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Gas supersaturation and its effect on the red abalone, Haliotis rufescens, (Swainson)

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GAS SUPERSATURATION AND ITS EFFECT ON THE RED ABALONE, *Haliotis rufescens*, (Swainson).

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ABSTRACT

The invertebrate aquaculture industry has undergone a rapid expansion over the last few decades in the United States which has often been accompanied by a series of problems inherent to a laboratory system. One such problem is when gas becomes supersaturated, a common occurrence in hatcheries that pump natural waters in to an intensive culture system. This can cause serious Gas Bubble Disease problems. Previous studies to identify the effects of lethal and sublethal gas supersaturation levels on finfish, bivalves, and crustaceans have been done, yet studies on the commercially raised mollusc, the red abalone (Haliotis rufescens Swainson) have not identified the levels of gas supersaturation that are both lethal and sublethal to the animal.

This experiment examines the effects of several levels of gas supersaturation on the behavior, growth and mortality of the red abalone. With some of the lethal and sublethal gas supersaturation levels and their effects identified, the abalone aquaculturist will have better water quality guidelines and health indicators to follow if and when gas supersaturation is suspected to be the cause of a problem. The culturist will also be better equipped to make a decision of whether or not to employ additional time and cost expenditures to de-gas the water. This should ultimately result in higher abalone survival rates and lower costs to the aquaculturist.

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INTRODUCTION

In the last decade the aquaculture industry has expanded globally to meet the growing nutritional needs of an increasing human population. Among these industries are marine invertebrate hatcheries that in addition to growing animals for food, often also intend to restock decimated natural populations. As most targeted species have suffered severe losses caused by "gas bubble disease" (Marsh et al., 1905; Hughes, 1968; Malouf et al., 1972; and Lightner et al., 1974), it is suspected that the abalone industry may not be meeting its potential due to these problems. Effects of exposure to seawater supersaturated with gas have been well documented in fin fish (Marsh et al., 1905; Crunkilton et al., 1980; and Weitkamp and Katz, 1980, reviewed in Harvey, 1975; and Marking, 1987). Marsh et al. (1905), were the first to label the pathological effect as the gas bubble disease. Bouck (1980) and Colt (1984) later attributed the symptoms to uncompensated hyperbaric pressure of dissolved gases that form bubbles in the tissues of aquatic organisms. However, this disease has only recently been recognized in aquatic invertebrates (Hughes 1968, Malouf et al., 1972; Goldberg, 1978; Elston et al., 1983; Bisker and Castagna, 1985; and Bisker and Castagna, 1986). Although the effects of exposure to gas supersaturated seawater on invertebrates have been documented, only Goldberg (1978) and Bisker et al.(1986) have attempted to define the levels of gas that are lethal to selected commercially important molluscs. With the proliferating interest in invertebrate aquaculture, the implications and expense of hatchery caused gas bubble disease on the struggling

aquaculture industry are significant. Progress in aquaculture can only be achieved as quickly as the problems that accompany the establishment of the industry are rectified. The primary objective of this study was to understand the effects of gas bubble disease on red abalone (Haliotis rufescens Swainson). Natural occurrences of gas supersaturation have been recognized in both seawater and fresh water (Marking, 1986; and Weitkamp and Katz, 1980). High pressure in underground aquifers or temperature increases due to the thermally heated groundwater that is pushed to the surface and mixed with air equilibrated water, has resulted in supersaturated water supplies in several fish hatcheries throughout the country that utilize freshwater springs and wells (Harvey, 1975). Another source of gas supersaturation is air entrainment which can be a result of turbulence in streams and waterfalls which traps air bubbles during the violent mixing of water (Harvey et al., 1961). When high velocity air-saturated water is forced below the surface into an increased pressure environment, the bubbles dissolve. This has resulted in supersaturation levels of up to 134% (Crunkilton et al., 1980). Primary productivity can also lead to gas supersaturation. For example, Renfro (1963) and Chan and Stephen-Hassard (1977), found that shallow ponds with heavy algal blooms exceeded dissolved oxygen saturation and were implicated as the cause of several "fish kills."

Artificial causes of supersaturated seawater have been a source of many gas bubble disease episodes since the turn of the century. For example, at Woods Hole in 1900, a gas bubble disease outbreak

occurred because a small leak in the intake pipe allowed air to be forced into solution causing water to become supersaturated (Marsh, 1903). More recently, Ebert (pers.comm.), Hughes (1968), Dawley et al.(1976) and Weitkemp et al.(1980), have noticed that supersaturation problems occurred as a result of leaks on the suction side of pumps. Rapid temperature increases can also cause gas supersaturation since gases are less soluble at higher temperatures. When culturing abalone and other molluscs, water temperature is often increased between 1-10°C to maintain the seawater at a temperature that is optimal for growth (Ebert, 1974). Malouf et al. (1972) hypothesized that this process was responsible for supersaturation of water within a bivalve hatchery. Other causes of gas bubble disease have also been noted. Three epizootic outbreaks occurred at the California Department of Fish and Game Laboratory at Granite Canyon within the last fifteen years (E. Ebert, pers. comm.). All of these outbreaks resulted in high mortality of juvenile and adult abalone, Haliotis rufescens (Swainson), and all occurred in the spring, when a 2-5°C decrease in temperature commonly occurs along the California coast. In all cases the exposed abalone had numerous lesions on the sole of the foot, and associated necrotic tissue that had elevated numbers of bacterial pathogens (Vibrio spp.). Bacterial counts from moribund animal tissue showed that bacteria were more abundant than usual. In addition, Elston (1983) reported that juvenile abalone exposed to short term supersaturated oxygen conditions showed systemic infection with the bacterium Vibrio alginolyticus. At the Granite

Canyon Laboratory, efforts were made to combat mortality by using sulfa drugs, fungicides, and bactericides. When these methods failed, it was suspected that bacteria played a secondary role and that stress, possibly gas supersaturation, was the precursor to the high mortality of abalone. In this study, I identified critical limits of gas supersaturation for H. rufescens growth and mortality and demonstrated that gas supersaturation ultimately results in abalone mortality. Concurrent measurements of water quality were made to detect possible correlative trends. My results show that gas supersaturation can be a prime cause of mortalities when culturing molluscs, and suggest that monitoring and adjustment of gas levels in a laboratory water system should be done to prevent major mortality.

MATERIALS AND METHODS

The experiments were done at the California Department of Fish and Game's Marine Resource Laboratory, at Granite Canyon which is located on the central California coast, 20 kilometers south of Monterey. The experiments began 1 May 1987 and ran continuously to 31 July 1987.

Experimental Seawater

At the Granite Canyon laboratory, seawater is pumped directly from the ocean to the main storage tank (105,000 l capacity) located approximately 3 m above the intake pipes. The water is then sand filtered (to approximately 50 um) by O&G Water Conditioners before entering the laboratory.

Gas supersaturation was accomplished by injecting air with a 1 / 10 horsepower, 7-gal / min centrifugal pump (Cole-Parmer Instrument Corporation) which was connected to a "pressure chamber" made of PVC pipe 5 m long and 25 cm diameter. The chamber was plumbed with outlet, inlet, compressed air injection valve, and overflow valves (Figure 1). The combined pressure capacity of the pump, the atmospheric pressure, and the head pressure provided approximately 30 psi of water pressure. The laboratory-supplied compressed air was maintained at 32 $\pm$ 1 psi, just slightly higher than the pressurized water. The higher pressure forced the air to dissolve in the seawater inside the pressure chamber.

All laboratory seawater in the experiment was filtered to 10 microns using a cartridge filtration system (Filtrex Inc.); half of this water was first diverted through the pressure chamber where it

was supersaturated to approximately 143% ± 3%. The supersaturated and ambient water supply lines were otherwise treated identically. Water hoses were rotated and complete filtration systems (one 25 micron and one 10 micron cartridge filter for each water line) were switched every other day for the first two weeks of the experiment and every two weeks thereafter to ensure that the results were not caused by factors inherent to the filters.

Seven different supersaturation concentrations were produced by mixing the appropriate amount of water from the pressure chamber (143% ± 3% supersaturation) with the ambient laboratory seawater (100% ± 0.45%). To eliminate significant variation, each treatment was mixed in a 15 l header tank before being introduced to the individual test containers. (Figure 2). Intermediate levels were maintained at supersaturation levels of 105%, 110%, 115%, 120%, and 130%. The gas supersaturation level of 115% was initiated thirty days into the experiment. The six treatments and the control were run simultaneously.

There were three replicate test container troughs for each gas level treatment. The water was supplied to each test container by gravity, with three identical sections of 4 mm plastic tubing that originated from the bottom of each corresponding header tank. Levels of supersaturation were determined for water within the header tanks every other day throughout the experiment to ensure the range was constant.

Water Quality Analyses

Several water quality parameters were monitored

throughout the experiment. Temperature was maintained at 10 - 14° C. Daily water samples were collected directly in front of the ocean intake lines for salinity analysis. An Orion Microprocessor Ionanalyzer (Model 901 Orion Research Inc., Cambridge, Mass.) was used for bimonthly pH measurements. Chlorophyll was analyzed weekly by filtering between 2 to 5 l of raw seawater (depending on the amount of chlorophyll needed to stain the filter) through a GF75 Borosilicate Microfiber Filter (Micro Filtration Systems, Dublin, Ca). The filter was then placed in a 2 cc cryotube (NUNC, Inter-med, Denmark); the oxygen was replaced with nitrogen by blowing compressed nitrogen gas into the cyrotube and swiftly closing the cap. These samples were then placed in the freezer until fluorometric analysis could be done. This process helped minimize the oxidation of the filtered cholorophyll. Analysis was done according to Parsons et al. (1984) using a Turner Fluorometer (Model 111, GK Turner Associates, Palo Alto, Ca.). A gas tensionometer was used (Common Sensing Inc. Total Dissolved Gas Meter, model FTR/TGO) to measure total gas (mmHg TG), nitrogen (mmHg N_2), oxygen (mmHg O_2), and percent total gas supersaturation levels (%TG) every other day throughout the experiment. A modified Winkler analysis was done once a month to calibrate the oxygen probe.

One large blade (approximately 44 cm long) of Macrocystis sp. was placed in each test container once a week; this quantity was more than could be consumed by the abalone. The test containers were decanted and rinsed out once a week to ensure that the detritus from both the abalone and Macrocystis sp. did not accumulate.

Test Containers

The three replicate test cylinders were constructed from grey 14.7 cm diameter PVC pipe sections which were cut into 27 cm lengths and glued onto PVC sheets that formed the container bottom. A 12 mm hole was drilled approximately 3 cm from the top of each container and a PVC coupling was placed into the opening and used as an outlet valve. A PVC elbow and a straight 4 cm pipe section of PVC (forming an upside-down "L") was then pushed into the outlet valve so that the effluent water was siphoned from the bottom of the test container (Figure 3). To alleviate the problem of working with abalone which seek refuge inside of the PVC pipe, a small piece of Nytex screen was fastened with cable ties around the intake side of the pipe. A PVC sheet was fitted around the PVC pipe approximately 3 cm below the surface of the water to create shade and additional surface area to reduce possible stress from both crowding and too much light. Total submerged surface area was 1350.5 cm , giving 67.5 cm /abalone. Each cylinder contained twenty 12 mm (± 1 mm) H. rufescens chosen from healthy laboratory stocks.

Bacteria Analysis

Several methods of estimating the abundance of bacteria were tested to determine the optimal counting procedure for this experimental system. After experimenting with the general bacteriological loop method (APHA et al., 1971) the swath method (Elston and Lockwood, 1983), and the logarithmic dilution method (Hamilton, 1984), a simple membrane filtration method was chosen (Patrick, 1978) because of its low risk to the juvenile abalone.

This entailed collecting 10 ml of effluent water and filtering it through a sterile Millipore, 0.45 mm gridded, 47 mm membrane filter. The membrane was placed on a layer of thiosulfate citrate bile salt (TCBS) agar (Difco Laboratories, Detroit, Michigan) in sterile, disposable plastic petri dishes. Every two to three days three replicate samples were taken from each gas level. The three test containers per treatment were sampled on a rotating schedule that allowed the sampling to cover all test containers every ten days. The filtrates were kept at 15°C for 48 hours to allow the bacteria colonies to develop. After the incubation period, colonies were distinctive and clearly visible to the naked eye. A dissecting microscope (10 X) was occasionally used to facilitate the counting of overlapping colonies. Vibrio was identified and recorded throughout the experiment.

Growth Measurements

On the first day of each month during the experiment, length and weight measurements of abalone were taken for each of the experimental groups and the control. The 20 animals were removed from each container, blotted dry with cheesecloth to remove excess water, and then collectively weighed. Total weight, mean weight and the range of weights were recorded monthly to the nearest 0.001 gm, for each treatment. Calipers were used to determine each animal's length to the nearest 1/100th of a mm. These data were used to compute mean length and ranges of each replicate group.

Weekly mortality and mortality rates were recorded weekly for each test container. Dying animals were lethargic and unresponsive

to stimuli, so these behavioral observations were used to identify moribund animals if other obvious symptoms were not present.

Qualitative Measurements

Several qualitative parameters were monitored three times a week throughout the experiment to detect any behavioral and/or physical changes in the experimental animals. Three times per week the amount of food consumed per container was given an index of 0 - 5 (0 = least eaten, 5 = most eaten) to classify the amount of Macrocystis blade consumed.

One characteristic of a healthy abalone is the ability to the animal to quickly "right" itself after being turned over; therefore, animals were timed to see how long they would take to "right" themselves. The range of time was between 1 and 15 seconds; it was considered a failed attempt if an animal had not righted itself within 15 seconds.

Another characteristic of a healthy abalone is the animal's ability to maintain a strong hold on the substrate, therefore an index of 0 - 5 (0 = weakest hold, 5 = strongest hold) was used to describe the animals' strengths. To accomplish this, I applied comparable force to try to remove five randomly chosen animals from each test container.

Other physical characteristics that were easily observed, such as air bubbles in the digestive gland and emaciation, were recorded using the same index of 0 - 5; the more severe the trait was the higher the number employed.

Statistics

Vibrio counts for the 100% and the 143% treatment levels were tested at two points in time using a one way analysis of variance (ANOVA) to determine whether bacteria were more abundant before or after abalone mortalities. Weight gain and length increase data were analyzed using an ANOVA. Scheffe's multiple comparison test was used for *a posteriori* evaluations to determine if significant differences existed among treatments. Variances of average survival time among saturation levels were examined using a one way ANOVA and Scheffe's multiple comparison test (Sokal and Rohlf, 1981).

RESULTS

Water Quality

Seawater salinity and pH measurements ranged between 33.45 to 34.17 ppt and 8.2-8.5, respectively, typical of the open ocean. Chlorophyll concentrations ranged widely from a mean of 0.012 mg/m ($\pm$ 0.006) to a mean of 0.450 mg/m ($\pm$ 0.072), most likely due to the natural changes of chlorophyll concentrations over the length of the experiment (Table 1). Means and ranges of mmHG TG, N_2, O_2, and %TG did not overlap among saturation levels, demonstrating that significant differences among treatments existed (Table 2).

Bacteriological Studies

Effluent water samples from each of the replicate test containers had detectable levels of *Vibrio* spp. during the entire experiment. There was no significant change at 100% saturation within the first month of the experiment (Table 3, Figure 4). In contrast, the 143% supersaturation level had significantly higher bacteria counts soon after the first sampling cycle (ANOVA, $p<.0001$; Table 3, Figure 5). Bacteria abundance toward the end of the sampling period for the 120%, 130%, and 143% supersaturation levels tended to decrease, but this was obviated by fewer abalone present (Table 4, and Figures 5 & 8). Other than the discrete separation of the bacteria counts in the lower and higher gas levels at the initial phase of the experiment, the treatments all fell within a similar range and showed no other significant trends in bacteria (Table 4, and Figures 4 & 5).

Sub-lethal Effects

A one-way analysis of variance (ANOVA) done on the initial length data was unnecessary because the abalone were chosen specifically for their similarity in length (12 mm $\pm$ 1 mm). However, for the final length measurements, there were significant differences between treatments (ANOVA, $p<.001$; Table 5a, Figure 6). Scheffe's multiple comparison test indicated that there were no significant differences in final lengths within the upper gas level group of 120% to 143%, and the lower gas level group of 100% to 115%, however, a significant difference was found between the two groups (Table 5b). The intermediate gas level of 115% showed a significant difference only with the treatment that had the greatest increase in shell growth, the 105% treatment (Scheffe, $p<.05$: Table 5c). The 120%, 130% and 143% levels were found to have significantly lower shell length increases than the lowest three saturation levels of 100%, 105%, and 110%, (Scheffe, $p<.05$; Table 5b, Figure 6), with the exception of the insignificant difference between the 100% and 120% treatments (Table 5a).

Although the initial weights among the treatments were statistically different (ANOVA, $p<0.0048$) (Table 6a,b, & c), Scheffe's multiple comparison test showed that the difference existed only between the 115% and the 143% gas levels (Table 6d). Changes in weights over the course of the experiment were significantly different among the treatments (ANOVA, $p<.0001$) (Table 7a). A posteriori tests indicated that two distinct groups existed (Scheffe, $p<0.05$) (Figure 7b). The abalone in the 100%,

105%, 110%, and 115% levels all gained weight, while the abalone in the 120%, 130% and 143% levels lost weight. Although the 115% gas level abalone were grouped with the heavier animals, they maintained a more intermediate level of weight change (Figure 7).

Lethal Effects

There were no mortalities in gas supersaturation treatments below 120% during the experiment, with the exception of one at the 115% level. A one-way ANOVA and Scheffe's multiple comparison test showed a significant difference in mortality rates among the 120%, 130%, and 143% supersaturation treatments (ANOVA, $p<0.0029$) (Table 9a & b). A posteriori tests indicated that significantly higher mortality occurred at the 143% level (Scheffe, $p< 0.05$) (Table 9b). The trend of increasing mortality rates with the increasing gas supersaturation levels is clearly shown by the cumulated mean mortality rates (Table 10, Figure 8).

Qualitative

Macrocystis consumption data show two conspicuous groupings previously found in the quantitative tests (Table 11). The control, 105%, and 110% levels had the highest consumption rates (Table 11). The 115% gas supersaturation level was intermediate, and the abalone in the more supersaturated treatment levels (120%, 130% and 143%) fed at substantially lower rates (Table 11).

Animals in the highest gas supersaturation levels were less tenacious (Table 11). Moreover, abalone in the control, 105%, and 110% treatment levels were usually able to "right" themselves in a few seconds, while abalone in 120% and 130% treatment levels often

took over fifteen seconds. In the highest two treatment levels, 130% and 143%, animals often detached and floated on the surface.

DISCUSSION

These experiments showed clear trends of the effects of gas supersaturation on the growth, behavior and mortality of red abalone, H. rufescens. Stress induced in the 120% and higher gas supersaturation treatments caused mortality in abalone.

Overall growth rates, including the control in my experiment were lower than expected. Hunt (in press) found 15 mm abalone to have a mean growth rate of 7.3 mg/day when using a considerably lower density than the density used in my experiment (67.5 cm /12 mm). Shibui (1972) has shown that growth rates are inversely correlated with density, perhaps accounting for the observed discrepancy. As my intention in these experiments was not to maximize growth, but to compare the relative growth rates within all gas supersaturation treatments, I chose a stocking density intermediate between growth optimization and growth inhibtion (Owens et al., 1984), because of the need to reconcile the demand of many replicates. Obvious growth trends were represented by the lowest gas level groups (100%, 105% and 110%) gaining significantly more weight than the higher gas level groups (120%, 130% and 143%). While there was a significant difference in initial mean weights between the 115% and 143% levels, that difference represented a value of only 0.01 gm. This, combined with a low standard deviation value ($\pm$ 0.002 gm) of the 115% treatment animals, disproportionately emphasized the initial weight difference, which is negligible when considering that the final weight difference is more than an order of magnitude of significance greater. The control animals in this experiment gained

weight at a rate of 2.4 mg/day. Each higher gas level had incrementally decreasing growth rates. At the intermediate level of 115%, the abalone showed a slow and steady growth increase of 0.8 mg/ day (Figure 8), which at best represents a maintenance rate (E. Ebert, pers. comm.). In the 120%, 130% and 143% supersaturation levels, the abalone actually lost weight. The weight loss was greater in the increasingly higher gas levels, with a maximum weight loss of 0.08 mg/day for the 143% treatment. The present growth data suggest that there is a supersaturation threshold beyond which the abalone will lose biomass. Apparently, the stress from supersaturation directly or indirectly causes loss of biomass which ultimately results in mortality.

Similar trends are apparent in the length data. For example, the control treatment abalone grew at a rate of 15.8 um/day. The highest mean growth rate of 22.1 um/day occurred in the 105% level. Owens et al. (1984) report a range of 37.3 um/day to 56.0 um/day for similarly sized red abalone. Differences among the 100%, 105% and 110% treatments may be a consequence of the large initial standard deviation of the abalone lengths ($\pm$ 1 mm), and not the absolute maximum difference of 0.78 mm for the final length value. Separating the two distinct groups, the 115% level gained a maximum of 11.8 um/day, an intermediate shell growth rate. In the higher gas supersaturation treatment groups the lengths did not change beyond the 12 mm $\pm$ 1 mm range; this is likely the result of stress induced starvation and is in accordance with the weight data. The intermediate gas level of 115% was statistically grouped with the

highest gas levels (120%, 130% and 143%) in the length data and grouped with the lowest gas levels (100%, 105% and 110%) in the weight data, and may be explained by punctuated inverse growth rates of length and weight which is thought to exist for abalone (E. Ebert, pers. comm.). This is likely represented by the 105% gas level, the 115% gas level and to a more severe degree, all of the higher gas levels groups (120%, 130% and 143%) that continued to increase in length although they did not gain weight.

In the three lowest gas level treatments of 100%, 105% and 110%, there were no mortalities, suggesting that these levels of supersaturation were not lethal. The intermediate gas level had only one mortality during the course of the experiment. However, this gas supersaturation level was initiated a month later than the other treatments. If the 115% level had continued for an additional month these animals may have minimally sustained themselves or possibly died.

Significant mortality occurred in the three highest gas supersaturation treatments. In the 143% treatment level, 50% of the abalone died by the 27th day; 50% mortality occurred on the 49th day in the 130% gas level and the 56th day in the 120% level. Total mortality occurred in these highly supersaturated treatment levels on the 49th, 73th and 90th day respectively. Stress induced emaciation was recognized as the main cause of mortality, although some mortalities were a result of animals that exploded due to the pressure exerted on the tissues by gas emboli. These gas levels are too high for successful abalone cultivation. However, the slower

mortality rates in the 120% and 130% levels suggest that once high gas supersaturation levels are detected, by degassing the water, additional mortalities may be averted.

Observation of abalone behavior in this study supported the quantitative data. Feeding rates, ability to hold on to a substrate, and motor skills decreased incrementally with increasing gas supersaturation values. This pattern was conspicuous throughout the experiment and helped define the lethal and sub-lethal levels of gas supersaturated seawater for H. rufescens.

Vibrio has been implicated previously as a common cause of mortality in laboratory raised shellfish (Elston and Lockwood, 1983; Elston, 1983, and Owens et al., 1984). Also, Hamilton (1984) found that the greatest survival of juvenile red abalone occurred under bacterialfree culture conditions. The results of Elston's (1983) experiments suggest that the exposure to hyperoxygenation reduced the abalones' immunity to disease. Epithelial rupture resulted in foot lesions and were symptomatic of potential vibriosis. Foot lesions such as those described by Elston (1983) have been seen at Granite Canyon Marine Resource Laboratory (pers. obs.) but none was observed during this investigation. In my experiment, the 143% treatment showed a positive correlation between the bacteria abundance and mortality (Figure 4, Table 10). However, the high numbers of bacteria found in the 143% gas treatment level were a result of opportunistic Vibrio on already moribund abalone.

To further deduce the role bacteria play in the abalone

mortalities, we need to understand the effects of different gas supersatuation levels on bacterial abundance without the presence of abalone.

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Table 1: Mean weekly chlorophyll measurements, (n = 3).

Sample Date days	(week #)	Chlorophyll (mg/m^3)
4	(0)	0.411
11	(1)	0.193
18	(2)	0.081
25	(3)	0.273
32	(4)	0.128
40	(5)	0.450
47	(6)	0.307
54	(7)	0.280
65	(8)	0.253
75	(10)	0.214
82	(11)	0.327
89	(12)	0.222

Table 2: Gas saturation levels measured over entire experiment (mean ± standard deviation and range). mmHg = hyperbaric gas pressure, %TG = percent total gas, $mmHgO_2$ = oxygen pressure, $mmHgN_2$ = nitrogen pressure, n = sample size.

Gas Treatment	mmHg	%TG	$mmHgO_2$	$mmHgN_2$	n
Ambient	765.97±3.46 (758-772)	100.17 ±0.38 (100-101)	147.84 ± 4.58 (136-156)	617.81 ±3.9 (612-630)	32
Control 1	767.30 ± 3.59 (763-777)	100.27 + 0.45 (100-101)	152.38 ± 4.16 (145-164)	614.82 ± 4.20 (606-622)	34
2	800.67± 5.87 (789-810)	104.97 ± 0.59 (104-106)	161.00 ± 6.20 (152-177)	642.88+ 4.31 (556-646)	33
3	840.85± 8.37 (827-874)	110.27 ± 1.04 (109-115)	171.13 ± 8.92 (158-184)	667.52 ± 8.00 (647-687)	32
4	879.16± 3.82 (872-887)	115.28 ± 0.46 (115-116)	187.53 ± 11.91 (170-216)	714.58 ± 9.83 (705-730)	19
5	916.27± 4.64 (911-927)	120.18 + 0.48 (119-121)	201.82 ± 10.94 (188-230)	770.52 + 10.01 (750-787)	27
6	992.40± 4.75 (982-1002)	130.24 + 0.54 (129-131)	218.29 ± 8.02 (206-225)	772.62 ± 12.97 (756-793)	22
7	1091.90± 2.69 (1021-1174)	143.14 ± 2.97 (134-145)	240.22 ± 9.83 (215-253)	851.22 ± 16.40 (805-880)	23

Table 3: Results of analysis of variance testing of bacteria abundance in the two extreme gas levels (100% and 143%). Three replicate samples of each gas treatment were used. SD = significant difference ($p \leq .05$), NSD = no significant diffference.

Source of Variation (100%)	DF	SS	MS	F	P	Result
Between Groups	1	2166	2166	1.195	3357	NSD
Within Groups	4	7249	1812			
Total	5	9415				

Source of Variation (143%)	DF	SS	MS	F	P	Result
Between Groups	1	47704	47704	639	.0001	SD
Within Groups	4	299	75			
Total	5	48002				

Table 4. Bacteria colony counts (mean ± standard deviation) throughout the 90 Day experiment. Each treatment is sampled once during each 10 day cycle. Sample size (n) changes according to survivorship of each replicate treatment.

	Gas Treatment Levels						
Cycle #	*100%	*105%	*110%	*115%	120%	130%	143%
1	17.0 (± 9.0)	57.7 (±71.9)	11.7 (±11.7)	---	12.3 (±6.8) n=59	83.0 (±92.7) *	32.3 (±29.1) *
2	10.3 (±14.6)	30.3 (±44.0)	8.0 (±6.1)	---	14.0 (±15.9) n=59	35.7 (±12.5) n=59	134.3 (±105.2) n=60
3	22.3 (±13.1)	110.3 (±78.7)	35.7 (±24.1)	---	51.0 (±32.6) n=59	73.7 (±58.0) n=58	80.3 (±100.8) n=56
4	76.7 (±56.9)	68.3 (±14.6)	85.0 (±24.1)	64.7 (±58.2)	137.7 (±44.8) n=58	115.7 (±31.1) n=53	179.0 (±36.4) n=46
5	89.0 (±100.9)	101.7 (±51.8)	125.3 (±64.7)	73.7 (±27.2)	138.7 (±24.2) n=52	178.7 (±35.2) n=39	139.0 (±41.8) n=31
6	111.7 (±78.5)	96.7 (±37.5)	92.3 (±68.1)	118.0 (±72.5)	99.3 (±26.0) n=38	88.3 (±32.0) n=31	8.7 (±15.0) n=13
7	90.3 (±33.9)	97.3 (±28.7)	110.3 (±18.2)	84.3 (±47.1)	125.0 (±38.9) n=25	---	---
8	122.3 (±31.3)	141.7 (±26.8)	89.3 (±30.0)	73.3 (±53.5)	---	---	---
9	95.0 (±22.9)	94.3 (±78.8)	86.7 (±60.1)	60.0 (±16.1)	---	---	---

* These replicate samples have a total of 60 animals/ treatment.
--- Refers to samples not taken : 115% treatment level started one month later . 120%, 130%, and 143% treatment levels did not contain sufficient numbers of animals for meaningful data analysis.

Table 5a: Analysis of variance of total length increase from initial to final shellgrowth. Each treatment was replicated 3 times. SD = significant difference ($p<.05$), NSD = no significant difference.

Source of Variation	DF	SS	MS	F	P	Result
Between Groups	6	9.54	1.59	23.10	.0001	SD
Within Groups	14	0.96	0.07			
Total	20	10.50				

Table 5b. Average length increase of experimenta animals in mm: mean (± standard deviation).*

100%	105%	110%	115%	120%	130%	143%
13.427	13.993	13.897	13.067	12.783	12.197	12.250
(±.0.374)	(±0.344)	(±0.155)	(±0.144)	(±0.208)	(±0.290)	(±0.226)

*All data points had an initial value of 12.00 mm ± 1 mm.

Table 5c: Scheffe's multiple comparison test of total length increase from initial to final shellgrowth data. Each treatment was replicated 3 times. Mean diff = final - initial length, SD = significant difference ($p < .05$), NSD = no significant difference.

Comparison	Mean Diff	Scheffe F- test	Result
100% vs. 105%	-0.567	1.167	NSD
100% vs. 110%	0.470	0.803	NSD
100% vs. 115%	0.360	0.471	NSD
100% vs. 120%	0.643	1.504	NSD
100% vs. 130%	1.230	5.497	SD
100% vs. 143%	1.177	5.031	SD
105% vs. 110%	0.097	0.034	NSD
105% vs. 115%	0.927	3.120	SD
105% vs. 120%	1.210	5.320	SD
105% vs. 130%	1.797	11.730	SD
105% vs. 143%	1.743	11.044	SD
110% vs. 115%	0.830	2.503	SD
110% vs. 120%	1.113	4.504	SD
110% vs. 130%	1.700	10.501	SD
110% vs. 143%	1.674	9.853	SD
115% vs. 120%	0.283	0.292	NSD
115% vs. 130%	0.870	2.750	NSD
115% vs. 143%	0.817	2.423	NSD
120% vs. 130%	0.587	1.251	NSD
120% vs. 143%	0.533	1.034	NSD
130% vs. 143%	-0.053	0.010	NSD

Table 6a: **Total weight gain data before and after exposure to 7 gas saturation levels throughout the 90 day experiment (or until mortality occurred), mean (± standard deviation), (n= 3).**

% TG	Initial Weight (mg.)	Final Weight (mg.)	Mean Diff.	n
100%	0.223(±0.001)	0.295(±0.008)	0.072	3
105%	0.228(±0.016)	0.292(±0.021)	0.064	3
110%	0.246(±0.018)	0.313(±0.027)	0.067	3
115%	0.207(±0.002)	0.260(±0.011)	0.053	3
120%	0.233(±0.003)	0.194(±0.012)	-0.039	3
130%	0.243(±0.015)	0.185(±0.005)	-0.053	3
143%	0.255(±0.015)	0.176(±0.014)	-0.079	3

Table 6b: Analysis of variance of initial weight data differences among the 7 gas treatments. Each treatment was replicated 3 times. SD = significant difference ($p \leq .05$), NSD = no significant difference.

Source of Variation	DF	SS	MS	F	P	Result
Between Groups	6	9.54	1.59	23.10	.0001	SD
Within Groups	14	0.96	0.07			
Total	20	10.50				

Table 6c: Average initial weight in mg: mean (± standard deviation).

100%	105%	110%	115%	120%	130%	143%
.223	.228	.246	.209	.233	.243	.255
(±0.001)	(±0.016)	(±0.018)	(±0.002)	(±0.003)	(±0.015)	(±0.015)

Table 6d: **Scheffe's multiple comparison test for the initial weight of all test abalone. Mean initial weight in mg. Each treatment was replicated 3 times. SD = significant difference ($p \leq .05$), NSD = no significant difference.**

Comparison	Mean Diff	Scheffe F-Test	Result
100% vs. 105%	-0.006	0.054	NSD
100% vs. 110%	-0.024	0.943	NSD
100% vs. 115%	0.016	0.413	NSD
100% vs. 120%	-0.010	0.168	NSD
100% vs. 130%	-0.020	0.674	NSD
100% vs. 143%	-0.033	1.797	NSD
105% vs. 110%	-0.018	0.546	NSD
105% vs. 115%	0.021	0.767	NSD
105% vs. 120%	-0.004	0.032	NSD
105% vs. 130%	-0.014	0.346	NSD
105% vs. 143%	-0.027	1.228	NSD
110% vs. 115%	0.039	2.606	NSD
110% vs. 120%	0.014	0.315	NSD
110% vs. 130%	0.004	0.023	NSD
110% vs. 143%	-0.009	0.136	NSD
115% vs. 120%	-0.026	1.110	NSD
115% vs. 130%	-0.036	2.143	NSD
115% vs. 143%	-0.048	3.935	SD
120% vs. 130%	-0.010	0.168	NSD
120% vs. 143%	-0.023	0.865	NSD
130% vs. 143%	-0.013	0.270	NSD

Table 7a: Analysis of variance of final weight gain differences among the 7 gas treatments. Each treatment was replicated 3 times. SD = significant difference ($p \leq .05$), NSD = no significant difference.

Source of Variation	DF	SS	MS	F	P	Result
Between Groups	6	0.061	0.01	41.084	0.0001	SD
Within Groups	14	0.004	0.0003			
Total	20	0.065				

Table 7b: Scheffe's multiple comparison test for final weight gain of all test abalone. Average final weight in mg. Each treatment was replicated 3 times. SD = significant difference ($p \leq .05$), NSD = no significant difference.

Comparison	Mean Diff	Scheffe F-Test	Result
100% vs. 105%	0.003	0.007	NSD
100% vs. 110%	-0.018	0.325	NSD
100% vs. 115%	0.035	1.229	NSD
100% vs. 120%	0.101	10.236	SD
100% vs. 130%	0.109	11.995	SD
100% vs. 143%	0.119	14.210	SD
105% vs. 110%	-0.021	0.429	NSD
105% vs. 115%	0.032	1.049	NSD
105% vs. 120%	0.098	9.703	SD
105% vs. 130%	0.107	11.417	SD
105% vs. 143%	0.116	13.580	SD
110% vs. 115%	0.053	2.819	NSD
110% vs. 120%	0.119	14.210	SD
110% vs. 130%	0.127	16.270	SD
110% vs. 143%	0.137	18.834	SD
115% vs. 120%	0.066	4.371	SD
115% vs. 130%	0.074	5.544	SD
115% vs. 143%	0.084	7.080	SD
120% vs. 130%	0.008	0.070	NSD
120% vs. 143%	0.018	0.325	NSD
130% vs. 143%	0.010	0.094	NSD

Table 8a: Analysis of variance testing of total weight gain among all treatments from initial to final weight gain data. Each treatment was replicated and sampled 3 times. SD= significant difference ($p \leq .05$), NSD = no significant difference.

Source of Variation	DF	SS	MS	F	P	Result
Between Groups	6	0.08	0.013	39.643	0.0001	SD
Within Groups	14	0.005	0.0003			
Total	20	0.084				

Table 8b: Average total weight gain in mg: mean (± standard deviation).

100%	105%	110%	115%	120%	130%	143%
0.072	0.064	0.066	0.053	-0.039	-0.057	-0.080
(±0.009)	(±0.008)	(±0.033)	(± 0.013)	(± 0.015)	(±0.010)	(±0.026)

Table 9a: Analysis of variance comparing the three highest supersaturation treatments and mortality levels. Each treatment was replicated and sampled 3 times. Samples were taken from a one week period (4th week) during the experiment. SD = significant difference ($p \leq .05$), NSD = no significant difference.

Source of Variation	DF	SS	MS	F	P	Result
Between Groups	2	48.2	24.11	18.083	.0029	SD
Within Groups	6	8.0	1.33			
Total	8	56.2				

Table 9b: Scheffe's multiple comparison test for mortality levels among the three higher supersaturation treatments. Each treatment was replicated 3 times. Samples were taken from a one week period (4th week) during the experiment SD = significant difference ($p \leq .05$), NSD = no significant difference. Mean diff.= group A mean mortality - group B mean mortality.

Comparison	Mean Diff.	Scheffe's F-Test	Result
120% vs. 130%	-1.00	0.562	NSD
120% vs. 143%	-5.333	16.000	SD
130% vs. 143%	-4.333	10.562	SD

Table 10: **Cumulated mean mortality data collected during experiment by sampling and removing the abalone in the 3 replicate containers of each gas level where mortalities were present. Samples were taken three times per week. Cumulated mean (± standard deviation).**

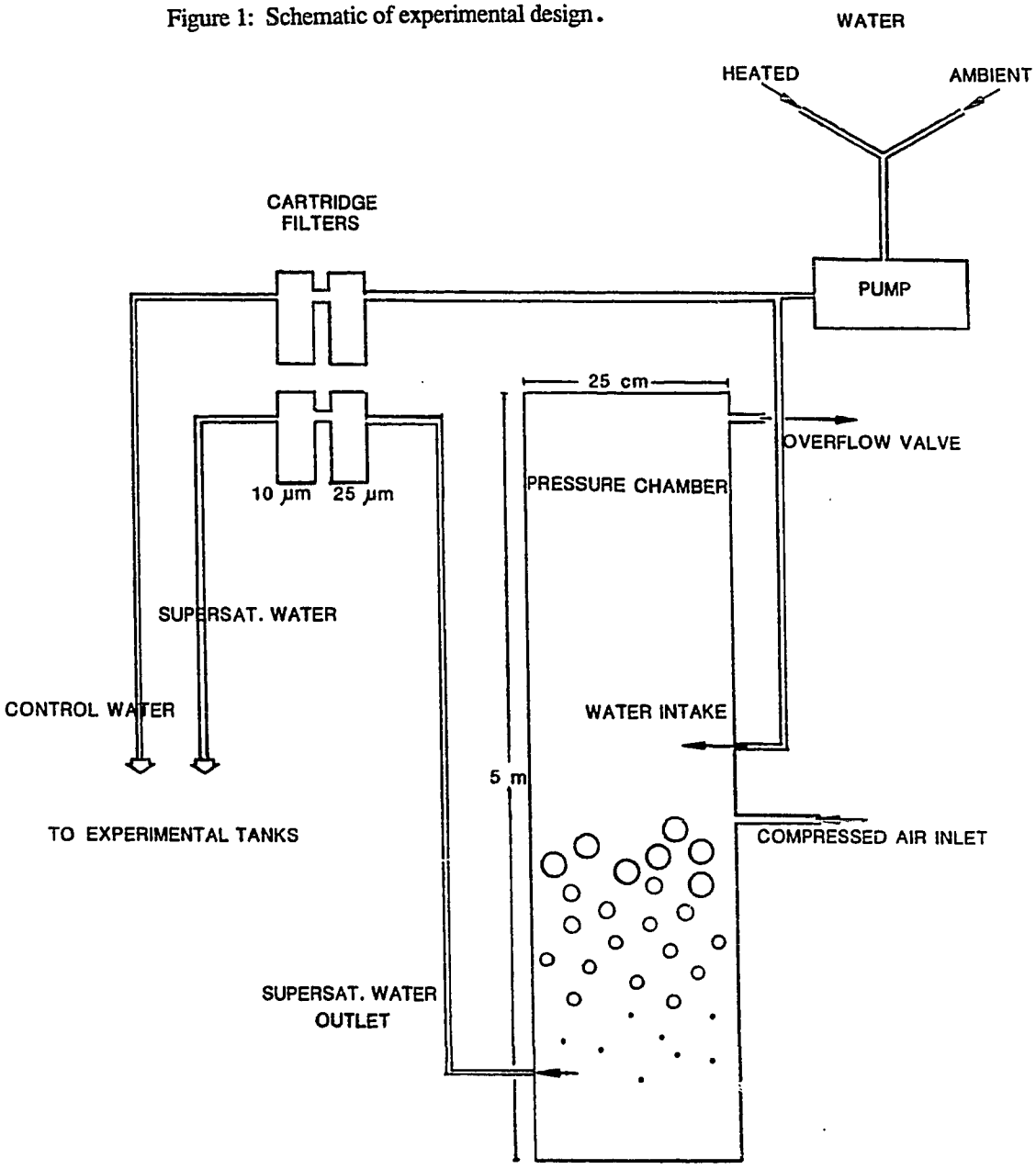
(Days)	120%	130%	143%
6	0.7 (± 0.6)	0	0
13	0	0.7(±0.6)	2.0(±2.0)
20	0	0.3(±0.6)	3.3(±1.2)
27	0	1.7(±1.2)	4.3(±3.1)
38	1.3 (±0.6)	2.7(±3.1)	4.3(±2.1)
45	2.3 (±2.1)	3.0(±2.7)	4.7(±1.5)
52	4.3(±2.5)	2.7(±0.6)	1.3(±1.5)
59	3.7(±3.1)	4.0(±2.0)	*
66	1.3(±0.6)	4.7(±1.5)	*
75	1.0(±1.0)	4.7(±1.5)	*
84	4.0(±2.0)	3.0(±2.7)	*
90	0.3(±0.6)	*	*

*** Complete mortality in test containers.**

Table 11: Qualitative values for food consumption and tenacity of abalone in all gas treatment levels throughout the experiment, (means, n=3).

Gas Level	Food Consumption mean (± s.d.)	Tenacity mean (± s.d.)	n
100%	4.9 (±0.3)	4.8 (± 0.3)	99
105%	4.6 (± 0.6)	4.5 (± 0.5)	99
110%	4.3 (± 0.6)	4.0 (± 0.5)	99
115%	3.2 (± 0.4)	3.1 (± 1.1)	63
120%	1.0 (± 0.8)	1.5 (± 0.8)	76
130%	0.1 (± 0.1)	0.5 (± 0.8)	69
143%	0.0 (± 0.0)	0.1 (± 0.0)	48

Figure 1: Schematic of experimental design.



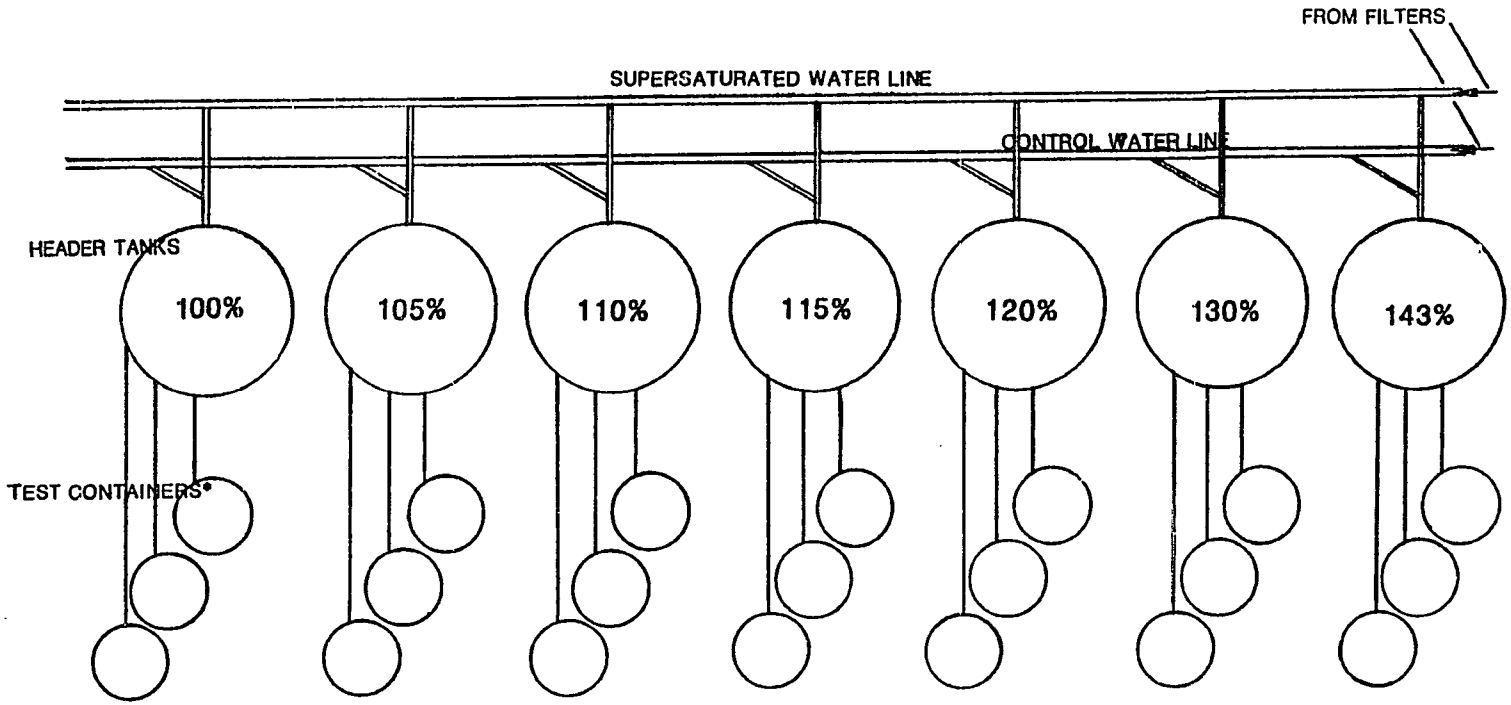


Figure 2: Schematic of experimental tank set-up (both header tank and test containers).

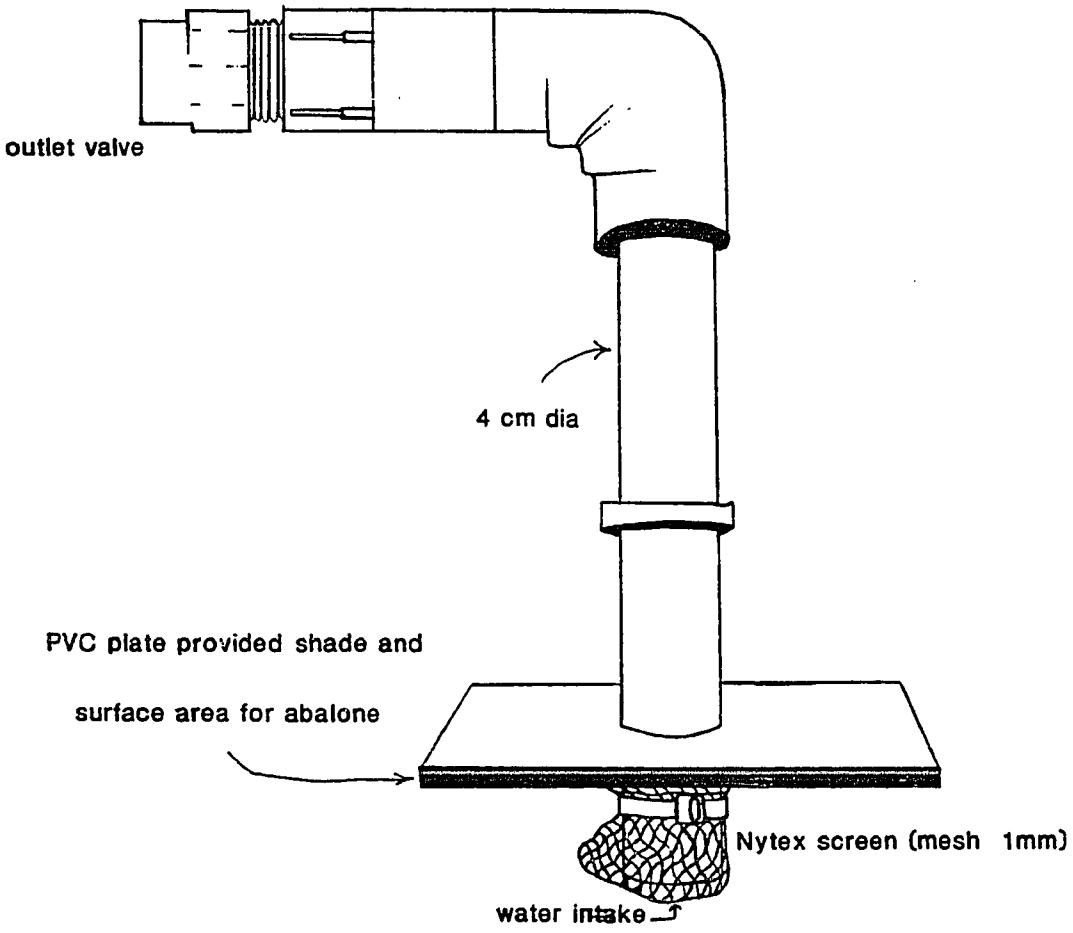


Figure 3:

Schematic of water intake apparatus on inside of test container.

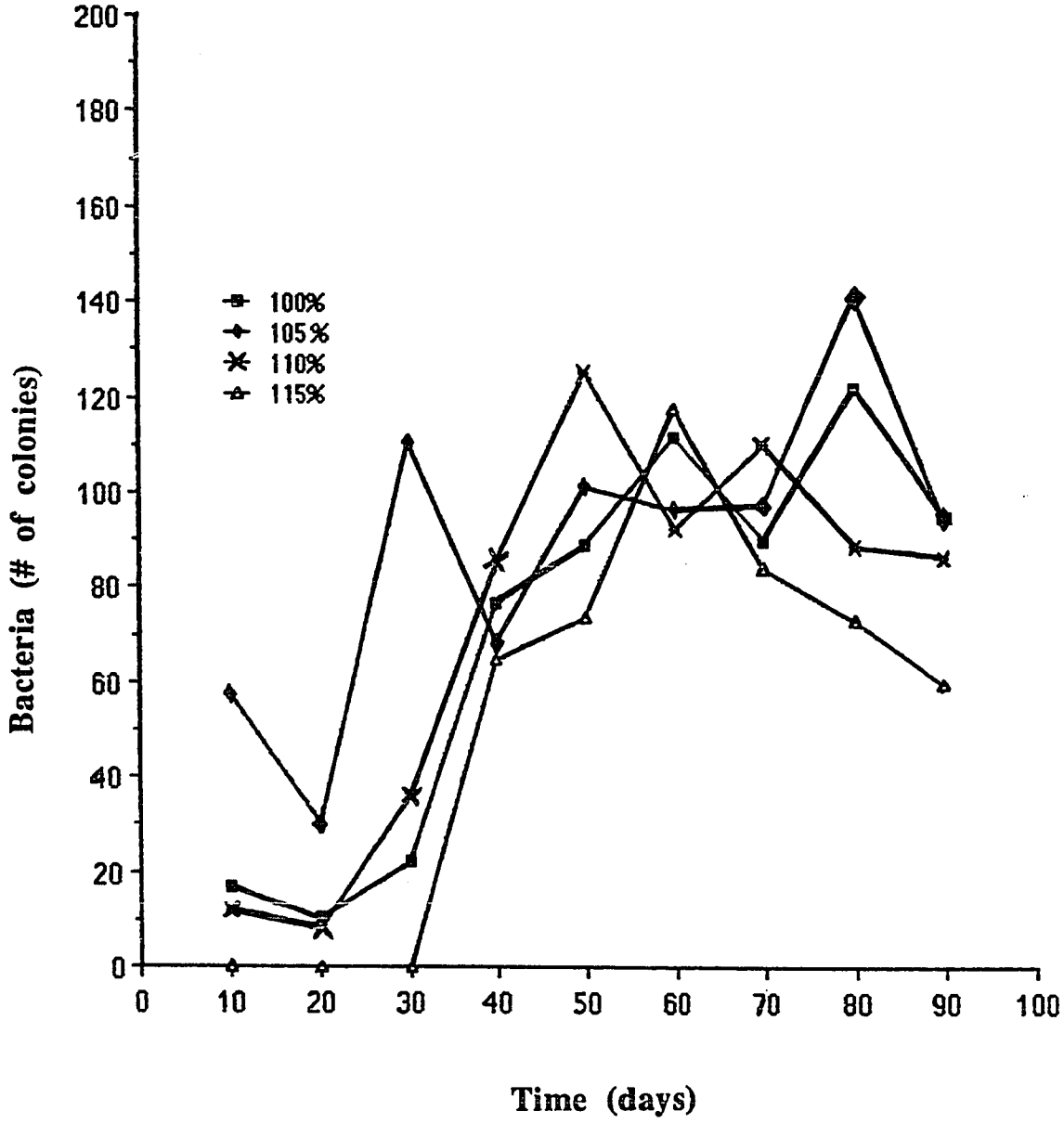


Figure 4: Bacteria abundance at four levels of gas supersaturation, (means, n = 3).

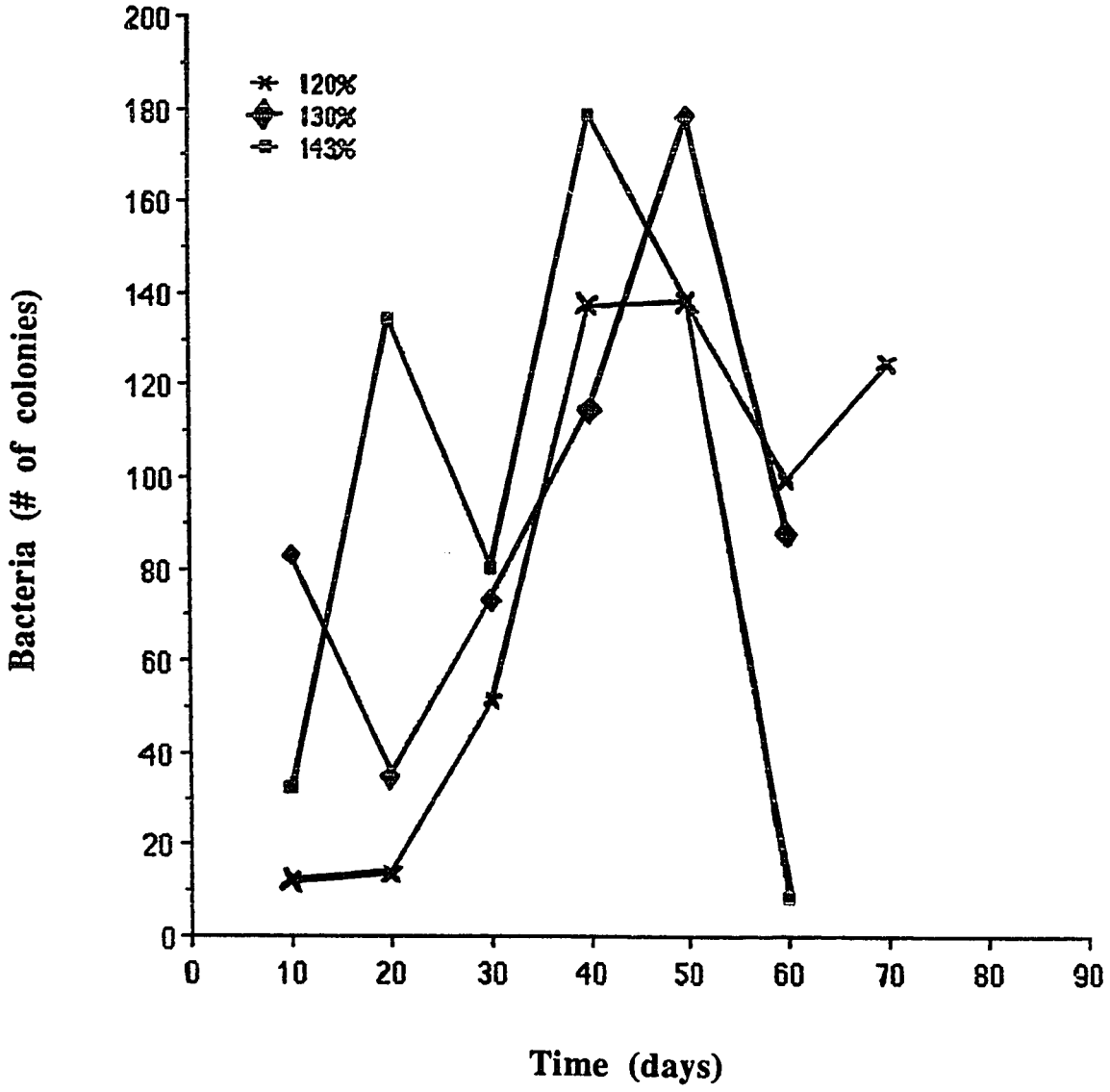


Figure 5: Bacteria abundance at three levels of gas supersaturation, (means, n = 3).

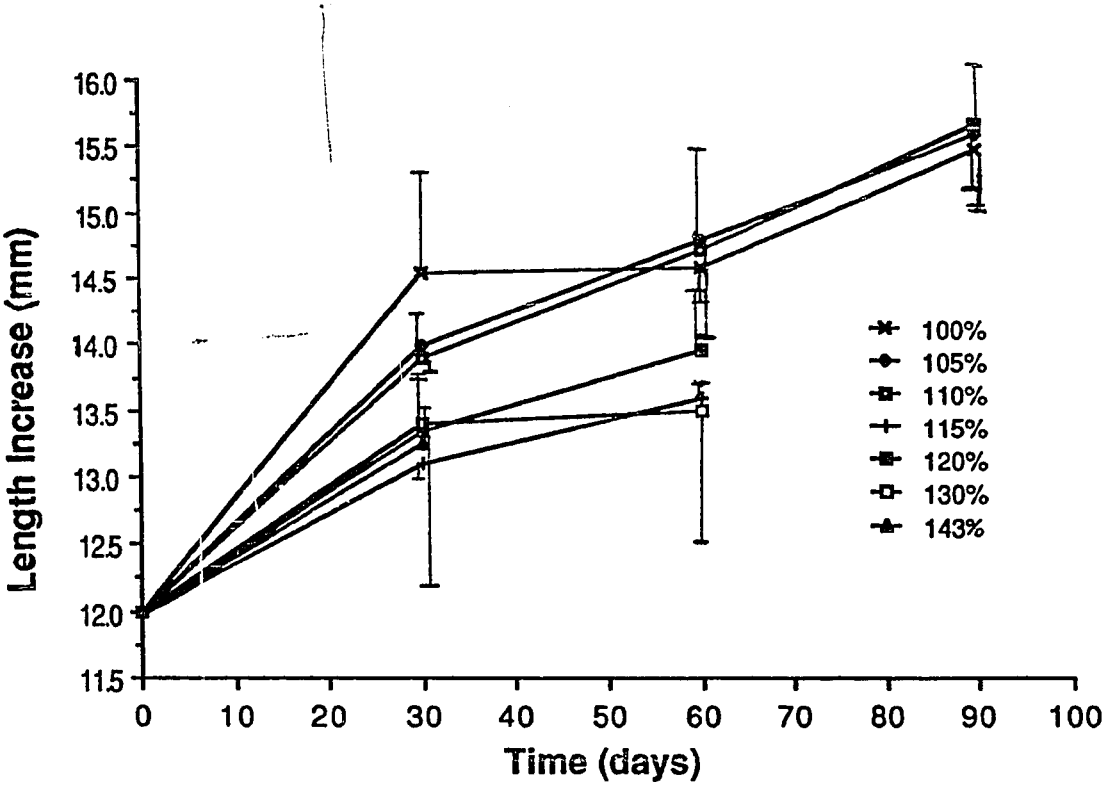


Figure 6: Length increase for all gas level treatments throughout the 90 day experiment (or until mortality occurred.) (mean, ±SD), (n = 3).

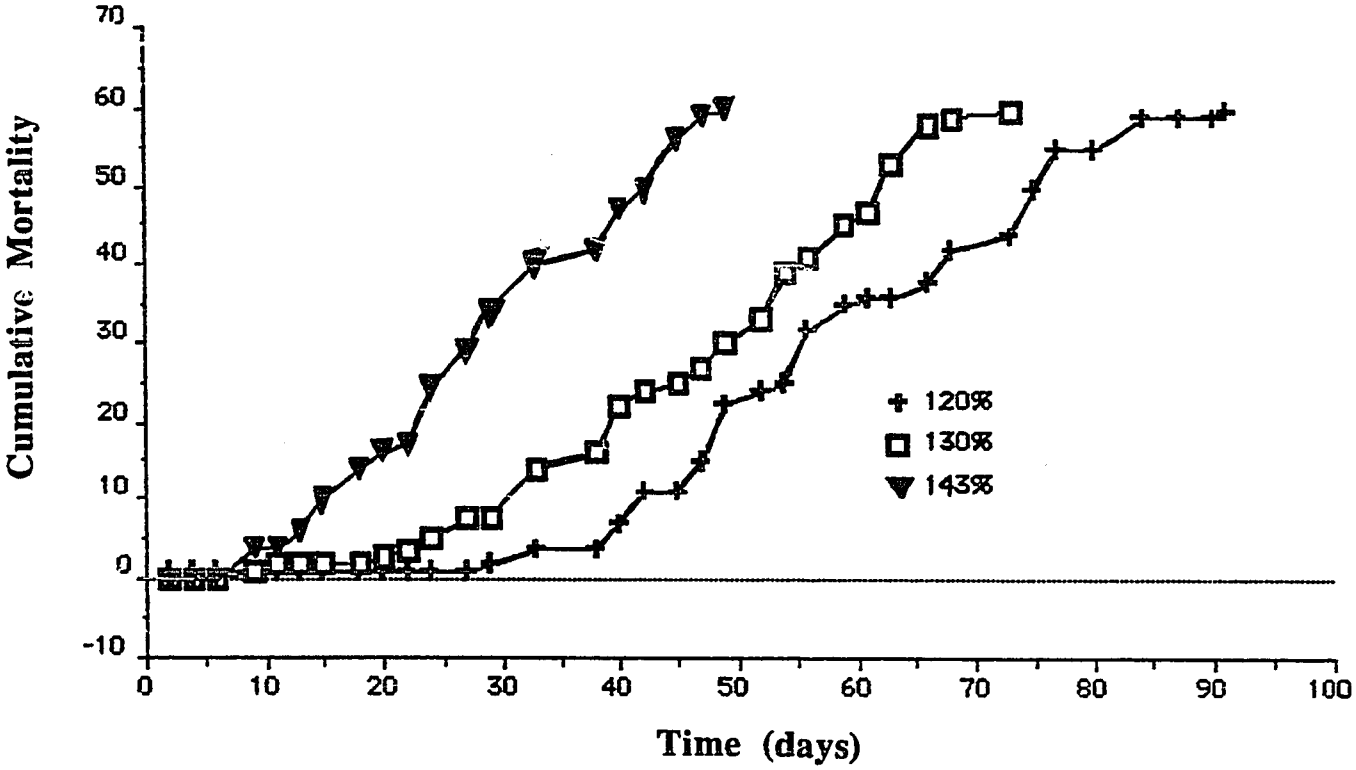


Figure 8: Cumulative mortality rates at three gas supersaturation levels. Each data point represents the mean mortality of three replicate tanks.