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Responses to Chemical Exposure by Foraminifera: Distinguishing Dormancy From Mortality

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Responses to Chemical Exposure by Foraminifera: Distinguishing Dormancy From Mortality

by

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Abstract

The Deepwater Horizon blowout in 2010 released an estimated 4.9 million barrels of crude oil into the Gulf of Mexico in the 83 days between the initial explosion and the capping of the well. Response included extensive use of Corexit® oil dispersant. Although South Florida was spared exposure by currents, this event highlights the need for effective bioassay organisms for coral reefs. *Amphistegina* spp. are benthic foraminifers that host diatom symbionts in a relationship similar to that of coral and their zooxanthellae. *Amphistegina* spp. occur abundantly in reef communities nearly worldwide, are easily collected and maintained in culture, and are a key component of the FoRAM Index, a indicator of water and sediment quality in coastal waters. The major goals of this project were to develop protocols to test the acute and chronic responses of *A. gibbosa* to potentially toxic organic chemicals.

Initial objectives were to determine lethal concentrations and effects ranges, as defined by the US Environmental Protection Agency, of two components of the Corexit® dispersants. Preliminary experiments indicated that many specimens exposed to propylene glycol (v/v) at concentrations of 2% or higher appeared to be dead following 48-hour exposure, resulting in apparent LC50 of 3% and an initial effects range of 2-4%. When placed in filtered seawater, after 72-hours the observed LC50 was 6%. All parameters assessed, including sub-lethal chronic effects (differences in growth and visible responses after 40 days), revealed an effects range of 0.5% to 12%, above which there was 100% mortality. For 2-butoxyethanol, the apparent LC50 after 48-hour

exposure was 0.2%; after 72-hour recovery the LC50 was 1%. In all experiments, a 72-hour recovery period was sufficient to determine acute effects. A key discovery was the observation of inactivity during exposure to toxic substances, followed by recovery when placed in filtered seawater. This observation indicates the potential for dormancy in adult foraminifers exposed to toxic substances that has not previously been reported.

1. Introduction

1.1 Deepwater Horizon Oil Spill

The Deepwater Horizon oil spill of 2010 released an estimated 4.9 million barrels of crude oil in the 83 days between the initial explosion and the capping of the well. The extent of the environmental damage is still being assessed, but this incident has clearly demonstrated the environmental dangers that can result from accidents with offshore drilling equipment. Fortunately, currents isolated the Florida reef tract from direct exposure to the Deepwater Horizon oil and the associated chemical dispersants, which could have had catastrophic effects on an already compromised reef ecosystem.

Coral reef communities worldwide are in decline, including those of the Florida reef tract. The Florida Keys National Marine Sanctuary Coral Reef Evaluation and Monitoring Project (CREMP) found that, between 1996 and 2010, stony coral species richness declined each year at all of their sampling sites throughout the sanctuary, and that overall stony coral cover has decreased approximately 50% (Callahan et al. 2006; Ruzika et al. 2010).

One of the primary threats to zooxanthellate corals is bleaching, which occurs when the coral, its algal symbionts (zooxanthellae), or both are stressed, resulting in either loss of algal chlorophyll or the loss of the symbionts through either digestion or expulsion. Photo-oxidative stress is the most common inducer of mass bleaching (Lesser and Farrell 2004, Lesser 2006), typically occurring with combined exposure to elevated

water temperatures and sunlight, especially the higher energy, shorter wavelengths. Moreover, bleaching can also occur when other physiological stressors such as toxic chemicals render the coral-algal system incapable of dealing with the reactive oxygen species produced under normal sunlight conditions (Weis 2008). For example, Negri et al. (2011) found that herbicides significantly increased the negative effects of thermal stress experienced by coral at 31 and 32º C.

Although Florida's reefs were spared the direct impacts of the Deepwater Horizon spill, the threat of oil and dispersant exposure on Florida reefs continues. The new joint Chinese-Cuban offshore drilling platform is closer to the reefs than the Deepwater Horizon site, and drilling continues in the Gulf. The threat is not isolated to Florida, either; in reality, coral reefs worldwide face the possibility of exposure to oil through either blowouts or, more commonly, spills from transport ships. Given this continued threat, a study of the effects that oil and associated chemicals have on reef organisms is clearly warranted, since oil and dispersant chemicals will continue to be a threat to coastal and estuarine waters. Improving our ability to assess and monitor the effects of these and other environmental pollutants in coastal waters is more important than ever, given the continued decline of coral reefs worldwide.

1.2 *Amphistegina* as bioindicators

Amphistegina spp., which are larger foraminifers (Class Foraminifera), are abundant nearly circumtropically in coral reef and open shelf environments, with *Amphistegina gibbosa* d'Orbigny being the species present in the Florida reef tract. *Amphistegina* host algal symbionts in a relationship very similar to that of reef corals and

their zooxanthellae (Lee 2006). Although these foraminifers are more tolerant of temperature changes than most reef-building corals (Talge and Hallock 2003), they are highly sensitive to light and water-quality stressors (Williams and Hallock 2004). *Amphistegina* are useful for experimental studies because their small size makes working with statistically robust sample sizes relatively easy, but at the same time, they are large enough to respond visibly to stressors, by color changes as well as changes in motility, growth rates, shell morphology, and reproductive success over time.

These characteristics make *Amphistegina* spp. useful bioindicators for water and sediment quality, both in the Florida Keys and tropical coastal regions worldwide. Indeed, both live samples and the protist's calcite shell, which remains in the environment after death, are currently used as bioindicators. The FoRAM (Foraminifera in Reef Assessment And Monitoring) Index uses the foraminiferal assemblage, as represented by the shells found in surface sediment samples, to calculate an index representing the environmental conditions and indicating whether water quality is conducive to reef growth (Hallock et al. 2003). When the shells of symbiont-bearing foraminifers represent at least 25% of the assemblage, conditions are likely favorable for proliferation of calcifying organisms with algal symbionts. A shift in species composition away from the symbiont-bearing foraminifers towards smaller, asymbiotic species indicates stressful conditions for reefs. Because of their circumtropical distribution and abundance, *Amphistegina* spp. are a key component of this index.

Examination of live *Amphistegina* populations can also be used as a relatively quick, low-cost method to assess environmental conditions on a reef (Hallock 1996, 2000). Previous research by Hallock et al. (2006) defined a protocol for sampling live

Amphistegina as an indicator of photo-oxidative stress on a coral reef that could precede mass bleaching. The utility of this method is based on *Amphistegina*'s higher tolerance to temperature changes, which allows them to be used as a bioindicator of photic stress independent of temperature changes. Another advantage is their fast reaction time; *Amphistegina* visually responds to acute photo-inhibition in hours to days, and to chronic stress over days to weeks. Given *Amphistegina*'s sensitivity to water-quality stressors, and their successful use as bioindicators, the next logical step is to develop bioassay methodologies utilizing *Amphistegina* to assess for pollutants.

1.3 Bioassay organisms for hydrocarbons and dispersants

Corexit® brand oil dispersant compounds, manufactured by Nalco®, are part of the Environmental Protection Agency's National Contingency Plan for treating oil spills, and were used in the cleanup efforts following the Deepwater Horizon blow out. Although Corexit® 9500 was the only product manufactured for use during the oil spill, Corexit® 9527, drawn from existing dispersant stockpiles, was also used, according to the Nalco website. The two compounds have different components, with Corexit® 9500 designed to work on a wider variety of oils (George-Ares and Clark 2000).

George-Ares and Clark (2000) reviewed previous publications that determined toxicological measures of Corexit® 9527 and 9500 on different organisms, and found that both compounds have low to moderate toxicity in most aquatic organisms, but that these estimates are significantly affected by experimental variables, including the species, life stage, length of exposure, and temperature. Duarte et al. (2010) found that, in fish exposed to either oil, Corexit® 9500, or a combination of the two, the effects were

heightened when the fish were exposed to the dispersant-oil combination, a likely occurrence in a situation where oil dispersants would be deployed.

1.4: Foraminifers as bioassay organism

Traditional descriptive toxicological bioassay measurements take place over a range of time scales. Short-term acute-lethality tests are carried out by exposing the animals to chemicals for a known time of exposure. The time scale can differ depending on the objectives of the test but, according to EPA guidelines, should be between 24 and 96 hours (EPA 2002). For aquatic organisms, the chemical is introduced by mixing it in the water medium. EPA protocols call for the determination of two measures based on mortality. The first is the Lethal Concentration 50, which is the concentration of the chemical in the water that causes death to 50% of the animals (Eaton and Klaasen 2003); this is generally written as LC50. The other measurement of interest is the No-Observed-Adverse-Effect-Concentration, or NOAEC, the highest concentration at which survival is not significantly different from control.

According to EPA guidelines (EPA 2002), for acute toxicity tests to be statistically rigorous, a minimum of 20 test organisms should be exposed to each concentration of interest. Foraminifers are thus quite an attractive option, owing to their small size and amenability to being maintained in laboratory conditions. In addition to these descriptive experiments, other short term indicators of stress, such as oxidative stress biomarkers, have also been examined in foraminifer (de Freitas Prazeres et al. 2011, 2012), contributing further to their potential as bioassay organisms.

Chronic bioassays take place over longer time scales and have different goals. They can be used to identify cumulative toxicity, but can also be used, as in Denoyelle et al. (2012), to examine sublethal effects of chemical concentrations that do not cause mortality, such as rate of chamber addition. Again, foraminifers are promising test subjects for such experiments. Size issues are compounded when one must keep an experiment running under controlled conditions for 30 days instead of 48 hours, so the small size of foraminifers is an ever greater benefit. In addition, their amenability to culture means that they can be kept for extended time periods so mortality or other effects can be attributed to experimental conditions, by comparison with control treatments.

Foraminifers also are well suited for identifying sublethal effects. Growth is easily tracked in foraminifers, either via methods like those of Denoyelle et al. (2012), employing calcein (a tracer which is integrated when new calcium carbonate is precipitated, and fluoresces, allowing the identification of chambers formed during the course of the experiment) or by simply measuring surface area or diameter using image analysis software. *Amphistegina gibbosa* has the added benefit of being symbiotic, allowing for symbiont loss (bleaching) or other color changes to be used as sublethal indicators of stress, and their ease of culture makes even longer term effects, such as altered reproductive response or anomalies in calcification, viable as indicators of sublethal exposure (Hallock et al. 2006).

Another important factor in any bioassay experiment is the appropriateness of the bioassay organism to the broader environments one needs to model, and to the overall objectives of the study. *Amphistegina* spp., along with other larger foraminifers that host algal symbionts, have already demonstrated applicability to monitoring and assessment of

water quality in coral-reef and other tropical coastal environments, which are somewhat neglected in bioassay research. Test organisms commonly used to measure acute toxicity in estuarine and marine systems (e.g., EPA 2002) are commonly estuarine fish, which are not associated with coral reefs, and even if they were, the effect of chemicals on relatively larger vertebrates would not tell us much about the effect of the experimental chemicals on benthic invertebrates, especially those like corals that host algal endosymbionts. Mysid shrimp are also commonly used in toxicology research; they have a cosmopolitan distribution and occupy benthic environments, and therefore are perhaps better models than the common fish species.

Amphistegina spp., on the other hand, are found in abundance living among corals in the reef ecosystem, and due to their small size, relatively limited mobility, and habitat preferences, would likely be directly exposed to any chemical stressors that would be affecting corals, their algal symbionts, and associated benthos. This suggests that the toxicological effects of these compounds on foraminifers such as *Amphistegina* may be very different than those seen in the most easily comparable species that have already been tested (i.e., small invertebrates such as *Artemia* spp.), and that additive effects may be anticipated.

A recent paper by Nigam et al. (2006) reviewed the use of foraminifers in pollution studies. Many of the qualities that make *Amphistegina* ideal for bioindicator studies hold true for other foraminiferal genera as well, including ease of collection and rapid response time, as well as the preservation potential of foraminiferal shells. Thus, studies utilizing foraminifers for marine pollution assessment have become more common in recent decades. Despite the wide range of pollutants that foraminifers have

been used to study, including sewage outfalls, heavy metals, industrial effluents, and pesticides, little is known about the effects of oil or oil dispersants on foraminifers. Nigam et al. (2006) note that Yanko and Flexer (1992) suggested using modifications in foraminiferal assemblages to monitor anthropogenic oil and gas slicks, while Mayer, in an unpublished PhD dissertation in 1980, reported ill effects of an oil spill on foraminiferal abundances and diversity. Ernst et al. (2006) showed similar effects in experimental oil exposures to benthic foraminifers from an intertidal mudflat in France. However, Nigam et al. (2006) also note that Vénec-Peyré (1984) observed no effects of hydrocarbons on relative abundance and species diversity, although she reported morphological abnormalities. These studies focused on changes in assemblages, not the toxicity of oil (or dispersant chemicals) on living foraminifers.

Although *Amphistegina gibbosa* and other foraminiferal taxa have been used as bioindicators in the field, there have been relatively few instances of bioassay experiments carried out using foraminifers. Hallock (2000) suggested that foraminifers have tremendous potential for use as bioassay organisms, particularly for coral-reef ecosystems, but they have been rarely employed in such a manner, on coral reefs or otherwise. This has been changing in recent years; for instance, Martinez-Colon et al. (2009) advocated the use of laboratory experiments to refine the use of foraminifers as bioindicators of potentially toxic elements, and recently Denoyelle et al. (2012) developed bioassay methods to test the chronic effects of cadmium, fuel oil and drilling muds on foraminifers, with a goal of increasing their potential in bio-monitoring studies. Bioassay research is an important step in determining the reactions of bioindicator organisms to potential toxins in a controlled setting, allowing for greater

effectiveness in their use as environmental indicators. As noted by many researchers (e.g., Shafer 2000, Martinez-Colon et al. 2009), foraminifers have a number of features that make them extremely useful in environmental monitoring and many of those features also contribute to their potential for use as bioassay organisms.

Common test organisms are also limited in their application as coral reef bioassays by their nutritional modes. Shallow-water corals, unlike any of the other invertebrates mentioned in the studies above, are mixotrophic, harboring algal symbionts that photosynthesize, while feeding at the same time. This mode of life likely has its own implications in terms of toxic exposure and uptake, as well as reactions. *Amphistegina* is also a mixotroph, and is known to react to stress by bleaching, a response analogous to that seen in coral, and a measureable response in laboratory bioassay experiments. This also gives *Amphistegina* another benefit as a bioassay; as noted in Hallock (2006), this visual representation of bleaching, which has previously been quantified by examining the percentage of bleaching within the shell, allows for the direct transfer of laboratory results to the field (see also Talge and Hallock 2003).

For these reasons, this thesis explores the applicability of *Amphistegina gibbosa*, the dominant western Atlantic and Caribbean species, as a bioassay organism for coral-reef ecosystems. As a result of unanticipated responses of these foraminifers to intermediate concentrations of test chemicals, my thesis also explores modifications in the design of bioassay experiments to account for these challenges. As noted by Hallock et al. (1986a), *A. gibbosa* is the sibling species for the ubiquitous Indo-Pacific *Amphistegina lessonii*, and these species have been shown to respond very similarly to

environmental stresses. Thus, my research on *A. gibbosa* should be widely applicable to Indo-Pacific reefs as well.

1.5. Objectives

My original objective for this research was to experimentally assess the effects of oil and dispersant chemicals on *A. gibbosa*. This objective required identifying useful measures of acute toxicity for these foraminifers. This included, as per EPA guidelines for toxicity testing (EPA 2002), establishing the LC50 and NOAEC. Additional goals were to define measures of longer term, sublethal effects of short term exposure, including differences in growth rates and in incidence of bleaching in exposed individuals. Following the discovery, during preliminary experiments, of *A. gibbosa*'s apparent ability to become dormant when exposed to toxic concentrations of test media, my objectives expanded, with a new goal of confirming this original observation, and developing methodologies to test acute exposures that take this response into account.

2. Methods

2.1. Standard collection and culture methods

Samples were collected from depths between 6 and 18m on Tennessee Reef in the Florida Keys. Tennessee Reef was chosen as the primary field site because there is a long history of studying the foraminiferal assemblage here (e.g,. Hallock et al. 1986a,b; Williams et al. 1997; Baker et al. 2009), and because the Keys Marine Laboratory (KML) allows relatively quick and easy boat access to the reef, as well as a land-based laboratory facility, which facilitates sample processing.

The collection methods for live foraminifers are standard procedures previously described by Hallock et al. (1986a, 1995, 2006) and others. In short, SCUBA divers either scrubbed reef rubble into re-sealable plastic bags at depth using soft brushes, bringing the resulting sediment and associated meio-and microfauna to the surface, or they brought the rubble to the surface for scrubbing. Once at the surface, the re-sealable bags were placed in a seawater-filled bucket, which was then covered by an opaque bag to protect the samples from elevated temperatures and irradiance during transport to the KML laboratory facilities.

On shore, the resultant sediment-organism slurry was decanted into 500 ml screw-top widemouth Nalgene jars, rinsed several times with seawater to remove excess organic debris, and covered with several centimeters of seawater for transport to the Reef Indicators Lab in the College of Marine Science, USF, in St. Petersburg, Florida, where

these bulk samples were maintained under standard culture conditions (Table 2.1) in one of three Thermo Precision® environmental chambers with dual temperature and light-bank controls, as used in numerous previous studies (e.g., Hallock et al. 1986, 1995; Talge and Hallock 2003).

Table 2.1: Standard conditions for bulk-sediment storage

Temperature	25 + 1° C
Light quality and intensity	Cool-white at ~10 μmol photon/m^{-2} s^{-1}
Photoperiod	12 hr light – 12 hr dark
Chamber size	500 ml widemouth jars with screw caps or petri plates
Culture medium	Filtered seawater, salinity 35-37 psu, pH 8.2
Nutrient source	None

2.2. Experimental methods

At least 24 hours prior to picking individual specimens for experimentation, stock samples were split into subsamples, and transferred to 150 x 20 mm petri dishes and allowed to "rest" undisturbed until examined under a stereo microscope. This procedure allowed the live *Amphistegina gibbosa*, which are negatively geotaxic, time to crawl to the sediment surface or up the sides of the petri dish, making them considerably easier to locate. Healthy *A. gibbosa* individuals were selected from these bulk-sediment samples using fine brushes or forceps, and transferred to a separate petri dish filled with filtered seawater. Health of each individual was visually assessed, based on the qualitative

assessment of the degree of bleaching used by Williams (2002). Only healthy individuals, as determined by a uniform golden-brown color and fully intact tests with no obvious deformities (see Fig. 2.1), were selected for experiments.

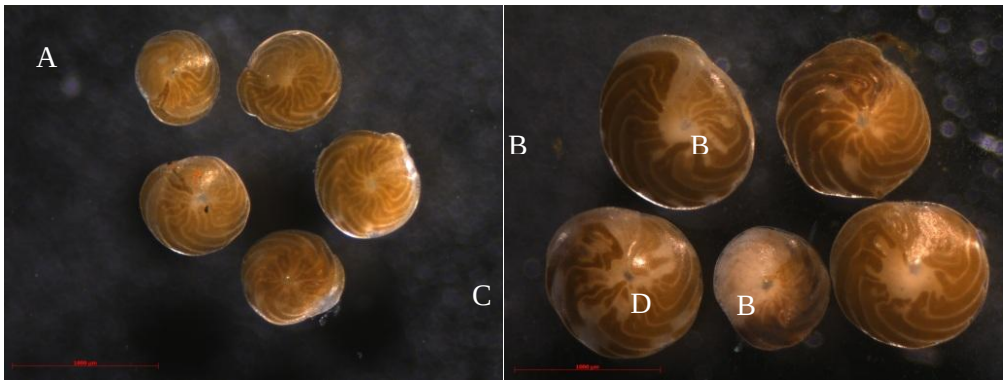

Figure 2.1: Visual appearance of *Amphistegina gibbosa* before and after exposure to 2.0% propylene glycol.
Left image shows the foraminifers before exposure.
Right image shows foraminifers 5 weeks after a 48-hr exposure. Those labeled *B* show slight mottling; *C* is very mottled; *D* exhibits an anomalous dark green coloration, in addition to some bleaching

At least 24 hours before the start of the experiment, the test media were mixed. All seawater used in the preparation of test media, including control medium, was first filtered through a 0.3 micrometer filter. Following filtration, pH of the water is reduced to 7.2-7.3, which is stressful to *A. gibbosa*. Therefore, before media preparation, all seawater used was pH corrected by the addition of 1-molar NaOH, until the pH of the water approached normal Keys seawater pH (~8.2). The test media were mixed in small glass beakers or flasks by volume, using seawater of the same temperature, salinity and pH as that used to keep the bulk samples in culture. The media were then sealed in the mixing vessel using Parafilm and allowed to rest in the incubators, to allow time for any

possible reactions to occur, and for the test media to arrive at the standard temperature used for culture.

2.2.1. *Preliminary experiments*

These initial experiments utilized propylene glycol as the chemical of interest. Propylene glycol is a component of both COREXIT® 9500 and 9527, making up 1.0-5.0% of both compounds by weight according to the material safety data sheets (Nalco 2005, 2008). Previous tests of the toxicity of propylene glycol on planktonic invertebrates found LC50 concentrations as low as 0.84% v/v for the shrimp *Penaeus japonicas*, to as high as >15% v/v for *Artemia tibetiana* (Tzovenis et al. 2004). Using these values as a baseline, I carried out 48-hour exposures of *Amphistegina gibbosa* to the concentrations of propylene glycol shown in Table 2.2.

Once the test media were prepared and test specimens had been selected from the bulk sediment, the test media were added to 15 ml glass petri dishes. Test specimens were then randomly assigned to different petri dishes; five individuals per petri dish, and five replicate dishes per concentration of test media, for 25 individuals per concentration. (Table 2.2).

Although initial concentrations were based on previous experiments with invertebrates, as noted above, these concentrations did not result in the necessary range of effects, causing too little mortality at the lower concentrations, and total mortality at the higher concentrations, making the identification of the LC50 unreliable. To provide a more complete range of effects, and to more precisely determine the LC50, as well as the NOAEC, a second round of experiments, using specimens from the same samples, was

performed after the initial experiment using a different range of concentrations, but including in that range another set of control and 1.5% propylene glycol replicates. The results were combined to give the entire range and as a result, there were 50 specimens in the control and 1.5% conditions (Table 2.2). Once the specimens were added to the test media, the petri dishes were placed in the environmental chambers for 48 hours.

Table 2.2: Concentrations of propylene glycol in the preliminary48-hour exposure experiments

Concentration of propylene glycol (v/v)		.05	.09	.19	.36	.75	.05	.5	.65	.95	.25	.85			2	4	8
Number of individuals	0	5	5	5	5	5	5	0	5	5	5	5	5	5	5	5	5

After 48 hours, the dishes were removed, and apparent mortality was assessed via visual inspection. Visual indicators of vitality were: obvious pseudopodial activity, attachment to either the bottom or side of the petri dish, or floating on the surface of the test media, indicating that the individual had climbed the side of the dish and along the surface tension of the water-air interface. If an individual showed no obvious pseudopodial activity and was detached, it was initially considered to be dead. After assessing the apparent mortality in each replicate, the foraminifers were rinsed, moved to petri dishes containing filtered seawater, and returned to the incubator for 24 hours. After this recovery period, apparent mortality was assessed again.

2.2.2. *Revised methods – propylene glycol*

The results of my preliminary experiments necessitated modification of methods for acute toxicity tests employing *Amphistegina gibbosa*. Sample collection, culture environment and preparation of test media remained the same as that presented above. Individuals were again selected for healthy appearance, as defined in the previous experiment, this time selecting a narrower size range, between 0.5 and 0.8 mm in diameter. Individuals in this size range are considered sub-adult, as large adults can reach in excess of 1 mm in diameter. Previous work with *A. gibbosa* have shown this to be an optimum starting size range for experiments in which both growth rates and bleaching will be assessed (e.g., Talge and Hallock 2003; Williams and Hallock 2004).

After selection for the experiment, specimens were transferred to the petri dishes containing test media, as before. When moving the foraminifers from this large petri dish to the smaller experimental petri dishes, care was taken to minimize cross contamination of the holding petri dish by the instruments used to move the individuals. In addition, I transferred specimens to the lowest concentration media first, so that no individual could possibly be exposed to concentrations higher than their treatment condition.

For this experiment, I utilized 48-hour exposures to propylene glycol using concentrations based on results of the preliminary experiments (Table 2.3) Following the exposures and rinsing, observations were made every 24 hours for 72 hours, and then every week (from the date of the first 24 hour recovery observation) for 5 weeks, for all concentrations except for 12.0% and 14.0% propylene glycol. After the first week, lack of recovery and discoloration made it clear that the specimens in the 12.0% and 14.0% replicates were completely dead, and were removed from further observations. As a

result, the time series used in the analysis covers the range from 0% propylene glycol to 10% propylene glycol.

Table 2.3: Concentrations of propylene glycol treatments in 48-hour exposure using refined methodology

Concentration of propylene glycol (v/v)	%	.5%	.0%	.0%	.0%	.0%	.0%	0.0%	2.0%	4.0%
Number of individuals	5	5	5	5	5	5	5	5	5	5

All LC50 were calculated by performing a linear regression on the portion of the dose response curve between the 0% mortality and 100% mortality thresholds, and using the resulting regression line to calculate the percentage propylene glycol that would cause the death of 50% of the specimens on the line. As in the preliminary experiments, the upper and lower thresholds were identified by performing an ANOVA, and then Tukey's Honestly Significant Difference test, to identify observations that were not significantly different from each other.

Once placed in the 15 ml petri dishes, the foraminifers were photographed using a digital camera mounted on a Zeiss stereomicroscope to record initial color, size, and test shape. After the foraminifers were photographed, the dishes were moved to the environmental chamber. After 24 hours, the samples were removed and checked for

apparent mortality, as per the assay described in Section 2.2. After assessment, the petri dishes were returned to the environmental chamber and left for another 24 hours, then examined again. Following examination, the foraminifers were photographed again to record size, color and condition immediately following the 48-hr toxic exposure.

After post-exposure photography, the specimens were rinsed using a very thorough rinsing regime. The foraminifers were first transferred to clean 15 ml petri dishes containing filtered and pH-corrected seawater. This seawater was decanted, and new seawater added. The petri dish was then moved to an orbital shaker and gently shaken for 5 minutes, at which point the water was again decanted and replaced. The dish was then shaken on the orbital shaker for another five minutes; the water was decanted once more, and this time replaced with an f/2-Si nutrient medium made using a 25% solution of NuSalts® Type 1 (Table 2.4) and moved to the environmental chamber. Although symbiotic algal photosynthesis provides enough energy for *A. gibbosa* to survive in plain seawater, without a source of nutrients they cannot grow. The use of nutrient-enriched medium provides adequate nutrients to allow the foraminifers to grow, enabling the comparative analysis of growth following exposures in different concentrations of test chemicals.

The foraminifers were visually assessed every 24 hours for 72 hours, with photographs taken again on the 3rd day. Following this initial 72 hours of daily observation, specimens were visually assessed, photographed, and nutrient media changed every week for five weeks from the day the specimens were rinsed. After 3 weeks, each replicate group was transferred to sterile 15 ml petri dishes, to combat algal growth (see, e.g., Hallock et al. 1986a).

To assess growth rates of the experimental specimens, I used the photographs taken immediately after removal from chemical exposure, and those taken each week for the next five weeks. I measured the longest (maximum) diameter on each individual using Zeiss Axiovision software (Fig. 2.2).

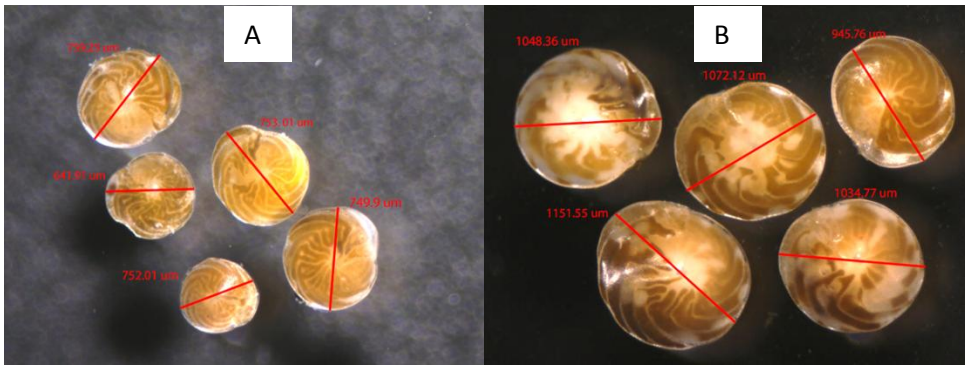

Figure 2.2: Measurements of longest diameter of individuals a) before chemical exposure and b) following a 48-hour exposure to 4% propylene glycol and 38-day recovery period (40 days total)

Analyses of visual changes utilized the same photographic time series employed to investigate growth rates. I modified the visual bleaching scale similar to that described by Hallock et al. (1995), Williams et al. (1997), and others. I used three categories of bleaching-related color change:. “Normal” individuals were a healthy golden or greenish-brown, with no white spots; “slightly mottled” individuals were brownish green, with a few white spots; “very mottled” individuals retained some brownish-green coloration, but were largely bleached. I observed no occurrences of completely bleached individuals. I also added two new categories. “Other” individuals were those whose color was unusual, often darker, without bleaching. The final category was “dead”, characterized by a lack of color, distinct from bleaching; dead individuals tend to be a dirtier white, sometimes with

slight non-green coloration, and devoid of evidence of pseudopodial activity such as attachment or movement. See Figures 2.1 and 2.2 for examples.

Table 2.4: Formula of NuSalts® Type 1 used as nutrient source during long term observation following 48-hour toxic exposures

$NaNO_3$	30.0%	**$MnCl_2.4H_2O$**	0.008%
$NaH_2PO_4.H2O$	2.0%	**$Na_2MoO_4.2H_2O$**	0.001%
Na_2EDTA	2.0%	**Biotin**	5ug/L
$FeCl_2.6H_20$	1.26%	**Cyanocobalamin**	5ug/L
$CuSO_4.5H_2O$	0.004%	**Thiamine HCl**	5 ug/L
$ZnSO_4.7H_2O$	0.0084%	**Inerts**	64.0%
$CoCl_2.6H_2O$	0.004%		

Due to a mislabeling of the NuSalts bottle on the formula, we initially thought that the formula contained no Thiamine HCl, and additional Thiamine HCl was added based on a formula used by others in the lab. Later communication with Argent Labs, the company which manufactures Nu-Salts, led to the discovery that the formula did in fact contain Thiamine HCl in the noted small amount. As a result, during the propylene glycol experiments under the new experimental methods, the nutrient media contained levels of Thiamine HCl elevated over those seen in Table 2.3.

2.2.3. *2-butoxyethanol toxicity*

Although I had confirmed the presence of a dormancy-recovery effect in *Amphistegina gibbosa* exposed to propylene glycol, I did not know whether this is a

common reaction of *A. gibbosa* when exposed to other waterborne toxic compounds. Propylene glycol is a relatively low-toxicity compound; its safety and resultant ease of use in laboratory experiments was one of the reasons why it was selected as the initial test compound. To investigate whether dormancy occurred in the presence of other chemicals, we chose as our compound of interest 2-butoxyethanol. Although not a component of Corexit® 9500, 2-butoxyethanol is a component of the (discontinued) Corexit® 9527, at 30-60% of the compound by weight. Although Corexit® 9500 was the compound specifically produced to combat the Deepwater Horizon oil spill, Corexit® 9527 drawn from existing reserves was deployed as well, making 2-butoxyethanol a test chemical of interest.

Methods are similar to those described for the previous propylene glycol experiment, with the exception that I did not follow mortality or growth over time. I carried out two separate experiments of 2-butoxyethanol exposures. The first was a small scale experiment to determine an appropriate concentration range. Limited data were available on the toxicity of 2-butoxyethanol to marine invertebrates, with LC50 values ranging from 1000 mg/liter for *Artemia salina* (24 hour exposure) to 5.4 mg/liter for the Grass Shrimp *Palaemonetes pugio* (96 hours) (Wess et al. 1998). Using these values as a guide, the concentrations in my initial experiment were too high. I carried out a second experiment, using considerably lower concentrations (see Table 2.5 for concentrations used in each experiment).

2.3: Statistical Analysis

Statistical tests employed in analyzing data included analysis of variance (ANOVA), nalysis of covariance (ANCOVA), Tukey's Honestly Significant Difference test (HSD), and the Kolmogorov-Smirnov Goodness of Fit test (Zar 1999). A significance level of 95% ($p \leq 0.05$) was used throughout.

Table 2.5: Concentrations of 2-butoxyethanol treatments in 48-hour exposure experiments

First experiment

Concentration of 2-butoxyethanol	0	0.32%	0.64	0.96	1.28	1.44
# of individuals	25	25	25	25	25	25

Second experiment

Concentration of 2-butoxyethanol	0	.05	.1	.15	.2	.25	.3	.35	.4
# of individuals	5	5	5	5	5	5	5	5	5

3. Results

3.1. Preliminary propylene glycol experiment

The results from the 48-hour exposure to selected concentrations of propylene glycol (Fig. 3.1a) and to the 24-hour recovery period in filtered seawater (Fig. 3.1b) revealed an unexpected response. In both cases, a clear linear dose-response could be calculated between the lower (0% mortality) and upper (100% "apparent" mortality) thresholds. The values within these thresholds were confirmed as being insignificantly different from each other by performing an ANOVA test, followed by Tukey's Honestly Significant Difference (HSD) posthoc test (see Table A1 in the appendix). I arrived at LC50 measures by performing a linear regression on the linear portion of the data, and using the regression formula to calculate the point at which 50% mortality would occur.

Based on the definition of mortality given above, the 50% lethal concentration (LC50) and the effects range (the range between concentrations causing no mortality and those causing 100% "mortality") of the observations immediately after the 48-hour exposure and following the 24-hour period in fresh seawater were different. The 48-hour exposure with no recovery period gave an apparent LC50 of 1.9% (Fig. 3.1a); after the 24-hour recovery period, however, the LC50 was 5.0% (Fig. 3.1b).

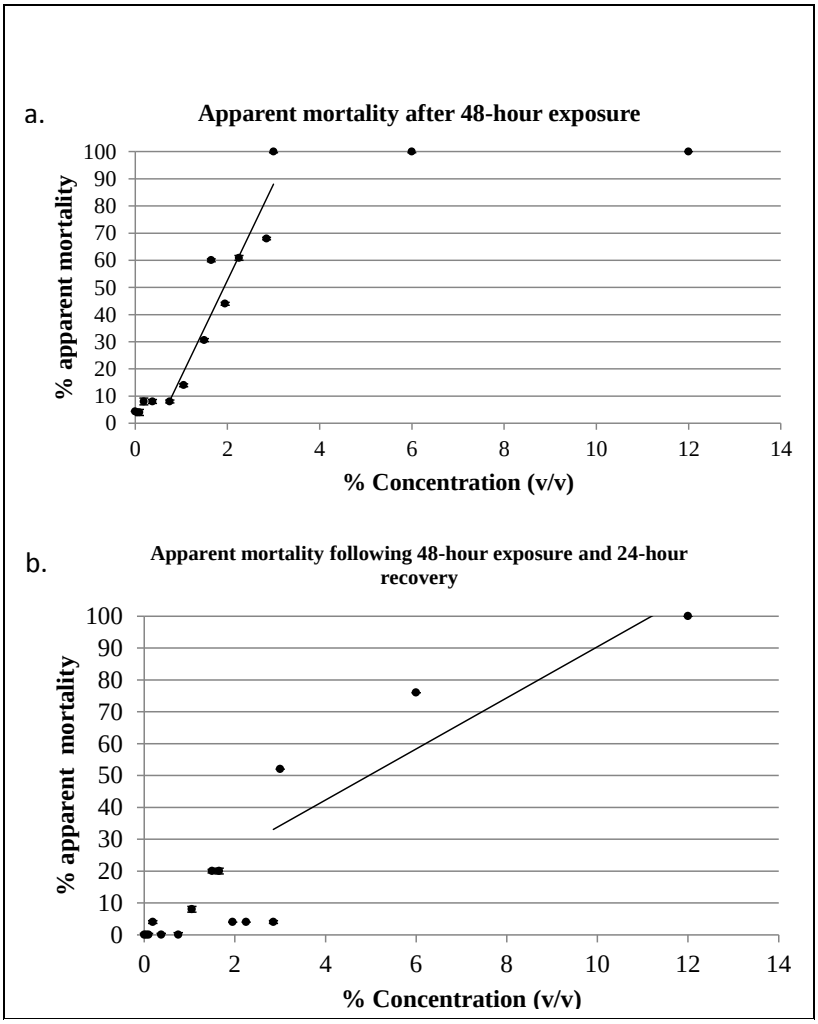

Figure 3.1: Apparent mortality following 48-hour propylene glycol exposure, before (a) and after (b) 24-hour recovery

a.: Apparent LC50: 1.9% propylene glycol (v/v); Apparent effects range: 1.05-3.0%
b.: Apparent LC50: 5.0% propylene glycol (v/v); Apparent effects range: 1.05-12.0%
Error bars indicate standard deviation

As there are threshold effects at both the upper and lower bounds of the test concentrations, instead of determining the NOAEC, I defined the "effects range", the difference between the NOAEC and the lowest concentration not significantly different

from 100% mortality, in each case; this is 0.75-3.0% following the 48-hour propylene glycol exposure (Fig. 3.1a), and 0.75 -12% following the 24-hour recovery period (Fig. 3.1b).

3.2. The second propylene glycol experiment

3.2.1 *Acute response*

The most striking observation is the appearance of a recovery effect similar to that that seen in the preliminary experiments. Figure 3.2 illustrates how the dose-response relationship changes over the daily-observed 72-hour initial recovery period. Up to 1% propylene glycol (v/v) concentrations had no apparent effect on the test specimens. Concentrations of 2% to 6% clearly affected some specimens, though the majority of test specimens eventually recovered.

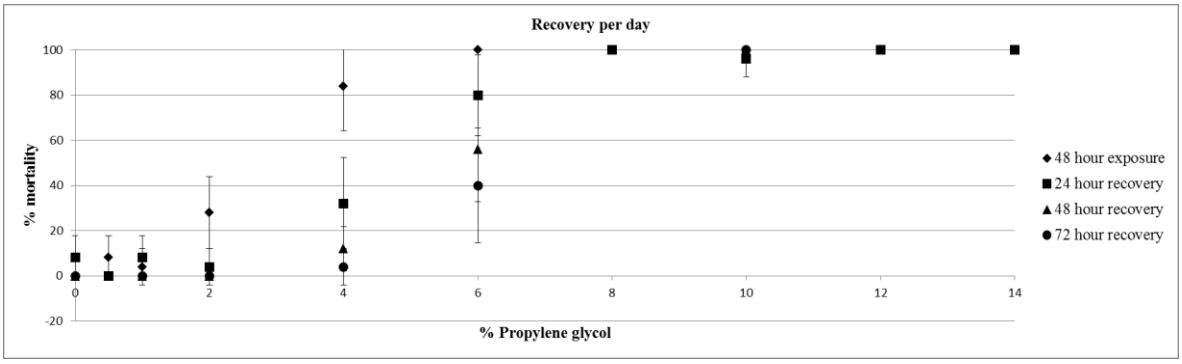

Figure 3.2: The "apparent" percent mortality by concentration of propylene glycol (v/v) in the test media during the 72 hour recovery period. Apparent mortality decreases at concentrations between 2.0% and 6.0% with recovery time. Error bars indicate standard deviation

In addition to confirming the dormancy-recovery effect, I was also interested in identifying an optimum observation period to differentiate acute effects, the combination of dormancy and mortality, and truly lethal effects, for future experiments. This would

allow for the determination of the acute concentration 50 (AC50), the concentration of exposure which causes acute effects in 50% of the sample population, as well as the LC50. As seen in Figure 3.2, I observed a definite change in the dose-response curve across 72 hours of observation. Comparing observations between mortality following the 72-hour recovery and 168-hour (one week) recovery revealed some additional, insignificant recovery near the upper threshold, as well as insignificant increases in mortality at low and intermediate concentrations (Fig. 3.3), while the LC50 and effects ranges remained constant, as seen in Figure 3.4 and Table 3.1.

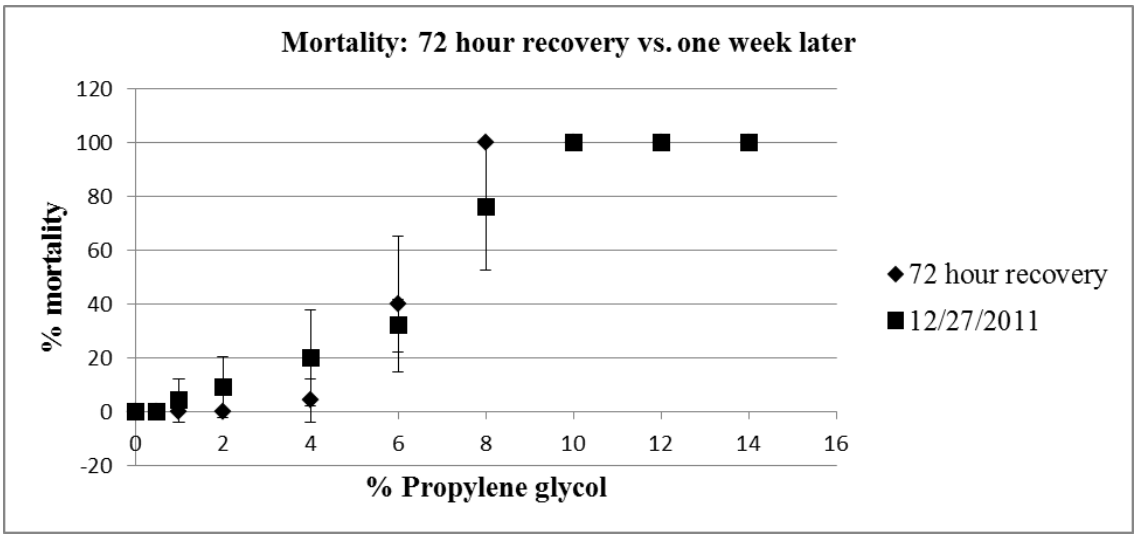

Fig 3.3: A comparison of mortality per concentration of propylene glycol between a 72-hour (3-day) recovery period, and a 168-hour (7-day) recovery, showing the increased mortality in low and intermediate concentrations. Error bars indicate standard deviation

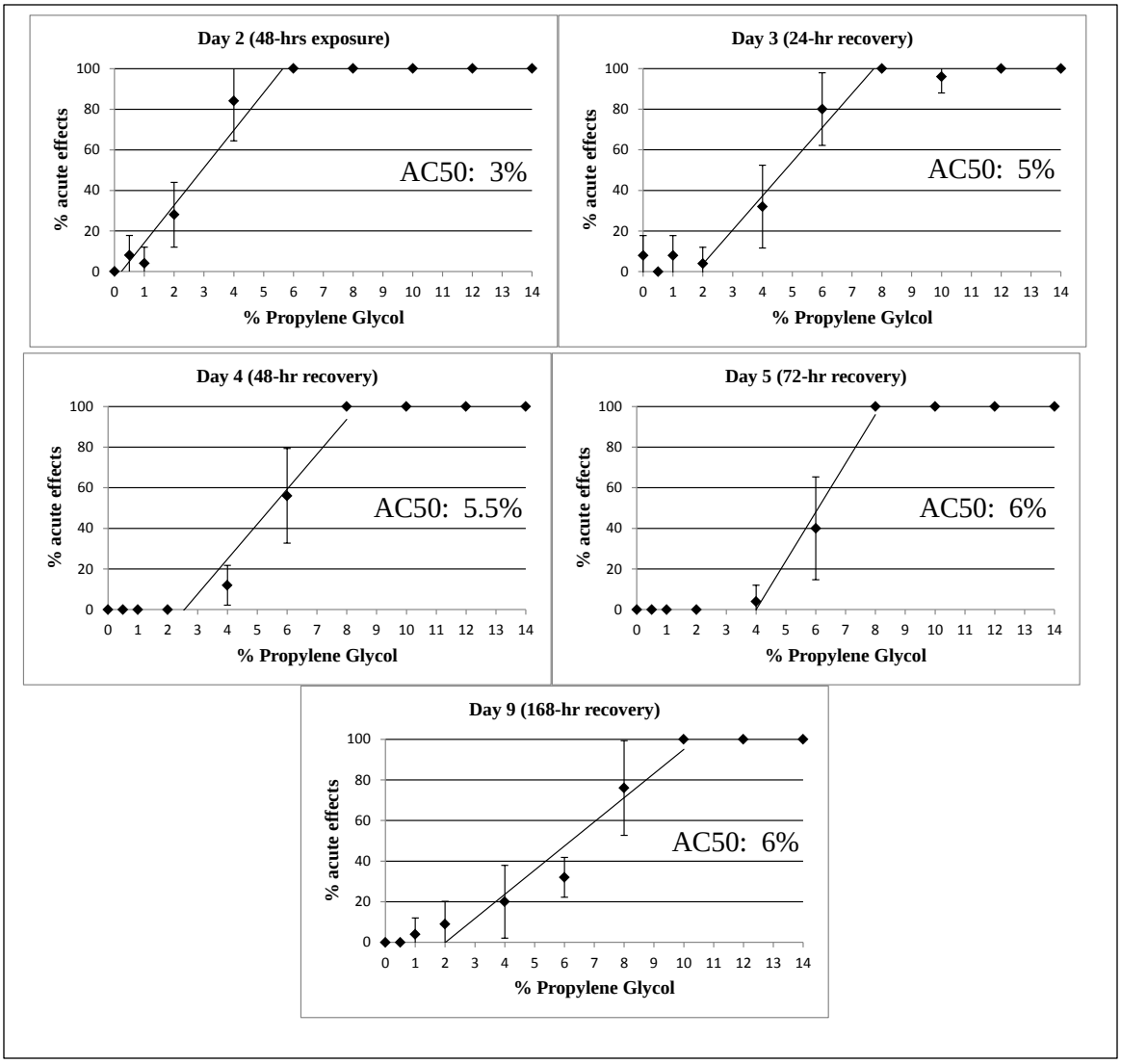

Fig. 3.4: The dose response curves of propylene glycol and associated 50% Acute Concentration 50/Lethal Concentration 50 across the first week of recovery. Error bars indicate standard deviation

Table 3.1: Summary of results from propylene glycol experiments

Preliminary: 48hr exposure	1.05-3%	1.9%
Prelim: 48hr exposure + 24hr recovery	1.05-12%	5%
Second: 48hr	2-4%	3%
Second: 48hr + 24hr recovery	2-6%	5%
Second: 48hr + 48hr recovery	2-8%	5.5%
Second: 48hr + 72hr recovery	2-8%	6%
Second: 48hr + 168hr recovery	2-8%	6%

3.2.2 *Chronic responses - mortality*

Observations of mortality over the five weeks following exposure to propylene glycol (Fig. 3.5) also revealed a surprising trend in which the specimens exposed to the two lowest concentrations (0.5 and 1% v/v) exhibited more mortality than the control and 2% treatments. In addition, a few individuals from the 8% and 10% exposure treatments that initially appeared dead continued to recover through the observation period.

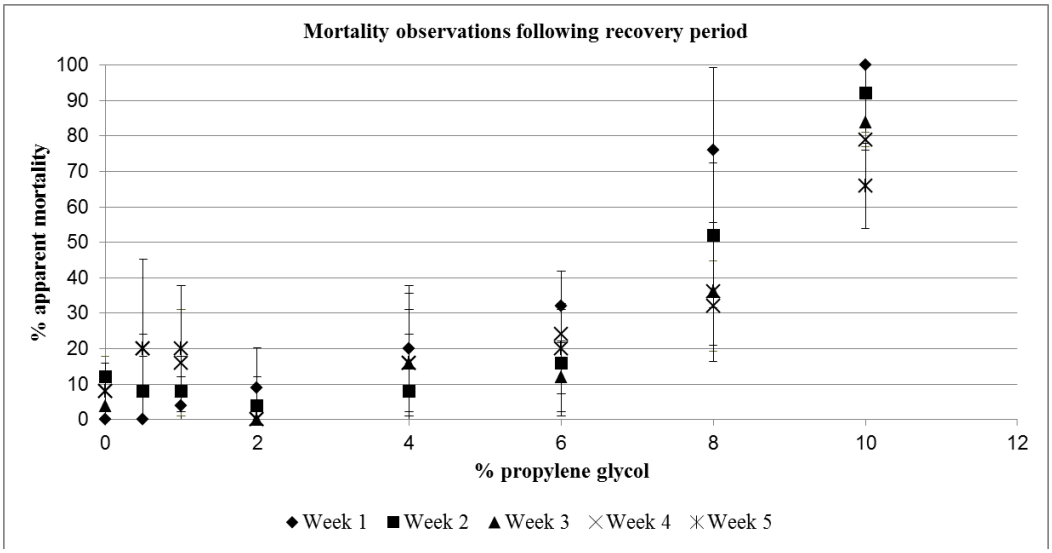

Figure 3.5: Mortality following 48-hr propylene glycol exposure, assessed weekly for 5 weeks. Error bars indicate standard deviation

3.2.3 *Chronic responses - growth*

Then I compared changes in the median diameter by treatment over time as an indicator of growth (Fig 3.6). Again, the results were unexpected, with the 2% treatment exhibiting the most growth. Median diameter was used to look for trends because, as mentioned previously, we did not remove individuals identified as dead from the samples. As a result, replicates with higher mortality would also show lower average growth, as dead *A. gibbosa* show no change in diameter between observations. To avoid this bias, I used median as a measure of central tendency. I further corrected for this effect in the 10% exposure replicates. In these cases, so many of the individuals were dead that, even using median as the measure of central tendency, little change was observable. However, the dead individuals in this treatment were more distinguishable as dead based on visual assessment than those in replicates exposed to lower concentrations. I removed those individuals from the growth analyses and instead present here the "10% corrected" values for diameter and growth rates.

Using MATLAB with the FATHOM toolbox (last retrieved November 14, 2012: http://seas.marine.usf.edu/~djones/matlab/matlab.html), I performed an ANCOVA on the raw diameter data, followed by pairwise comparisons of growth for each percentage of exposure to determine which were statistically different. I again used the "corrected" 10% data series, and also removed one set of data from the control replicate from the analysis, after determining that the pictures for that replicate on that day had been incorrectly calibrated. The removal of this replicate did not alter the median diameter as presented in Figure 3.6.

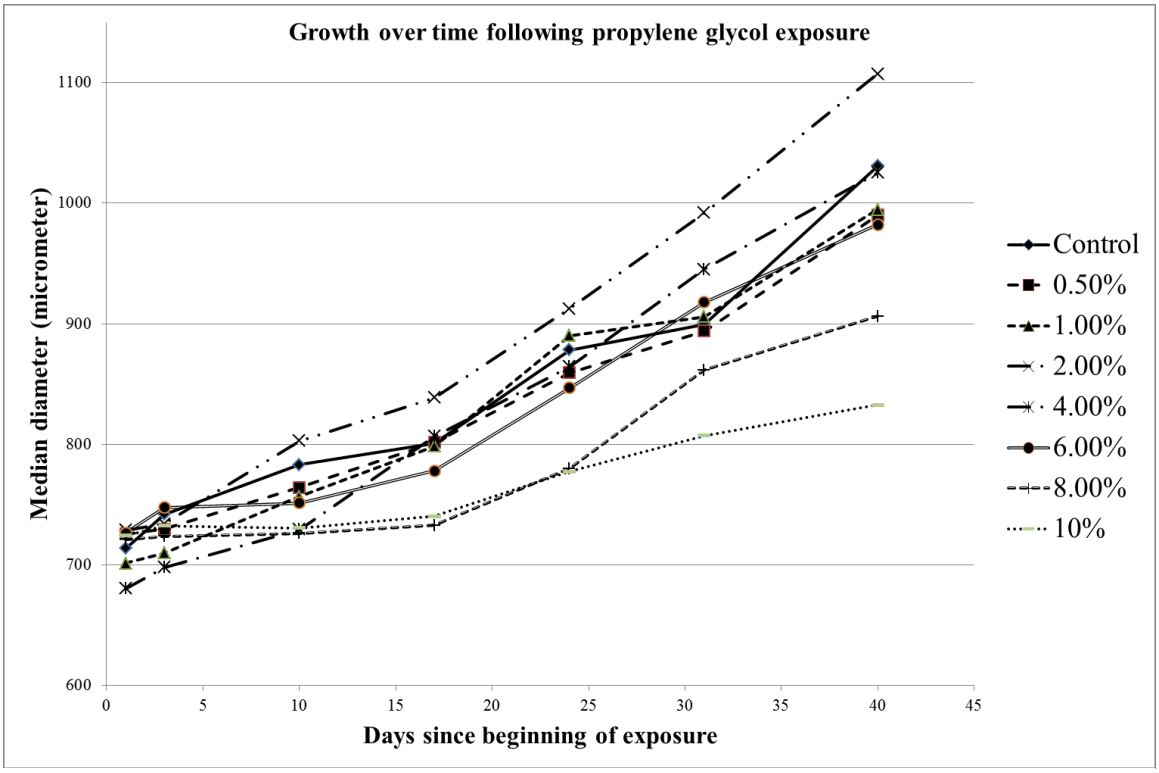

Figure 3.6: Change in median longest diameter over time following 48-hour exposure to indicated concentrations of propylene glycol

The ANCOVA determined that growth, as measured by the slope of the regression line for each concentration of exposure, was heterogeneous. Further pairwise testing, using Holmes-adjusted p-values, determined which concentrations were significantly different from each other, and provided somewhat surprising results. Although growth in the 8% treatment group was significantly lower than in the 2% and 4% treatments, and growth in the 10% treatment was lower than growth in the 1%, 2%, 4% and 6% treatments, neither group exhibited a significant difference in growth

compared to the control or 0.5% treatment groups. In fact, no group exhibited significantly different growth from the control (Table 3.3).

Table 3.2: Median diameter (micrometers) and range of diameters per day before and after 48-hour exposure to propylene glycol

		0%	0.5%	1%	2%	4%	6%	8%
Pre-exposure	Median	714	726	701	729	680	727	721
	Range	416	320	617	516	687	444	397
48-hour exposure	Median	742	730	710	734	698	748	724
	Range	450	337	448	351	345	444	405
168-hour (1 week) recovery	Median	783	764	757	803	730	751	727
	Range	384	427	431	377	415	387	404
336-hour (2 week) recovery	Median	823	802	799	839	813	778	733
	Range	449	491	427	453	414	430	391
504-hour (3 week) recovery	Median	878	860	890	912	888	857	780
	Range	554	533	357	508	517	352	473
672-hour (4 week) recovery	Median	900	894	906	992	948	927	862
	Range	511	566	406	613	556	402	516
888-hour (37 day) recovery	Median	1031	990	995	1107	1030	988	878
	Range	566	617	516	687	615	538	602

Table 3.3: Pairwise comparisons of differences in growth rates of *A. gibbosa* following exposures to various concentrations of propylene glycol

	0%	0.5%	1%	2%	4%	6%	8%	10%
0%	-							
0.5%	-	-						
1%	-	-	-	-				
2%	-	+	-	-				
4%	-	-	-	-	-			
6%	-	-	-	-	-	-	-	
8%	-	-	-	+	+	-	-	
10%	-	-	+	+	+	+	-	-

- indicates no statistical difference in growth rates of the foraminifers exposed to the propylene glycol concentrations in the pairs. + indicates a significant difference

Because the initial variability in diameters of individual foraminifers was substantial (Table 3.2), I also performed a series of ANOVA tests, focusing on the absolute difference in sizes between the foraminifers on the first day of the experiment and on the 40th and final day of the experiment.

To perform these statistical tests, I first placed the diameters of the foraminifers exposed to each concentration in ascending order. Although I had no way to label the individual foraminifers in order to compare absolute difference in diameter, I assume here that growth was homogenous between individuals, and that, i.e., the smallest individual in each treatment group on day 1 was compared with the smallest individual in each treatment group on day 40. Because Tukey's HSD test, as used in the analysis of previous experiments, requires homogenous sample sizes, in those cases where individual foraminifers had been lost, I replaced their diameter with the median diameter of that treatment group. For this reason, I also excluded the corrected 10% propylene glycol treatment group where only 7 specimens were alive on day 40.

First, it is important to note that an ANOVA of the starting diameters showed no significant difference between the size distributions for each treatment group. However, an ANOVA of the 40th day ending diameters does show a significant difference. The mean increase in diameter for each treatment is shown in Figure 3.7, and the Tukey's HSD pairwise comparisons in Table A19 in the appendix.

Mean increases in diameter in the control, 1%, 2%, and 4% groups were not significantly different; specimens exposed to 6% propylene glycol increased significantly less than those exposed to 2%, but were not significantly different from the control, 1%

or 4% treatments. Mean increases in diameter in the 0.5% and 8% treatment groups were significantly lower than that seen in the control, 1%, 2% and 4% groups.

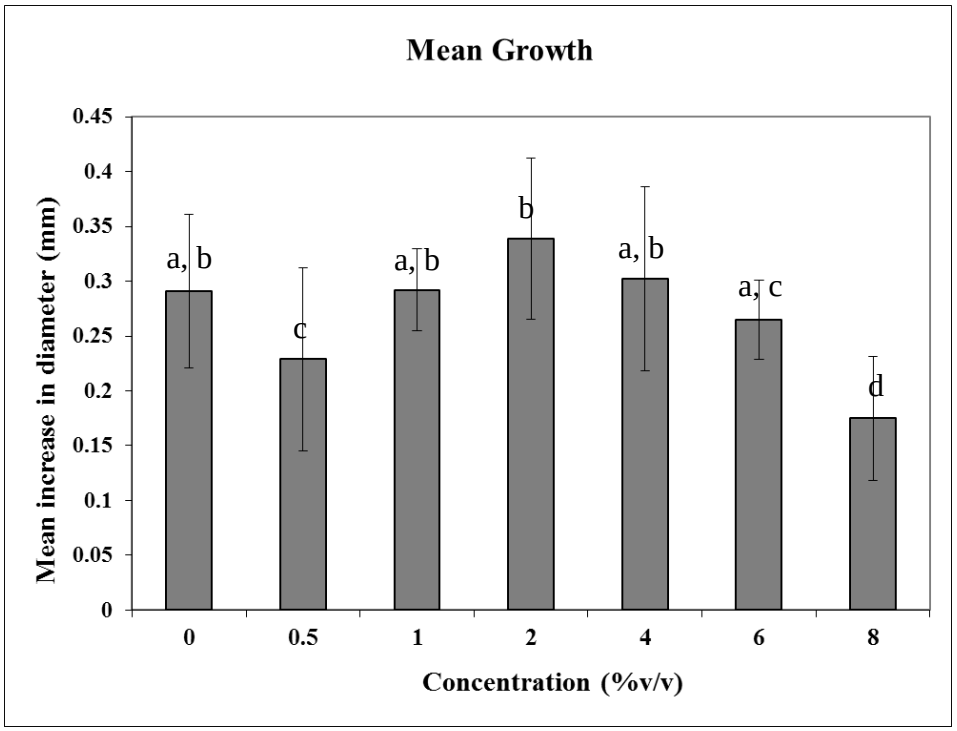

Figure 3.7: Mean increase in diameter of *Amphistegina gibbosa* after 40 days in culture following 48-hour exposures to the concentrations of propylene glycol shown. Letters above the bards indicate which treatments are significantly different from each other. Error bars indicate standard deviation

3.2.4 *Chronic responses – visual*

At the end of the five week observation period, the percentage of normal, slightly mottled, very mottled, other, and dead *Amphistegina gibbosa* were calculated for each propylene glycol treatment (Fig. 3.8). The differences in the distribution can be seen more clearly in Figure 3.9, which shows the cumulative percentage of individuals in each propylene glycol treatment group which fall into all of the categories under the curve at each point.

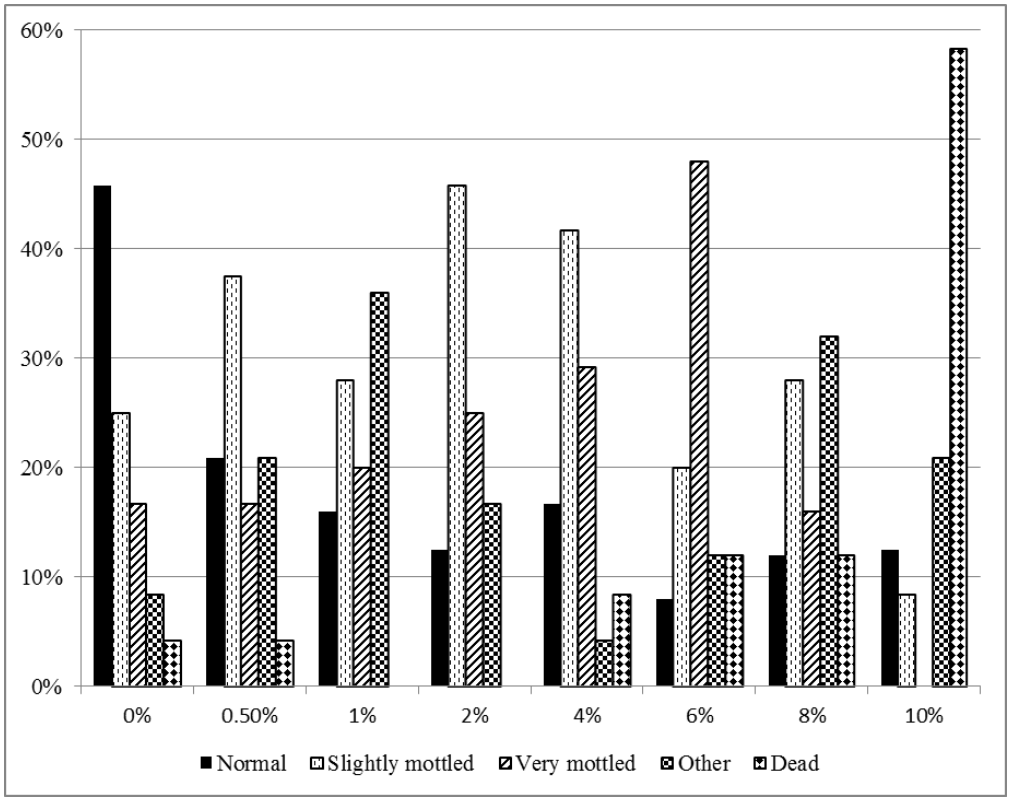

Figure 3.8: Visual assessment 40 days after propylene glycol exposure

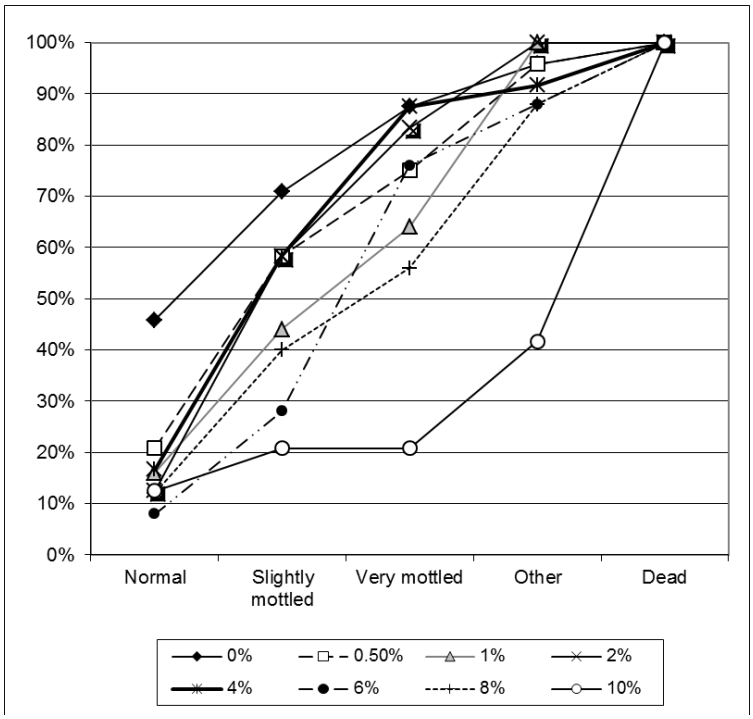

Fig. 3.9: Cumulative frequency graph of visual assessment 40 days after propylene glycol exposure

Differences in the cumulative distributions were tested using Kolmogorov-Smirnov Goodness of Fit Tests (Table 3.4 and 3.5), a pairwise comparison between two distributions (Zar 1999). Table 3.4 compares all of the distributions to that of the control. For a p-value of 0.05, k = 5 categories, and a conservative n of 20 (since some treatment groups were missing individuals and had less than 25), the critical d_{max} is 6. If the difference between any 2 categories in any 2 treatment groups is equal to or greater than d_{max}, the distribution is significantly different. Table 3.4 shows that all of the treatment groups show significantly different distributions of visual assessment classes from the control. Table 3.5 compares nearest neighbors. The 0.5%, 1%, 2% and 4% treatments have similar distributions, as do the 6% and 8% treatments, and 10% and 12% are dissimilar. Differences are significant between the 4% and 6% treatments, 8% versus 10%, and 10% versus 12%.

Table 3.4: Kolmogorov-Smirnov Goodness of Fit tests comparing maximum differences in cumulative frequencies of all treatments against the control, based on visual assessments

Comparing all treatments with Control								
	0.5%	1.0%	2.0%	4.0%	6.0%	8.0%	10.0%	12.0%
Normal	**6**	**7**	**8**	**7**	**9**	**8**	**8**	**11**
Slightly mottled	3	**6**	3	3	**10**	**7**	**12**	**17**
Very mottled	3	5	1	0	2	**7**	**16**	**21**
Other	0	-2	-1	1	1	1	**13**	**23**
Dead	0	-1	0	0	-1	-1	0	-1

Bold numbers indicate significant differences between the test frequency distributions and the control frequency.

Table 3.5: Kolmogorov-Smirnov Goodness of Fit tests comparing nearest neighbors, based on cumulative frequencies of visual assessments

Comparing nearest neighbors								
Concentration:	0.5 vs 0%	1.0vs 0.5%	2.0 vs 1.0%	4.0 vs 2.0%	6.0 vs 4.0%	8.0 vs 6.0%	10.0 vs 8.0%	12.0% vs 10.
Normal	**6**	1	1	-1	2	-1	0	3
Slightly mottled	3	3	-3	0	**7**	-3	5	5
Very mottled	3	2	-4	-1	2	5	**9**	5
Other	0	-2	1	2	0	0	**12**	**10**
Dead	0	-1	1	0	-1	0	1	-1

Bold numbers indicate significant differences between frequency distributions.

Comparing increase in diameter with visual response by Day 40 shows minimal relationship (Fig. 3.10). Although growth rates in foraminifers exposed to 1, 2 and 4% propylene glycol were not significantly different from the control, these treatments also had fewer normal, healthy-appearing individuals than the control and 0.5% treatments.

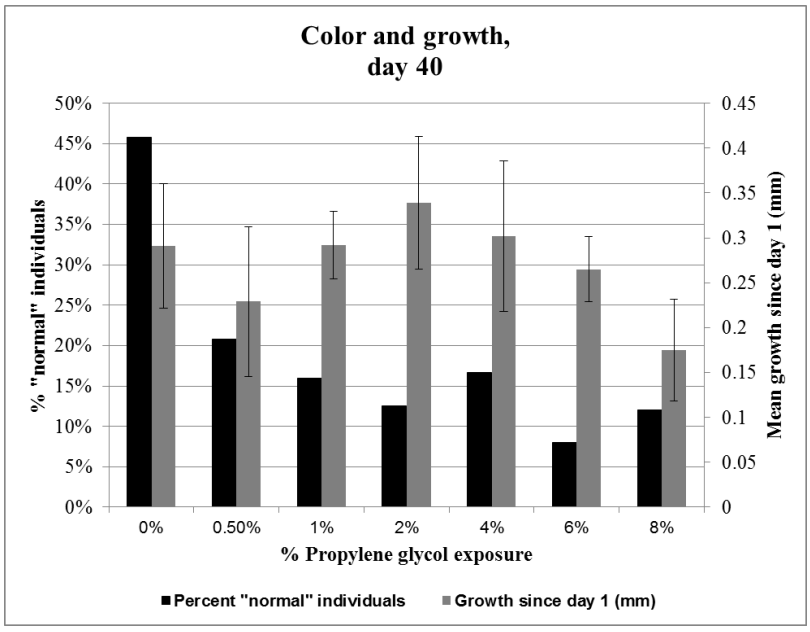

Figure 3.10: Percent normal-appearing specimens and mean increase in diameter after 40 days, following exposure to propylene glycol. Error bars indicate standard deviation

3.3. Experiments with 2-butoxyethanol

The results of the preliminary experiment with 2-butoxyethanol clearly showed a dormancy-recovery effect in *Amphistegina gibbosa* similar to that observed in treatments with propylene glycol (Fig. 3.11). Every test concentration of 2-butoxyethanol produced 100% acute effects. However, recovery occurred in specimens exposed to the lower and intermediate concentrations following the 72 hour recovery period (Fig. 3.12). Following recovery, it was possible to estimate the LC50 at 1% and an effects range of 0.32 -1.28% (Fig. 3.12) using the same statistical techniques as used in the previous experiment.

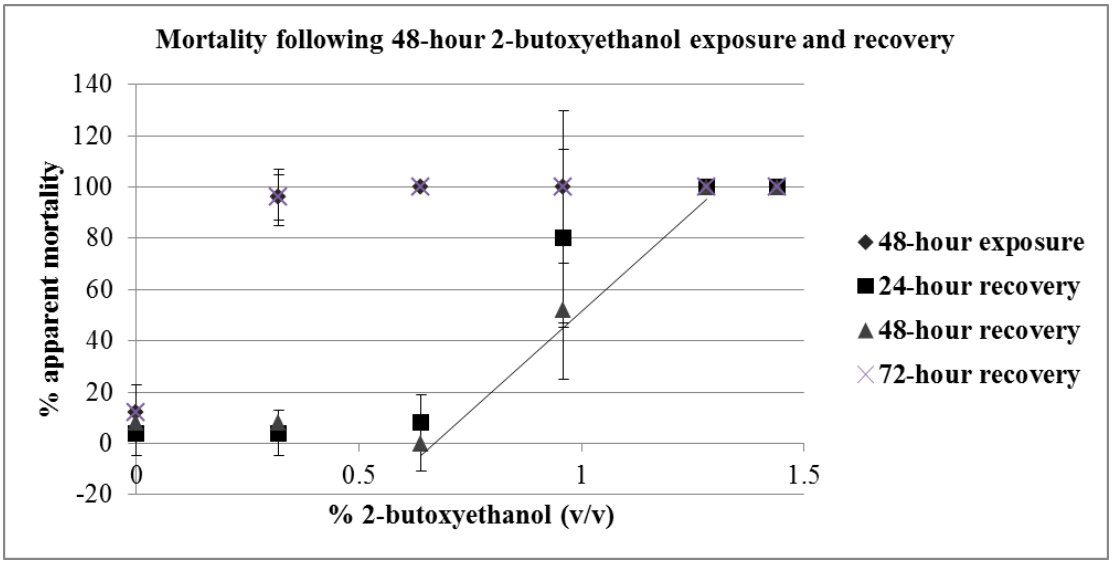

Fig. 3.11: Mortality vs. concentration of 2-butoxyethanol, 48-hour exposure and after three recovery periods. Error bars indicate standard deviation

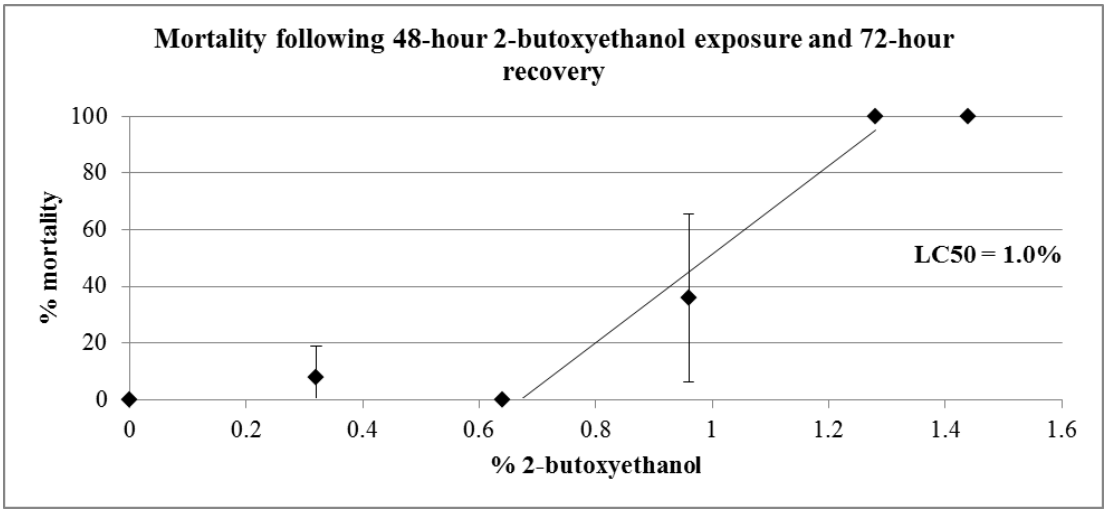

Fig. 3.12: Mortality following 48-hour 2-butoxyethanol exposure and 72-hour recovery. LC50 = 1.0%; Effects range = 0.3-1.3%. Error bars indicate standard deviation

A second round of exposures determined the AC50. Unfortunately, inclement weather kept me from accessing the lab and assessing the mortality at our established 72-hour recovery period, so recovery was over a period of 96 hours instead, and I was not able to follow the recovery daily. Nevertheless (Fig. 3.13), I was able to establish the AC50 of 0.2% and the effects range of 0.15-0.3% following the 48-hour exposure, using the same statistical techniques previously employed (Fig. 3.14).

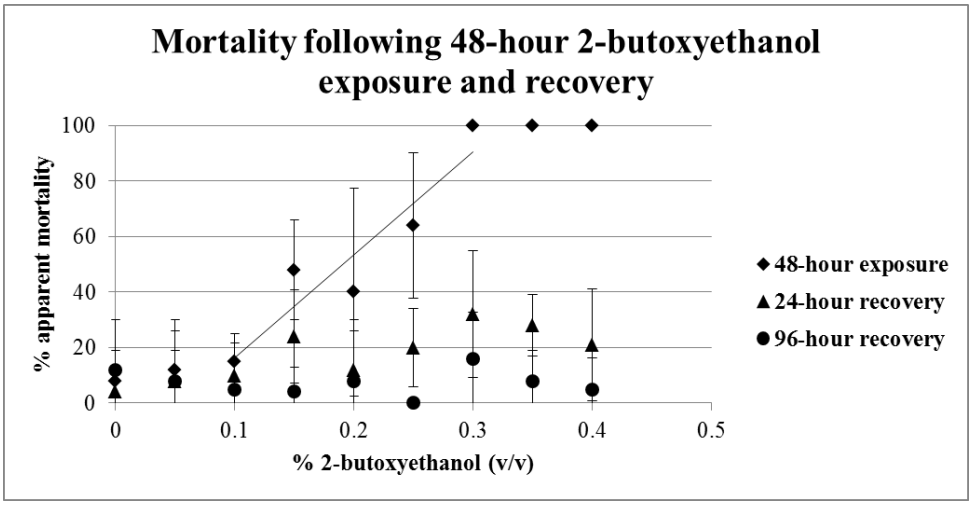

Figure 3.13: Mortality vs. concentration of 2-butoxyethanol, 48-hour exposure and recovery. Error bars indicate standard deviation

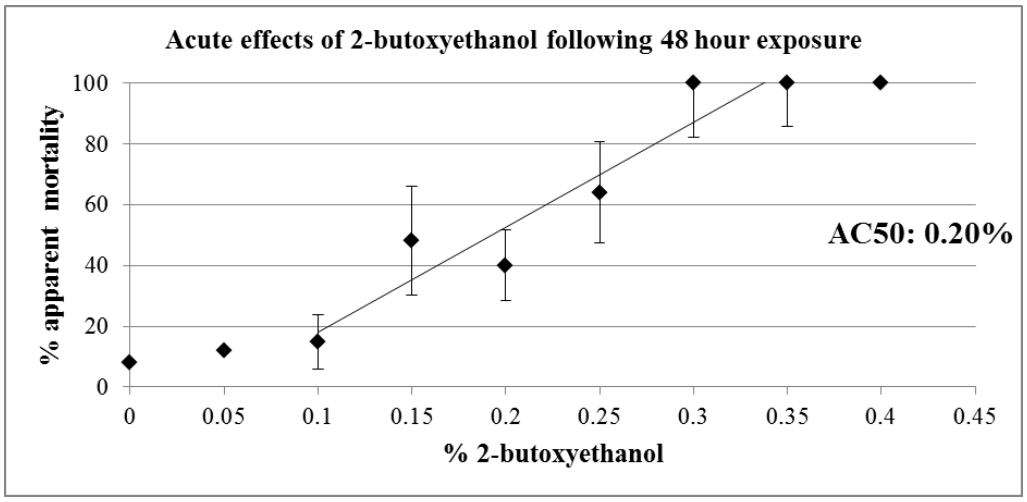

Figure 3.14: Mortality following 48-hour exposure to 2-butoxyethanol. AC50 = 0.20%; Effects range = 0.2-0.3%. Error bars indicate standard deviation

4. Discussion

4.1 Dormancy as a response to toxic exposure

The initial surprise, and ultimately unifying theme of this set of experiments, was the discovery that *Amphistegina gibbosa* respond to exposure to a potentially toxic chemical in their culture medium by becoming inactive by withdrawing their rhizopodia into their shells, detaching from the substratum, and apparently rearranging their endoplasm in ways that result in abnormal coloration. While a similar response had been observed in *Amphistegina* spp. previously (e.g., Smith and Hallock 1992), the trigger for the response primarily had been prolonged darkness. These foraminifers were observed to recover from this inactive condition after more than one year in total darkness, and normal reproduction was observed after nine months in total darkness. While one earlier observation indicated that *A. lessonii* and *A. lobifera* could survive anoxia in the dark, the assumption had been that the foraminifers ceased activity in the darkness and were able to survive the anoxia because they were already inactive (Hallock personal communication).

The appropriate terminology for this inactivity can be considered dormancy or quiescence in a broad sense, and more narrowly, perhaps diapause is the best term. Reasonable working definitions for dormancy in organisms include: a) a condition of biological inactivity characterized by cessation of growth or development, and the suspension of many metabolic processes (http://www.thefreedictionary.com/dormancy)

and b) a state when organisms are in unfavourable conditions and slow down their metabolic processes to a minimum to retain resources until conditions are more favourable (http://www.biology-online.org/dictionary /Dormancy). The latter definition is also a working definition for diapause. O'Farrell (2011), reviewing "quiescence", which is a subset of dormancy, noted that quiescence is likely a primitive biological process, that appears in many distinct biological settings. One example of the diversity of possible mechanisms within a taxon was reported by Guidetti et al. (2011), who noted that tardigrades have evolved a variety of dormant stages that can be ascribed to diapause (encystment, cyclomorphosis, resting eggs) and cryptobiosis (anhydrobiosis, cryobiosis, anoxibiosis). In tardigrades, diapause and cryptobiosis can occur separately or simultaneously, and thus are not mutually exclusive.

Dormancy in foraminifers is not well understood, and relatively little has been published on the phenomenon. Alve and Goldstein (2003) note that, although dormancy has not yet been documented in benthic foraminifers, there are several lines of evidence suggesting the potential for quiescence in adult specimens. Previous studies that have provided evidence of dormancy in benthic foraminifers tend to fall into two broad categories: a) papers which suggest dormancy in combination with encystment as a form of juvenile dispersal (i.e., cryptobiosis), and b) papers which suggest it as a survival mechanism in reaction, primarily, to anoxic conditions or, in the case of foraminifers that host algal endosymbionts, prolonged darkness.

Evidence for cryptobiosis has primarily been presented by Alve and Golstein (2003, 2010, Goldstein and Alve 2011), who have seen the appearance and growth of shallow-water species of benthic foraminifers under simulated shallow-water conditions

in sediment collected from deeper water sites (i.e., 320 m water depth). The species that appeared were not found in the live assemblage of foraminifers collected at the site, instead only appearing as juveniles after conditions had changed to favor their growth. This appearance was correlated with the presence in the sediment samples of hollow agglutinated "cysts". The presence of both microspheric and megalospheric juveniles suggests that both sexually- and asexually-produced juveniles were capable of creating these cysts, and the emergence of juveniles up to 2 years after initial sediment collection (Alve and Goldstein 2010) suggests that these foraminiferal propagules are biologically dormant while in conditions unsuitable for growth. Alve and Goldstein primarily consider cryptobiosis in juvenile propagules as a mechanism for dispersion

Linke and Lutze (1993), however, reported encystment of *Elphidium incertum* and *Sacchoriza ramosa* as a survival mechanism in anoxic conditions. Although they did not specifically mention any evidence of dormancy, the presence of sedimentary encystment in adult specimens suggests that this may be a viable survival mechanism in adult, as well as juvenile, foraminifers.

The link to anoxia is interesting in that this is the other major condition for which evidence indicates dormancy in foraminifers. Hannah and Rogerson (1997) reported that live foraminifers buried in anoxic benthic sediment may survive in an "inactive" state, and are capable of survival if passively relocated in a relatively short amount of time, although due to a reliance on Rose Bengal staining, which is not very reliable at fine time scales, no definite conclusions could be drawn. Bernhard (1993) suggested dormancy as a mechanism to explain survival observed following anoxia experiments, and Bernhard and Alve (1996) used an ATP assay to analyze foraminiferal survivorship under anoxic

conditions. They found that, in some species, survival was not affected by anoxic conditions, but that ATP was significantly depleted. The conclusion was that these species were surviving anoxic conditions by becoming "dormant", as opposed to groups that showed a decrease in both survival and ATP (dying or damaged under anoxic conditions) or no change in either (apparently capable of anaerobic respiration).

Adult specimens becoming dormant in reaction to an environmental stress is more similar to the response I observed in my experiments. Furthermore, one of the species exhibiting the pattern of survivorship and ATP change associated with dormancy, *Stainforthia fusiformis*, was apparently performing chloroplast husbandry, which may have contributed to its ability to remain alive while not actively feeding, and in lowering ATP requirements (Bernhard and Alve 1996). This is interesting, in that it suggests something similar could be occurring in the endosymbiotic *Amphistegina gibbosa*, in reaction to periods of environmental toxicity as well as to anoxia.

4.2 Responses of *Amphistegina gibbosa* to toxic exposures

Difficulties arise when trying to reconcile inactive behavior responses with standard measures of toxicity, such as the LC50. Since little known about the physiology of dormant foraminifers, little is known about the length of time specimens can remain dormant before dying, especially in the presence of light, nor how rapidly individual specimens can enter the dormant state, or whether they are still taking up toxins in some amount from the environment while dormant. Although there are a number of assays which may be able to answer some of these questions, or provide a more accurate

definition of vitality vs. mortality, they were beyond the scope of this project. As a result, I instead focused on redesigning the original methodology to take the dormancy effect into account, as well as to allow for longer term observations of mortality and sub-lethal effects, including growth rates and bleaching.

In addition to confirming the dormancy-recovery effect, I was also interested in identifying an effective observation period to differentiate acute effects and lethal effects for future experiments. As seen in Figure 3.2, I observed a definite change in the acute dose-response curve across 72 hours of observation. Although 72 hours was originally my target recovery time, I had planned to continue observing for the duration of the first week; unfortunately, outside events kept me from performing another mortality assessment until the specimens had been in recovery for the full week. Comparing observations from these two days (Fig. 3.3) produced some interesting observations. Although there was some slight recovery near the upper threshold, there was also an increase in mortality at low and intermediate concentrations, while the AC50/LC50 remained consistent, as seen in Fig. 3.4.

In all of the 48-hour exposure experiments, some *A. gibbosa* specimens, which appeared to be functionally dead following exposure to the test media, were able to recover when moved to clean seawater. As seen in Figure 4.1, the difference in the AC50/LC50 between a 72-hour recovery and a 168-hour recovery is negligible. At the same time, the observed increase in mortality at low and intermediate concentrations suggests that the mortality may be a result of chronic effects following the chemical exposures. The lack of a measureable difference in LC50 after a further 4 days of recovery, especially considering the larger daily changes during the 72-hour recovery

period, leads me to conclude that 72 hours is a reasonable amount of time in clean seawater to allow recovery from the dormant state, without allowing individuals exposed to low and intermediate concentrations to die from chronic effects of exposure.

The AC50 estimates were similar in both the preliminary and full experiments with propylene glycol, with the AC50 following 48-hour exposure calculated at 2% in the initial experiments and 3% in the subsequent experiment. Moreover, although the foraminifers in the initial experiments were given 24 hours of recovery in filtered seawater instead of 72 hours, the apparent LC-50 following the 24-hour recovery in both series of experiments is the same, 5%. This suggests that sample populations of *A. gibbosa* collected at different times and selected using visual assessments of health as a basis of inclusion will produce consistent results.

Documenting the dormancy-recovery phenomena in *Amphistegina gibbosa* exposed to propylene glycol underscores the challenges in defining a 50% lethal concentration following chemical-exposure experiments, an observation which is important both in what it reveals about *A. gibbosa*'s reactions to the chemical, and in understanding comparisons of the effects of pollutants on *A. gibbosa* with other organisms. Given the method of determining vitality, exposed individuals must be allowed to recover to differentiate inactivity from mortality. Moreover, the higher mortality at low and intermediate concentrations seen between the 72-hour recovery and 7-day (168-hour) recovery assessments reveals the challenges in distinguishing mortality as an acute effect of the 48-hour exposure, and not a chronic effect. This differentiation requires determining the minimum recovery time while allowing as full a recovery as possible. Given my observations (Fig. 4.1), I conclude that 72 hours as an effective

amount of recovery time, at least in reaction to propylene glycol. As seen in Figure 3.4, recovery continued every day of assessment for the first 3 days, as indicated by the increasing AC50; but by the seventh day, I began to see increased mortality at low and intermediate concentrations.

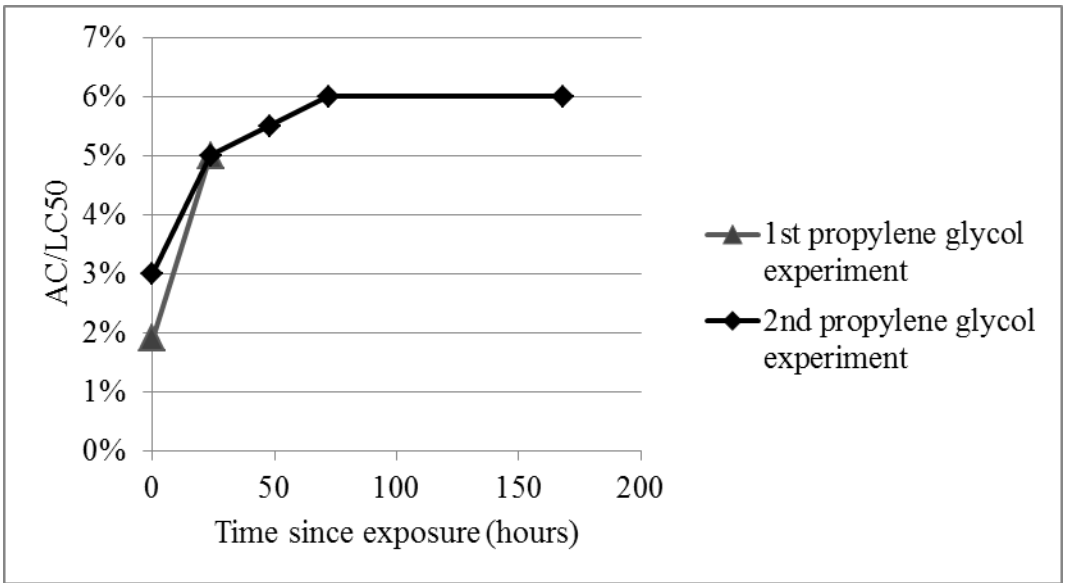

Figure 4.1: Change in AC50/LC50 following 48-hour exposures to propylene glycol and recovery periods shown

I observed slightly higher mortality in the foraminifers exposed to low (0.5 and 1%) concentrations of propylene glycol after 5 weeks of observation than in those exposed to 2%, which may suggest longer term chronic effects of the chemical exposure in specimens that did not become inactive (Fig. 3.5). However, there is also an increase in variability of the observations between weekly observations compared to the daily observations during the recovery period. The variability may be a result of the qualitative nature of the vitality assessments. Because I was investigating recovery times, individuals identified as dead were not removed from the petri dishes, except in a few cases where they had asexually reproduced and therefore were unable to recover. As a

result, individuals, especially those that were less healthy and less active, might have been counted as dead one week and live the next, depending on their activity level on the day of observation. With no way to label individuals, I was not able to compare weekly observations at a scale smaller than by replicate. Because of the variability arising from my working definition of vitality, more precisely identifying chronic effects requires a more precise measure of vitality. Thus, identifying a more accurate and efficient vitality measure is a future research goal.

Another observation that is somewhat incongruous is that, in specimens in the treatments exposed to higher concentrations of propylene glycol (8 and 10%), some continued recovery occurred over time, at the same time as specimens exposed to low concentrations (0.5 and 1%) exhibited increased mortality. These observations may be the result of algal contamination. There was a difference in the visual appearance of individuals between the concentrations, with those exposed to lower concentrations tending to appear "healthier" than those exposed to higher concentrations (see section 2 and Figure 2.1 for a definition of visual criteria). This difference also made it easier to accurately determine the vitality of individuals exposed to the lower concentrations, as they also tended to be more active and more likely to extrude pseudopodia. Those individuals in the higher concentrations, in addition to having a markedly unhealthy appearance, also tended to be less active. Pseudopodial extrusion, when it occurred, was less common than in the lower concentration exposures, and more difficult to identify. As a result, attachment to the petri dish became a more important indicator of vitality. Although efforts were taken to limit algal growth in the petri dishes (including filtering the seawater and rinsing the specimens before the start of the long term observations), it

still occurred. Algal growth was especially marked around the specimens, where it was also difficult to identify, especially when it was just beginning. It is possible that some of the individuals which appeared to be attached were actually anchored by algal growth and recorded as alive due to their apparent attachment. This could explain the pattern of apparent recovery in individuals which appeared to be quite unhealthy, if not completely dead, as judged by color. As a result, the long term mortality observations are likely not as reliable. Because the algal growth occurred relatively slowly over a number of weeks, it did not affect the observations during the 72-hr recovery period. Any further research aimed at differences in chronic mortality effects following exposure will need to be highly cognizant of the danger of algal growth and its potential to disrupt mortality assessment; brushing of the specimens, weekly transfers to fresh petri dishes, and more frequent changing of the nutrient media may help to combat algal growth. However, as algae and microbes facultatively associated with the host can bloom on the food and nutrient sources provided by the decay of the host cytoplasm, future protocols should be designed to take this into account to avoid misidentifying dead individuals as live.

Exposure to propylene glycol also had a somewhat ambiguous influence on growth rates. There is evidence for enhancement of growth at 2% and possibly also at 4%, in the latter case after initial inhibition (Fig. 3.6). Likewise, grow was significantly inhibited at 0.5%, 6 and 8 %. It is important to note, however, that this pattern of intermediate enhancement of growth is not unprecedented. The pattern of change in median growth by the percent of propylene glycol exposure (Fig. 4.2) is comparable to the pattern of growth rates of the chlorophyte *Dunaliella tertiolecta* (relative u_{max}) in different concentrations of propylene glycol (Tzovenis et al. 2004), and, in exposures of

Amphistegina gibbosa to arsenic (McCloskey 2009). Mean growth of these foraminifers, when plotted against the concentration of arsenic exposure also revealed a significant enhancement of growth at intermediate levels. Plotting the mean growth rate of *A. gibbosa* against the concentration of propylene glycol exposure gives a pattern with a very similar shape, as seen in Figure 4.2. McCloskey (2009) suggested that the effect seen in his research may have been caused by arsenic's role as a microbioherbicide, eliminating algal growth that could limit *A. gibbosa* growth. Propylene glycol is not known to have the same effect, so the source of this growth pattern may be different. One possibility is that foraminifers initially took up propylene glycol and were able to use it as a food source. Thus, the similarity of all three examples suggests that the observed patterns are valid.

When all parameters from my 48-hour propylene glycol exposure experiments are compared to those parameters from the control treatments, at least one chronic effect is evident in all treatments. Interestingly, specimens in the lowest treatment concentration, 0.5%, grew significantly slower than the control and exhibited significantly more visual changes, primarily bleaching. In the 1% treatment, specimens also exhibited significantly more bleaching but growth was not affected. In the 2%, 4%, and 6% treatments, many specimens exhibited acute responses of detachment and color changes, though most recovered and growth rates were not affected. However, approximately 70% of the specimens in those three treatments exhibited bleaching, with the percentage of very mottled specimens nearly doubling between the 2% treatment and the 6% treatment. Exposure to higher concentrations produced both acute and chronic responses in all categories, with nearly 100% mortality at 12% and 14% concentrations.

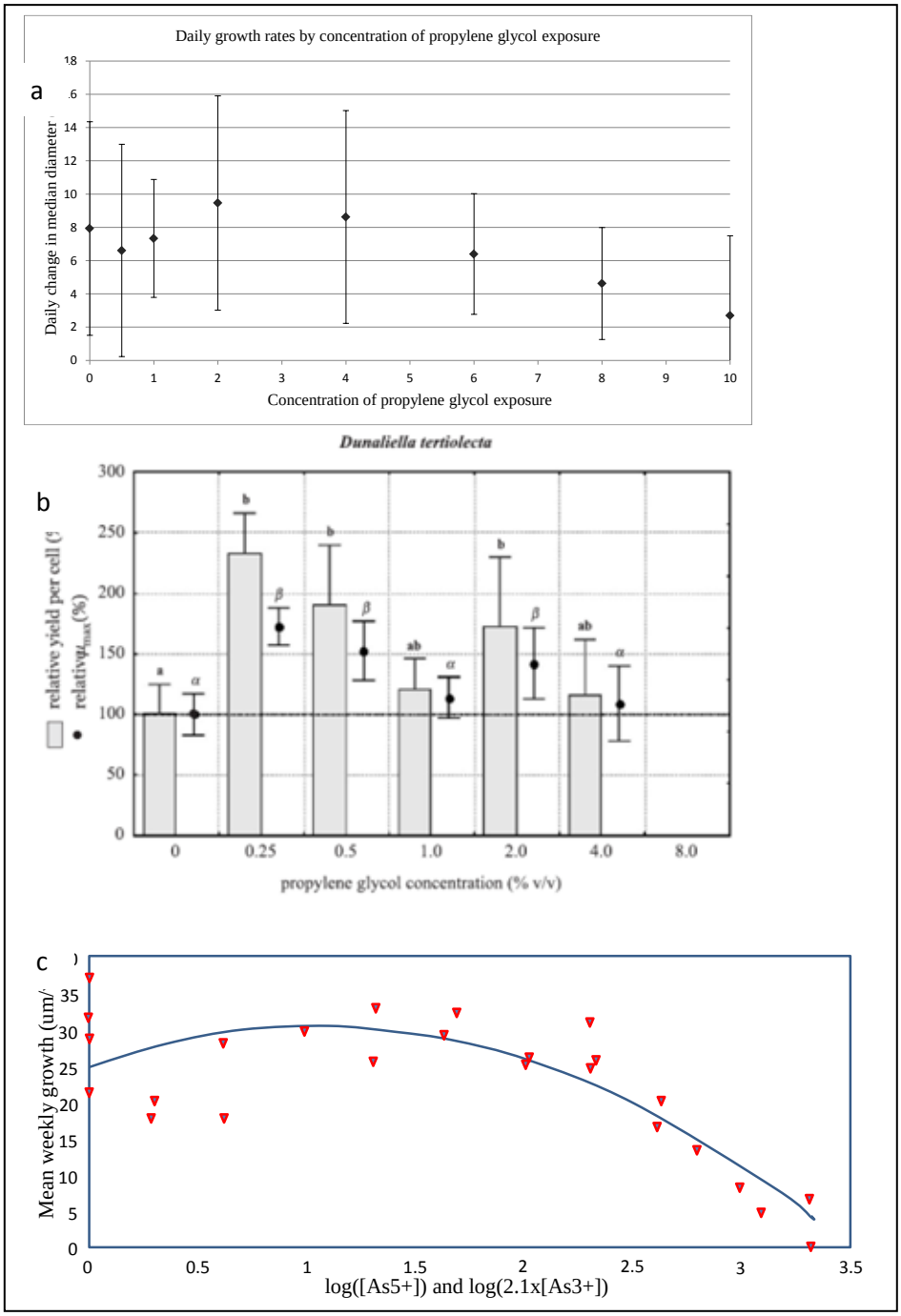

Fig. 4.2: Comparison of average growth rates. (a) *Amphistegina gibbosa* exposed to propylene glycol (error bars indicate standard deviation), (b) *Dunaliella tertiolecta* exposed to propylene glycol (modified from Tzovenis et al. 2004), and c) *Amphistegina gibbosa* (McClosky 2009) exposed to arsenic

Table 4.1: Comparison of effects of propylene glycol exposure treatments to control treatments

	Acute Effects (after 48 hours)	Mortality (after 72-hour recovery)	Growth (after 40 days)	Visual health (% normal individuals after 40 days)
0.5%	↔	↔	↓	↓
1.0%	↔	↔	↔	↓
2.0%	↑	↔	↔	↓
4.0%	↑	↔	↔	↓
6.0%	↑	↑	↔	↓
8.0%	↑	↑	↓	↓
10.0%	↑	↑	↓	↓
12.0%	↑	↑	N/A	N/A
14.0%	↑	↑	N/A	N/A

Arrow indicates whether the concentration produced a significant increase (↑) in the measurement compared to control, a significant decrease (↓), or was not significantly different (↔)

Table 4.1 makes it easy to recognize the broad effects which the 48-hour propylene glycol exposures had. Firstly, although I hypothesized that chemical exposure would have a sublethal inhibiting effect on growth, this only occurred in the 0.5, 8 and 10% treatment groups; in fact, some increase in growth at 2 and 4% was suggested by Figures 3.6 and 3.7, although it was not significant compared to the control. This suggests that short-term exposure of *Amphistegina gibbosa* to propylene glycol may have

a sublethal effect on growth at low (~0.5%) concentrations. In the 8 and 10% treatment groups the presence of growth inhibition is accompanied by significantly higher mortality rates symptomatic of direct toxicity.

Secondly, Table 4.1 illustrates that although there were no significantly acute effects below 2% propylene glycol exposure, and no significantly higher mortality below 6%, visual assessments, expressed here in terms of the proportion of *A. gibbosa* in each treatment group appearing "normal" 40 days after exposure to propylene glycol – shows significant negative change even at concentrations that do not cause significant increases in acute effects or mortality. The lack of a relationship between growth and proportion of normal individuals matches previous research, which found that partly bleached individuals continued to grow (Hallock 1986a). Moreover, the presence of effects on percent normal individuals at low concentrations of exposure suggests that even low concentrations can stress the foraminifer, inducing bleaching and other stress responses. This is also promising in terms of *A. gibbosa*'s potential as a bioindicator of chemical exposure on coral reefs, given that visual assessment is the simplest indicator of chronic response.

Amphistegina gibbosa has already seen use as a bioindicator of photoxidative stress on coral reefs (Hallock et al. 2006). Corals, too, may become more susceptible to photoxidative stress when exposed to organic compounds in dispersants. Knowing that sublethal concentrations of the propylene glycol will cause increased bleaching in *A. gibbosa* would make this response ideal for tracking contamination on a reef, and with further research it may be possible to determine similar reactions to other water-borne

pollutants, suggesting potential for its use as an indicator for organic chemical exposure in general.

The greatest challenge in this study as a whole was something that sounded deceptively simple: determining mortality. The 24-hour recovery period in clean seawater following the toxicological exposures was originally expected to show the recovery of a few borderline individuals. Instead, early results saw almost complete recovery in some treatments where "mortality" was originally recorded as 100%. Thus, the visual characteristics assumed to indicate mortality, i.e., lack of rhizopodial activity including attachment and the alteration of color, cannot distinguish between an acute "dormancy" response and mortality. This dormancy effect, as discussed previously, must be considered when attempting to determine levels for acute toxicity tests. Moreover, future studies to determine physiologically and cytologically how these foraminifers are responding, and how long they can survive exposure to otherwise toxic substances, requires further study. However, it is interesting to note that, despite surviving exposure to 2-6% concentrations of propylene glycol by becoming dormant, after return to clean seawater they grew seemingly normally but bleached quite extensively. Hallock et al. (1995) demonstrated that bleached *A. gibbosa* are much less likely to produce normal asexual broods. Thus, exposure to propylene glycol at all of the test concentrations caused at least chronic effects that would reduce reproductive potential.

Using attachment as an indicator of condition at the end of the 48-hour exposure time means that, because of the potential for dormancy, my original experimental design would have overstated the true mortality in the test population. Because of this, I developed a method to account for this difficulty, while still being as accurate in as short

a time as possible. Although perhaps not as precise as some other methods, one of my ongoing goals is to develop protocols which use reef foraminifers as "low-cost" bioindicators. Many coral reefs are located in areas that lack the equipment and resources to employ more advanced laboratory methods. There are a number of techniques which have the potential to more accurately differentiate dormant and dead individuals via physiological observations but these methods require specialized equipment, expensive chemicals and training that may make the cost of the methods untenable for many independent groups interested in monitoring reef health. Reef-damaging environmental practices are more common in countries that lack the resources for education and the building of infrastructure that combats such behavior, and there is great potential for research and conservation groups working in those areas to greatly benefit from assay methods that can be performed with a minimum of technological requirements. Thus, although there are shortcomings associated with the subjective measure of mortality employed in my methods, they have the potential to be very useful in situations where more technologically advanced methods are not feasible.

In addition, although I developed methods to determine toxicity measures based on "true" mortality, information on the "acute effects" (the combination of mortality and dormancy), is not without merit. From the point of view of the foraminiferal assemblage in an area, dormant *A. gibbosa* are functionally dead. With their pseudopods retracted and all activity appearing to cease, they are functionally cut off from the environment. More importantly, they also detach from the substrate, and are therefore highly susceptible to wave motion and transport away from the affected area. This can be a deadly problem; many foraminifers tend to have a strong preference for specific habitats,

especially substrate types, so simply being moved from hard bottom to sandy sediment could make recovery and survival difficult (Hohenegger et al. 1999). Furthermore, it is possible that the foraminifers could be moved much farther, and to deeper or more turbid waters, where they may be unable to survive due to light limitation. Even though dormancy may allow the foraminifers to survive the initial toxic event, if they are not able to maintain their physical positions, at least some mortality will result. The sudden forced dormancy of a large portion of the population, as seen in our initial toxicology tests, would seem likely to have an effect on the foraminiferal population of an area as a whole. If dormancy can be maintained for the extent of a toxic exposure in the environment, then for as long as the pollutant is present, the dormant individuals are effectively dead. If dormancy cannot persist, and the individuals either expire while dormant, or are exposed when forced to actively feed to survive, then they are truly dead. Because of this, acute effects, as well as lethal effects, are an effective measure of toxic exposure.

The presence of the same dormancy-recovery effect in 2-butoxyethanol as in propylene glycol suggests that this is a much more common survival strategy than previously recognized. The 2-butoxyethanol is more toxic than propylene glycol, which is clear when comparing LC50 values (1% 2-butoxyethanol vs. 6% propylene glycol), and from comparing previous research and the Material Safety Data Sheets for each (Sciencelab.com 2010a,b). These results show that the dormancy effect was not simply enabled by the relatively low toxicity of propylene glycol.

One caveat is that, despite differences between the two compounds in terms of chemical composition and toxicity, they are both alcohols, and can both act as solvents.

To better understand dormancy in *A. gibbosa* and other foraminifers, future research will need to look for evidence of the reaction when exposed to a wider variety of chemicals and other water-quality stress factors.

4.3 Designing a bioassay protocol for *A. gibbosa*

4.3.1 *Bioassay protocol based primarily on visual observations*

As discussed above, I feel that the defining a bioassay protocol for *A. gibbosa* based primarily on visual observations has the potential to be beneficial owing to its low cost and technology requirements. The refined methods presented in section 2.2.2 provide an ideal starting point, accounting for the dormancy response, and allowing for observation of chronic and sublethal toxicity. However, as discussed above, a number of difficulties arose in my experiments which must be addressed in order to develop a more reliable bioassay protocol.

There were a number of difficulties related to the visual identification of dead individuals. This affected my observations of differences in mortality over time following chemical exposure, making the results unreliable, as well as complicating analysis of growth rates over time due to the presence of dead specimens. Visual assessments of vitality are commonly used in experiments with foraminifers (e.g., Talge and Hallock 2003, Schmidt et al. 2011, de Freitas Prazeres et al. 2012). Adapting additional visual measures of vitality, such as the presence of food waste indicating active metabolism, may allow more effective identification of low-activity individuals that are still alive.

To avoid complications related to identifying individuals as dead one week, and live the next, I suggest that dead individuals be removed from the replicate. As discussed previously, 72 hours seems to be an appropriate amount of time to allow for recovery from dormancy and, following this period, individuals identified as dead are unlikely to recover. If the goal of the study is to identify long term recovery from dormancy or to identify borderline low-activity individuals, I suggest removing them to a separate petri dish for observation. Removing dead individuals will help to combat variability in long-term observations, and avoid skewing growth data. For the purposes of visual analysis of chronic response, these individuals would be considered dead. Similarly, I found that low to moderate algal growth complicates the identification of dead individuals by anchoring them to the bottom of the dish, simulating attachment. To combat algal growth, I recommend weekly movement of the specimens to sterile petri dishes. By making these changes to the bioassay methods employed in this experiment, the results should become less subjective and more consistent during observations over time, providing a strong basic protocol for use in future studies.

4.3.2 *Bioassay protocol based primarily on physiological observations*

Although bioassay methods based on visual observations are effective, some researchers may need methods that are less subjective. The development of bioassay methods employing physiological observations will enable greater precision. There are a number of techniques which have the potential to be adapted for use in *A. gibbosa* bioassay methods as measures of physiological response to chemical exposure. The measurement of respiration rates has been used as an indicator of stress in foraminifers

(e.g., Geslin et al. 2011, Sinutok 2012). Similarly, Pulse Amplitude Modulated (PAM) fluorometry has been used to identify photodamage in *A. gibbosa* (e.g., Nobes et al. 2008). My experiment have shown that even sublethal exposure to toxic chemicals can cause increased expression of visual stress indicators, including bleaching, and since both of these techniques have been used to explore the physiology of bleaching, adapting the methods for use in bioassays is very reasonable.

On an even more basic level, assessment of respiration or photosynthesis may simplify the identification of dead individuals, and the differentiation from dormant specimens. Although further research is needed, if dormant individuals are respiring at measureable levels, measures of respiration could be used to differentiate between live and dead specimens, although this could be complicated by microbial respiration in dead specimens . Similarly, if dormancy affects the photosynthetic capabilities of *A. gibbosa*'s algal symbionts, PAM fluorometry would indicate differences between dead and dormant individuals, and by performing fluorometry on individuals that visually appear dead, could enable differentiation.

Another option for determining dormant from dead individuals may be the use of CellTracker Green CMFDA, a fluorogenic probe, which passes across the cell membrane, and fluoresces at certain wavelengths when cleaved by enzymes present in living cells (Bernhard et al. 2006). Further work will be required to determine whether it is appropriate for differentiating dormant and dead individuals, and to develop the correct methods given that, once labeled, the individuals will continue to fluoresce indefinitely. Methods for using CellTracker green to identify dead individuals will necessitate adding the tracer at the correct time to avoid labeling dying individuals or microbial activity.

Adapting protocols to include ATP assay would also allow greater differentiation between live and dead specimens. Bernhard (1988,1989) suggests that is considerably more precise than Rose Bengal, another common indicator of vitality. Bernhard and Alve (1996) used ATP assay to identify dormant individuals sampled under anoxic conditions. A drawback of ATP assay is that extracting the ATP from the foraminifer will kill the specimen, but bioassay protocols designed to employ this method could be considerably more precise in determining mortality.

In addition, calcein is an option for examining growth over time by identifying the process of chamber formation, instead of using the longest diameter to measure growth. Calcein is added to the test media, and taken up when calcium carbonate is deposited. This will cause calcareous growth to be recognized with an epifluorescence microscope, and has been used in a chronic bioassay protocol developed by Denoyelle et al. (2012). However, in rotallid foraminifers that deposit new calcite over the entire test during chamber addition, calcein has limited application.

4.4 Recommendations for further documentation of dormancy in *A. gibbosa*

Very little is known about foraminiferal dormancy in general, and more research is required to understand the phenomenon in *A. gibbosa*. A logical first step would be experiments to determine how long *A. gibbosa* can remain dormant. I recommend experiments to investigate the length of time *A. gibbosa* can remain dormant in dark and anoxic conditions, as well as how long it can remain dormant when exposed to chemicals and still successfully recover. These three conditions have all been linked to foraminiferal

dormancy and investigation of the longevity of dormancy in each case will be useful in the study of dormancy in field conditions, and any difference in survival may shed light on the mechanisms of dormancy in reaction to each condition.

Investigating the physical characterists of dormancy is also integral to understanding the phenomenon. The use of CellTracker green may shed light on whether seawater, or chemicals in the seawater, are able to enter a dormant foraminiferal cell. If the CellTracker green is added to the media after individuals have gone dormant, they will only fluoresce if the CellTracker is enters the cell, despite the foraminifer being dormant. This could be especially important in assessing the effectiveness of dormancy as a survival mechanism in response to chemical exposure.

The use of Transmission Electron Microscopy will allow for the examination of cytological features. Talge and Hallock (2003) defined protocols for TEM imaging of foraminiferal cells, and by employing these methods it will be possible to investigate whether *A. gibbosa* produces detectable cytological barriers to toxic substances in the environment as part of the dormancy response.

Measures of respiration and photosynthetic capability are physiological observations which have the potential to provide insights into dormancy in *A. gibbosa.* Respiration and oxygen production, measured with oxygen sensors, can be used to identify oxygen consumption rates in the absence of photosynthesis (i.e., specimens held in the dark) and at varying levels of photosynthetic efficiency, based on light exposure and saturation. Comparing these measures in active and dormant foraminifers is a logical first step to investigating the effects that dormancy has on metabolism. Similarly, PAM

fluorometry can be used to investigate photoefficiency, and an effective complement to oxygen respiration observations. These methods could also be used to test specific hypotheses; for instance, if, as suggested by Bernhard and Alve (1996) in the case of *Stainforthia fusiformis*, photosynthetic activity is providing energy for life while the foraminifer is dormant, these methods should allow the identification of photosynthesis in dormant individuals.

Observations on the magnitude of metabolic activity via ATP assay (e.g., Bernhard and Alve 1996, Bernhard 2000, McIntyre-Wressnig 2012) is another method that will be important in understanding the dormancy reaction. ATP analyses have been used to identify dormant individuals from anoxic conditions (Bernhard and Alve 1996), and if dormancy does involve a change in metabolic activity, this will be reflected in a change in the amount of ATP present. ATP assay requires specialized equipment, and is relatively expensive and results are sometimes difficult to interpret (e.g., Talge 2002, McIntyre-Wressnig 2012). It may also be difficult to separate metabolic effects resulting from dormancy and those resulting from changes in the health of specimens. However, changes in metabolism are an important factor in understanding dormancy, and ATP analyses have the potential to allow the observation of these changes. A bonus to investigations using ATP assay, as well as respiration measures, PAM fluorometry and CellTracker green, is the potential for adapting these methods for use in bioassay protocols, as discussed above, allowing for more efficient identification of mortality following exposure experiments.

5: Conclusions

One of my goals was to define measures of the acute toxicity of propylene glycol, and later 2-butoxyethanol, to *A. gibbosa*. Using the methods I developed, I was able to establish that the apparent LC50 for *A. gibbosa* after a 48-hour exposure to propylene glycol is 3%; the initial effects range is 2-4%. After a 48-hour exposure to propylene glycol, followed by a 72-hour recovery period in filtered seawater, the LC50 was 6%, and the effects range was 2-8%.

For 2-butoxyethanol, the apparent LC50 following a 48-hour exposure was 0.2%, and the effects range 0.15-0.3%. After a 48-hour exposure to propylene glycol, and a 72-hour recovery period in filtered seawater, the LC50 was 1% and the effects range 0.32 - 1.28%.

Another goal was the establishment of measures for chronic sub-lethal effects. Although differences in the increase in mean diameter over time suggested some chronic effects, visual assessments of health indicated chronic effects in all concentrations of propylene glycol above control. This suggests that visually assessed health is a sensitive measure of chronic effects. It also means that the actual effects range of 48-hour exposure to propylene glycol on *A. gibbosa* was at least 0.5-12%.

I also wanted to confirm the observation of dormancy made in the first propylene glycol experiment. The observed dormancy effect in *A. gibbosa* was confirmed in repeated experiments, and during exposure to both propylene glycol and 2-butoxyethanol,

despite differences in the relative toxicity of each chemical. This is the first experimentally observed occurrence of dormancy in a foraminifer in response to chemical exposure and, in the context of previous observations, suggests that dormancy may be a general response among the Foraminifera to acutely unfavorable conditions.

My results show that *Amphistegina gibbosa* has the potential to be a bioindicator of water-borne pollutants on coral reefs. Exposure to chemicals causes measurable acute and lethal effects in *A. gibbosa*. In addition to acute effects and mortality, chemical exposure also produced sub-acute effects, causing foraminifers exposed to propylene glycol to express increasing visual signs of stress, with increasing concentration of exposure. Although growth in exposed individuals did not prove to be linearly related to the concentration of exposure, making it seem unsuitable as a direct measure of the toxicity of exposure in the long term, the effects on foraminiferal health as determined by the increased visual stress markers may fill this role. Although further development of bioassay and bioindicator protocols will be required, the promise is there.

My refined methods are capable of differentiating between dormant and dead individuals, allow the study of both acute effects and lethal effects, and are shown to be effective for experiments exposing *A. gibbosa* to two different alcohols. These methods appear to be appropriate when using these protists in acute toxicology experiments, and strengthen the case for the use of *Amphistegina* as a low-cost, low-technology chemical bioassay.

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APPENDIX

Table: A1: Preliminary propylene glycol experiment:
Significant differences in mortality following 48-hour exposure to propylene glycol

Concentration	0.00%	0.00%	0.05%	0.09%	0.19%	0.38%	0.75%	1.05%	1.50%	1.50%	1.65%	1.95%	2.25%	2.85%	3.00%	6.00%	12.00%	24.00%	48.00%
0.00%	0.00																		
0.00%	0.77	0.00																	
0.05%	0.77	0.00	0.00																
0.09%	0.77	0.00	0.00	0.00															
0.19%	1.54	0.77	0.77	0.77	0.00														
0.38%	1.54	0.77	0.77	0.77	0.00	0.00													
0.75%	1.54	0.77	0.77	0.77	0.00	0.00	0.00												
1.05%	**5.39**	4.62	4.62	4.62	3.85	3.85	3.85	0.00											
1.50%	**5.39**	4.62	4.62	4.62	3.85	3.85	3.85	0.00	0.00										
1.50%	**6.16**	**5.39**	**5.39**	**5.39**	4.62	4.62	4.62	0.77	0.77	0.00									
1.65%	**11.56**	**10.79**	**10.79**	**10.79**	**10.02**	**10.02**	**10.02**	**6.16**	**6.16**	**5.39**	0.00								
1.95%	**7.71**	**6.93**	**6.93**	**6.93**	**6.16**	**6.16**	**6.16**	2.31	2.31	1.54	3.85	0.00							
2.25%	**10.79**	**10.02**	**10.02**	**10.02**	**9.25**	**9.25**	**9.25**	**5.39**	**5.39**	4.62	0.77	3.08	0.00						
2.85%	**13.10**	**12.33**	**12.33**	**12.33**	**11.56**	**11.56**	**11.56**	**7.71**	**7.71**	**6.93**	1.54	**5.39**	2.31	0.00					
3.00%	**19.26**	**18.49**	**18.49**	**18.49**	**17.72**	**17.72**	**17.72**	**13.87**	**13.87**	**13.10**	**7.71**	**11.56**	**8.48**	**6.16**	0.00				
6.00%	**19.26**	**18.49**	**18.49**	**18.49**	**17.72**	**17.72**	**17.72**	**13.87**	**13.87**	**13.10**	**7.71**	**11.56**	**8.48**	**6.16**	0.00	0.00			
12.00%	**19.26**	**18.49**	**18.49**	**18.49**	**17.72**	**17.72**	**17.72**	**13.87**	**13.87**	**13.10**	**7.71**	**11.56**	**8.48**	**6.16**	0.00	0.00	0.00		
24.00%	**19.26**	**18.49**	**18.49**	**18.49**	**17.72**	**17.72**	**17.72**	**13.87**	**13.87**	**13.10**	**7.71**	**11.56**	**8.48**	**6.16**	0.00	0.00	0.00	0.00	
48.00%	**19.26**	**18.49**	**18.49**	**18.49**	**17.72**	**17.72**	**17.72**	**13.87**	**13.87**	**13.10**	**7.71**	**11.56**	**8.48**	**6.16**	0.00	0.00	0.00	0.00	0.00

Pairwise comparisons using Tukey's Honestly Significant Difference test. For p = 0.05, v (degrees of freedom = 76), and k (number of groups) = 19, the critical value of the *q* distribution is ~5.16. For all pairs where the difference in the group average (x_1-x_2) divided by the standard error is greater than the critical q value, the difference between groups is significant. Here, significant values are bold.

Table A2: Preliminary propylene glycol experiment:
Significant differences in mortality following 48-hour exposure to propylene glycol and 24-hour recovery

Concentration	0.00%	0.00%	0.05%	0.09%	0.19%	0.38%	0.75%	1.05%	1.50%	1.50%	1.65%	1.95%	2.25%	2.85%	3.00%	6.00%	12.00%	24.00%	48.00%
0.00%	0.00																		
0.00%	0.96	0.00																	
0.05%	0.96	0.00	0.00																
0.09%	0.96	0.00	0.00	0.00															
0.19%	0.00	0.96	0.96	0.96	0.00														
0.38%	0.96	0.00	0.00	0.00	0.96	0.00													
0.75%	0.96	0.00	0.00	0.00	0.96	0.00	0.00												
1.05%	0.96	1.93	1.93	1.93	0.96	1.93	1.93	0.00											
1.50%	0.96	1.93	1.93	1.93	0.96	1.93	1.93	0.00	0.00										
1.50%	1.93	2.89	2.89	2.89	1.93	2.89	2.89	0.96	0.96	0.00									
1.65%	3.85	4.81	4.81	4.81	3.85	4.81	4.81	2.89	2.89	1.93	0.00								
1.95%	0.00	0.96	0.96	0.96	0.00	0.96	0.96	0.96	0.96	1.93	3.85	0.00							
2.25%	0.00	0.96	0.96	0.96	0.00	0.96	0.96	0.96	0.96	1.93	3.85	0.00	0.00						
2.85%	0.00	0.96	0.96	0.96	0.00	0.96	0.96	0.96	0.96	1.93	3.85	0.00	0.00	0.00					
3.00%	**8.66**	**9.63**	**9.63**	**9.63**	**8.66**	**9.63**	**9.63**	**7.70**	**7.70**	**6.74**	4.81	**8.66**	**8.66**	**8.66**	0.00				
6.00%	**17.33**	**18.29**	**18.29**	**18.29**	**17.33**	**18.29**	**18.29**	**16.37**	**16.37**	**15.40**	**13.48**	**17.33**	**17.33**	**17.33**	**8.66**	0.00			
12.00%	**23.11**	**24.07**	**24.07**	**24.07**	**23.11**	**24.07**	**24.07**	**22.14**	**22.14**	**21.18**	**19.25**	**23.11**	**23.11**	**23.11**	**14.44**	**5.78**	0.00		
24.00%	**23.11**	**24.07**	**24.07**	**24.07**	**23.11**	**24.07**	**24.07**	**22.14**	**22.14**	**21.18**	**19.25**	**23.11**	**23.11**	**23.11**	**14.44**	**5.78**	0.00	0.00	
48.00%	**23.11**	**24.07**	**24.07**	**24.07**	**23.11**	**24.07**	**24.07**	**22.14**	**22.14**	**21.18**	**19.25**	**23.11**	**23.11**	**23.11**	**14.44**	**5.78**	0.00	0.00	0.00

Pairwise comparisons using Tukey's Honestly Significant Difference test. For p = 0.05, v (degrees of freedom = 76), and k (number of groups) = 19, the critical value of the *q* distribution is ~5.16. For all pairs where the difference in the group average (x_1-x_2) divided by the standard error is greater than the critical q value, the difference between groups is significant. Here, significant values are bold.

Table A3: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol

Concentration	0%	0.5%	1%	2%	4%	6%	8%	10%	12%	14%
0%	0									
0.5%	1.788854382	0								
1%	0.894427191	0.89443	0							
2%	**6.260990337**	4.47214	**5.36656**	0						
4%	**18.78297101**	**16.9941**	**17.8885**	**12.522**	0					
6%	**22.36067977**	**20.5718**	**21.4663**	**16.0997**	3.57771	0				
8%	**22.36067977**	**20.5718**	**21.4663**	**16.0997**	3.57771	0	0			
10%	**22.36067977**	**20.5718**	**21.4663**	**16.0997**	3.57771	0	0	0		
12%	**22.36067977**	**20.5718**	**21.4663**	**16.0997**	3.57771	0	0	0	0	
14%	**22.36067977**	**20.5718**	**21.4663**	**16.0997**	3.57771	0	0	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A4: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and 24-hour recovery

Concentration	0%	0.5%	1%	2%	4%	6%	8%	10%	12%	14%
0%	0									
0.5%	1.557	0								
1%	0	1.557	0							
2%	0.7785	0.7785	0.7785	0						
4%	4.67099	**6.22799**	4.67099	**5.44949**	0					
6%	**14.013**	**15.57**	**14.013**	**14.7915**	**9.34199**	0				
8%	**17.9055**	**19.4625**	**17.9055**	**18.684**	**13.2345**	3.89249	0			
10%	**17.127**	**18.684**	**17.127**	**17.9055**	**12.456**	3.114	0.7785	0		
12%	**17.9055**	**19.4625**	**17.9055**	**18.684**	**13.2345**	3.89249	0	0.7785	0	
14%	**17.9055**	**19.4625**	**17.9055**	**18.684**	**13.2345**	3.89249	0	0.7785	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A5: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and 48-hour recovery

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%	12%	14%
0%	0									
0.50%	0	0								
1%	0	0	0							
2%	0	0	0	0						
4%	3	3	3	3	0					
6%	**14**	**14**	**14**	**14**	**11**	0				
8%	**25**	**25**	**25**	**25**	**22**	**11**	0			
10%	**25**	**25**	**25**	**25**	**22**	**11**	0	0		
12%	**25**	**25**	**25**	**25**	**22**	**11**	0	0	0	
14%	**25**	**25**	**25**	**25**	**22**	**11**	0	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A6: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and 72-hour recovery

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%	12%	14%
0%	0									
0.50%	0	0								
1%	0	0	0							
2%	0	0	0	0						
4%	0.95346	0.95346	0.95346	0.95346	0					
6%	**9.53463**	**9.53463**	**9.53463**	**9.53463**	**8.58116**	0				
8%	**23.8366**	**23.8366**	**23.8366**	**23.8366**	**22.8831**	**14.3019**	0			
10%	**23.8366**	**23.8366**	**23.8366**	**23.8366**	**22.8831**	**14.3019**	0	0		
12%	**23.8366**	**23.8366**	**23.8366**	**23.8366**	**22.8831**	**14.3019**	0	0	0	
14%	**23.8366**	**23.8366**	**23.8366**	**23.8366**	**22.8831**	**14.3019**	0	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A7: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and 168-hour recovery

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%	12%	14%
0%	0									
0.50%	0	0								
1%	0.74665	0.74665	0							
2%	1.67997	1.67997	0.93332	0						
4%	3.73327	3.73327	2.98661	2.0533	0					
6%	**5.97323**	**5.97323**	**5.22657**	4.29326	2.23996	0				
8%	**14.1864**	**14.1864**	**13.4398**	**12.5064**	**10.4531**	**8.21319**	0			
10%	**18.6663**	**18.6663**	**17.9197**	**16.9864**	**14.9331**	**12.6931**	4.47992	0		
12%	**18.6663**	**18.6663**	**17.9197**	**16.9864**	**14.9331**	**12.6931**	4.47992	0	0	
14%	**18.6663**	**18.6663**	**17.9197**	**16.9864**	**14.9331**	**12.6931**	4.47992	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A8: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and recovery on 1-3-2012

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%
0%	0							
0.50%	1.20605	0						
1%	1.20605	0	0					
2%	0.60302	0.60302	0.60302	0				
4%	1.20605	0	0	0.60302	0			
6%	2.41209	1.20605	1.20605	1.80907	1.20605	0		
8%	**7.83929**	**6.63325**	**6.63325**	**7.23627**	**6.63325**	**5.4272**	0	
10%	**13.8695**	**12.6635**	**12.6635**	**13.2665**	**12.6635**	**11.4574**	**6.03023**	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A9: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and recovery on 1-10-2012

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%
0%	0							
0.50%	0.70711	0						
1%	0.70711	0	0					
2%	0.70711	1.41421	1.41421	0				
4%	2.12132	1.41421	1.41421	2.82843	0			
6%	1.41421	0.70711	0.70711	2.12132	0.70711	0		
8%	**5.65685**	**4.94975**	**4.94975**	**6.36396**	3.53553	4.24264	0	
10%	**14.1421**	**13.435**	**13.435**	**14.8492**	**12.0208**	**12.7279**	**8.48528**	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A10: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and recovery on 1-17-2012

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%
0%	0							
0.50%	1.8065	0						
1%	1.20434	0.60217	0					
2%	1.20434	3.01084	2.40867	0				
4%	1.20434	0.60217	0	2.40867	0			
6%	2.40867	0.60217	1.20434	3.61301	1.20434	0		
8%	1.8065	0	0.60217	3.01084	0.60217	0.60217	0	
10%	**10.6885**	**8.88198**	**9.48415**	**11.8928**	**9.48415**	**8.27981**	**8.88198**	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A11: Second propylene glycol experiment: Significant differences in mortality following 48-hour exposure to propylene glycol and recovery on 1-26-12

Concentration	0%	0.50%	1%	2%	4%	6%	8%	10%
0%	0							
0.50%	2.05704	0						
1%	2.05704	0	0					
2%	0.51426	2.5713	2.5713	0				
4%	1.54278	0.51426	0.51426	2.05704	0			
6%	2.05704	0	0	2.5713	0.51426	0		
8%	4.11408	2.05704	2.05704	4.62834	2.5713	2.05704	0	
10%	**7.97102**	**5.91398**	**5.91398**	**8.48528**	**6.42824**	**5.91398**	3.85695	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A12: First 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol

Concentration	0%	0.32%	0.64%	0.96%	1.28%	1.44%
0%	0					
0.32%	**79.6894**	0				
0.64%	**83.4841**	3.79473	0			
0.96%	**83.4841**	3.79473	0	0		
1.28%	**83.4841**	3.79473	0	0	0	
1.44%	**83.4841**	3.79473	0	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A13: First 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol and 24-hour recovery

Concentration	0%	0.32%	0.64%	0.96%	1.28%	1.44%
0%	0					
0.32%	0	0				
0.64%	1.39497	1.39497	0			
0.96%	**26.5045**	**26.5045**	**25.1095**	0		
1.28%	**33.4793**	**33.4793**	**32.0843**	**6.97486**	0	
1.44%	**33.4793**	**33.4793**	**32.0843**	**6.97486**	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A14: First 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol and 48-hour recovery

Concentration	0%	0.32%	0.64%	0.96%	1.28%	1.44%
0%	0					
0.32%	0	0				
0.64%	3.15135	3.15135	0			
0.96%	**17.3324**	**17.3324**	**20.4838**	0		
1.28%	**36.2406**	**36.2406**	**39.3919**	**18.9081**	0	
1.44%	**36.2406**	**36.2406**	**39.3919**	**18.9081**	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A15: First 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol and 72-hour recovery

Concentration	0%	0.32%	0.64%	0.96%	1.28%	1.44%
0%	0					
0.32%	3.39411	0				
0.64%	0	3.39411	0			
0.96%	**15.2735**	**11.8794**	**15.2735**	0		
1.28%	**42.4264**	**39.0323**	**42.4264**	**27.1529**	0	
1.44%	**42.4264**	**39.0323**	**42.4264**	**27.1529**	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A16: Second 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol

Concentration	0%	0.05%	0.10%	0.15%	0.20%	0.25%	0.30%	0.35%	0.40%
0%	0								
0.05%	1.47011	0							
0.10%	2.5727	1.10259	0						
0.15%	**14.7011**	**13.231**	**12.1284**	0					
0.20%	**11.7609**	**10.2908**	**9.18821**	**2.94023**	0				
0.25%	**20.5816**	**19.1115**	**18.0089**	**5.88046**	**8.82068**	0			
0.30%	**33.8126**	**32.3425**	**31.2399**	**19.1115**	**22.0517**	**13.231**	0		
0.35%	**33.8126**	**32.3425**	**31.2399**	**19.1115**	**22.0517**	**13.231**	0	0	
0.40%	**33.8126**	**32.3425**	**31.2399**	**19.1115**	**22.0517**	**13.231**	0	0	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A17: Second 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol and 24-hour recovery

Concentration	0%	0.05%	0.10%	0.15%	0.20%	0.25%	0.30%	0.35%	0.40%
0%	0								
0.05%	1.63908	0							
0.10%	2.45861	0.81954	0						
0.15%	**8.19538**	**6.5563**	**5.73676**	0					
0.20%	**3.27815**	1.63908	0.81954	**4.91723**	0				
0.25%	**6.5563**	**4.91723**	4.09769	1.63908	3.27815	0			
0.30%	**11.4735**	**9.83445**	**9.01491**	3.27815	**8.19538**	**4.91723**	0		
0.35%	**9.83445**	**8.19538**	**7.37584**	1.63908	**6.5563**	3.27815	1.63908	0	
0.40%	**6.96607**	**5.32699**	4.50746	1.22931	3.68792	0.40977	4.50746	2.86838	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A18: Second 2-butoxyethanol experiment: Significant differences in mortality following 48-hour exposure to 2-butoxyethanol and 96-hour recovery

Concentration	0%	0.05%	0.10%	0.15%	0.20%	0.25%	0.30%	0.35%	0.40%
0%	0								
0.05%	2.09165	0							
0.10%	3.66039	1.56874	0						
0.15%	4.1833	2.09165	0.52291	0					
0.20%	2.09165	0	1.56874	2.09165	0				
0.25%	**6.27495**	4.1833	2.61456	2.09165	4.1833	0			
0.30%	2.09165	4.1833	5.75204	**6.27495**	4.1833	8.3666	0		
0.35%	2.09165	0	1.56874	2.09165	0	4.1833	4.1833	0	
0.40%	3.66039	1.56874	0	0.52291	1.56874	2.61456	**5.75204**	1.56874	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in mortality between concentrations of exposure

Table A18: Significant differences in diameter after 40 days

	0%	0.5%	1%	2%	4%	6%	8%
0%	0	51.7579	11.2219	61.8445	12.1607	13.1919	121.358
0.5%	51.7579	0	40.536	113.602	39.5972	38.566	69.6
1%	11.2219	40.536	0	73.0664	0.9388	1.97	110.136
2%	61.8445	113.602	73.0664	0	74.0052	75.0364	**183.202**
4%	12.1607	39.5972	0.9388	74.0052	0	1.0312	109.197
6%	13.1919	38.566	1.97	75.0364	1.0312	0	108.166
8%	121.358	69.6	110.136	**183.202**	109.197	108.166	0

Pairwise comparisons using Tukey's HSD. Bold values indicate significant differences in diameter between concentrations of exposure