of the above contaminants in seawater.

In the following study, I examined the effect of two common biocides on settlement of the brooding coral, *Leptastrea purpurea.* The chemicals of interest were Irgarol® 1051, an antifouling agent used on water craft, and Roundup®, a popular herbicide used to control weeds and other undesirable plants on land.

Irgarol® 1051

Fouling organisms, such as bacteria, algae and invertebrates, attach and grow on the bottom of boats, on underwater aquaculture equipment, and other submerged surface, causing increased frictional resistance or reduced water flow. Callow (1986) demonstrated that only a 1 mm thick slime film attached to a ship bottom decreased the speed by 15 %. Irgarol® 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine), a triazine herbicide used in copper-based antifouling paints, was introduced in the early 1980s to replace tributyltin (TBT) in Europe. TBT-containing antifouling paint was first introduced in the 1960s and was found to be highly toxic to some non-target organisms such as Dogwhelk, *Nucella lapillus* (Bryan and Gibbs, 1991). Irgarol® 1051 has become one of the more popular antifouling paints because it is more biodegradable than TBT and has a lower affinity for sediment adsorption (Thomas *et al.*, 2002). It has

an octanol/water coefficient (log $_{Pow}$) of 2.8 and, thus, has a low bioaccumulation potential (Ciba, 1999). Irgarol® 1051 is slowly degraded in sediments and has a reported half-life of between 100 - 200 d in aerobic environments, and even longer under anaerobic conditions (Ciba, 2003).

Concentrations of Irgarol® 1051 detected in marinas, harbors, and coastal waters range from 1 - 1,700 ng/L (Table 1); these concentrations have already been shown to have detrimental effects on non-target organisms in the marine environment (Table 2).

Table 1. Reported Irgarol® 1051 concentrations in marine environments.

Country	Concentration (ng/L)	Observed year	Reference
Europe	316 (marinas) 19 to 41 (coastal waters) 133 (90% of pooled stations)	1992 to 1997	Hall *et al.* (1999)
- U.K.	2 to 500 (coastal waters) 1421 (marinas) max 10 to 592 (marinas)	1998	Gough *et al.*(1994) Thomas *et al.* (2001) Connelly *et al.* (2001)
- France	5 to 1700 (marinas)	1992	Readman *et al.* (1993)
- Mediterranean sea	1 to 640 (coastal waters)	1995	Tolosa *et al.* (1996)
- Sweden	30 to 405 (marinas)		Tolosa *et al.* (1996)
Germany (North Sea & Baltic Sea)	440 (marinas)	1997	Biselli *et al.* (2000)
U.S.			
- Hamilton Harbor, Bermuda	Up to 590 (marinas)	1995	Connelly *et al.* (2001)
- Florida Keys, Bermuda, St.Croix	3 to 294 (coastal waters, marinas, harbors)	2000, 2001	Owen *et al.* (2002)

Table 2. Ecological effects of Irgarol® 1051. LC_{50} is the concentration required to kill 50% of the test animal in a given period of time. EC_{50} is the concentration at which 50% of test organisms exhibit an effect not seen in the control.

Species	LC_{50}/EC_{50}	Reference
Fish		
Bluegill	LC_{50} (96h) 2.9 mg/L	Ciba (2003)
Rainbow trout	LC_{50} (96h) 0.86-0.94 mg/L	Ciba (2003)
Inland silverside	LC_{50} (96h) 1.8 mg/L	Ciba (2003)
Sheepshed minnow	LC_{50} (96h) 3.5 mg/L	Ciba (2003)
Zebra fish	LC_{50} (96 h) 4 mg/L	Ciba (2001)
Golden orfe	LC_{50} (96 h) 6 mg/L	Ciba (2001)
Invertebrates		
Daphnia magna	LC_{50} (48h) 8.1 mg/L	Ciba (2003)
Mysid shrimp	LC_{50} (96 h) 0.4 mg/L	Ciba (2003)
Chironomus riparius	LC_{50} (10d) 2.6 mg/L	Hall *et al.* (1999)
Oyster	LC_{50} (48h) 3.2 mg/L	Ciba (2001)
Aquatic plants		
Porphyra yezoensis	EC_{50} (4-d) 0.6 µg/L (growth)	Okamura *et al.* (2000)
Eisenia bicyclis	EC_{50} (7-d) 2.0-2.1 µg/L (growth)	Okamura *et al.* (2000)
Fucus vesiculosus	EC_{50} 0.8 µg/L (germination of zygote)	Ciba (2001)
Lemna gibba	EC_{50} (14 d) 1.78 µg/L (growth)	Hall *et al.* (1999)
Selenastrum capricornutum	EC_{50} (72 d) 7.43 µg/L (growth)	Hall *et al.* (1999)
Scenedesmus subspicatus	EC_{50} (72 d) 4.03 µg/L (growth)	Hall *et al.* (1999)
Anabaena flos-aquae	EC_{50} (5 d) 2.07 µg/L (growth)	Hall *et al.* (1999)
Enteromorpha intestinalis	EC_{50} (72 h) 2.5 µg/d/m (photosynthetic efficiency)	Scarlett (1997)

Concentrations of Irgarol® 1051 as low as 0.6 µg/L, for example, significantly reduced the growth of the algae, *Porphyra yezoensis*, after a 4-d exposure period (Okamura *et al.*, 2000). Irgarol® 1051 acts as a Photosystem II inhibitor (Scarlett, *et al,*.1997) in plants. In Photosystem II, oxygen, hydrogen ions and electrons are derived from the splitting of water molecules. The energy from these electrons is used to facilitate the reduction of CO_2 to organic molecules during photosynthesis and is critical for plant growth and survival. Thus, when Irgarol® 1051 blocks the electron transport system in Photosystem II, the plants starve to death (Hall *et al.*, 1999).

Irgarol® 1051 has a low water solubility of 7 mg/L (Ciba, 2003) and when applied to marine surfaces slowly dissolves in the water column, providing moderate exposure to potential fouling organisms. The rate of leaching from newly painted boats is high in the first couple of years and rapidly decreases thereafter. The average rate of leaching is about 25 $\mu g/cm^2/d$, although this value can be affected by environmental factors such as pH, temperature, and salinity (Hall *et al.*, 1998). The level of Irgarol® 1051 in the environment also varies with season. High concentrations are typically found during summer months when a greater number of recreational boats are launched, but decreases rapidly during the winter due to a decrease in boat density (Comber *et al.*, 2001). Higher concentrations of Irgarol® 1051 can also be found in areas with limited water exchange, such as in harbors and marinas (Scarlet *et al.*, 1997). Worldwide usage for Irgarol® 1051 was not available at the time of writing this thesis.

Roundup®

Roundup® is a broad-spectrum (nonselective) herbicide used to control annual and perennial plants including grasses, weeds, sedges, and woody plants. It was introduced by Monsanto in 1974 and has become one of the most widely used herbicides for personal and professional use. From 1995 to 2001, annual usage of Roundup® in the U.S. has gone up from 25 to 30 million pounds, to 85 to 90 million pounds (Aspelin and Grube, 1999; Kiely *et al.*, 2004). Roundup® is the second most widely used herbicide in the U. S. after 2, 4-D (2,4-dichlorophen oxyacetic acid) (Kiely *et al.*, 2004).

Roundup® contains the active ingredient isopropylamine (IPA) salt of glyphosate (hereafter referred to as glyphosate), the surfactant polyethoxylated tallowamine

(referred to as POEA), and water[1]. POEA is added to increase the effectiveness of glyphosate by facilitating its penetration of plant cell membranes. In the literature, POEA has been found to be more toxic than glyphosate. For example, Martinez and Brown (1991) reported that the acute lethal dose of POEA for rats was lower than glyphosate. Another study showed that POEA was about 30 times more toxic to fish than glyphosate (Servizi *et al.*, 1987). Moreover, the combination of these two products is generally considered to be more toxic than each product separately (Perkins *et al.*, 2000).

The mechanism of action for this herbicide was not known until quite recently (U.S. EPA, 1993). Research by Giesy *et al.* (2000) has reported that after application, Roundup® is absorbed through the foliage and is rapidly translocated. Glyphosate inhibits EPSPS (5-enolpyruvyl shikimate-3-phosphate synthase), which is involved in aromatic amino acid synthesis. This reaction occurs in the chloroplast via the shikimic acid pathway. As a result, the production of proteins in plants is reduced and their growth and survival is inhibited. It takes two to three days to see the visible effects (e.g. yellow leaves) on the plant and another seven to ten days for the plant to die.

Roundup® is usually applied by aerial spraying from a helicopter, truck, or backpack spray. Thus, there is potential for the herbicide to drift to areas where application was not intended, including adjacent forests, reef flats, and rivers. Although the measurement of drift distance is difficult in the natural environment, several studies

1. Monsanto does not provide a complete list of ingredients for its Roundup® Ready to Use Weed and Grass Killer product. However, studies by Freedman (1991) and Giesy *et al.* (2000) indicated that Roundup® contains 41% glyphosate, 15% POEA and 44% water. However, this product requires dilution, unlike Roundup® Ready, which can be used as purchased.

have estimated the drift distance of glyphosate. Freedman (1991) reported that the amount of offsite spray drift from four helicopter treatments was between 14% - 78% of the original application amount. Yates *et al.* (1978) found that glyphosate drifted up to 800 m away from both aerial and ground application sites, depending on prevailing wind conditions. Roundup® applied by tractor was found to damage the seedlings of sensitive non-target plants located 40 m downwind (Marrs *et al.*, 1993). Drift models showed the potential damage to native plants located 80 m from the application site (Breeze *et al.*, 1992). Riley *et al.* (1991) demonstrated that glyphosate drifted up to 200 m off site after helicopter application. Thus, there is a potential that Roundup® may drift to the marine environment contaminate the seawater and affect marine organisms.

The persistence of glyphosate in soil is classified as moderate, with an average half-life of 47 d depending on climate conditions, soil type, and organic matter content. Half-life values reported under field conditions ranged from 1 - 174 d (EXTOXNET, 1996), though a study performed in northern Sweden detected glyphosate three years after a single application (Torstensson *et al.*, 1989). Microbes are primarily responsible for the breakdown of glyphosate and, therefore, the half-life of this chemical in soil is likely to be shorter in warm climates than cold climates (Torstensson *et al.*, 1989). Glyphosate is considered to be immobile (i.e. tightly-bound) in most soils and normally stays within the top six inches (U.S. EPA, 1993). Thus, even though glyphosate has a high water solubility of 12 g/l at 25 °C (EXTOXNET, 1996), the potential for the herbicide to contaminate ground water is generally considered low. The results of several studies reported concentrations after direct application ranging from 0.013 - 2.27

mg/L in surface water and from 0.15 - 25.3 mg/L in bottom sediments (summarized in Giesy *et al.*, 2000).

Recent research has shown that glyphosate is readily desorbed from some soils and can leach into the underlying groundwater (Hallberg, 1989). Piccolo *et al.* (1994) reported that soils lacking vermiculite or amorphous hydrous oxides do not adsorb glyphosate well and readily desorb 72 - 80 % of the adsorbed chemical. Glyphosate can be detected in surface water near treated areas as a result of run-off and spray drift (Giesy *et al.*, 2000) and it is tightly adsorbed by suspended solids and mineral matter (EXTOXNET, 1996).

The toxic effect of glyphosate to aquatic species varies depending on its formulation; glyphosate is slightly toxic to aquatic microorganisms, while Roundup® is slightly to highly toxic (Table 3). Studies examining the relationship between toxicity and pH found that an alkaline pH increases the toxicity to the crustacean *Ceriodaphnia dubia* (Tsui and Chu, 2003), the zooplankton *Simocephalus vetulus*, and tadpoles of the frog *Rana pipiens* (Chen *et al.*, 2004). The alkalinity of saltwater environments would, therefore, suggest that marine organisms are at greater risk than their freshwater counterparts.

Monsanto originally claimed that Roundup® was an environmentally safe herbicide. However, in 1996, the New York Attorney General's office filed a lawsuit against the company for false statements such as 'biodegradable' and 'environmentally friendly,' in reference to Roundup® and all other glyphosate - formulated products. Monsanto agreed to discontinue use of those statements and paid $50,000 for the state's cost of pursuing the case (Pesticide Action Network North America, 2002).

Table 3. Ecological effects of glyphosate and Roundup®.

Species	LC_{50}	Test Chemicals	Reference
Bacteria			
- *Vibrio fischeri*	(15min.) 216 mg/L	G	Tsui and Chu (2003)
	(15min.) 33.2 mg a.i./L	R	
Macroalga			
- *Selenastrum capricornutum*	(96hr) 33 mg/L	G	Tsui and Chu (2003)
	(96hr) 7.75 mg a.i./L	R	
- *Skeletonema costatum*	(96hr) 3 mg/L	G	Tsui and Chu (2003)
	(96hr) 2.47 mg a.i./L	R	
Protozoa			
- *Tetrahymena pyriformis*	(40hr) 864 mg/L	G	Tsui and Chu (2003)
	(40hr) 39.3 mg a.i./L	R	
- *Euplotes vannus*	(48hr) 13.5 mg/L	G	Tsui and Chu (2003)
	(48hr) 31.3 mg a.i./L	R	
Invertebrates			
- *Ceriodaphnia dubia*	(48hr) 196 mg/l	G	Tsui and Chu (2003)
	(48hr) 7.19 mg a.i./L	R	
- *Acartia tonsa*	(48hr) 47.1 mg/L	G	Tsui and Chu (2003)
	(48hr) 2.36 mg a.i./L	R	
- *Palaemonetas vulgaris*	(96hr) 281 mg/L	G	U.S. EPA (1993)
- *Uca pugilator*	(96hr) 934 mg/L	G	U.S. EPA (1993)
- Daphnia (water flea)	(48hr) 780 mg/L	G	U.S. EPA (1993)
	(96hr) 962 mg/L	G	
- Atlantic oyster	(96hr) 10 mg/L	G	U.S. EPA (1993)
Fish			
- Fathead Minnow	(48hr) 84.9 mg/L	G	U.S. EPA (1993)
- Bluegill sunfish	(48hr) 120-140 mg/L	R	U.S. EPA (1993)
	(96hr) 120 mg/L	G	
- Rainbow trout	(48hr) 10-197 mg/L	R	U.S. EPA (1993)
	(96hr) 86 mg/L	G	
- Coho salmon	(48hr) 27-174 mg/L	R	IPCS (1994)

G = glyphosate (mg/L); R = Roundup® (mg a.i./L, or mg glyphosate/L).

Although Roundup® is only intended for use on land, some countries outside North America use it directly in the aquatic environment to control aquatic plants (Giesy *et al.*, 2000). The amounts of glyphosate-formulated products used on Guam are not

known (Guam EPA pers. comm.). However, usage has rapidly increased in recent years on local golf courses, hotels, and around the home (Total Chemical Resources Inc.; K-mart, and Ace Hardware pers. comm.). Two local suppliers estimate average monthly sales of 40 – 50 gallons of glyphosate products rising to about 100 gallons per month during peak season (Total Chemical Resources Inc., pers. comm.; Ace Hardware, pers. comm.). Since Roundup® product can be used on golf courses and private gardens adjacent to coastal waters, its toxicological effects on non-target organisms in or near the marine environment requires investigation. Nothing is known about the impact of this particular herbicide on coral reefs.

Objective of the study

The objectives of this study were to examine the effects of a popular antifouling agent (Irgarol®1051), and herbicide (Roundup® Ready-to-Use Weed and Grass Killer, referred to as Roundup® Ready for the remainder of this thesis), on mortality rates and larval settlement of the brooding coral, *L. purpurea.* The effects of the individual components of Roundup® Ready – POEA and glyphosate– were also investigated.

L. purpurea was used for the bioassays because this species releases larvae daily and has a high rate of settlement success. In addition, the species commonly occurs on shallow reef flats, where the potential for exposure to Irgarol® 1051, Roundup® Ready, POEA, and glyphosate is high.

MATERIALS AND METHODS

Test organism

Description

Leptastrea purpurea is a common encrusting coral that can be found from the Red sea and the West African coast to the Marquesas Islands in the Pacific Ocean (Veron, 2000). Colonies occur as either thin, encrusting sheets or rounded lobes, and are usually pale brown, greenish, or purple in coloration (Veron, 2000); (Figure 1). They are brooders, releasing zooxanthellate planula larvae everyday throughout the year (McCormick, pers. comm.).

Colony collection

Coral colonies of *Leptastrea purpurea* attached to dead *Acropora* branches were collected from Luminao reef flat in Guam (Figure 2) on several occasions between July 2004 and August 2005. Specimens were placed in a cooler with seawater and transported to the University of Guam Marine Laboratory, where groups of approximately 30 to 40 colonies were put in individual plastic containers (10.7 L) and floated in a water bath. The average size of colonies used in the present study was 3 x 4 cm. Water was changed daily and aerated constantly. Approximately 1500 colonies were collected throughout the duration of the study and colonies were returned live to the collection site after one larvae collection cycle.

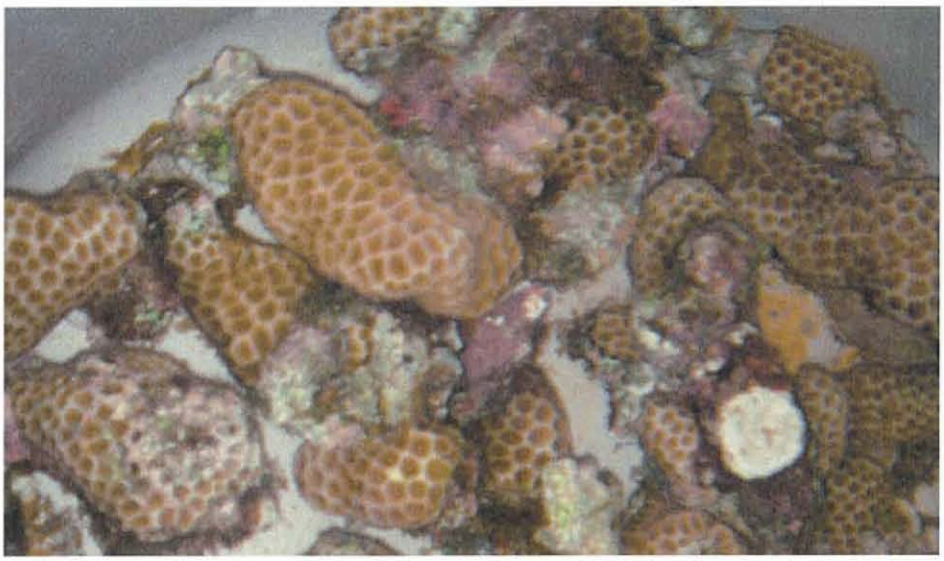


Figure 1. Adult colonies of *L. purpurea* in laboratory aquaria

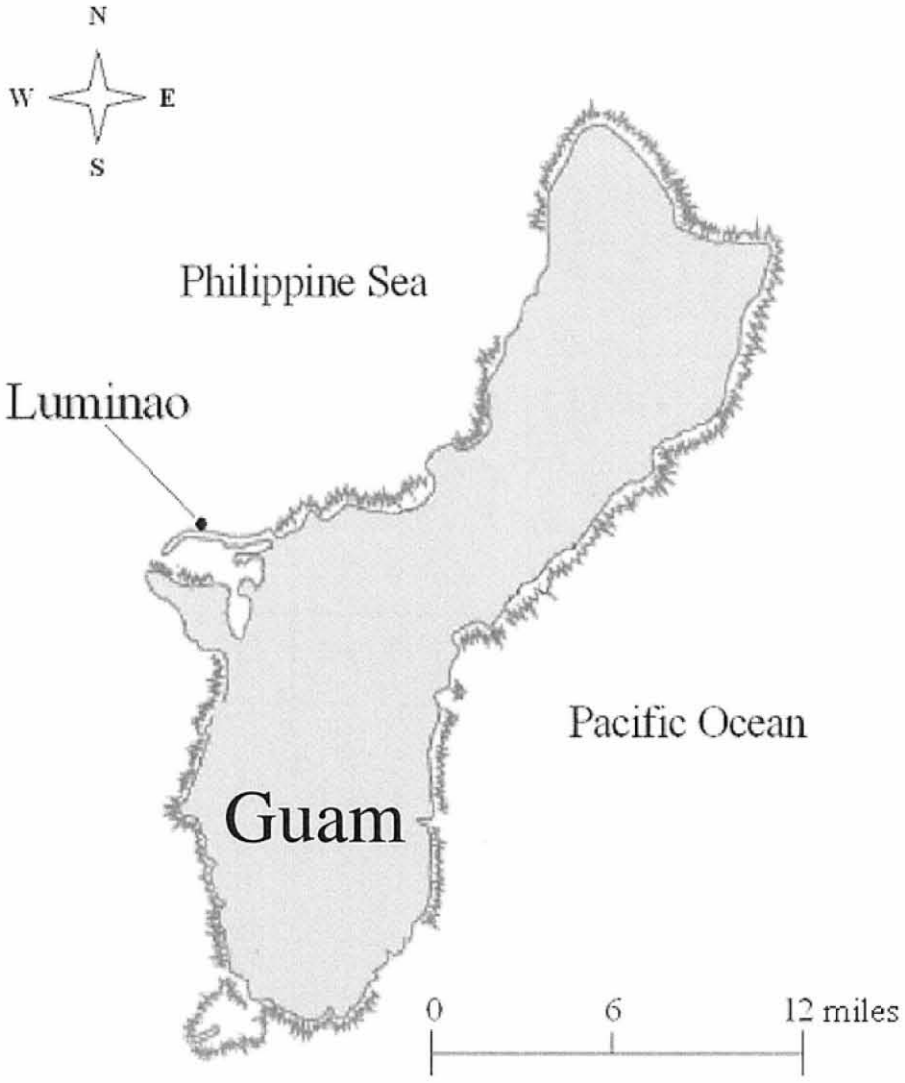


Figure 2. Map indicating the collection site for *L. purpurea*

Larval collection

The larvae were not buoyant and, thus, needed to be separated from their parent colonies using a gentle, pulsating water stream generated from a pipette. After separation, the contents of the container, including the larvae, sand, and water, were poured into a plastic cup through a 90 μm mesh filter. Only water passed through the mesh. The retained sand and larvae were then rinsed from the filter with seawater into a 250 mL glass dish. Individual larvae were captured with a glass Pasteur pipette under a dissecting microscope and transferred into another 250 mL glass dish with fresh seawater. Approximately, 70 larvae were usually collected from one container containing 30 to 40 adult colonies. The glass dishes containing the larvae were left outside in the shade until required for testing the following day. The ambient temperature of the outside shaded area in the lanai ranged from 28 - 31°C. Larvae from different parents were polled in assays as the number of larvae per parent was too small to allow for adequate sample size and previous studies showed consistently higher settlement for all colonies (McCormick, pers. comm.).

Bioassay experiments

Seawater used for the bioassays was filtered through a 90 μm mesh sieve. The bioassays were performed in 20 mL plastic petri dishes. The petri dishes were washed with detergent and soaked with 10 % HCI for an hour, and were then rinsed three times with tap water and left to dry. Crustose coralline algae (CCA), the substrate preferred by *L. purpurea* larval (McCormick, pers. comm.), was used for the bioassays conducted in this study. Coral rubble covered with CCA was collected from the same site as the adult

colonies of *L. purpurea*. The CCA-covered rubble was kept in a large, aerated tank that was constantly supplied with fresh seawater. The experiments were performed between July 2004 and August 2005.

Test chemicals

The biocides Irgarol® 1051 and Roundup® Ready were used in this study. Irgarol® 1051 (CAS# 28159-98-0); (Ciba, 2003), in powder form, was obtained from Ciba Special Chemical Inc., Switzerland. Roundup® Ready is a clear, liquid herbicide produced by Monsanto; it contains 1.92 % glyphosate and 98.08 % other unlisted ingredients (Monsanto Company, 2002) and was obtained from a local hardware store. The ingredients of Roundup® Ready were also tested separately to determine their individual potency. Glyphosate was purchased as a liquid concentrate from Sigma-Aldrich (Saint Louis, MO, USA) in a 40 % pure form (CAS# 38641-94-0). Although Monsanto does not indicate that Roundup® Ready contains the surfactant POEA (CAS # 61791-26-2), the literature suggests that all Roundup® products contain POEA (Tsui and Chu, 2003; U.S. EPA, 1993), with the exception of Roundup® Bioactive (a recent glyphosate-formulated product). POEA was purchased from the Chem Service Company (West Chester, PA, USA).

Experimental design of acute toxicity tests

The acute toxicity of Irgarol® 1051, Roundup® Ready, POEA, and glyphosate was examined over a 96-h exposure period. Notes on the preparation of stock solutions and concentration ranges of each chemical tested are summarized in Table 4 together

with the appropriate controls used. In each experiment, five replicates (10 larvae/replicate) were used for each concentration of test chemical and for the control(s). Larval mortalities were identified and counted under a binocular microscope after 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h, 20 h 24 h and every 12 h thereafter up to 96 h when the experiment was terminated. The criterion for death was larval immobility and opaqueness (white). Dead larvae were removed immediately when observed. The test concentrations of each chemical were changed daily. This was accomplished by carefully transferring the surviving larvae to the fresh solutions using a glass Pasteur pipette.

Table 4: Experimental concentrations of test chemicals used to determine acute toxicity to *L. purpurea*

Chemical (Stock Solution)	Nominal Test Concentration	Controls
Irgarol® 1051 (100,000 mg/L) 10 g powdered formulation in 100 ml acetone	1, 10, 100 mg/l	seawater seawater/acetone
Roundup® Ready (10,000 mg a.i./L) 5.2 ml liquid formulation in 4.8 ml seawater	5.6, 10, 18, 32, 56 mg a.i./L	seawater
POEA (1,000 mg/L) 0.1 ml liquid concentrate in 99.9 ml acetone	0.56, 1.00, 1.35, 1.80, 3.20 mg/L	seawater seawater/acetone
Glyphosate (1,000 mg/L) 250 µl of liquid concentrate in 100 ml seawater	1.0, 3.2, 5.6, 10, 18, 32 mg/L	seawater

All stock solutions were made up within 2 h prior to use. All nominal test concentrations were made up in 100 ml seawater. All bioassays were conducted in 20 ml seawater

Data analysis

The toxicity data was analyzed according to the procedures described by Litchfield (1949). This involved plotting the percentage mortality against time on logarithmic probability paper for each test concentration. A best-fit line was drawn by eye and the median lethal time (LT_{50}: defined as the time required for a particular test chemical concentration to kill 50% of the test population) was read from the graph. The 95% confidence limits, the slope function (S) and its confidence limits were then calculated nomographically. The toxicity response curve for each chemical was constructed from the LT_{50} estimates and the 24-h LC_{50} (defined as the concentration of test chemical required to kill 50% of the test population in 24-h) and threshold concentration (defined as the maximum concentration of test chemical below which 50% of test population will survive indefinitely) were read from the graph.

Experimental design of settlement tests

Two experimental treatments were set up to test the effect of Irgarol® 1051, Roundup® Ready, POEA, and glyphosate on settlement of *L. purpurea* larvae on CCA chips. In the first treatment, larvae were pre-exposed to the chemicals of interest for 24 h before the settlement bioassays were conducted. The second treatment monitored larval settlement on CCA that had previously been exposed to the chemicals of interest over the same time period.

The settlement bioassays ran for 24 h in the shade on the lanai at an ambient air temperature of 28-31 °C. As with the acute toxicity tests, five replicates (10 larvae/replicate) were used for each chemical concentration tested and all controls.

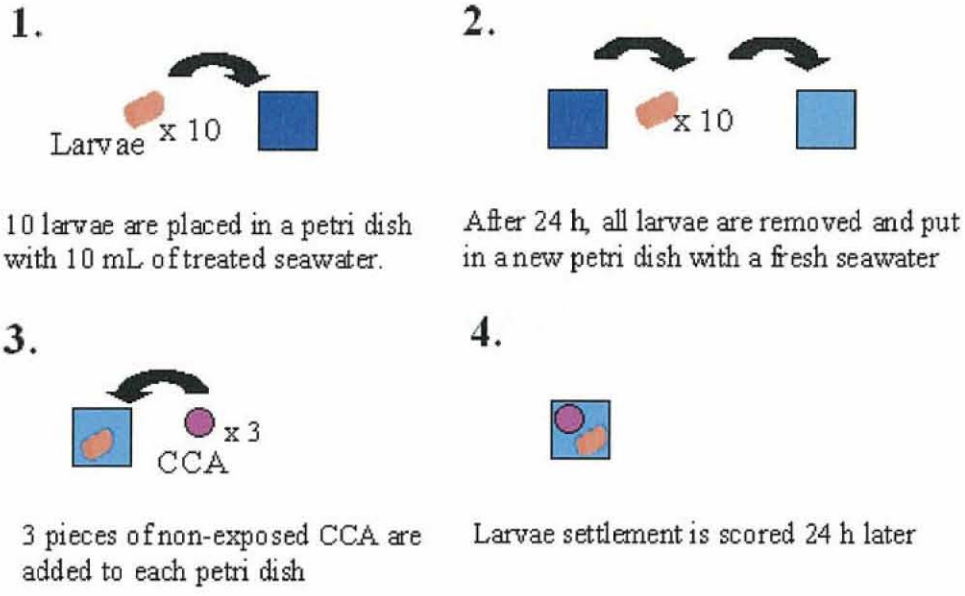
At the end of the exposure period, exposed larvae were placed in new 20-mL petri dishes containing 10 mL of freshly filtered seawater and three CCA chips (average size of 5x5 mm). Each dish was inspected 24 h later to determine settlement rates (Fig 3.A). In the second treatment, the exposed CCA chips were added to 10 mL of seawater (three chips/dish) and 10 previously unexposed larvae were introduced into each dish. Again, settlement rates were determined 24 h later (Fig 3.B). Larval *attachment* in this study was taken to be when the larvae make contact with the substrate but show no signs of metamorphosis (Figure 4). Larvae can easily unattach from the substrate and swim away during this stage of the settlement process. True *settlement* was therefore recorded only if the attached larvae had commenced metamorphosis.

The ranges of test chemical concentrations used for this part of the study are shown in Table 5. POEA concentrations were based on amounts present in the Roundup® Ready concentration range tested. Since the % POEA in Roundup® Ready was not provided on the container label by the manufacturer, it was assumed that the ratio of POEA to glyphosate was the same as that reported by Kiely *et al.* (2004) for Roundup®, i.e., 1 part POEA to 2.73 parts glyphosate (vol:vol).

Data Analysis for larval settlement tests

The effects of the treatments and the corresponding controls were tested using One-way ANOVA and Tukey-Kramer Multiple Comparison Tests. All statistical analyses were performed using Number Crunching Statistical Software 2000 (NCSS 2000).

A. Pre-exposure of larvae to chemical contaminants:



B. Pre-exposure of CCA to chemical contaminants:

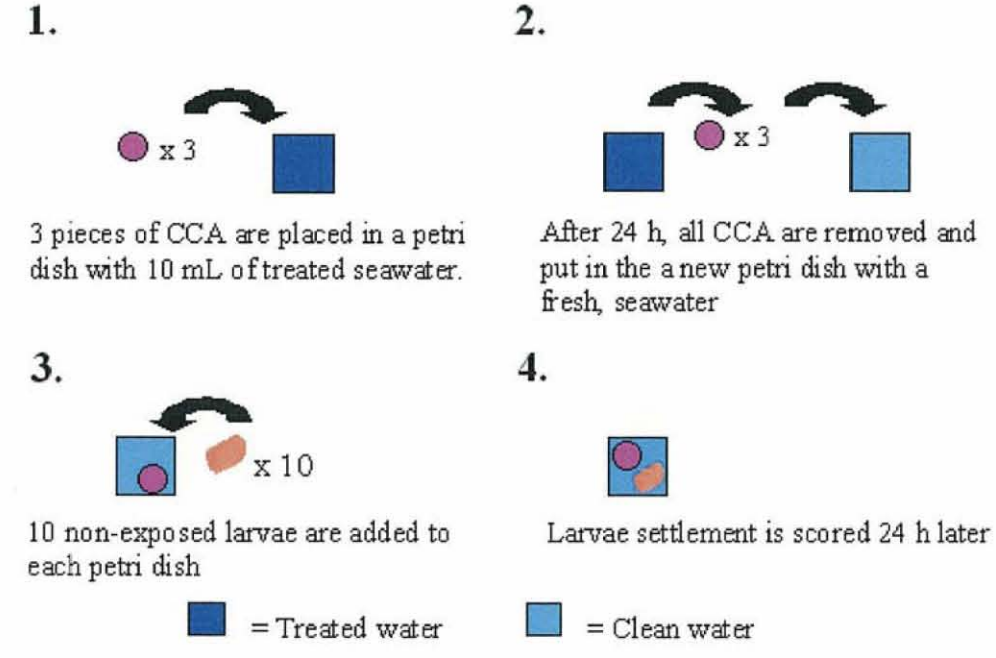
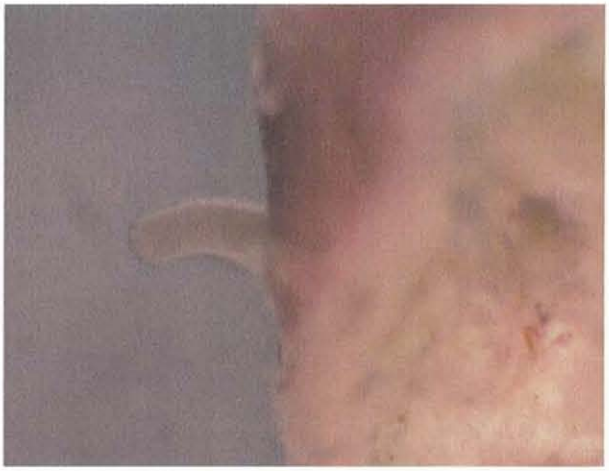
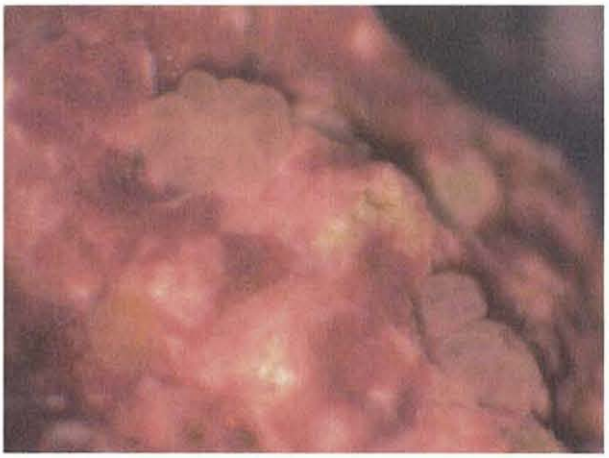


Figure 3. Procedural steps for the preparation of exposed to *L. purpurea* larvae and CCA

Swimming stage of larva



Larva primary attachment stage



Larval secondary settlement and metamorphosis

Figure 4. The different settlement stages of *L. purpurea* larva.

Table 5: Experimental concentrations of test chemicals used to determine effects on *L. purpurea* larval settlement

Chemical (Stock Solution)	Nominal Test Concentration	Controls
Irgarol® 1051 (100,000 mg/L) 10 g powdered formulation in 100 ml acetone	1, 10, 100 mg/l	seawater seawater/acetone
Roundup® Ready (10,000 mg a.i./L) 5.2 ml liquid formulation in 4.8 ml seawater	0.1, 1.0, 10, 100 mg a.i./L	seawater
POEA (100,000 mg/L) 10 ml liquid concenrate in 90 ml acetone	0.036, 0.36, 3.6, 360 mg/L	seawater seawater/acetone
Glyphosate (1,000 mg/L) 250 µl of liquid concentrate in 100 ml seawater	0.1, 1.0, 10 mg/L	seawater

All stock solutions were made up within 2 h prior to use. All nominal test concentrations were made up in 100 ml seawater.
All bioassays were conducted in 20 ml seawater

RESULTS

Acute toxicity tests

The mortality data derived from the acute toxicity tests with *Leptastrea purpurea* larvae are included on the appendices (A, B, and C) together with all time response curves used to derive the LT_{50} values, the slope of each line, and the 95% confidence limits of both parameters. Toxicity response curves for Roundup® Ready, POEA and glyphosate were constructed from their respective LT_{50} values (Figures 5, 6, and 7) and suggested a single mode of toxic action in each case. The toxicity response curves were also used to provide estimates of the 24-h LC_{50} and incipient threshold concentration for each chemical (Table 6). Based on the 24-h LC_{50} values, POEA was the most toxic compound, examined followed by Roundup® Ready and glyphosate. Irgarol® 1051 was the least toxic chemical tested.

Behavior and morphological changes recorded during the tests included reduced swimming activity and larval deformities and are summarized in Table 7. These preliminary observations suggest that the prevalence and severity of larval deformations were concentration dependent. In an attempt to quantify this further, a second experiment was conducted with Roundup® Ready in which the larvae were individually photographed before and after exposure. Morphological changes were recorded using the Motic Image Plus Version 2.0 MI computer program (Motic China Group Co. Ltd.) and were ranked on a scale of 1-4 with 1 being non deformed and 4 being severely deformed (Figure 8). The results are summarized in Figure 9 and clearly show a concentration dependent relationship exists above 5.6 mg a.i./L with 80% of larvae severely deformed at 10 mg a.i./L. The narrow range of the effective Roundup® Ready concentrations

highlights the potency of this particular herbicide to *L. purpurea* larvae.

Table 6. The acute toxicity of Roundup® Ready, POEA, and glyphosate to *L. purpurea* larvae.

Chemicals	Conc. (mg/L)	Median lethal time (h) + 95% confide. limits	Slope function (S) + 95% confide. limits
Irgarol® 1051	100	>96 h	-
	24-h LC_{50} > 100 mg/L	Threshold concentration >100 mg/L	
Roundup® Ready	56	1.8 (1.22 – 2.66)	4.04 (3.06 – 5.33)
	32	3.4 (2.39 – 4.85)	3.47 (2.7 – 4.46)
	18	8 (6.48 – 9.88)	2.08 (1.79 – 2.41)
	10	22 (18.72 – 25.85)	1.8 (1.61 – 2.02)
	5.6	Zero mortality after 24 h exposure	
	SW control	Zero mortality after 24 h exposure	
	24-h LC_{50} = 9.6 mg a.i./L	Threshold concentration = 6.2 mg a.i./L	
POEA	3.2	5.7 (4.94 – 6.58)	1.7 (1.53 – 1.89)
	1.8	10.5 (9.05 – 12.18)	1.72 (1.55 – 1.91)
	1.35	30.5 (25.96 – 35.84)	1.78 (1.59 – 1.99)
	1	Zero mortality after 24 h exposure	
	0.56	Zero mortality after 24 h exposure	
	Acetone/ SW control	Zero mortality after 24 h exposure	
	SW control	Zero mortality after 24 h exposure	
	24-h LC_{50} = 1.5 mg/L	Threshold concentration = 1.1 mg./L	
Glyphosate	32	55 (45.45 – 66.55)	1.96 (1.72 – 2.23)
	18	9.7 (8.47 – 11.1)	1.65 (1.48 – 1.83)
	10	20.5 (17.23 – 24.4)	1.87 (1.66 – 2.11)
	5.6	105 (83.33 – 132.2)	2.34 (2.03 – 2.69)
	3.2	Zero mortality after 24 h exposure	
	1	Zero mortality after 24 h exposure	
	SW control	Zero mortality after 24 h exposure	
	24-h LC_{50} = 9.3 mg/L	Threshold concentration = 5.7 mg/L	

Table 7. Summary of the larval swimming activities and deformities during the Acute Toxicity of Roundup® Ready, POEA, and glyphosate to *L. purpurea* larvae

Chemicals	Conc. (mg/L)	Swimming behavior	Deformities
Roundup® Ready	56	Movement ceasesed immediately	Elongate to spherical after 1 h
	32	10 %: swim in circles slowly after 1 h 90%: no moment after 1hr	No data
	18	0% swimming slow after 15 min 50% no movement after 15min.	No data
	10	Several swim in circles after 4 h	10% deformed after 4 h
	5.6	No data	2% deformed after 48 h
	SW control	Swim actively after 96 h	No effect
POEA	3.2	Swim very slow immediately	26% deformed after 1 h
	1.8	Swim slow after 30 min.	16% deformed after 1 h
	1.35	No data	14% deformed after 2 h
	1	No data	4% deformed after 12 h
	0.56	Swim actively	No effect
	Acetone/SW control	Swim actively	No effect
	SW control	Swim actively	No effect
Glyphosate	32	No data	42% deformed after 1 h
	18	No data	36% deformed after 1 h
	10	No data	18% deformed after 2 h
	5.6	No data	14% deformed after 8 h
	3.2	No data	2% deformed after 36 h
	1	No data	No effect
	SW control	No data	No effect

Figure 5. The toxicity response curve for Roundup® Ready with *L. purpurea*

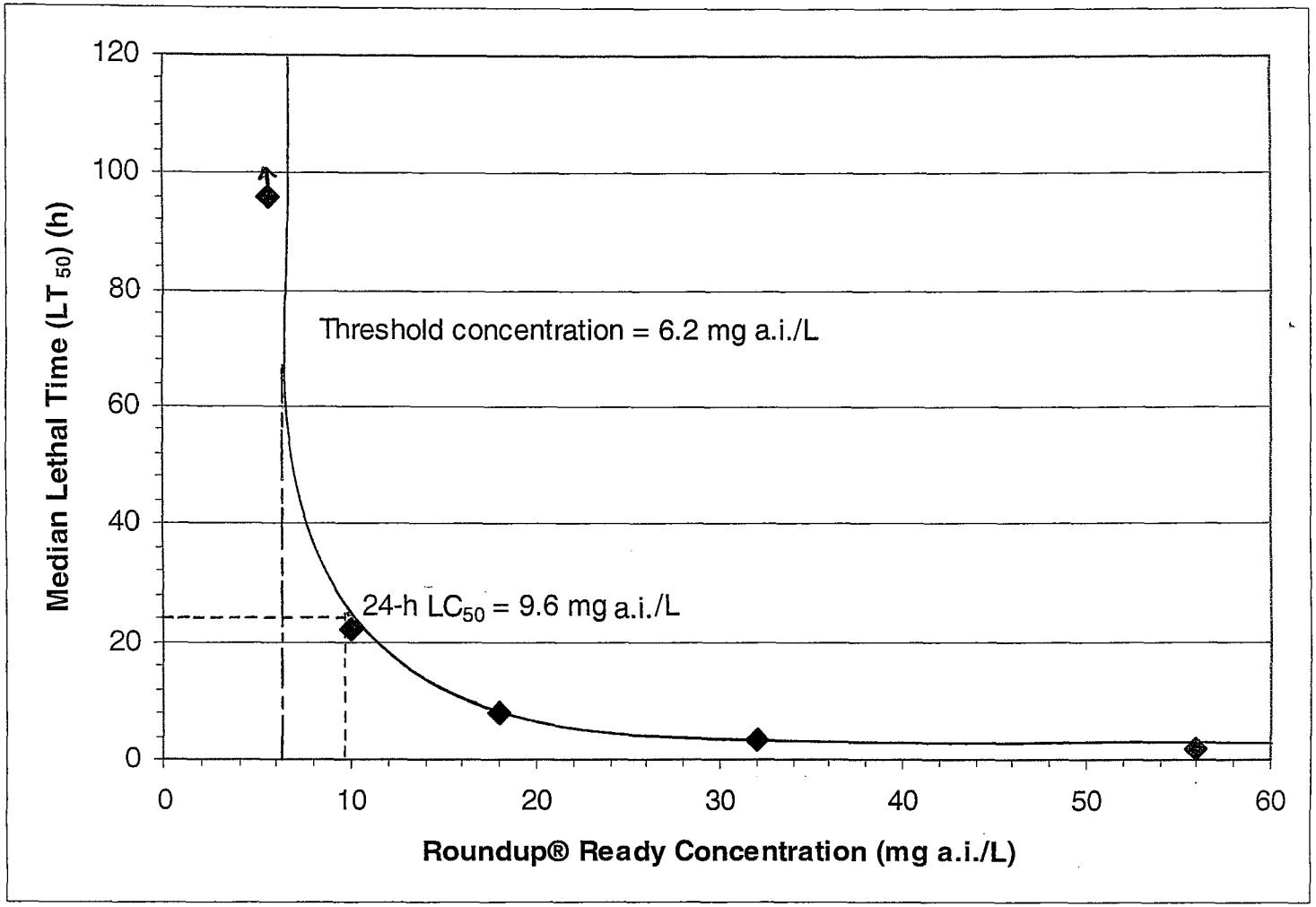


Figure 6. The toxicity response curve for POEA with *L. purpurea*

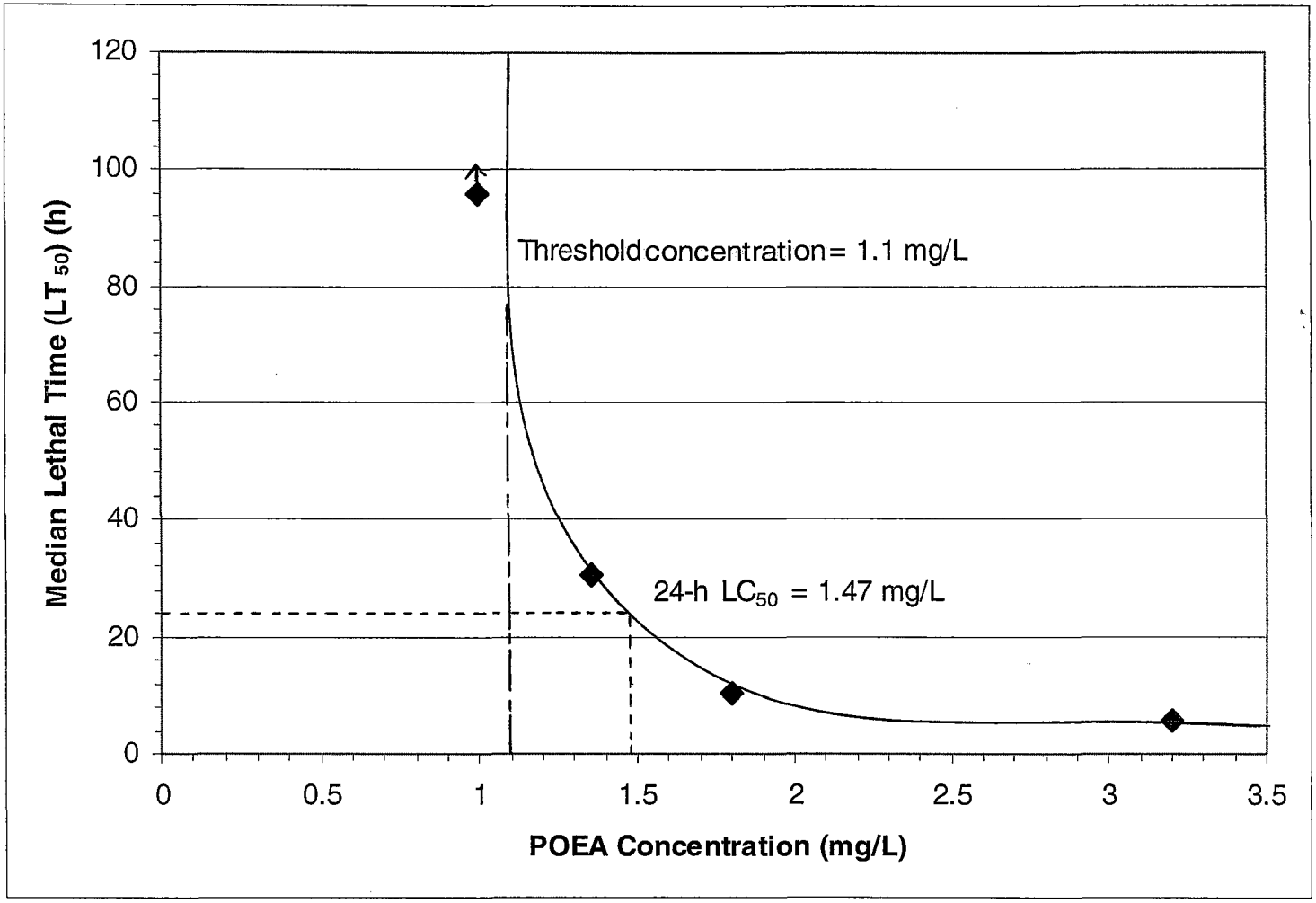
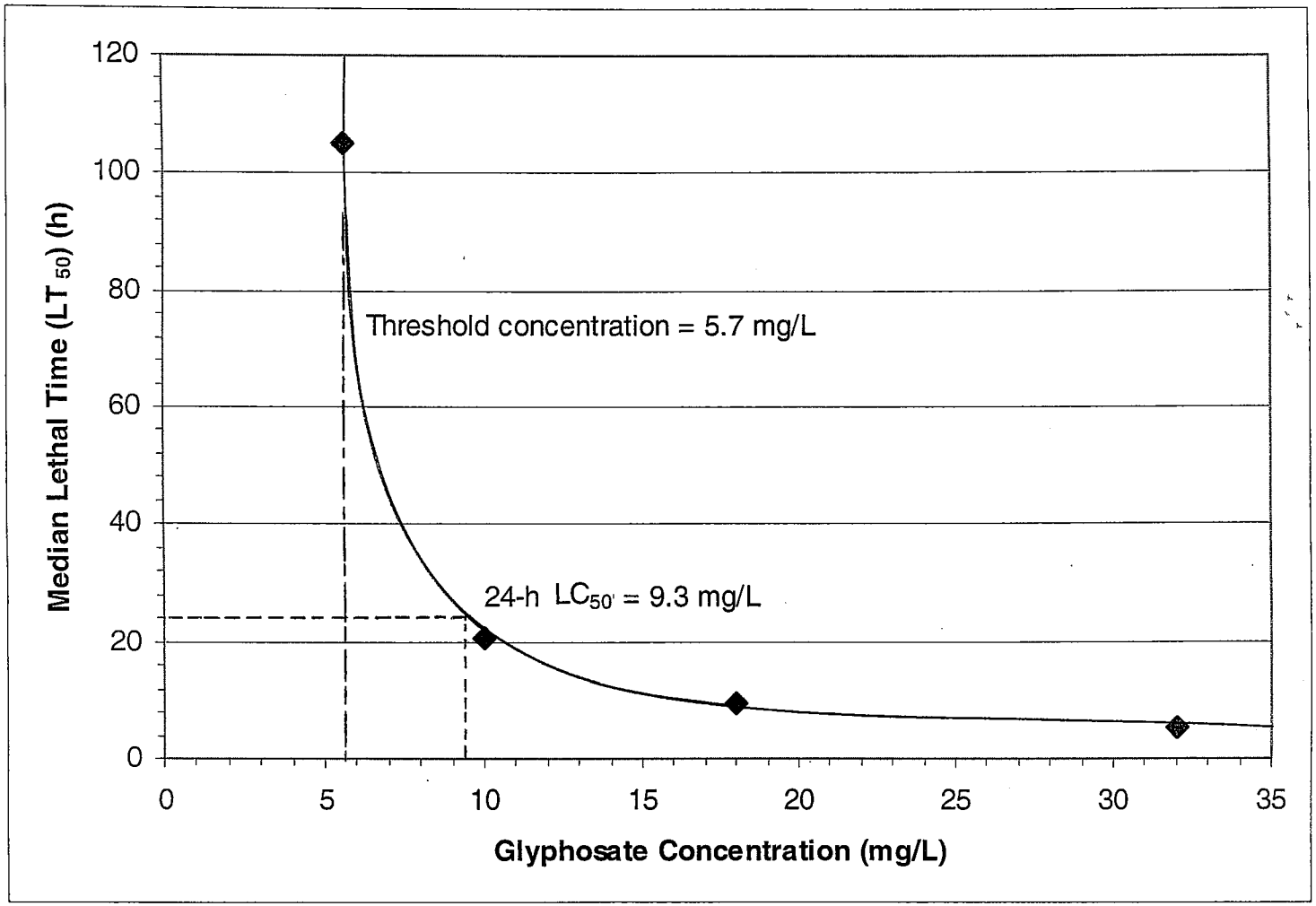
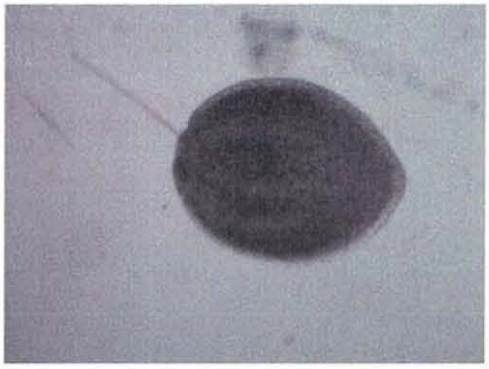
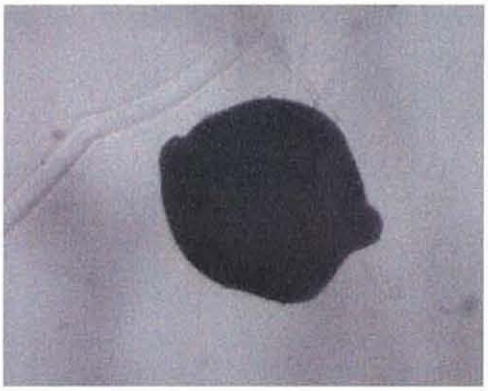


Figure 7. The toxicity response curve for glyphosate with *L. purpurea*





Rank 1: Normal
- Cigar or capsule shaped
- Active directional swimming
- Zooxanthellae can be seen inside of body



Rank 2: Slightly deformed larva
- Slightly irregular
- Round shape with one or two projections
- No directional swimming behavior
- Larvae swim in circles

Rank 3: Moderately deformed larva
- Moderately irregular
- Swim in circles very slowly
- Very slow movement

Rank 4: Severely deformed larva
- Apparent necrotic membrane
- Practically no movement
- Small body size compared to control
- Individual zooxanthellae visible

Figure 8. Ranks for the degree of *L. purpurea* larval deformation.

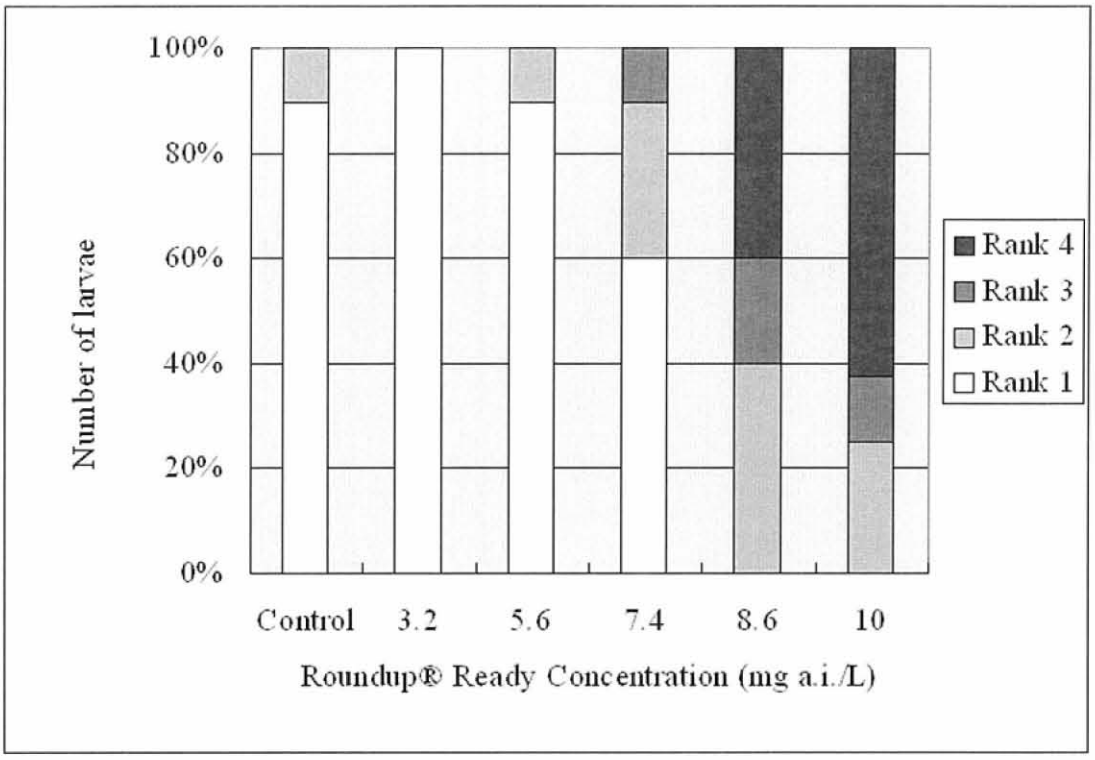


Figure 9. Deformation of *L. purpurea* larvae vs. Roundup® Ready concentration. Each control and test concentration contained 1 larva and was replicated 10 times. Exposure time was 24 h. Rank 1 = normal shape; Rank 2 = slightly deformed; Rank 3 = moderately deformed (few lumps); and Rank 4 = severely deformed (membrane destruction).

Settlement tests

Irgarol® 1051

Experiments with Irgarol® 1051were repeated twice. Hence, the results presented in Figure 10 and 11 show the average settlement of 10 replicates at each concentration and both controls.

Settlement of pre-exposed larvae on CCA

A preliminary test performed with Irgarol® 1051 at environmentally realistic concentrations of 0.1 to 10 μg/L revealed no significant effect on larval settlement. The test was therefore, repeated at the concentrations of 1, 10, and 100 mg/L. A dense, white precipitated was noted at 100 mg a.i./L (water solubility ~7 mg/L). Larval settlement was lower than that of controls at all concentrations but only significantly so ($P < 0.05$) at 100 mg/L (Figure 10).

Settlement of larvae on pre-exposed CCA

Settlement rates on CCA previously exposed to 1 and 10 mg/L Irgarol® 1051 were very similar to that seen in the acetone control. A significant decrease in settlement was shown only for the 100 mg/L test concentration (Figure 11) and likely reflects the precipitation of Irgarol® 1051 on the CCA substrate.

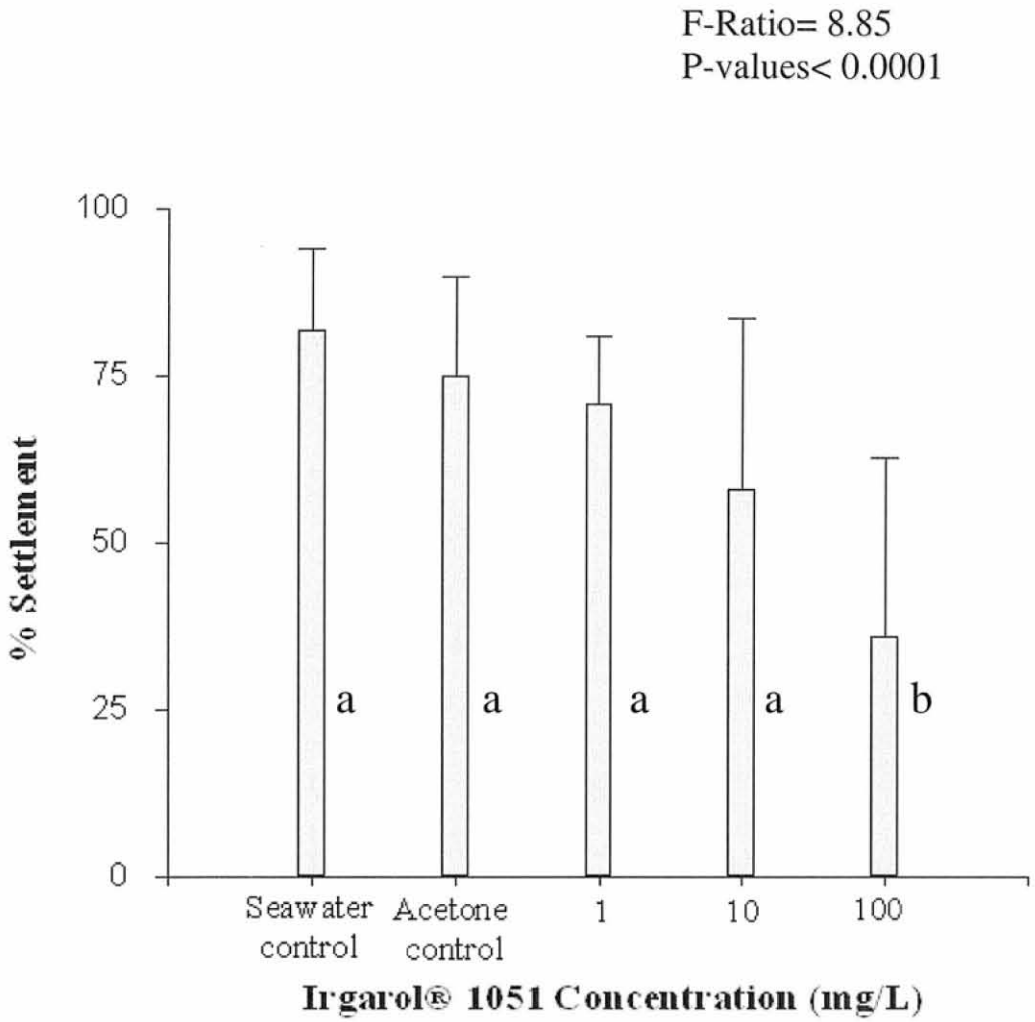


Figure 10. Average settlement on CCA substrates of *L. purpurea* larvae that were pre-exposed to Irgarol® 1051 for 24 h. Each control and test concentration contained 10 larvae and was replicated 10 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

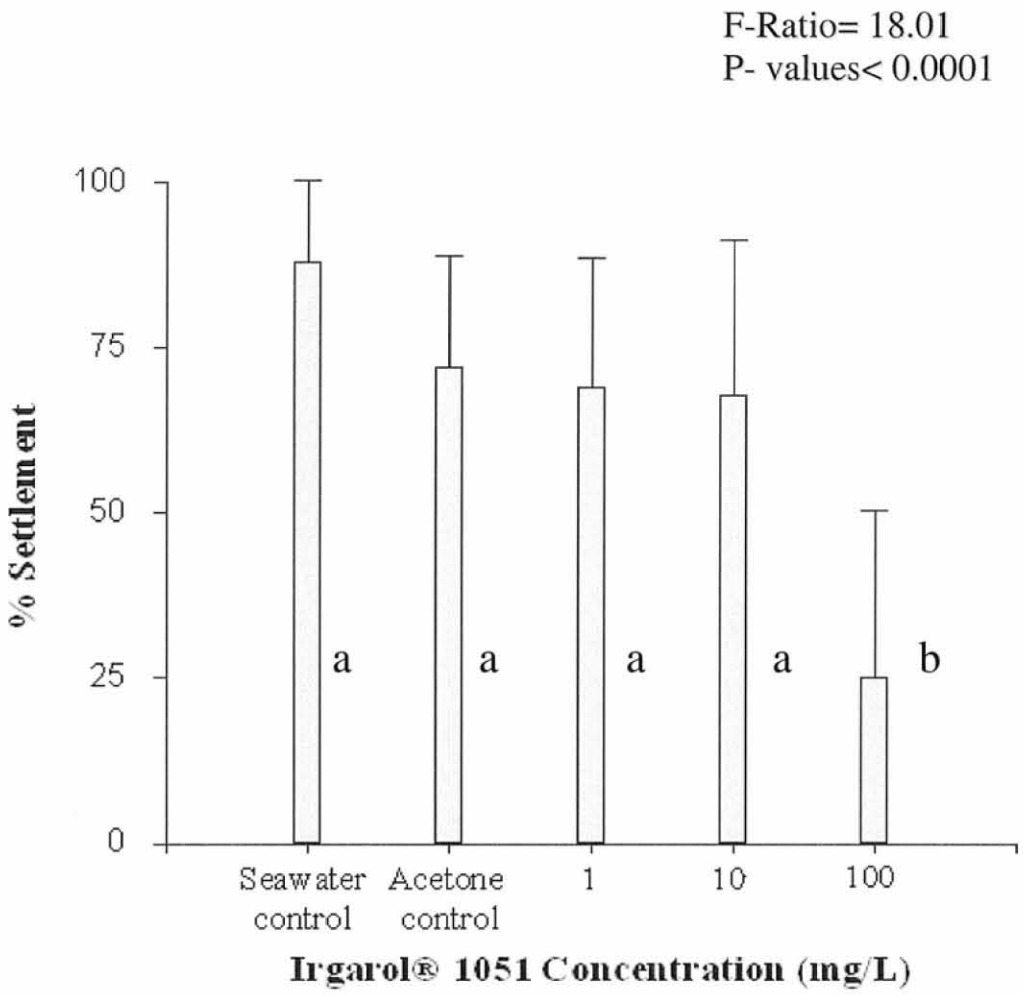


Figure 11. Average settlement of *L. purpurea* larvae on CCA substrates pre-exposed to Irgarol® 1051 for 24 h. Each control and test concentration contained 10 larvae and was replicated 10 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

Roundup® Ready

Settlement of pre-exposed larvae on CCA

Roundup® Ready significantly influenced larval settlement at concentrations of 0.1 mg a.i./L and above. At 1 mg a.i./L, larval settlement was decreased by 20%, while no settlement was observed at the 10 mg a.i./L and at 100 mg a.i./L test concentrations (Figure 12). Most of the larvae were either dead or severely deformed at 10 mg a.i./L while all were dead after a 24 h exposure period at 100 mg a.i./L.

In a followup experiment, larvae exposed to 7.4 mg a.i./L of Roundup Ready showed mild deformation in 50% of the test population. The remaining 50% appeared normal. Both deformed and non-deformed larvae were presented with uncontaminated CCA and settlement was monitored for 24 h. Only 5% of the deformed larvae settled, compared with 64% and 96% for un-deformed larvae and controls respectively. Differences between all treatments were statistically significant (Figure 13).

Settlement of larvae on pre-exposed CCA

CCA chips exposed to Roundup® Ready concentrations of 0.1, 1, 10 mg a.i./L had no effect on larval settlement (Figure 14). Settlement on CCA chips exposed to a 100 mg a.i./L concentration of Roundup® Ready was significantly lower than that of the control and the other test concentrations.

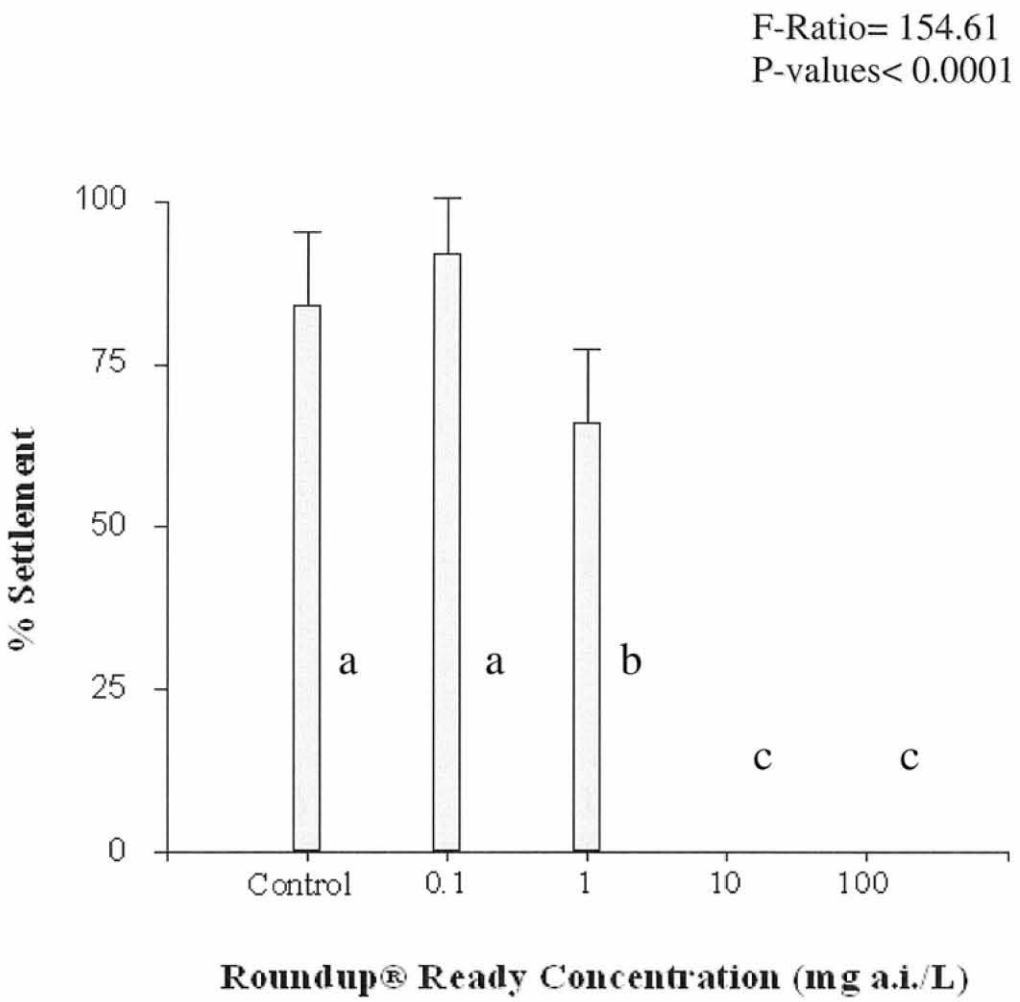


Figure 12. Average settlement on CCA substrates of *L. purpurea* larvae that were pre-exposed to Roundup® Ready for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

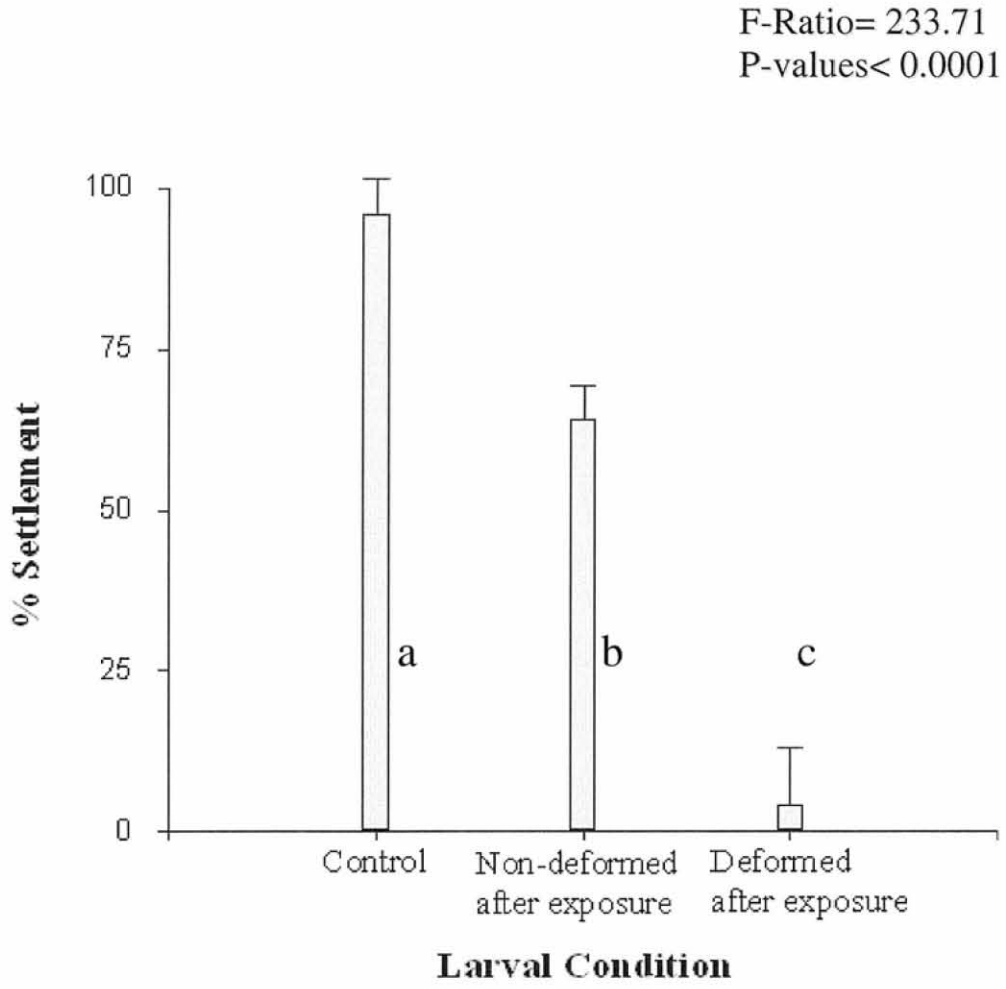


Figure 13. Average percent larval settlement on CCA of non-deformed and deformed *L. purpurea* larvae following 24 h exposure to a Roundup® Ready concentration of 7.4 mg a.i./L. Five replicates were used for each larval condition, with 10 larvae in each replicate. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

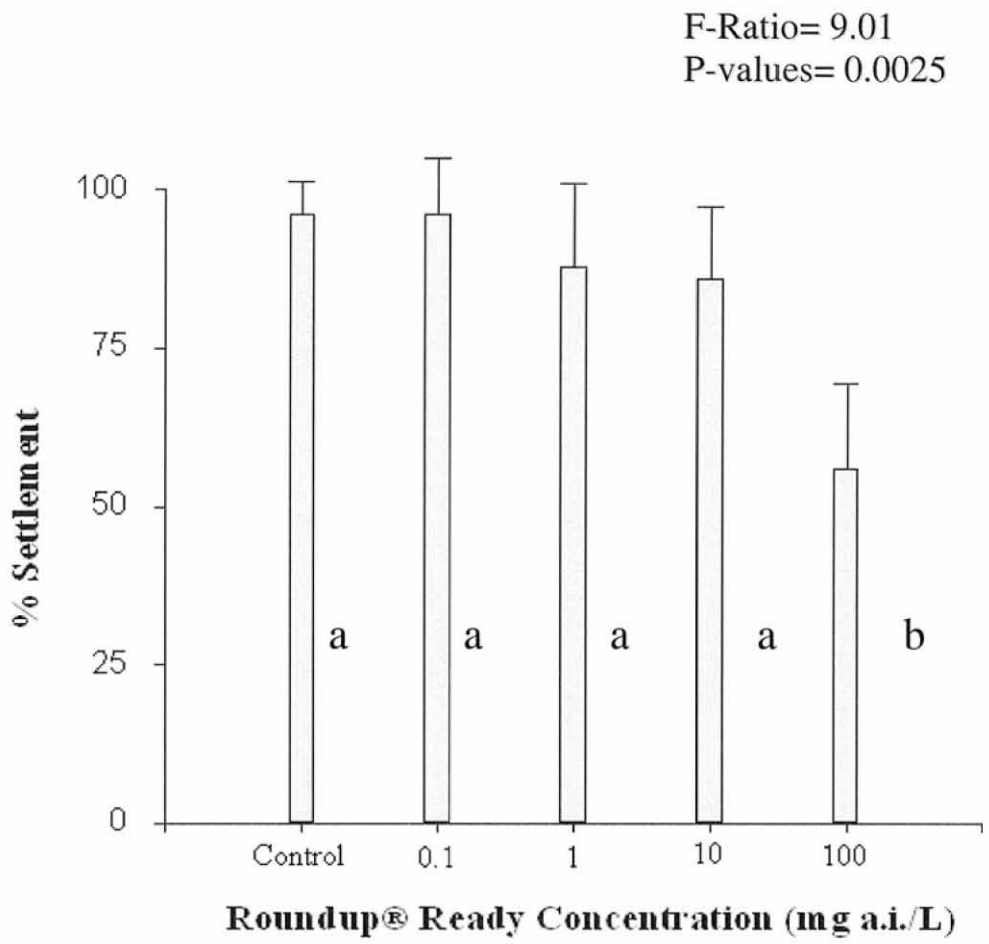


Figure 14. Average settlement of *L. purpurea* larvae on CCA substrates pre-exposed to Roundup® Ready for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

POEA

Settlement of pre-exposed larvae on non-exposed CCA

POEA concentrations of 3.66 mg/L and above caused 100% larval mortality after 24 h exposure. Of the lower test concentrations, only larvae exposed to 0.366 mg/L showed a significant reduction in settlement and only against the seawater control (Figure 15).

Settlement of larvae on pre-exposed CCA

Larvae settlement was not significantly different between the test concentrations or between test concentrations and controls (Figure 16).

Glyphosate

Settlement of pre-exposed larvae on non-exposed CCA

Larvae exposed to 10 mg/L glyphosate showed 100% mortality after a 24 h exposure period. Settlement was not significantly different in the remaining test concentrations or the controls (Figure 17).

Settlement of larvae on pre-exposed CCA

The settlement of larvae on CCA chips previously exposed to a range of glyphosate concentrations are provided in Figure 18. Larval settlement was only significantly different in the 100 mg/L test concentration.

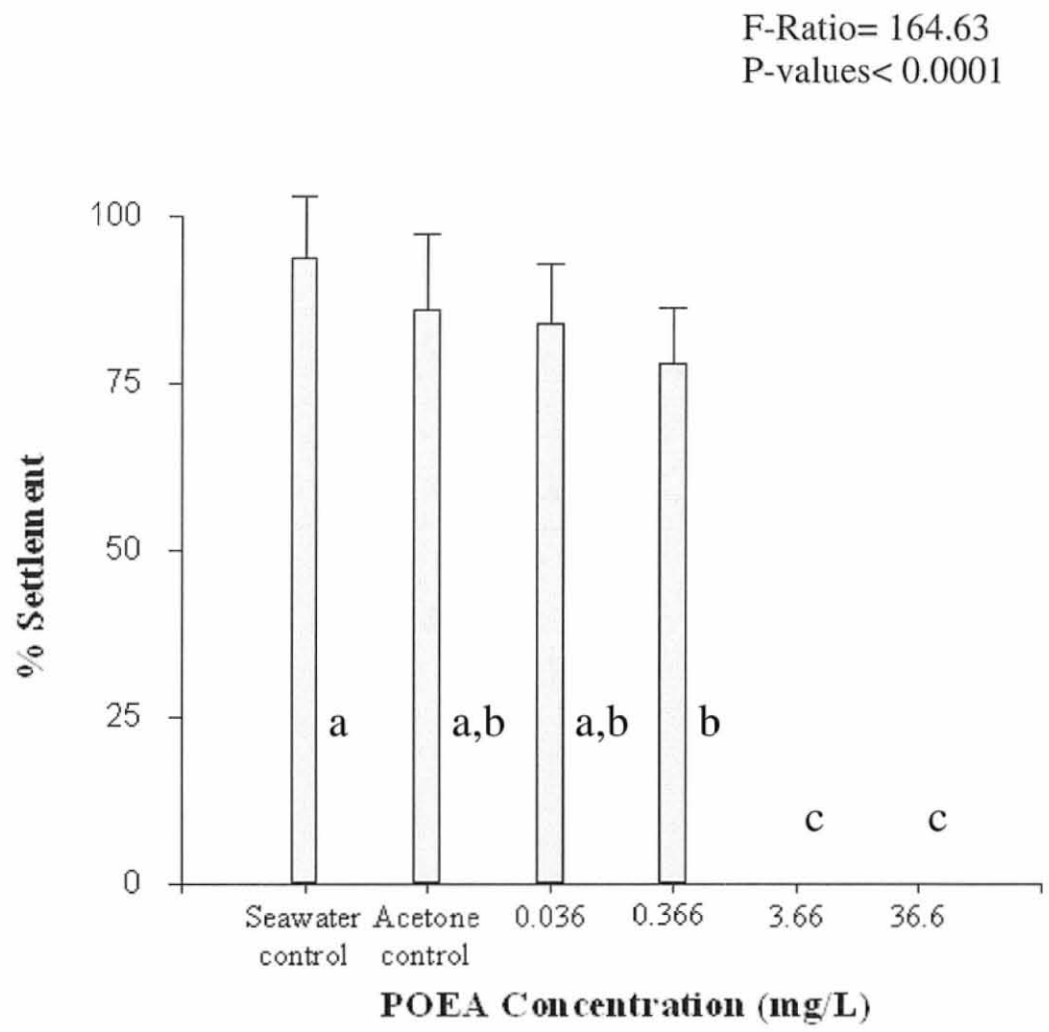


Figure 15. Average settlement on CCA substrates of *L. purpurea* larvae pre-exposed to POEA for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

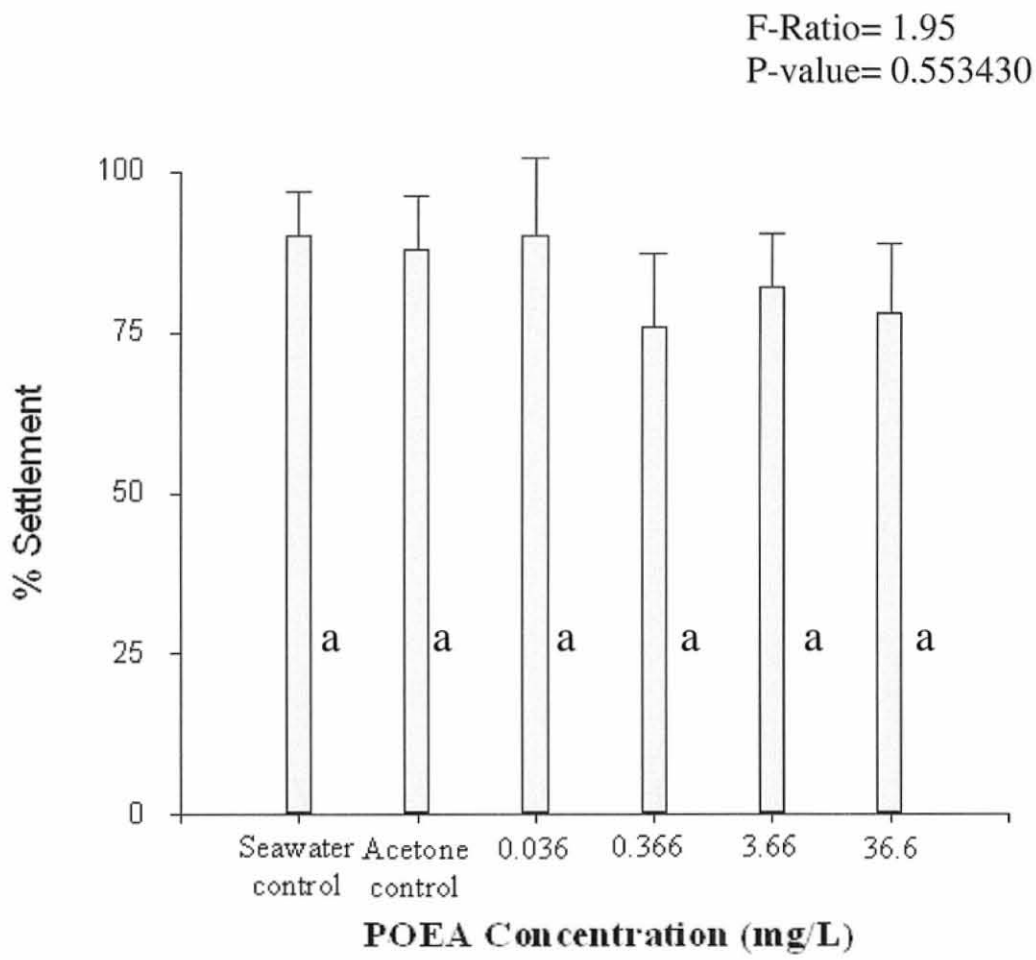


Figure 16. Average settlement of *L. purpurea* larvae on CCA substrates pre-exposed to POEA for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

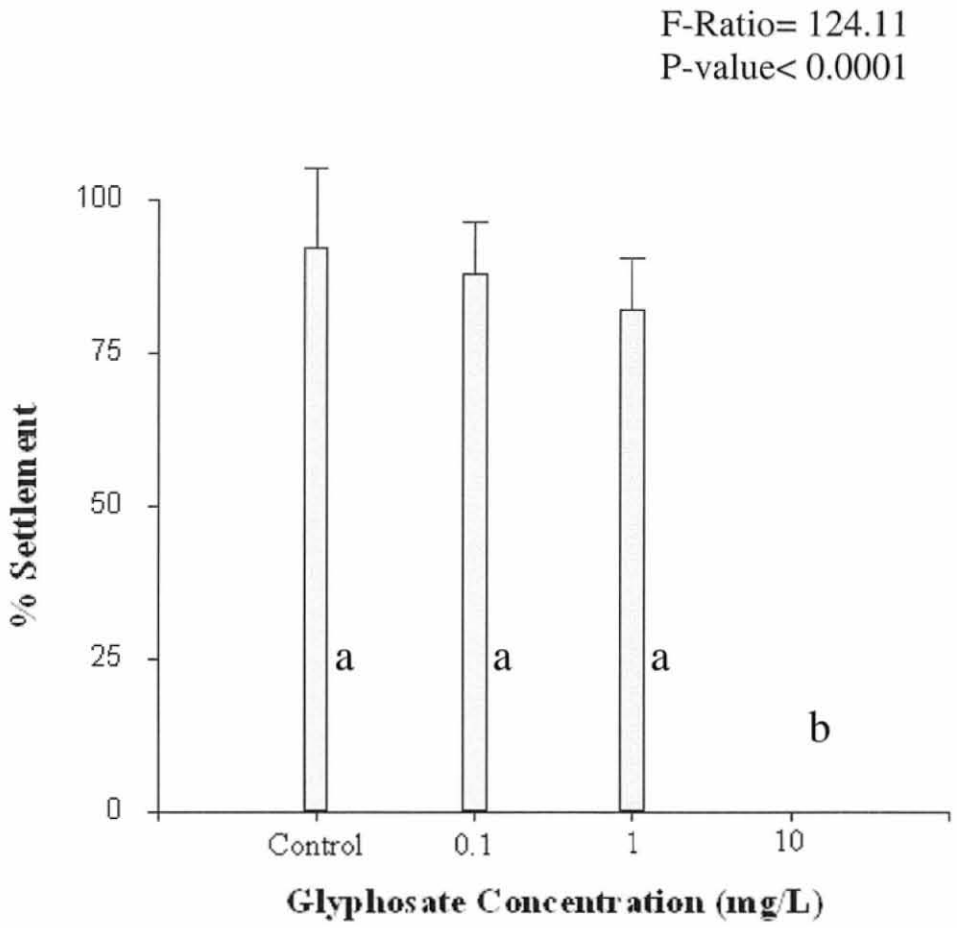


Figure 17. Average settlement on CCA substrates of *L. purpurea* larvae pre-exposed to glyphosate for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

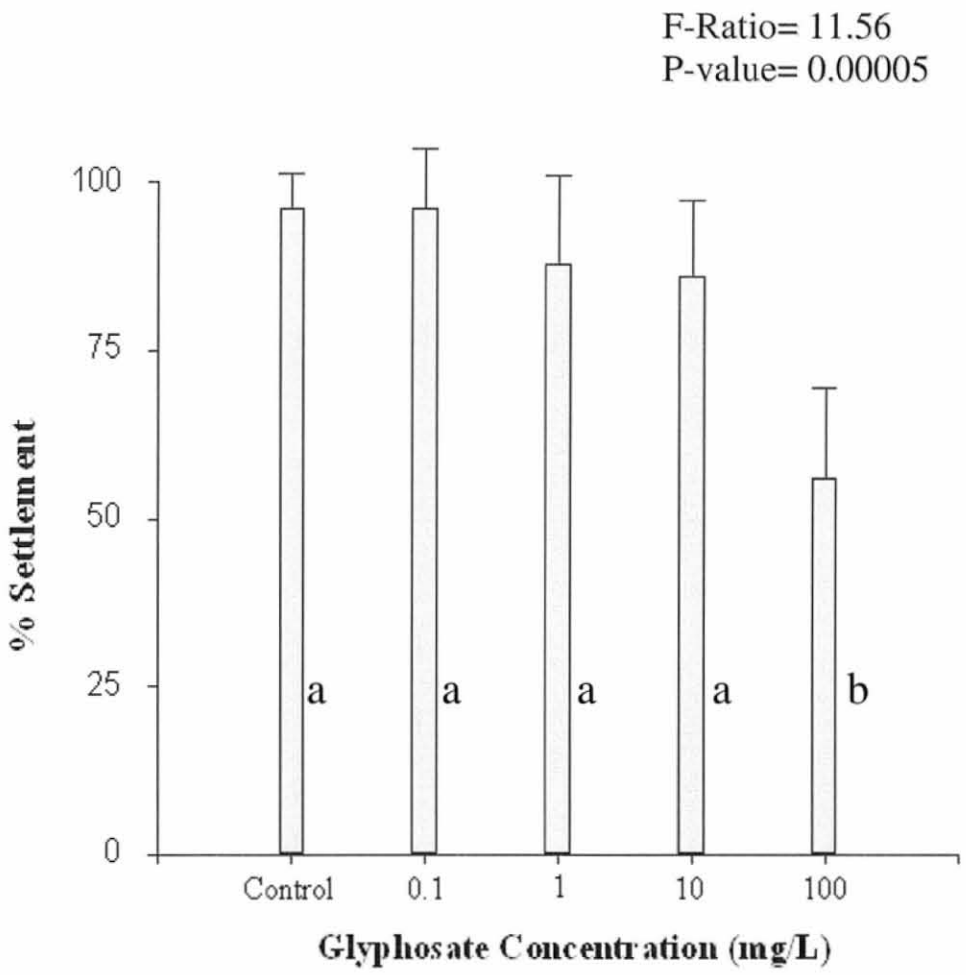


Figure 18. Average settlement of *L. purpurea* larvae on CCA substrates pre-exposed to glyphosate for 24 h. Each control and test concentration contained 10 larvae and was replicated 5 times. Error bars represent the standard deviation. The letter next to each bar shows significant grouping by Tukey-Kramer Multiple-Comparison Test.

DISCUSSION AND CONCLUSION

The herbicide, Roundup® Ready, and antifouling agent, Irgarol® 1051, are commonly used throughout the world, but their impact on tropical reef organisms remains to be fully evaluated. The present study is the first to examine the effects of both chemicals on planulae larvae of the stony coral *Leptastrea purpurea.* The following discussion integrates the results of the acute toxicity test and settlement bioassays performed in this study with available literature to provide an estimate of the likely effects Irgarol® 1051 and Roundup® Ready have on the settlement and morphological integrity of *L. purpurea* larvae in their natural environment.

Irgarol® 1051

Treatment concentrations of Irgarol® 1051 that fall within the range detected in ports, harbors and coastal waters (1 to 1,700 ng/L) do not adversely effect *L. purpurea* larval settlement. Although the solubility of Irgarol® 1051 is 7 mg/L, a 100 mg/L test concentration, though not acutely toxic, was found to significantly lower settlement rates in both treatments, while lower concentrations had no effect. The heavy precipitate of Irgarol 1051 noted in the 100 mg/L test concentration undoubtedly influenced the reduction in settlement because larvae would have come into direct contact with the pure compound rather than the diluted form. Since the dissolved fraction of Igarol® 1051 does not appear to detrimentally affect *L. purpurea* larval settlement, this chemical is unlikely to cause problems in coastal areas unless organisms come in direct contact with it in antifouling paints or paint chips.

In contrast with the results found here, other researches have shown that

environmentally realistic concentrations of Irgarol® 1051 (< 7mg/L) are highly toxic to the symbiotic algae (zooxanthellae) in certain corals. For example, Owen *et al.* (2002) tested the effect of Irgarol® 1051 on zooxanthellae isolated from the coral, *Madracis mirabilis*. The rate of photosynthesis was reduced significantly after the zooxanthellae were exposed to 0.1 μg/L of the chemical for 1.5 h and little or no photosynthesis was detected after 2 to 8 h of exposure at 1.0 μg/L. Also, Jones and Kerswell (2003) reported that the density of zooxanthellae in the coral, *Seriatopora hystrix*, was reduced by 30% after exposure to 0.03mg/L Irgarol® 1051 for two h a d over a four-d period.

Owen *et al.* (2002) suggested that the inhibition of photosynthesis in coral zooxanthellae by Irgarol® 1051 may affect coral growth and reproduction. Since the energy provided by zooxanthellae is essential for the survival of some corals (Rinkevich, 1989; Muscatine, 1990), any reduction in the photosynthetic output of zooxanthellae would likely have detrimental effects on coral growth and reproduction. Therefore, even though environmentally realistic concentrations of Irgarol® 1051 do not appear to effect *L. purpurea* larval settlement, the reported adverse effects of even very small Irgarol® 1051 concentrations on the zooxanthellae of other coral species suggest that concentrations of Irgarol® 1051 previously detected in the marine environment may indirectly interfere with coral reproduction and settlement.

Roundup® Ready

Roundup® Ready is readily available in Guam and elsewhere and is often mistakenly believed to be safe and rapidly biodegraded in the environment. However, glyphosate can enter the aquatic environment as spray drift, accidental spillage, and in terrestrial runoff adsorbed to soil particles. Recent studies have shown that Roundup® and other glyphosate-based products, such as Accord® or Rodeo®, are relatively persistent in the environment and can detrimentally affect non-target organisms such as fish, macroalga, and crustaceans (Table 3) (Torstensson *et al.*, 1989; U.S. EPA, 1993; Tsui and Chu, 2003).

Results of the settlement assays performed in this study showed a reduction in larval settlement on CCA chips when the larvae were exposed to Roundup® Ready concentrations of 1 mg a.i./L and above. Interestingly, the reduction in larval settlement at 1 mg/L of the active ingredient, glyphosate, was greater than that seen with a 10 mg/L test concentration of glyphosate alone (see Figure 12 and Figure 17). These different results indicate a possible synergistic effect between POEA and glyphosate in the commercial mixture as previously indicate by Perkins *et al.* (2000). Glyphosate-only products without POEA (or any other surfactant), such as Accord® and Rodeo®, could therefore be less toxic than Roundup® and Roundup® Ready. However, according to the manufacturers' instructions, Accord® and Rodeo® products must be mixed with a nonionic surfactant before they are applied. This may well exacerbate their toxicity and requires further evaluation before the precise impact of these chemicals can be unequivocally determined.

In the current study, Roundup® Ready inhibited larval settlement at concentrations as low as 1 mg/L and caused deformities at a concentration a little over 5 times higher. Larvae exhibiting such deformities virtually lost all ability to settle. An empirically derived estimate for the maximum concentration of Roundup® Ready in aquatic environments stands at 3.7 mg a.i./L (Giesy *et al.* 2000). This is well above that shown here to cause a decline in settlement of *L. purpurea*, but lower than that required to cause deformities. Thus, except under the most extreme conditions (i.e. limited tidal water exchange and the direct application of Roundup® to surface waters), larval deformation resulting from exposure to Roundup® Ready is unlikely to occur, although settlement may well be affected. However, the possible toxic effects of prolonged exposure to lower concentration cannot be ruled out and requires further study.

Roundup® products usually need to be diluted with water before use, unlike Roundup® Ready, which comes ready to use. Based on the 100% larval mortality seen at a POEA test concentration of 3.66 mg/L and the assumption that Roundup® Ready contains the same ratio of glyphosate to POEA as Roundup® (i.e., 2.73 to 1), 100% larval mortality should have occurred following a 24 h exposure to the 10 mg a.i./L test concentration of Roundup® Ready (Figures15 and Appendix A and B). Instead, only 40% of exposed individuals were killed which suggests that the percent composition of POEA in Roundup® Ready is actually lower than that found in undiluted Roundup®. A comparison of the 24-h LC_{50} values for Roundup® Ready, glyphosate and POEA (Table 8) supports this conclusion. Alternatively, POEA has been substituted in Roundup® Ready by a comparatively non-toxic surfactant. Either that, or the data anomaly could mean that glyphosate in some way ameliorates POEA toxicity. This would certainly be

Table 8. Summary table of acute toxicity and effects of Irgarol® 1051 Roundup® Ready, POEA, glyphosate on settlement of *L. purpurea* larvae

Test chemical	mg/L			Treatments
	24 h LC_{50}	Threshold conc.	Minimum conc. effecting settlement	
Irgarol® 1051	-	100		1
		100		2
Roundup® Ready	9.6	6.2	10	1
			100	2
POEA	1.5	1.1	3.66	1
			>3.66	2
Glyphosate	9.3	5.7	1.0	1
			100	2

1 = Larvae pre-exposed to test chemical for 24 h prior to 24 h settlement period on CCA; 2 = CCA expected to test chemicals for 24 h prior to 24 h settlement period with non-exposed larvae; Dashes indicate no data

unusual considering that POEA appears to have the exact opposite effect on glyphosate toxicity with respect to settlement of pre-exposed *L. purpurea* larvae (Table 8).

Even though the constituents of Roundup® Ready may be proportionally or qualitatively different than the more concentrated version of Roundup®, less settlement was observed after larvae were exposed to Roundup® Ready than when exposed to glyphosate alone at the same concentrations. This result suggests that the surfactant in Roundup® Ready, whether POEA or some other chemical, still contributes significantly to the inhibition of larval settlement on CCA compared to glyphosate alone.

There are few data available on the ecotoxicological significance of Roundup® in the marine environment; the literature that is available focuses mainly on freshwater environments. Concentrations of Roundup® in a contaminated freshwater pond were reported to be around 2.3 mg a.i./L (Newton *et al.*, 1984), which is very close to the maximum environmental level of 3.7 mg/L predicted by Giesy *et al.* (2000).

A study by Paveglio *et al.* (1996) used Rodeo® to estimate glyphosate persistence in a Washington estuary. When the recommended concentration (4.7 l/ha) was applied to the estuarine waters, the glyphosate concentration declined by 73% (from 9.77 μg/L to 2.62 μg/L) between the first two high tides immediately following application. Therefore, if recommended concentrations are used and there is sufficient tidal exchange, the effect of the Roundup® on *L. purpurea* larval settlement is likely to be minimal or absent in the natural environment. However, POEA has been found to be the more toxic component of Roundup® and the persistence of POEA in the natural environment following Roundup® applications has not yet been examined. Thus, a conclusive statement about the effect of Roundup® Ready on *L. purpurea* larval settlement in the natural environment cannot be

made at this time. Also, the effects of environmental realistic concentrations of Roundup® on larval settlement in other coral species and on other aspects of coral biology (e.g. fecundity, photosynthetic activity of zooxanthellae, gametogenesis) have yet to be determined.

The spraying of Roundup® Ready during and immediately after prolonged, heavy rain could still affect larval settlement. Edwards *et al.* (1980) found that the concentration of glyphosate in runoff was highest when there was heavy rain the first day following Roundup® application. On tropical islands such as Guam, squalls often occur throughout the year, and runoff in the coastal areas is commonplace during heavy rain. Thus, if rain occurs immediately after the application of Roundup®, it is possible that the chemical may reach reef areas in sufficient concentrations to affect larval settlement and other aspects of coral biology, particularly during spawning periods.

Edwards *et al.* (1980) detected 5.2 mg/L of glyphosate in the runoff when 7 times the recommended concentration of Roundup® was applied, which they concluded was unlikely to happen under normal condition. However, Monsanto, as well as Roundup® users, have acknowledged that four times the recommended concentration may be required for some types of weeds (Relyea, 2005). Under such conditions Roundup® concentrations reaching coastal waters during storm events may be sufficient to adversely effect coral larval settlement.

FUTURE RESEARCH

A major difficulty encountered during the current work was minimizing the effect of extraneous variables (temperature and solar radiation) and limiting the amount of stress imposed on the test organisms during handling. Most of these variables are difficult to avoid completely, but their effects can be minimized with due care and attention. The transfer of larvae from one petri dish to another during water changes required to use a glass pipette, which could easily have damaged test individuals and influenced their response to the treatment investigations. It was necessary to perform this procedure daily in order to maintain relatively constant treatment concentrations and oxygen levels in the experimental petri dishes. However, an improved bioassay method allowing test concentrations of the chemicals of interest and oxygen levels to remain constant without causing larvae additional stress could be developed in order to improve the reliability of the bioassay.

Irgarol® 1051

Ecologically realistic concentrations of Irgarol® 1051 do not appear to have an effect on larval settlement of the coral, *Leptastrea purpurea.* However, Owen *et al.* (2002) reported that concentrations well below the solubility limit of 7 mg/L interfered with the photosynthetic activity of zooxanthellae in the coral, *Madracis mirabilis*, in Bermuda and the Florida Keys. Irgarol® 1051 toxicity on zooxanthellae from the coral, *L. purpurea,* may differ from the results seen with *M. mirabilis* due to a different zooxanthellae community composition and differences in coral physiology, membrane

permeability, as well as differing environmental factors (e.g. temperature changes a rate of chemical release) between Bermuda, the Florida Keys, and Guam. Therefore, the sensitivity of zooxanthellae in adult colonies of *L. purpurea* to Irgarol® 1051 should be investigated.

Roundup® Ready

Results of the larval settlement bioassays with glyphosate showed that levels detected in the environment are not likely to affect larval settlement of *L. purpurea.* However, there is a possibility that after larvae successfully settle in areas where glyphosate concentrations are sufficiently high, sustainable reef growth may be inhibited through reduced coral growth rates, fecundity, etc. When Shick *et al.* (1999) examined the effect of UV-A and UV-B on the coral *Stylophora pistillata*, with and without the addition of glyphosate, they reported that glyphosate reduced the UV-induced accumulation of most mycosporine-like amino acids (MAA), which act as a natural sunscreen protecting coral from UV radiation. They also found that UV-A and UV-B radiation decreased the density of zooxanthellae in *S. pistillata, Acropora* sp., and *Seriatopora hystrix* by one-third compared to the controls after 15 d of exposure. It may be possible that *L. purpurea* cannot survive from exposure of glyphosate even if they could settle. Therefore, the effects of chronic exposure of *L. purpurea* to glyphosate after their settlement should be considered for future research.

While brooding corals (e.g. *L. purpurea*) release developed zooxanthellate larvae from the parent colonies, most spawning corals release their eggs and sperm into the water column where fertilization takes place; thus, water quality is especially critical for

reproduction, settlement, and early development in spawning corals. For example, Richmond (1993) reported that a salinity of 28.5‰ and a red clay concentration of 1.28 g/L in runoff at a river mouth reduced fertilization by 53% and prevented 51% of the viable embryos from developing into planulae. Fertilization of spawning coral is practically vulnerable in nearshore waters, where higher boats densities and spray drifts from the land may elevate aqueous environmental concentrations of Irgarol® 1051 and Roundup® Ready respectively. Therefore, the effects of these biocides on fertilization and embryonic development of coral species inhabiting this area should be examined.

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Appendices

APPEDIX A. Mortality of *L. purpurea* to Roundup® Ready over a 96-h exposure period

Concentration (mg a.i./l)	15min.	30min.	1 hr	2hr	4hr	8hr	12hr	16hr	20hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr
SW control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.6	0	0	0	0	0	0	0	0	0	0	0	0	4	6	8	12
10	0	0	0	0	0	10	22	24	34	46	72	90	98	99	100	-
18	0	0	0	0	24	42	64	78	88	100	-	-	-	-	-	-
32	0	0	0	46	64	68	90	100	-	-	-	-	-	-	-	-
56	0	0	0	58	74	84	100	-	-	-	-	-	-	-	-	-

APPENDIX. B. Mortality of *L. purpurea* to POEA over a 96-h exposure period

Concentration (ppm)	15min.	30min.	1 hr	2hr	4hr	8hr	12hr	16hr	20hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr
SW control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetone/SW .	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	6	6	12	14	18
1.35	0	0	0	0	0	0	8	18	22	30	50	72	92	100	-	-
1.8	0	0	0	0	0	36	56	78	90	92	100	-	-	-	-	-
3.2	0	0	0	8	12	72	96	100	-	-	-	-	-	-	-	-

APPENDIX. C. Mortality of *L. purpurea* to glyphosate over a 96-h exposure period

Concentration (ppm)	15min.	30min.	1 hr	2hr	4hr	8hr	12hr	16hr	20hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr
SW control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.6	0	0	0	0	0	0	0	2	4	6	10	18	26	32	40	44
10	0	0	0	0	0	6	16	38	54	72	76	100	-	-	-	-
18	0	0	0	0	4	34	64	88	94	100	-	-	-	-	-	-
32	0	0	0	10	16	66	86	98	100	-	-	-	-	-	-	-

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