OCS Study
MMS 2002-050

Effects of Produced Water on Complex Behavior Traits of Invertebrate Larvae

Final Technical Summary

Final Study Report

U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

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Authors

Peter Raimondi
Principal Investigator
and
Anthony Boxshall

Prepared under MMS Cooperative
Agreement No. 14-35-0001-30758
by
Coastal Marine Institute
Marine Science Institute
University of California
Santa Barbara, CA 93106

U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

Camarillo
January 2002

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Minerals Management Service
Pacific OCS Region
770 Paseo Camarillo
Camarillo, CA 93010
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Suggested Citation

The suggested citation for this report is:

Raimondi, P. T. and Boxshall A. Effects of Produced Water on Complex Behavior Traits of Invertebrate Larvae. MMS OCS Study 2002-050. Coastal Research Center, Marine Science Institute, University of California, Santa Barbara, California. MMS Cooperative Agreement Number 14-35-0001-30758. 38 pages.

Table of Contents

List of Tables

List of Figures

FINAL TECHNICAL SUMMARY

STUDY TITLE: Effects of Produced Water on Complex Behavior Traits of Invertebrate Larvae

REPORT TITLE: Effects of Produced Water on Complex Behavior Traits of Invertebrate Larvae

CONTRACT NUMBER: 14-35-0001-30758

SPONSORING OCS REGION: Pacific

APPLICABLE PLANNING AREA: Southern California

FISCAL YEAR(S) OF PROJECT FUNDING: FY 96, FY 97, FY 98

COMPLETION DATE OF THE REPORT: December 2001

COST(S): FY 96 - $25,000; FY 97 - $33,910; FY 98 - 63,819, FY 99 – no cost

CUMULATIVE PROJECT COST: $122,729

PROJECT MANAGER: Peter Raimondi

AFFILIATION: University of California, Santa Cruz

ADDRESS: Department of Biology, University of California, Santa Cruz, CA 95460

PRINCIPAL INVESTIGATOR: [1]Peter Raimondi

POST DOCTORAL RESEARCHER: [1,2]Anthony Boxshall

ADDRESSES: [1]Department of Biology, University of California, Santa Cruz, CA 95460; [2]Marine Sciences Unit, EPA Victoria, Ernest Jones Drive, Macleod VIC, Australia, 3085

KEY WORDS: ecotoxicology, produced water, oil drilling, invertebrate larvae, bryozoan, *Watersipora* spp*, Bugula neritina, Haliotis rufescens, Schizoporella unicornis*, sub-lethal impact, pulsed exposure, carry-over effects.

BACKGROUND: Produced Water (PW) is a by-product of oil drilling and in the Southern California region a common form of disposal of PW is by direct discharge into the ocean from a platform-based diffuser. Many compounds and elements in PW are known to have toxic effects

on a number of organisms however these toxic effects are not always lethal, even in static conditions.

Short, spiked exposure sees larvae exposed to a pulse of PW for a short period of time and is unlikely to produce lethal effects, however it may result in a series of important sub-lethal effects for organisms in various stages of their lifecycles. It is now generally believed that the discharge of produced water can cause severe, generally sub-lethal, effects to organisms over distances well beyond that predicted by plume dilution models. Plume measurement and modeling has shown that it is more than possible for larvae in the water column 1km away from a diffusion source to contact PW at 1% of it's original concentration.

The early life history stages of invertebrate larvae are very important developmental phases. Some invertebrates and algae have been shown to be particularly susceptible to any negative effects of contaminants during these developmental phases. The impacts seen on larval behavior have included swimming behavior, cue-recognition, settlement and larval survival. Little work has been published that follows the sub-lethal impacts on larvae through to their adult phase. The ecological consequence of carrying-over impacts to the adult phase from sub-lethal impacts on larvae have been rarely tested.

OBJECTIVES: One of the major aims of this project was to test the assumption that sub-lethal impacts on larvae can, and do, carry-over into the adult phase of the invertebrate life-cycle. By exposing invertebrate larvae at an early developmental stage and following their development through to adulthood and beyond, it is possible track the impact of early exposure to PW.

Other aims were more broad: to expand the number of larvae exposed to, and types of sub-lethal impacts assessed from exposure to PW.

DESCRIPTION: By exposing different types of larvae to PW at various stages of their development, we had a number of outcomes:

- First, we expanded the number and type of larvae tested for sub-impacts from pulsed exposure to PW.

- Second, we tested the types of sub-lethal impacts that can occur on invertebrate larvae from exposure to PW. By doing so, we tested for the impact of PW on a number of sub-lethal endpoints, including swimming behavior, cue-recognition, attachment and metamorphosis.

- Finally, we followed some species of larvae through their development into adults and tested for any carry-over impacts for a range of endpoints, including growth, competitive ability, reproduction and survival.

The project tested a wide range of invertebrate larvae, including the bryozoans *Watersipora* spp*, Bugula neritina, Schizoporella unicornis*, the red abalone *Haliotis rufescens*, the sea star *Asterina miniata* and the ascidian *Botrylloides* spp. for carry-over effects from the sub-lethal impact of exposure to produced water as larvae. This broad range represents four different phyla of both

introduced and local species as well as a broad range of types of larval development. All exposures were short-term, spiked exposure in still water conditions in the lab.

SIGNIFICANT CONCLUSIONS:

- All exposures were short-term, spiked exposure in still water conditions in the lab.
- Not all experiments on all invertebrate larvae were successful.
- Those experiments that were successful showed, in broad terms, that there was little evidence for strong sub-lethal effects on the growth, competitive ability or reproductive output of those invertebrates successfully studied. However sub-lethal impacts did occur.
- Where mortality occurred, it tended to be larger in colonies of bryozoans in treatments exposed to concentrations of 10% PW.
- Many and varied sub-lethal impacts were found from exposing larvae to a range of PW concentrations (with 10 % PW having the greatest and most consistent impact).
- Sub-lethal impacts include decreases in swimming capacity, slowed metamorphosis, altered attachment and settlement behaviors, and delayed opercula development.
- Not all invertebrate larvae showed all impacts, nor were tested for the full range of endpoints.
- There was little evidence for strong carry-over effects to adulthood of larval PW exposure, with some caveats.

STUDY RESULTS: The results of this study are fully contained within the Report "Effects of Produced Water on Complex Behavioral Traits of Invertebrate Larvae" presented to MMS.

STUDY PRODUCTS: A number of presentations (6) were given during this work as both invited and conference seminars. Three posters were also given at a range of conferences. The details of all these were contained in the annual reports written for MMS.

There are three manuscripts in preparation from work down during this project:

> Boxshall, AJ and PT Raimondi, The sub-lethal impact on *Watersipora subtorquata* adults from exposing larvae to a toxicant. *In prep. A*
>
> Boxshall, AJ, and PT Raimondi, Carry-over effects on adults from exposing *Bugula neritina* and *Schizoporella unicornis* larvae to sub-lethal toxicant. *In prep. B*
>
> Boxshall, AJ and PT Raimondi, Impacts on adult *Phragmatapoma californica* of exposing larvae to a sub-lethal toxicant. *In prep. C*

FINAL STUDY REPORT

Introduction

Produced Water (PW) is a by-product of oil drilling and in the Southern California region a common form of disposal of PW is by direct discharge into the ocean from a platform-based diffuser. The actual content of PW is very variable on both the spatial (regionally, locally) and temporal (daily from the same field) scales. The salinity of many PWs in California is generally around the mid- to high-20's ppt. PWs can contain a large number of different compounds and elements, including polycylic aromatic hydrocarbons (PAH - e.g., benzene, toluene, naphthalene, phenols), metals (e.g., As, Cr, Ni, Ag, Cd, Cu, Pb, Se, Ba), and other compounds (e.g., cyanides and ammonia). Many of these compounds and elements are known to have toxic effects on a number of organisms however these toxic effects are not always lethal, even in static conditions (e.g., Ray and Engelhardt 1993).

If larvae are entrained in a low concentration plume of PW, exposure could be on-going. However, plume dilution models suggest that many invertebrate larvae are likely to undergo spiked, rather than on-going, exposure to PW (e.g., Washburn *et al* 1999). A short, spiked exposure sees larvae exposed to a pulse of PW for a short period of time. This form of exposure is unlikely to produce lethal effects, however it may result in a series of important sub-lethal effects for organisms in various stages of their lifecycles (Raimondi and Schmitt 1993, Reed and Lewis 1994).

Field studies in the early 1990s in the southern California Bight challenged the belief that the discharge of produced water from oil drilling platforms had little or no effect on organisms in the water column (e.g., Raimondi and Schmitt 1993, Krause *et al* 1993, Reed and Lewis 1994, Reed *et al*. 1994). This belief was based, in part, on the idea that the harmful components of PW have relatively short residence times in the water column. Based on acute (lethal) laboratory tests, the water-soluble contaminants in PW are believed to be diluted rapidly to levels well below those suspected to cause meaningful biological responses. However, the results of the studies noted above support the idea that the discharge of produced water can cause severe, generally sub-lethal, effects to organisms over distances well beyond that predicted by plume dilution models.

Washburn *et al* (1999) modeled and measured the movement of diffused PW in the Santa Barbara channel. They found that the minimum initial dilution from the diffuser was approximately 100 times in summer in a zone within approximately 80m of the diffuser source. The dilution rate increased in winter to approximately 500 times. However, from modeling work they also found it was possible to get patches of PW up to 1000m from a diffusion source with time-averaged dilution factors of 100 and 1000 for summer and winter (respectively). Hence, it is more than possible that larvae in the water column even 1km away from a diffusion source could contact PW at 1% of it's original concentration.

The early life history stages of invertebrate larvae are very important developmental phases. Some invertebrates and algae have been shown to be particularly susceptible to any negative effects of contaminants during these developmental phases (Capuzzo 1987, Raimondi and Reed

1995). Bryozoan (Raimondi *et al* 1997) echinoid (Krause *et al*. 1993) and molluscan larvae (Raimondi and Schmitt 1993), and even algal spores (Reed and Lewis 1994) that are contaminated during this developmental phase show behavioral effects later in the larval phase.

The impacts seen on larval behavior have included swimming behavior, cue-recognition, settlement and larval survival. Little work has been published that follows the sub-lethal impacts on larvae through to their adult phase. There is a suggestion that the metamorphosis that occurs between the invertebrate larval and adult phases can be thought of as "a new beginning" (e.g., Pechenik 1999, Pechenik *et al*, 2001). Clearly, if this is the case, sub-lethal impacts thought to be important for larvae may not be as important from the perspective of the adult organisms. Which the carry-over of impacts to the adult phase, the ecological consequence of sub-lethal impacts on larvae may be diminished. This question has been rarely tested.
One of the major aims of this project has been to test the assumption that sub-lethal impacts on larvae can, and do, carry-over into the adult phase of the invertebrate life-cycle. By exposing invertebrate larvae at an early developmental stage and following their development through to adulthood and beyond, it is possible track the impact of early exposure to PW.

By exposing different types of larvae to PW at various stages of their development, we tested a number of outcomes.

- First, we expanded the number and type of larvae tested for sub-impacts from pulsed exposure to PW.

- Second, we tested the types of sub-lethal impacts that can occur on invertebrate larvae from exposure to PW. By doing so, we tested for the impact of PW on a number of sub-lethal endpoints, including swimming behavior, cue-recognition, attachment and metamorphosis.

- Finally, we followed some species of larvae through their development into adults and tested for any carry-over impacts for a range of endpoints, including growth, competitive ability, reproduction and survival.

By following invertebrates from release through metamorphosis to adulthood as a competing member of a marine assemblage, we can measure the impact of early PW exposure on a different component of the invertebrate's growth or interactions. Despite surviving the initial exposure to PW, the ecological impact of PW exposure on both the organism itself and surrounding organisms is relatively unknown (e.g., see Schüürmann and Market 1997).

In this project, we tried to use a wide range of invertebrate larvae, including the bryozoans *Watersipora* spp*, Bugula neritina, Schizoporella unicornis*, the red abalone *Haliotis rufescens*, the sea star *Asterina miniata* and the ascidian *Botrylloides* spp (see Methods for details on each). This broad range represents four different phyla of both introduced and local species as well as a broad range of types of larval development. Not all tests listed above were done with each invertebrate.

Methods

General Comments

In general, we did similar experiments on various invertebrate larvae. Details of the invertebrates are below (see "Organisms") where a broad overview of the culturing techniques and origins of the various animals is presented.

In all cases, all larval exposure to PW was short between 45 and 90 minutes and occurred in the laboratory at the Long Marine Laboratory (LML) at the University of California, Santa Cruz. Larval culturing facilities were available at LML, which included constant temperature, cultured algal food and flow through water systems. When larvae culture was required, two methods were used. A sunken, constant-flow method (similar to that for molluscs discussed in Strathmann, 1987) was used as well as raising small batches (>1 larva/ml) of larvae in ≥1l beakers of 0.2 µl filtered seawater. Batches were cleaned and fed cultured algae of various species every other day.

In the following section, details of each experimental are discussed. At many times the same method was used for different larvae. This has been noted where it occurred.

Produced Water

The produced water (PW) used in this project was supplied by the Minerals Management Service (MMS) in California. We did not analyze the exact composition of the PW we used in these experiments. Under our agreement with the MMS, we do not know the exact origin of the platform/s from which the PW was taken. We do know there were two samples collected from different platforms on different days in southern California, most likely from within or near the Santa Barbara channel. There was one collection in 1997 and another in 1999. Most experiments used the 1997 collection. After collection of the PW, it was stored on ice. In the laboratory, it was frozen in small aliquots and stored at –80°C within 24 hours of collection. The PW used is not representative of all PW, or even all southern Californian PW (see papers in Ray and Engelhardt, 1993 for discussions of the variation in PW composition). The lack of variation in the samples of PW is a potential a source of experimental error.

In all cases, we exposed the larvae to various concentrations of PW ranging from 0% to 10% (and 25% in some pilot tests) of pre-diffuser levels. Note that all PW concentrations are expressed in % of the pre-release concentrations of PW (i.e., as obtained from the platform downstream of the WEMCO) prior to release via a diffuser. PW was diluted in all experiments using 0.2 µm filtered seawater (0.2SW). The 10% PW treatment was included as a positive control as pilot studies indicated we could expect a sub-lethal larval response to this treatment. We did not expect this concentration to be lethal, however we believed it would elicit negative behavioral responses, such as altering swimming and settlement behaviors. For the same reason we included the 25% PW treatment in some pilot studies. A 10% treatment is quite a high concentration of PW and would generally only be found in close proximity to the diffuser array

of an oil platform (< ~10 m; *pers. comm*. Bill Ford, Chevron, 1997). Washburn *et al* (1999) modeled the movement of diffused PW in the Santa Barbara channel near where we understand the test PW was collected. They showed that the minimum initial dilution was about 100 times (i.e., 1% concentration of the raw PW) in summer (about 500 times in winter) in a zone within approximately 80m of the diffuser source. Hence, the 10% treatment should only be present quite close to the diffuser. A 25% concentration would be unlikely more than 1m from the diffuser array and was only used in pilot studies to produce a known larval response. Larvae are extremely unlikely to encounter PW at 25% in the water column.

Organisms

The bryozoan *Watersipora subtorquata,* is now a common introduced fouling organism in sheltered subtidal waters along the California coast (e.g., Rees 2000). Larvae of *W. subtorquata* were used for a large proportion of this project. The bryozoan *Schizoporella unicornis* is a common native species found in sheltered subtidal areas along the Californian coast (Ricketts *et al* 1985). Both bryozoans brood larvae, which are released after exposure to light. Both have an encrusting, clonal growth form, making them a good target species to follow through settlement and subsequent growth as an adult.

Larvae of *W. subtorquata* and *S. unicornis* were individually collected and stored in a communal beaker until exposed to PW within the first 2 hours of release. For all experiments using these bryozoans, the larvae came from multiple unrelated colonies collected from at least 3 different sites within the Santa Cruz Harbor, Santa Cruz, California, USA. At least 40 different adult colony fragments were used per experiment for both bryozoans. The adult *W. subtorquata* used for spawning in different experiments were from very different stocks and from three different seasons, thus reducing the chance we were re-testing the progeny of the same adults three times. *S. unicornis* were collected for a single experiment.

The upright bryozoan *Bugula neritina* is ubiquitous across the globe (Keough 1989) and found commonly in bays, harbors and sloughs in California. Larvae *of B. neritina* are brooded and released from adults after exposure to bright light. Larvae were individually collected and stored in a communal beaker until exposed to PW within the first 2 hours of release. Adults used in these experiments were collected from Elkhorn Slough, Monterey Bay for use in both 1998 and 1999.

The colonial ascidian *Botrylloides* spp broods larvae which can be released after exposure to light. The adults are encrusting and common in sheltered subtidal areas of the California coast (Ricketts *et al* 1985). A similar protocol to that used for collecting the *W. subtorquata* and *S. unicornis* adults was used with *Botrylloides* spp. The adults used came from the Santa Cruz Harbor. We attempted experiments with the *Botrylloides* spp larvae twice in the summer of 1998 and once in 1999 with limited success.

The sea star *Asterina miniata* is common along the Californian coastline in rocky and sandy areas from the intertidal to >250 m (Ricketts *et al* 1985). We did a series of pilot experiments with the sea star in late 1997. These larvae were cultured using the standard 1-methyladenine method (see

Strathmann 1987 for details). Multiple adults used in these experiments were collected from the intertidal around Santa Cruz and raised in aquaria at LML, UCSC.

The red abalone *Haliotis rufescens* is found subtidally in California. Culturing techniques are well-established (see Boxshall 2000 for information). *H. rufescens* larvae do not feed and are in the plankton for 7 days before they are competent to settle. They cue to a peptide associated with the phycobillins in coralline red algal species, which is a mimetic of the neurotransmitter, GABA (Morse and Morse, 1984).

Specific Methods

Watersipora subtorquata

We ran three experiments with similar methods (see Boxshall and Raimondi*, in prep. A*). The methods used for *Watersipora subtorquata* are a template for the methods used for other larvae. Differences will be noted when they occur.

Larvae were exposed for between 50 and 65 minutes to PW of four concentrations (0%, 0.1%, 1% and 10%). We grew the settlers in the lab for between 8 and 12 days and transferred them to the field for monitoring for between 40 and 150 days. For the first two experiments we pooled all larvae within a treatment into one beaker for the short duration of exposure. This is not the most ideal situation and was forced onto us by low numbers of larvae. It can be argued this results in pseudo-replication, particularly as larval behavior can be quite variable (see any paper in McEdwards 1995). It can also be argued that this practice reduces variation in application of a potentially variable toxicant. Except for this 50 - 65 minute period, the larvae and subsequent adults were raised and monitored individually for the duration of the experiments.

To test if this short-term pooling during exposure in the first two experiments resulted in the loss of important information on the variability of larval reactions to PW we artificially formed batches of larvae for the 3rd Experiment and in some experiments with other invertebrates. If exposing larvae in batches resulted in treatment effects that were different between in batches (i.e., a treatment x batch interaction), we need to be careful with interpretations of the first two experiments. There were very few batch x treatment effects across a range of endpoints (see Results for details).

During Exposure

All larvae were exposed in 100ml of 0.2µm filtered seawater (0.2 SW) and PW (at the required dilution) in 250ml plastic beakers (Markson Lab Supplies) in static water conditions in a water bath at 15 -18°C. All beakers were swirled every 10-15 minutes to discourage settlement during exposure. For the three main experiments, PW concentrations ranged between 0% (the Control) and 10% of the raw PW concentration (see Table 1 for details). In experiment 1, there were 5 treatment levels, but in experiments 2 and 3, there were 4 treatment levels. We did a separate behavioral trial in which the highest concentration was 25%. During exposure, the salinity (30.9 to 31.1 ppt), pH (6.6 to 6.8) and dissolved O_2 (4.35 - 4.5 mg/l) in the different treatments was within the narrow range of those seen in the 0.2SW used as a control, however the pH and dissolved O_2 was at the lower end of this range in the 10% PW treatments.

After Exposure

After exposure, the larval were transferred into beakers containing a 105µm mesh. We used the sunken filter technique (Strathmann 1987) at all times so as not to expose larvae to air. We flushed all beakers at least twice with 0.2SW to remove traces of PW. Larvae were transferred from exposure in the same order in which they were added. After flushing, larvae from each beaker were carefully washed into 0.2SW in 15ml Petri dishes and stored. When all larvae had been flushed and transferred (generally a 10-15 minute process), they were transferred individually into growth beakers.

Growth beakers were 10ml disposable plastic beakers (Fisher Scientific) filled with ~8ml of 0.2SW and contained only one larva each. The larval behavior (see "*Behavioral Endpoints"*) was noted within the first hours and at a number of times while being grown in the lab. Larvae were fed a 1ml mixture of phytoplankton (*Isochyris* and *Rhodomonas*) after they settled and water was changed at least every other day.

Field Outplanting and Monitoring

At outplanting, the colonies were generally only the ancestrula plus some of the first zooid. We gently cut out the plastic beaker around the colonies and glued it a PVC back-board (sizes of the boards ranged up to 75cm x 36 cm depending on the space required in each experiment), which was hung from floating docks in the Santa Cruz Harbor.

For experiments 2 and 3 and days 110 and 150 only of experiment 1 (see Table 1 for details), the outplanted adults were assessed using a camcorder (Sony Hi-8 TR 400: x12 optical zoom) with close up filters (total possible magnification ~ x20). The video images were captured in the lab and analyzed using NIH Image for area, number of zooids and perimeter of colony (NIH Image is a public domain program developed at the US National Institutes of Health and available at http://rsb.info.nib.gov/nih-image/). For all census times before day 110 in experiment 1, colonies were counted using a field microscope. No data about colony size were taken at these census times.

Table 1. A summary of the experimental conditions for *W. subtorquata*

Experiment, Start & Exposure Batches	**Exposure Duration**	**Concentrations**	**Outplanted**	**Census Days**	**Larvae**
1: November 1997 (No Batches)	50 mins	0%, 0.01%, 0.1%, 1%, 10%	After 8 Days For 150 Days	Day 10,20, 30, 39, 81, 110, 150	89 total 70 used
2: February 1998 (No Batches)	50 mins	0%, 0.1%, 1%, 10%	After 8 Days For 80 Days	Day 20, 25 40, 60, 80	46 total 39 used
3: August 1998 (4 Batches)	65 mins	0%, 0.1%, 1%, 10%	After 12 Days For 40 Days	Day 10, 20, 40	323 total 192 used

Behavioral Endpoints

At each stage, we measured mortality and any visible abnormalities in the larvae or adults. Following are the endpoints for each phase of the experiments with *W. subtorquata* but many are common to the experiments with other larvae.

During Exposure

- The swimming behaviour of the larvae. This was assessed as either swimming or not. Other experiments ran parallel to the exposure experiments to quantify larval swimming (see "Behaviour Trial").
- The number of larvae settled.

After Exposure

In this phase, larval behavior was placed into one of 7 categories:

- Swimming;
- Searching - temporarily attached;
- Not moving;
- Metamorphosed;
- Operculum visible (in later counts, this became the number of opercula visible);
- Dead (clearly dead with a evidence remaining);
- and unknown (this included larvae that disappeared).

Some of the categories were pooled for analysis (e.g., the categories: 'searching - temporarily attached' and 'metamorphosed' are both a part of the settlement process and were often lumped together as 'settling'.

We measured:

- Larval behaviour soon after exposure (within <1 to 3 hours).
- Larval behaviour one day (24 hours) after exposure.
- Larval behaviour at various times during the lab growth phase (details are shown where data are reported).

Field Outplanting and Monitoring

The colony size was measured as both the number of zooids and zooid size (mm^2). We measured a number of endpoints.

- The number of zooids at outplanting.
- The adult growth was measured as the change in the number of zooids between census dates (see Table 1 for census details).
- The zooid size (average zooid size in per mm^2) was also calculated for each census date. This measure of colony size allows the zooids counts to be scaled for differential growth in the colonies by taking into account the area the colony occupies.
- The competitive ability of the colonies. This experiment was only attempted with *Watersipora subtorquata.* The competitive load on the colonies was assessed for each treatment. Competitive ability was measured as the ability of colonies to maintain themselves against competing neighbours ("draws"), the inability to withstand overgrowth by neighbours ("losses"), or the ability to overgrow neighbours ("wins"). A drawn situation is a

stalemate where neither the target colony nor the neighbour has the upper hand and there is a change in growth pattern of both competitors. A loss is when the target colony has been overgrown, whereas in a win the target colony has overgrown its neighbours. From a *W. subtorquata* colony perspective, these situations are not independent interactions as most *W. subtorquata* colonies will experience at least two of these conditions at one time. A loss results in the reduction of biomass and hence potential reproductive output due to overgrowth, and should be more detrimental to a colony than a draw. In a loss situation, we estimated the proportion of total area overgrown on the target colony. This was done on the computer screen with captured images by predicting the growth pattern in the absence of the ascidian based on the growth at this and previous census dates. It is an estimate as it is hard to define exactly where a colony is underneath a competitor. However, due to the controlled nature of the surface in this experiment, even after 150 days the colonies were quite regularly shaped. The competitors that were in large enough numbers to analyse were colonial ascidians of two genera: *Botryllus* and *Botrylliodes*.

Analyses

Mortality and behavior

To analyze most behaviors and mortality, we used hierarchical, log linear modeling with batch, PW concentration (treatment) and the behavior as categorical variables (Sokal and Rohlf 1995). Initially, a model is fitted with all the interaction terms and used to calculate the G^2 statistic. To find the significance of each term, you remove that term, re-run the model and calculate a ΔG^2, which is compared to a distribution similar to the χ^2 distribution.

In Experiment 3, we exposed larvae to PW in four artificially allotted batches as opposed to the pooled exposure in experiments 1 and 2. We tested for an effect of batch on larval reactions to the treatments at various stages:

- The swimming behaviour of the larvae 1 hour after being removed from PW.
- The larval metamorphosis 24 hours after being removed from PW.
- The mortality 24 hours after being removed from PW.
- The number of colonies with opercula by Day 4.
- The mortality at outplanting.
- The size of colonies at outplanting.
- Mortality of the colonies at Day 10, Day 20 and Day 40.
- the size of the colonies at Day 10, Day 20 and Day 40.

Of particular interested are any batch x treatment effects on larval behaviors: swimming, settled/metamorphosed, operculum development and survival. Some of the interactions are biologically meaningless and so we have not included them in the results. We have detailed the results for the batch x treatment x "behavior", treatment x "behavior" and batch x "behavior". If there is a significant batch x "behavior" interaction, it simply shows that larvae in different batches had different behaviors, regardless of treatment. This interaction may be biologically interesting but is not important in the context of these experiments as we are only interested in interactions of behaviors with the treatments. A significant batch x treatment x "behavior" indicates that the difference in behavior with treatment depends on the batch of larvae used. When there was a batch x treatment x 'behaviour' effect, we checked the frequencies for a

pattern. If there was a clear pattern, we removed that batch and re-ran the analysis. As this is technically an unplanned comparison (Sokal and Rohlf, 1995), we corrected the alpha level for the unplanned test. Generally there was only one extra test and hence we I used an error rate of $\alpha = 0.025$. This is a very conservative error correction (Sokal and Rohlf, 1995).

A significant treatment x "behavior" indicates that the larval behavior differs between treatments. To ascertain which treatments were important, we made 3 planned comparisons using the Fisher's Exact χ^2 or the Yate's corrected χ^2, whichever was appropriate (Sokal and Rohlf, 1995).

Growth and competitive ability

All data measuring growth and competitive ability of adults or larvae were analyzed using variations of ANOVA. Where necessary the data were transformed to maintain homogeneity of variances and normality (Underwood 1997). We analyzed the growth data with a repeated measures ANOVA where growth was the repeated measure (assessed as the change in number of zooids between census dates).

The data in experiments 1 and 2 were analyzed using ANOVA with Treatment (fixed factor; 4 or 5 levels) as the single factor. The data in Experiment 3 were analyzed using 2-way ANOVA with Batch (4 levels) and Treatment (4 levels) as fixed factors. The treatment effects in this experiment were compared between batches first and if no Batch x Treatment interaction was found, the data were pooled across batch for further analysis of the treatment effect. For each significant treatment effect, we compared the control to each treatment level in a pairwise comparison using a two-sided Dunnett test (Underwood 1997) as this was the most biologically interesting. Some marginally non-significant results with low power were also tested using this method.

Due to low sample sizes in some experiments possibly resulting in increased Type II errors, we checked the power of all non-significant tests to pick up a change using Pass 6.0 (www.ncss.com/pass). We based the effect size on natural levels of variation in this system and the alpha level was set at 0.05.

Behavioral Trial

We ran one experiment separately from the exposure-outplant experiments to detail the behavior of the larvae during exposure. The larvae used in the behavior trial came from a subset of the batch of adults used in Experiment 3 and the trial started the day before the release of larvae for Experiment 3.

We did the behavior trial in 20 ml disposable plastic beakers (Fisher Scientific) filled with at least 10 ml of 0.2SW and PW. In this trial, there were 5 treatments: 0%PW, 0.1%PW, 1%PW, 10%PW and 25%PW with 5 replicates of each. We used 186 larvae placing multiple larvae in each beaker (4-8 larvae per beaker) and calculated the % swimming after 15, 30, 45, 75 minutes and 23 hours. We also noted if any larvae attached or metamorphosed. These larvae were never washed from the PW and never outplanted. These data did not require analysis for the swimming behaviors at 15 and 75 minutes, however we used a 1-way ANOVA to analyze the proportion

attached after 75 minutes and a two-sided Dunnett test to compare between treatments. After 23 hours of constant exposure, we analyzed the proportion of larvae metamorphosed in each treatment with a similar one-way ANOVA and two-sided Dunnett test.

For extra behavioral information we also quantified the swimming behavior of the larvae used in Experiment 3 during the first 10 minutes of exposure and up to 75 minutes of being transferred to clean 0.2SW. There were 4 replicate batches of each treatment in this test with between 14 and 25 larvae in each. The analysis was the same as the main behavioral trial but data were analyzed for the first 5 to 10 minutes during exposure and at 75 minutes after exposure only.

Schizoporella unicornis

We ran one experiment in June 1998 with methods similar to those used for *Watersipora subtorquata.* The larvae were raised in the lab for 17 days after exposure to the 4 standard PW concentrations (Boxshall and Raimondi, *in prep. B*). Larval development was followed in parallel studies with similar behavioral endpoints measured as with *W. subtorquata*. The colony size was measured at outplanting. There was extraordinary growth on the outplanted boards for this experiment, which obscured much further analysis. Analyses were the same as those used above.

Bugula neritina

We ran a series of experiments in late 1999 using the larvae of the upright bryozoan, *Bugula neritina* (Boxshall and Raimondi, *in prep. B*). The general methods were very similar to those used for *W. subtorquata.* One important difference is in the method for assessing the size of the colony. As *B. neritina* have an upright growth form, the standard way to assess growth is to count the number of bifurcations in the colony (Keough 1989). The size of the colonies was measured at outplanting and at Day 70 when the experiment ended. Another difference was that the number of ovicells present on the colonies was counted at the end of the experiment. For analysis, the number of ovicells was scaled for size of the colony (i.e., number of bifurcations) and analyzed as $\log_{10}$ (ovicell density+1). This enables some estimate of reproductive differences between treatments. *B. neritina* were batched for all experiments. There were no differences between batches that affected the treatments (Boxshall, unpubl. data).

***Botrylloides* spp.**

We ran a series of experiments in mid 1998 using the larvae of the colonical ascidian, *Botrylloides* spp. The general methods were very similar to those used for *W. subtorquata.* These experiments were not successful as the larvae only swim for approximately 20 minutes before settling. We obtained preliminary data on the swimming behavior of *Botrylloides* spp (Boxshall, unpubl. data).

Asterina miniata
The behaviors of these larvae to a biofilm cue were far less specific than we had hoped, making them untractable for use in PW experiments. Although an extensive series of pilot tests were complete, we were unable to find a tractable, cued surface to which this larvae would attach. *Asterina miniata* show very interesting swimming behaviors and would be very useful for future work into the detailed impacts of PW on swimming behaviors of invertebrates.

Haliotis rufescens
The abalone larvae were tested for settlement ability as competent larvae after exposure to four treatments of PW in 0.2SW (0%, 0.01%, 0.1% and 10%) for one hour. As a cue to settlement, 10^{-6} GABA was used. The proportion of larvae settled in 20ml disposable beakers was assessed and compared to control beakers without GABA. The proportion of larvae settled was tested using a two-way ANOVA (Treatment: 4 levels; Cue: 2 levels). Data did not require transformation.

Results

General Comments

This results section is a summary of many of the important results from the project. Further detailed results and analyses are to be published in a series of papers (Boxshall and Raimondi, *in prep A, B and C*).

Specific Results

Watersipora subtorquata
<u>Batched Exposure vs Pooled Exposure</u>
In summary there was no effect of batching the larvae during exposure on larval activities very early in life (i.e., swimming (table 2), metamorphosis at 23 hours (table 3), survival in the first 24 hours (out of 185 larvae, only one had clearly died and one other had disappeared from different batches) or later as adults (growth at outplanting, and both growth and survival at Days 10 and 40 (table 4)). However, there was a difference b/n batches in the development of opercula (Day 4) in the lab and the survival of juveniles to outplanting (table 5 and 6). These are early stages of development, but after metamorphosis.

Table 2: *W. subtorquata* swimming behaviors. Swimming 75 minutes after being washed from PW.

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x sw (full model)	9.2970	9	0.410			
treatment x sw	15.77	12	0.202	6.47	3	0.091
batch x sw	17.47	12	0.133	8.17	3	**0.043**

Table 3: *W. subtorquata* larval metamorphosis 23 hours after being washed from PW.

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x met (full model)	10.4019	9	0.319			
treatment x met	26.38	12	0.010	15.98	3	**0.001**
batch x met	13.31	12	0.347	2.90	3	0.407

0% Vs 0.1% (i.e., without 1% and 10%)

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x met (full model)	1.375	3	0.771			
treatment x met	10.18	4	0.037	8.81	1	**0.003**
batch x met	5.33	6	0.502	3.96	3	0.266

0% Vs 10% (i.e., without 0.1% and 1%)

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x met (full model)	4.142	3	0.247			
tmt x met	12.85	4	0.012	8.71	1	**0.003**
batch x met	4.46	6	0.614	0.32	3	0.956

Table 4: Survival of *W. subtorquata* colonies to the final day in Experiment 3 (Day 40).

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x surv (full model)	11.195	9	0.263			
tmt x surv	13.39	12	0.342	2.19	3	0.533
batch x surv	11.58	12	0.480	0.39	3	0.943

Table 5: *W. subtorquata* survivorship to outplanting in Experiment 3, comparing the batches. We have included mean survival for comparison.

Treatment	% Total Survival				
	Mean	Batch 1	Batch 2	Batch 3	Batch 4
0%	98	92	100	100	100
0.1%	95	100	100	100	80
1%	87	73	92	82	100
10%	84	92	83	92	67

Table 6: *W. subtorquata* survivorship to outplanting in Experiment 3.

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x surv (full model)	**17.415**	**9**	**0.043**			
tmt x surv	24.06	12	0.020	6.64	3	*0.084*
batch x surv	19.32	12	0.081	1.91	3	0.592

Re-analyzed without Batch 4. Note an alpha level of 0.025 this analysis.

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x surv (full model)	**6.478**	**6**	**0.371**			
tmt x surv	**17.65**	**9**	**0.039**	**11.18**	**3**	**0.011**
batch x surv	7.95	8	0.439	1.47	2	0.479

Re-analyzed survival as planned comparison s without Batch 4 to test which tmt differed from the control.

	Pearson χ^2	df	*p*	Fisher's Exact (*p*)
0 % vs 10 %	1.934	1	0.354[#]	*0.357
0% vs 1%	8.016	1	**0.013**[#]	***0.006**
0% vs 0.1% ^				

[#] We have used a corrected alpha level of 0.017 to test the null hypotheses due to previous comparisons of these data.

* Due to small number of frequencies in some cells, I have used the conservative Yate's corrected χ^2 in the 1% and 10% comparison (Sokal and Rohlf, 1981). I have also shown the Fisher's Exact test *p* for comparison.

^ There is no difference in survival between batches 1,2 and 3 for this treatment.

Behaviour during and after exposure

The larvae consistently swam less (Figure 1), settled less (Table 7) and showed less movement of any kind under the influence of higher concentrations of PW (Boxshall, unpubl. data). Not surprisingly, the 25% PW impacted heavily on larvae (table 7). There was little difference in the activity of larvae within hours of exposure to PW if they were washed in clean, filtered seawater (Table 8).

After 24 hours, most larvae ×± sd : 72 ± 18%) had metamorphosed and there was no difference between treatments (df= 3, G^2 = 4.96, *p*= 0.176). By day 3 (66 ± 12%) or day 4 (77 ± 8%) the number of colonies with opercula also did not differ between treatments (Day 3: df= 3, G^2 =1.49, *p*= 0.684; Day 4: df= 3, G^2 = 0.86, *p*= 0.836).

There was no difference between all the treatments for the number of colonies that had developed opercula by either 3 (× ± sd : 73 ± 7%) or 4 (86 ± 6%) days after exposure (Day 3: df= 4, G^2 =1.42, *p*= 0.840; Day 4: df= 4, G^2 = 1.76, *p*= 0.779).

Table 7.

A. The proportion of *W. subtorquata* larvae attached (settling) after 75 minutes of exposure. These data did not require transformation.

Source	**df**	**MS**	**F-ratio**	***p***
Treatment	4	0.249	12.808	**0.000**
Error		20	0.019	

Two-sided Dunnett test

Tmt	**Mean differences from control**	***p***
0.1%	-0.018	0.999
1%	-0.159	0.245
10%	-0.449	**0.000**
25%	-0.452	**0.000**

B. The proportion of *W. subtorquata* larvae metamorphosed after 23 hours of constant exposure. These data were arcsine transformed due to the high number of cells with a value of 100%.

Source	**df**	**MS**	**F-ratio**	***p***
Tmt	4	1.207	12.722	**0.000**
Error	20	0.095		

Two-sided Dunnett test

Tmt	**Mean differences from control**	***p***
0.1%	-0.024	1.000
1%	0.043	0.998
10%	-0.257	0.501
25%	-1.127	**0.000**

Figure 1. Proportion of *Watersipora subtorquata* swimming during exposure to PW after 15 minutes and 75 minutes.

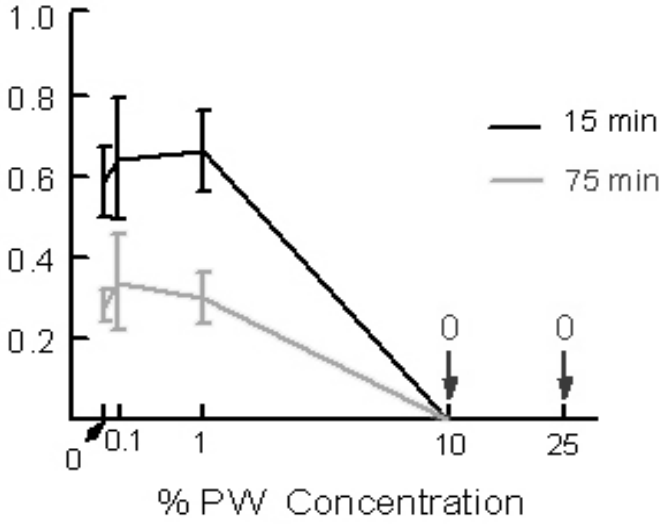


Table 8. The percentage of *W. subtorquata* larvae showing each behavior 3 and 31 hours after exposure. The numbers in brackets are the actual number of each larvae showing each behavior.

Treat-ment	Percent Post-exposure behavior								
	Percent 3 hours after exposure					Percent 31 hours after exposure			
	total larvae	swim	settlement activities	not moving	dead	swim	settlement activities	not moving	dead
0%	**10**	10 (1)	90 (9)				100 (10)		
0.01%	**15**		80 (12)	7 (1)	13 (2)		87 (13)		13 (2)
0.1%	**15**	13 (2)	80 (12)		7 (1)		87 (13)		13 (2)
1%	**15**	7 (1)	73 (11)	20 (3)			93 (14)		7 (1)
10%	**15**		67 (10)	20 (3)	13 (2)		80 (12)		20 (3)

Mortality

When larval mortality occurred, it tended to be in higher concentrations of PW and was visible after the first hours of exposure rather than during exposure. There was no mortality during exposure in Experiments 1, 2 or 3.

In Experiment 1, there was no mortality at all in the control (0%) during the laboratory phase of this experiment. What mortality that did occur was in PW treatments. It was not possible to test these data due to small sample sizes (low mortality in >1/3 of the cells). However, there was little mortality and very little difference between treatments with PW at any census time in the lab. At outplanting, there had been a uniform 20% mortality in all the PW treatments.

Most mortality in the field occurred between Day 39 and Day 81. However by day 81there was no difference in mortality between the treatments (df=4 , G^2 =1.82 , p=0.768). By Day 150, there was no difference in mortality between the treatments (table 3; df=4 , G^2 =2.04 , p=0.728) but note that the there was more mortality in the 10% treatment than in the 0%. The highest survivorship of colonies that were outplanted was in the 1% treatment.

In Experiment 2, there was no difference in mortality between the treatments 15 minutes after exposure (only 3 larvae dead: 2 in 0.1%, 1 in 1%), 24 hours after exposure (total of 4 larvae dead: 3 in 0.1%, 1 in 1%) or 8 days after exposure, prior to outplanting (df= 3, G^2 = 1.27, p= 0.736). The small numbers in this experiment meant that testing for mortality differences was generally not possible (low mortality in >1/3 of the cells). There was little difference in mortality as the colonies grew in the field. The only sizeable mortality occurred in the 10% treatment and mainly between Day 40 and Day 60. In experiment 2, the highest mortality was in the 10% treatment and the highest survivorship was the 1% treatment, as in experiment 1.

In experiment 3, there was little early mortality in this experiment in the lab. There was no mortality within an hour of transferring the larvae from PW to clean 0.2SW. The mortality 24 hours after being removed from PW was very low. Two larvae out of 185 were dead or missing. There was no batch x treatment effect on early mortality.

There was increased mortality at outplanting in treatments with higher PW concentrations. However, there was no clear pattern amongst batches. There was a significant batch x treatment x survival interaction but the reasons are not that clear (Table 6). Batch 4 seemed to be driving that result, as evidenced by the lack of batch x treatment x survival interaction in the analysis without batch 4 (table 6). When batches 1,2 and 3 were pooled and re-analyzed, there was no statistical difference in survival between the control and 10 % treatment, however there was statistically greater mortality in the 1% treatment than in the control (table 6).

There was very little difference in mortality of the colonies at day 10 or day 20, between the treatments or within the batches in each treatment and no statistical differences at day 40. At days 10 and 20 it was not possible to test for a batch x treatment interaction, or even treatment effects as not enough larvae had died. Only 8 larvae out of 128 were dead at day 10 (15/128 at day 20). There was little clear pattern to this slight mortality, but the mortality in the 10% treatment was always greater than the control. Most mortality occurred from day 20 to day 40. When the mortality from day 20 to day 40 was compared, there was no difference in survival between treatments or within batches (Table 9).

Table 9. Experiment 3. *W. subtorquata* survival from Day 20 to Day 40.

	G^2	df	*p*	ΔG^2	df	*p*
batch x tmt x surv (full model)	14.460	9	0.107			
tmt x surv	16.22	12	0.181	1.76	3	0.624
batch x surv	14.55	12	0.267	0.09	3	0.993

Growth

In experiment 1, the colonies were the same size in all treatments at outplanting ($F_{4,53}$ =1.040, *p*=0.396), but given the variability in the sizes, the power was low (26%). From outplanting to day 150, the growth trajectory was similar between all treatments (figure 2, Table 10). There was no effect of PW exposure at all on the growth rates over time.

Table 10. Experiment 1. Repeated Measures of *W. subtorquata* growth rates from Day 0 to Day 150. Given the p values, I have only included the univariate results and given the epsilon values, we have included the GG corrected p-values.

Between Subjects

Source	**df**	**MS**	**F-ratio**	***p***
Tmt	4	0.468	2.026	0.120
Error	26	0.231		

Within Subjects

Source	**df**	**MS**	**F-ratio**	***p***	**G-G**
Time	6	11.787	76.591	0.000	0.000
Time*Tmt	24	0.086	0.557	0.953	0.804
Error	156	0.154			
Greenhouse-Geisser Epsilon:		0.3261			
Huynh-Feldt Epsilon:		0.4066			

The final size of the colonies, measured simply as the number of zooids, was not different between treatments at day 150 ($F_{4,30}$ = 1.320, *p*=0.285), however the power in this test was low (32%) and there is a trend for smaller final size in the 10 % PW. The average final size of the zooids at Day 150 was not different between treatments (table 11, figure 3). As the result was marginal at an α =0.05 level (*p*=0.061) and the power was low (power=58%), we ran a pairwise comparison of the PW treatments with the control. There was no difference between any of the treatments and the control (table 19). Due *a priori* decisions, we only tested the PW concentrations against the control. Constrained by degrees of freedom, we can only speculate that the effects of exposure may be stronger at intermediate concentrations (Figure 3).

Table 11. The size of *W. subtorquata* zooids in Experiment 1 at day 150. These data did not require transformation.

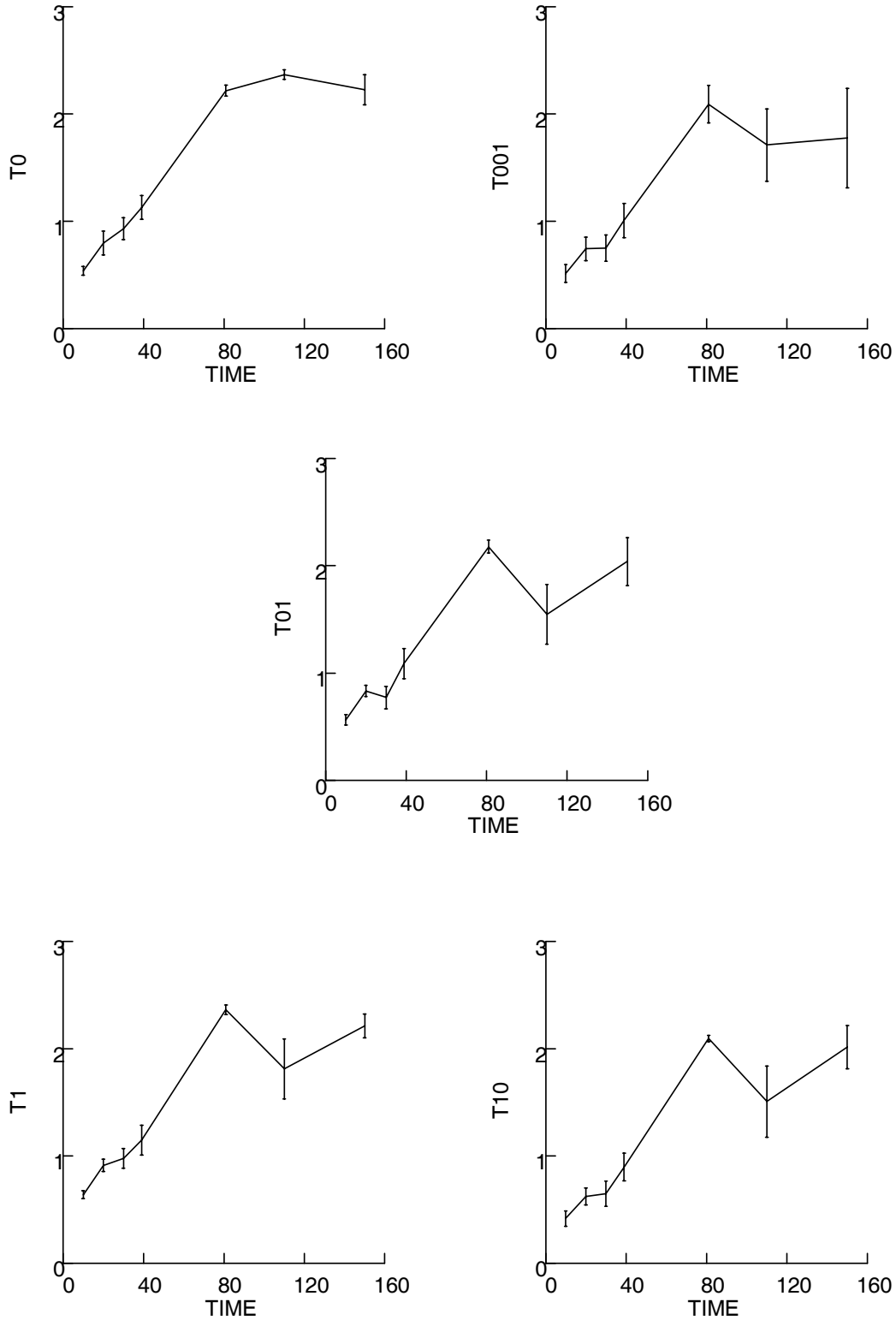
Source	**df**	**MS**	**F-ratio**	***p***
Treatment	4	0.572	2.541	0.061
Error	29	0.225		

Two Sided Dunnett Test

Tmt	**Mean differences from control**	***p***
0.01%	-0.277	0.696
0.1%	0.439	0.292
1%	0.288	0.598
10%	-0.106	0.984

In experiment 2, all the colonies surviving to outplanting were the same size ($F_{3,34}$ =0.242, p=0.867), as were the subset chosen to ouplant ($F_{3,22}$ =0.850, p=0.482). From outplanting to Day 80, there was no difference in the growth trajectory due to the treatments (table 12). The final size of the colonies (day 80), measured as the number of zooids ×± se : 521 ± 50) was not different between treatments ($F_{3,17}$ =0.321, p=0.810). Nor was the average size of the zooids (× ± se:0.617 ± 0.03 mm^2) different between treatments at day 80 ($F_{3,22}$ =0.282, p=0.838).

Figure 2. Experiment 1. Adult growth in the field for *W. subtorquata* over the entire duration of the experiment. Growth is measured in Log_{10} (growth+1), where growth = the number of zooids added between census dates. T0 to T10 represent the different PW treatments and time is measured in days.



In experiment 3, all colonies were statistically the same size (measured as the number of zooids) at outplanting. There was no interaction with, or difference due to the main effect of, batch when the colonies were outplanted (Table 13). Also colonies had a similar number of zooids regardless of treatment.

From outplanting to Day 40, there was no difference in the growth trajectory for any treatment or due to any batch x treatment interactions (Table14). The average size of zooids at day 40 differed between treatments. At the alpha = 0.05 level, there was no batch x treatment interaction, however there was a significant effect of treatment (Table 15). The test of batch x treatment (p=0.056) has a power of 100% so we are comfortable there was no batch x treatment effect at an alpha = 0.05 level. There is no consistent pattern amongst treatments. I pooled the batches and have analyzed the zooid size between the treatments (table 15). There is a statistical difference between some of the treatments however none are different to the control.

Figure 3. Experiment 1. The final size of *W. subtorquata* colonies at Day 150. Both axes are displayed as logs.

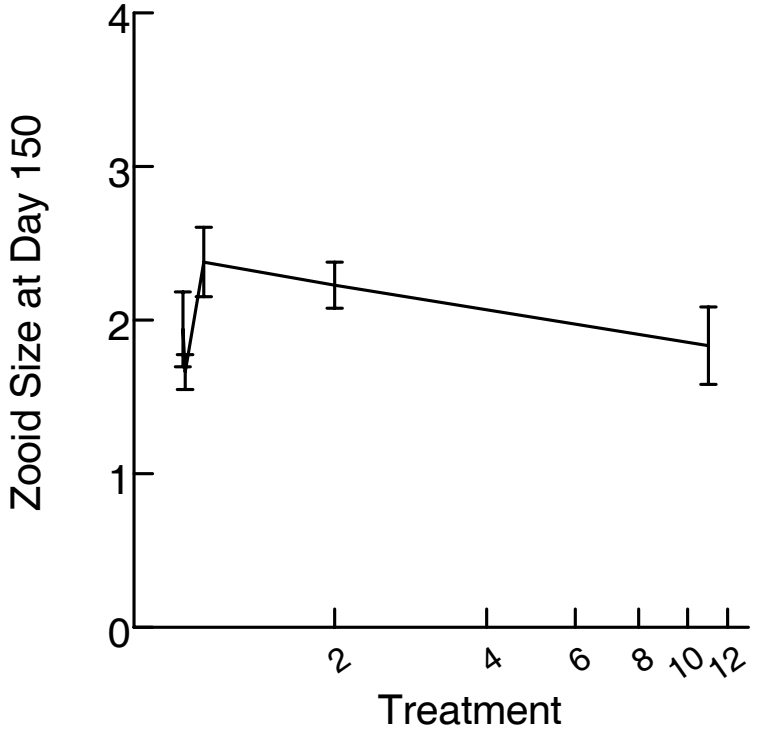


Table 12. Growth rates of *W. subtorquata* throughout Experiment 2. The univariate results from a repeated measures ANOVA. These data have been $\log_{10}$ transformed.

Within Subjects	**df**	**MS**	**F-ratio**	***p***	**G-G**
Growth	4	10.815	505.340	**0.000**	**0.000**
Growth x Tmt	12	0.022	1.021	0.445	0.439
Error	48	0.021			
Greenhouse-Geisser ξ	0.608				
Huynh-Feldt ξ:	0.964				

Table 13. The size of *W. subtorquata* colonies in Experiment 3 at outplanting.

Variable	**df**	**MS**	**F-ratio**	***p***
Batch	3	0.104	0.529	0.663
Tmt	3	0.063	0.322	0.810
Batch*Tmt	90.169	0.858	0.564	
Error	149	0.197		

Table 14. Growth rates of *W. subtorquata* throughout Experiment 3. This repeated measures reports the unadjusted *p*-values as the G-G and H-F ξ values are 1 or close to it .

Within Subjects	**df**	**MS**	**F-ratio**	***p***
Growth	2	37.294	922.053	**0.000**
Growth x Batch	6	0.057	1.421	0.211
Growth x Tmt	6	0.039	0.975	0.445
Growth x Batch x Tmt	18	0.049	1.223	0.251
Error	134	0.040		
Greenhouse-Geisser ξ:	0.982			
Huynh-Feldt ξ:	1.000			

Table 15

A. Experiment 3. Average *W. subtorquata* zooid size (log_{10}(zooid size+1)) at the final census date (Day 40). This test has a power of 100%.

Source	**df**	**MS**	**F-ratio**	***p***
Batch	3	0.001	1.518	0.217
Tmt	**3**	0.006	5.843	**0.001**
Batch x Tmt	9	0.002	1.973	**0.056***
Error	69	0.001		

B. Experiment 3. Average *W. subtorquata* zooid size (log_{10}(zooid size+1)) at the final census date (Day 40) pooled across batches.

Source	**df**	**MS**	**F-ratio**	***p***
Tmt	**3**	**0.007**	**6.485**	**0.001**
Error	81	0.001		

Two Sided Dunnett Test

Tmt	**Mean differences from control**	***p***
0.1%	-0.020	0.117
1%	0.022	0.062
10%	0.008	0.795

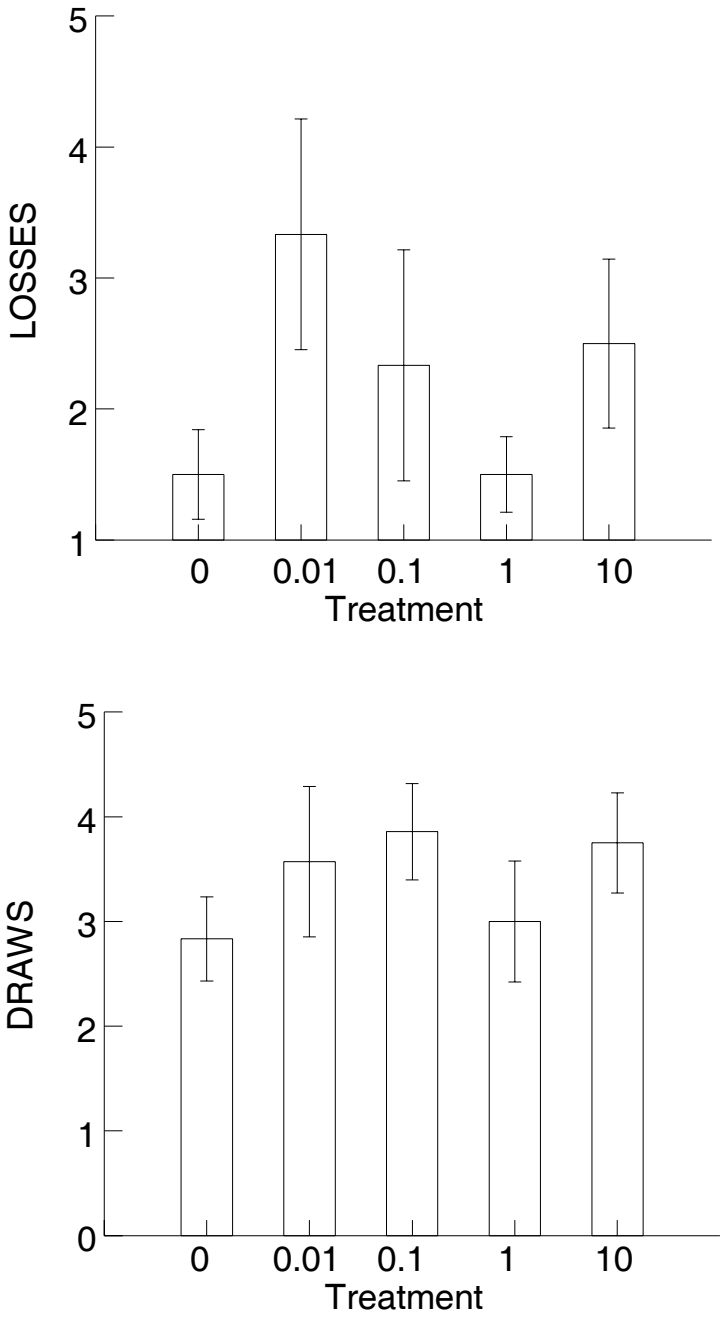
Competitive ability

In experiment 1, we could identify a total of 7 deaths in the entire experiment due directly to overgrowth. There was no real pattern, with each treatment represented once except for 3 from 0.1%. At day 150, every colony except one was interacting on average with at least 3 colonial ascidians. There was no difference in the number of ascidians (×± se : 4.3 ± 0.4) interacting with target colonies across treatments ($F_{4,31}$ = 0.744, p=0.569) although there was only 17.5% power. This measure can be considered a crude indicator of the overall competitive load on the colonies due to interactions with colonial ascidians. There were never situations where the *W. subtorquata* colony clearly overgrew the ascidian (a "win"). Almost every colony was involved in a "draw" with multiple ascidian competitors but there is no clear pattern from these data ($F_{4,26}$ = 0.648, p=0.633; Figure 4). Most colonies had a "loss" but again there was no clear pattern across treatments ($F_{4,13}$ = 1.263, p=0.334, power = 12%; Figure 4). There was no difference in the proportion of estimated area lost through overgrowth ($F_{4,14}$ = 0.728, p=0.587) and, again, the power was low (17%). These data were log_{10} transformed to maintain normality for analysis.

Of the 21 colonies remaining in experiment 2 at day 80, ten were interacting with neighboring colonial ascidians however there was no clear pattern from these interactions. Eleven colonies had no neighbors at the time of census, including all remaining colonies from the 10% treatment. No colonies were being overgrown by colonial ascidians (Loss), 3 were overgrowing colonial ascidians (Wins: 1 x 0%, 2 x 1%) and 7 had 1 or more colonial ascidians touching them without a clear overgrowth by either the bryozoan or ascidian (Draws). The only colonies that clearly died from overgrowth were both from the 10% treatment (1 at Day 40, 1 at Day 80). Overall,

again there was no clear decrease in competitive abilities across treatments. Due to the shorter duration of experiment 3, there were not enough competitive interactions for analysis.

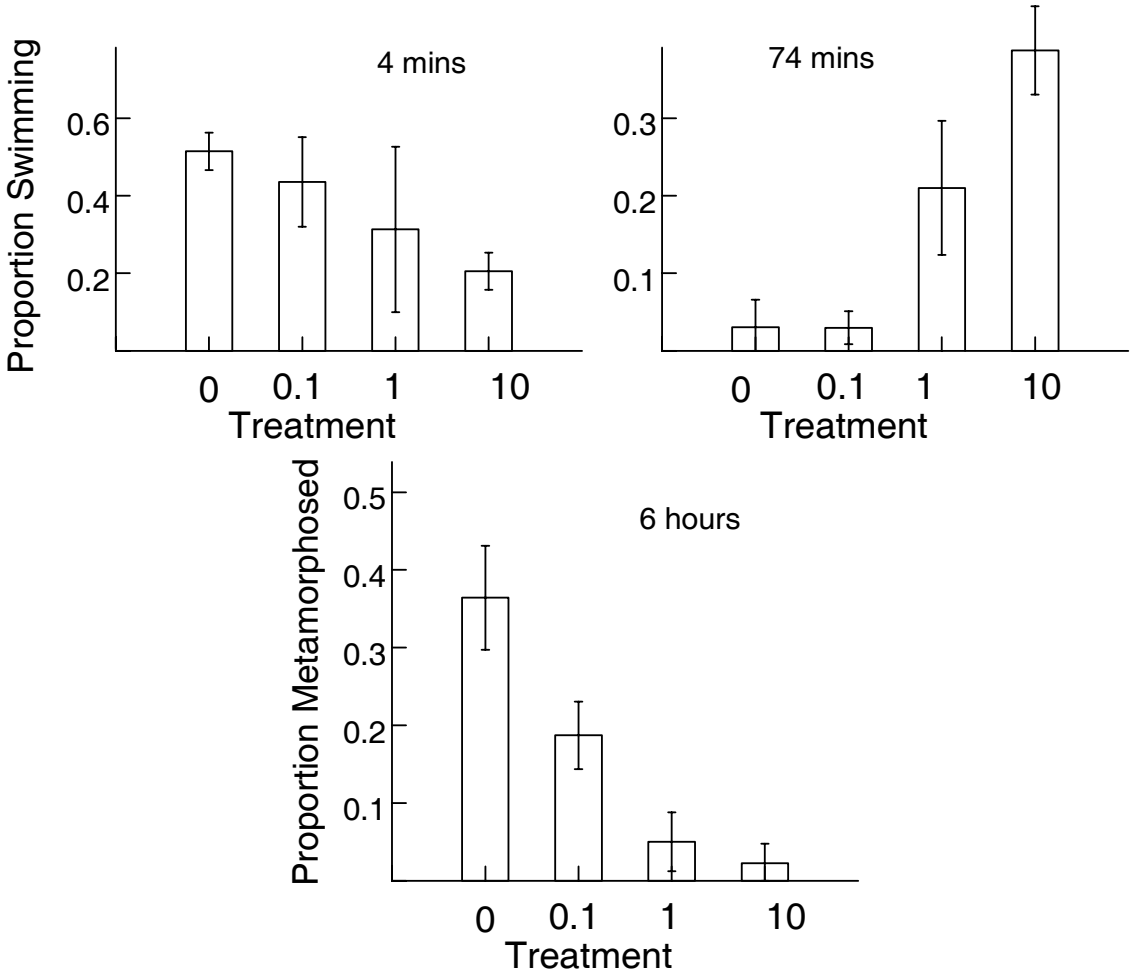
Figure 4. Competitive interactions of *W. subtorquata* with colonial ascidians. Note the difference in scales. For details of what constitutes 'Losses' and 'Draws', see text.



Schizoporella unicornis

The *Schizoporella unicornis* larvae showed an interesting pattern similar to *W. subtorquata* larvae. After four minutes of exposure to PW, the trend was for larvae to be swimming more in the lower PW concentrations. Those not swimming were searching on the surface or not moving. More were in the "not moving" category at 10% PW concentration (Figure 5). After 74 minutes of exposure, there were more larvae swimming in the higher concentrations. However, at this time, those in the lower concentrations had started to attach and metamorphose. By 6 hours of exposure, many more *S. unicornis* larvae had metamorphosed in the control than any other PW treatment, especially when compared to 10% PW (figure 5). Mortality was negligible during this exposure test, and after exposure in other tests while larvae were in the laboratory.

Figure 5. The proportion of *S. unicornis* larvae swimming and metamorphosed at different times after exposure to PW. Note the different scales.

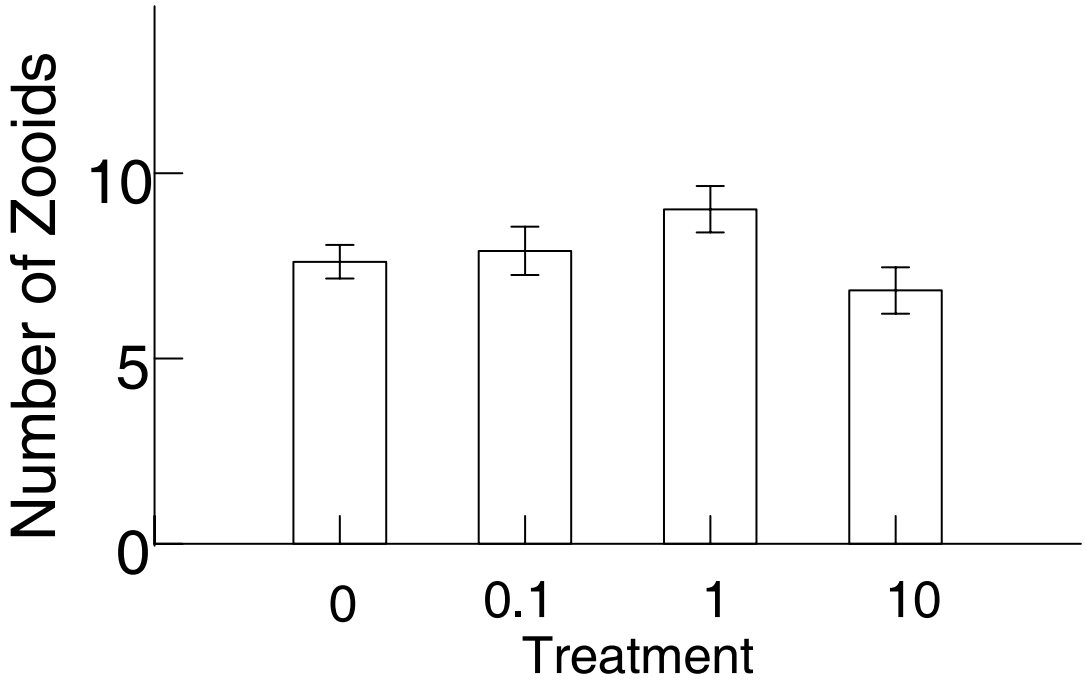


By the time for outplanting the *S. unicornis* colonies, there was no difference in the size of the colonies as measured by the number of zooids, between the treatments. There was no difference between the four batches of larvae, however there was a trend for the colonies in 10% PW to be smaller than the other colonies (Table 16, Figure 6).

Table 16. The size of *S.unicornis* colonies at outplanting to the field.

Source	**df**	**MS**	**F-ratio**	***p***
Batch	3	9.093	0.865	0.461
Tmt	3	25.870	2.462	0.066
Batch x Tmt	9	11.891	1.132	0.347
Error	109	10.509		

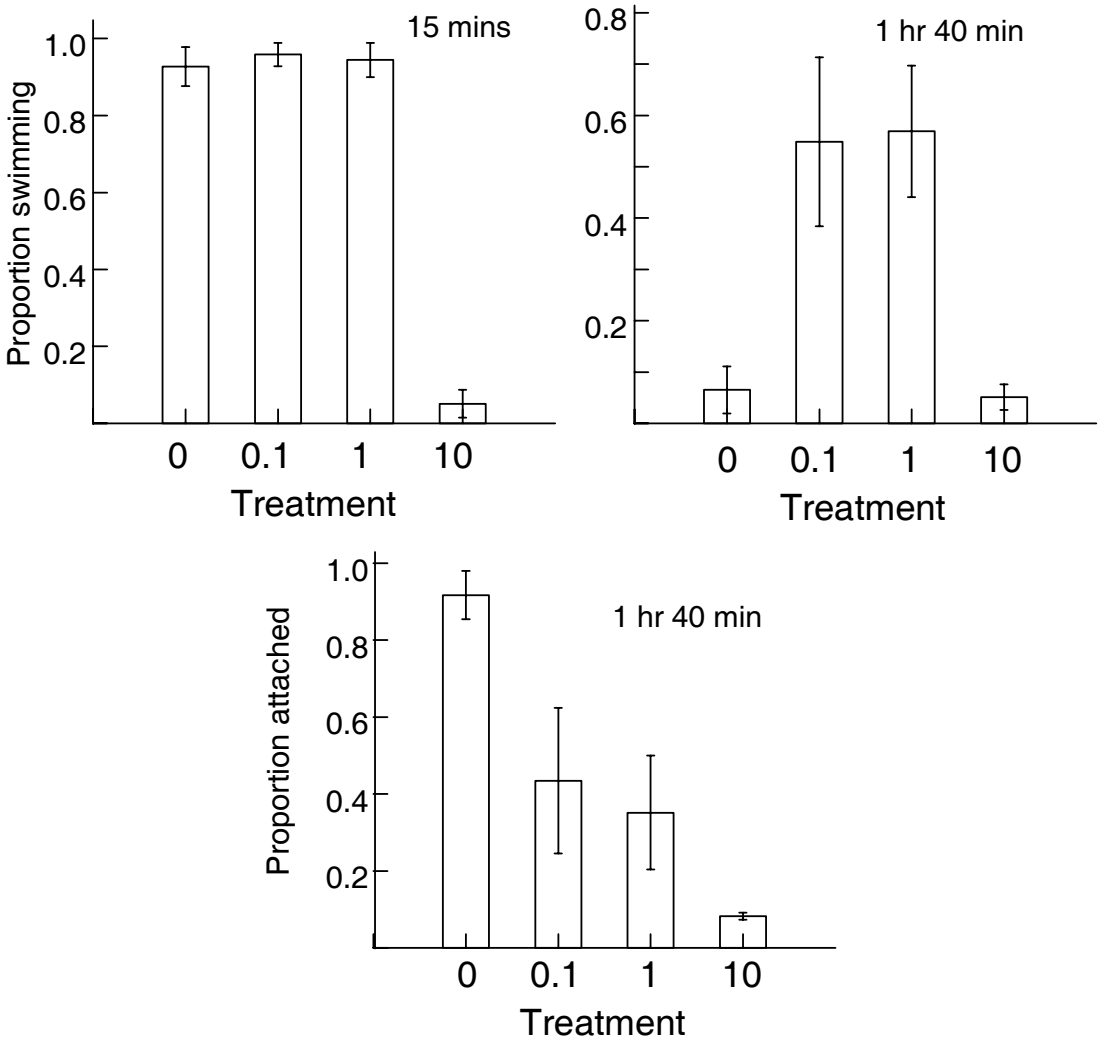
Figure 6. The size of *Schizoporella unicornis* colonies at outplanting.



Bugula neritina

After 15 minutes of exposure to PW, almost all the larvae in the 0%, 0.1% and1% treatments were swimming, compared to <10% of larvae in the 10% PW treatment. After 1 hour and 40 minutes of exposure, most larvae were still swimming in the 0.1% and1% treatments but most of the larvae in the control had metamorphosed. This was not the case for the 10% PW treatment. Most larvae were not moving (figure 7). Mortality was negligible while larvae were being exposed to PW. This results was mirrored in the parallel experiment which exposed larvae ready for outplanting of the adults.

Figure 7. Proportion of *Bugula neritina* larvae swimming after 15 minutes and 1 hour 40 minutes of exposure to PW. The proportion of larvae attached at 1 hour and 40 minutes is also shown. Note the difference in scales.



By the time for outplanting the *B. neritina* colonies, there was no difference in the size of the colonies as measured by the number of zooids (not bifurcations), between the treatments (table 17). On average colonies were 5.5 ± 0.14 ($\times \pm$ SE) zooids in size. Despite a difference in the size of the batches of larvae, there was no interaction between treatment and batch.

Table 17. The size of *Bugula neritina* colonies at outplanting to the field.

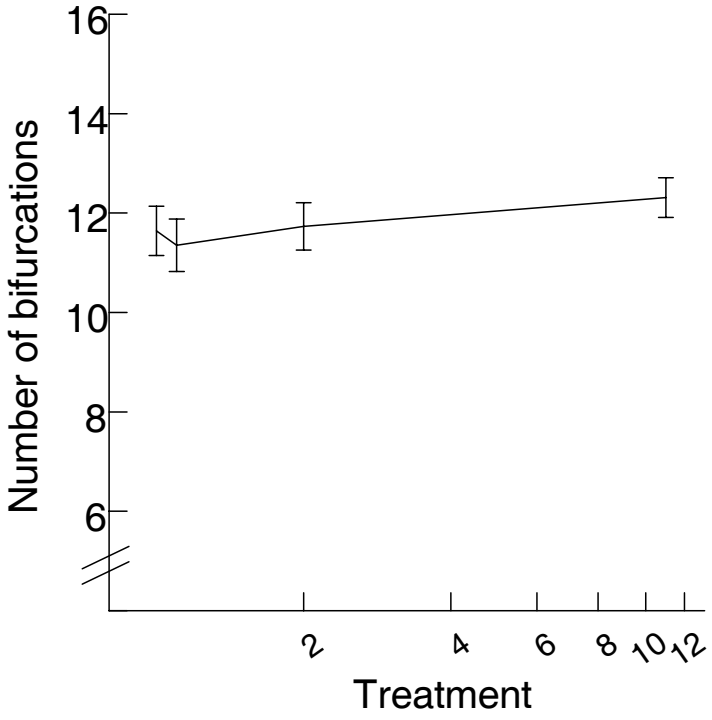
Source	**df**	**MS**	**F-ratio**	***p***
Batch	3	11.510	4.574	0.005
Tmt	3	0.280	0.111	0.953
Batch x Tmt	9	2.248	0.894	0.533
Error	119	2.516		

After 70 Days in the field, there was no difference in the size of the colonies as measured by the number of bifurcations (table 18, figure 8).

Table 18. The size of *Bugula neritina* colonies after 70 days in the field.

Source	**df**	**MS**	**F-ratio**	***p***
Tmt	3	2.634	0.790	0.505
Error	58	3.336		

Figure 8. The size of *Bugula neritina* colonies after 70 days in the field, measured by the number of bifurcations present on colonies.

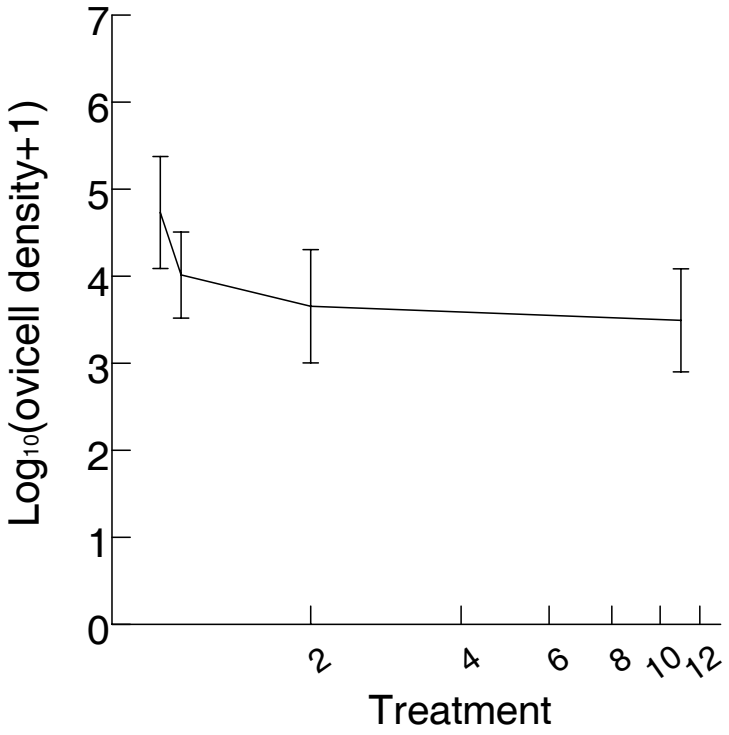


Interestingly there was no difference in the density of ovicells present on the colonies after 70 days in the field (table 19, figure 9). The number of ovicells were scaled for the size of colonies (number of bifurcations). Not surprisingly, there was a great deal of variability in the number and density of ovicells (note figure 9 is on a log scale). Despite this variability, there is a trend to decreased reproductive output as PW concentration increases. We counted all ovicells (full and empty).

Table 19. The size of *Bugula neritina* colonies after 70 days in the field.

Source	**df**	**MS**	**F-ratio**	***p***
Tmt	3	4.429	0.875	0.460
Error	**58**	**5.064**		

Figure 9. The density of ovicells present on *Bugula neritina* colonies after 70 days in the field, measured as Log_{10}(ovicell density+1).



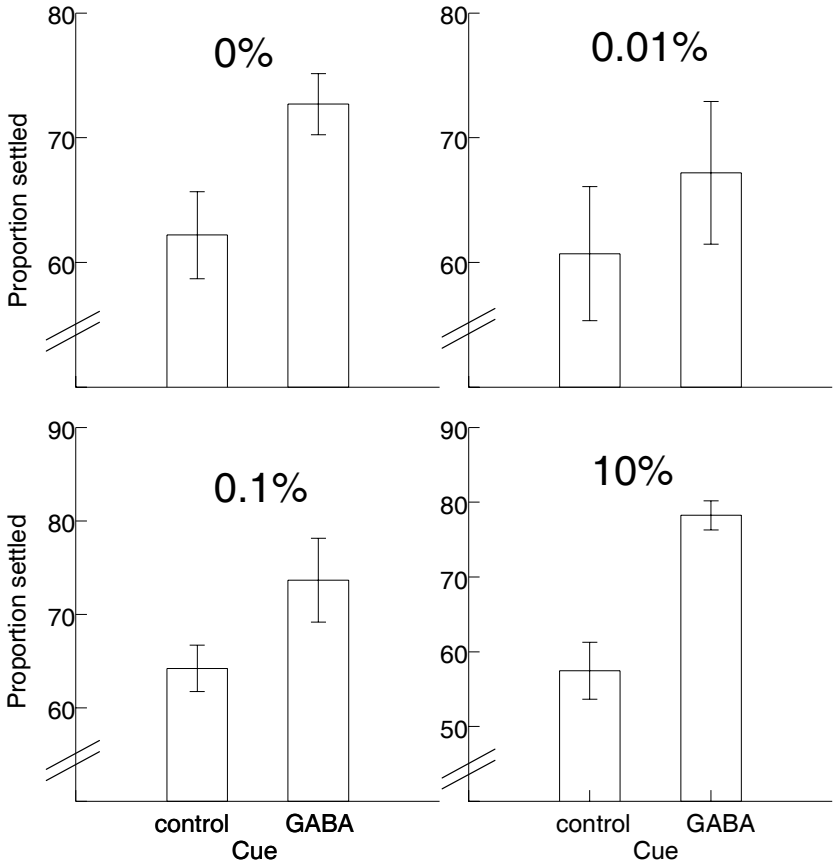
Haliotis rufescens

We did a small experiment using the larvae of *Haliotis rufescens* comparing the complex behavior of settlement cue recognition amongst larvae exposed to four levels of PW for one hour. Not surprisingly, proportionally more larvae settled when the cue (GABA) was used than in the control. After 32 hours had elapsed since exposure, there was no difference between the behavior of larvae in all PW treatments (table 20, figure 10).

Table 20. The proportional settlement of abalone larvae in four PW treatments (05, 0.01%, 0.1% and 10%) with and without a settlement cue. These data did not require transformation.

Source	**df**	**MS**	**F-ratio**	***p***
Cue	1	1070.249	27.314	0.000
PW	3	33.548	0.856	0.478
Cue*PW3	74.645	1.905	0.157	
Error	23	39.183		

Figure 10. The proportional settlement of abalone larvae in four PW treatments (05, 0.01%, 0.1% and 10%) with and without a settlement cue.



Discussion

Overall, this project found there was little evidence for strong sub-lethal effects that carried over from the larval phase and impacted the growth or competitive abilities of the subsequent *Watersipora subtorquata* adults (see also Boxshall and Raimondi, *in prep. A*). The studies of *W. subtorquata* were done three times across three different seasons. Each time, the results were the same. There is certainly evidence for (at times) quite strong sub-lethal impacts on the larvae of *Watersipora subtorquata* and the other larvae studied. Although not as extensively studied, the other invertebrates in this study showed similar patterns for the adults (see also Boxshall and Raimondi, *in prep. B and C).* Although this is an important finding for the protection of some planktonic organisms from impacts of the release of PW, there are a number of caveats.

One important caveat is that although mortality was generally low and not statistically significant, as would be expected from tests deigned to have deliberately sub-lethal impact, there were small differences seen in mortality of adults from larvae exposed to different concentrations of PW. When mortality occurred, it tended to be larger for *Watersipora subtorquata* colonies in treatments with spiked exposure to concentrations of and above 10% pre-release levels. These may be biologically interesting. Marine assemblages are highly variable, where there can be large variations in natural mortality from many sources (e.g., Connell 1978). Such small changes in adult mortality due to PW could simply be swamped by the natural high variability of marine populations. This caveat should be addressed by further work, possibly modelling of impacts (see Forde *et al.* in press).

Higher concentrations of PW affected larval behaviour of *Watersipora subtorquata* during exposure. Larvae generally swam less, settled less and showed less movement of any kind under the influence of higher concentrations of PW. This may be due to a narcotic effect from an unidentified constituent of PW. This speculation is based on the evidence that larvae placed into clean, filtered seawater immediately began to show a range of 'normal' behaviours. Generally there was little difference in the activity of the larvae within hours of exposure to PW, if they were washed in filtered seawater. Where there was larval mortality it tended to be in higher concentrations and was visible after the first hours of exposure. Although, as stated above, mortality in most of these experiments was low.

One important question that arises from these data is that if PW does not greatly impact the growth rates of invertebrates as adults, what is the fate, in a field situation, of those larvae that have had their behaviours altered? It is important to note that the all the larvae in this study were exposed in a laboratory situation. In this benign environment, individual larvae could be studied, followed, cleaned of PW and continue to live. If larvae pass through a cloud of PW in the ocean, stop swimming and sink, do they start to swim again once out of the PW? Or is there another fate? Are they more susceptible to predators? What about delayed metamorphosis and development? Does this leave them open to other natural impacts in the field? These remain unanswered questions.

Information from the outplanting of *Schizoporella unicornis* colonies after the exposure of larvae to PW was unfortunately truncated due to field logistics however there are some data being

reconstructed (Boxshall and Raimondi, *in prep. B*). At the time of outplanting there was little statistical difference in the size of *S. unicornis* colonies, although the pattern of increased size with increasing PW concentration was not continued through to the 10% PW concentration. Further study will be needed on the impact on subsequent adults to strengthen conclusions. There were very clear sub-lethal impacts on the larvae of *S. unicornis* that tracked increasing PW treatment quite well. The higher the concentration of PW, the less the swimming ability of newly released *S. unicornis* larvae. When these same larvae began to attach and metamorphose, the pattern of metamorphosis similarly tracked the PW concentration.

Like the other two bryozoans, there were strong sub-lethal impacts of PW on the behavior of larvae of *Bugula neritina*. Also like the other bryozoans, the carry-over impacts from the larval phase to adulthood were not strong. In the case of *Bugula neritina*, we were able to track the adults for 70 days in the field and follow their growth as well as gain some insight into their reproductive capacity. It is important to note that this measure of reproductive output can only be viewed as a single, snap-shot impression of the reproductive output from *Bugula neritina*. If it would be possible logistically, a better estimate of reproductive output would be to count the life-time output from adult colonies. From our single estimate of reproduction, there was no strong impact carried-over from larval exposure to different concentrations of PW. This result should be viewed as a precursor to more analysis. Very late in the period of this project, access was granted to new supplies of PW by MMS. With this access, it was possible to re-visit the question of carry-over impacts on reproductive success of *B. neritina*. As a consequence, more data was generated after the project time had concluded. This is being further analyzed and will be published in future (i.e., Boxshall and Raimondi, *in prep. B*).

Given the sub-lethal impacts previously seen in field studies using the abalone *Haliotis rufescens* (Raimondi and Schmitt 1993) it was surprising to note that the complex behavior of cue recognition by the abalone was not impacted by PW exposure. The swimming capacity of *Haliotis rufescens* larvae were impacted during other pilot tests (Boxshall, unpubl. data).

On the topic of PW concentrations, as previously discussed, exposure to 10% PW concentrations for one hour is unrealistically high exposure based on current plume dilution studies. This concentration was deliberately chosen to elicit a behavioural response (which it did) and to allow us to follow these larvae/adults through life to look for long-term effects. The 25% PW was used for similar reasons but no adults of any species were tracked through life. One important source of error in this project was the origin of the PW stocks. As noted, PW used for experiments came from 2 collections on single days at single (unknown) platforms. If there is to be the capacity to generalise about the impacts of PW (*per se*) on the ecology of marine organisms, studies must be done using a range of PW from a range of sources. The composition of PW is known to be very variable (see multiple papers in both Ray and Engelhardt, 1993 and Schuurmann and Market, 1997).

There were very few interactions with treatment produced by exposing the larvae to PW in batches or as one large pool. When larvae were not batched, except for this 50 - 65 minute period, the larvae and subsequent adults were raised and monitored individually for the duration of the experiment. Of course, the exposure is an important time. The practice of batching larvae

is in sharp contrast to common procedure in many LC50-96 tests where organisms are treated and monitored as pooled groups throughout the study. The behaviors of larvae are inherently variable (see any paper in McEdwards, 1995) and so this issue should not be dismissed. However, it should also be seen within the context of the previous discussion about the lack of replication in sources of PW.

When effects from batching larval exposure were seen, they tended to occur in later in the early stages of young adult development, not in the early larval swimming, metamorphosis or survival in the first 24 hours. Batch effects were also generally not present later as adults (e.g., growth at outplanting, and both growth and survival later). However, there were batch effects in the development of opercula of the bryozoan *W. subtorquata* (Day 4) in the laboratory and the survival of juvenile *W. subtorquata* to outplanting. These are early stages of development, but after metamorphosis. This is an interesting pattern worth noting but for which we have no present speculative solutions.

The competitive ability of *W. subtorquata* adults were assessed after 150 days in the field. This time was required for enough competitors to grow around the colonies. The overall competitive load was no different amongst colonies in different PW treatments. The competitors were mainly ascidians. No PW treatment showed a diminished ability to compete. The unfortunately low power in these tests is difficult to overcome due to the variable and unpredictable nature of settlement in the field. The design of this experiment is ultimately controlled by where and when competitors settle.

This project was successful in addressing the question of carry-over impacts from exposed invertebrate larvae to the adult phase of their life-cycle. A number of important further questions were raised as a result of this study. During this project, various personnel were involved, gaining valuable learning experience. They are listed in Appendix 1.

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The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

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As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Royalty Management Program** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.