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DEVELOPMENT OF METHODS FOR BIOLOGICAL INJECTION AND SAMPLING FROM FLUID LINES

FINAL REPORT
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N O T I C E

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16. Abstract (MAXIMUM 200 WORDS)

A critical factor in providing consistent and accurate organism loads to test ballast water treatment equipment is the injection of surrogate organisms. Additionally, it is necessary to acquire representative samples from flowing pipes to assess the treatment's effects on the number and viability of organisms. As a result of the interaction between mechanical processes and living organisms, there are inevitably both mortality and recovery issues. The current document presents data and a discussion regarding the design, construction, operation and relative performance of various types of pumps for inserting *Artemia* into flowing seawater pipes eight inches in diameter. The effects of several sampling configurations on organism viability and recovery are documented. These include both assemblies for obtaining continuous samples from flowing pipes and configurations for receiving, holding, and concentrating samples. The effect of analysis time on organism viability and recovery is also discussed. Recommendations are made for the best injection, sampling, and holding configurations.

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Executive Summary

Non-indigenous aquatic nuisance species are introduced into waters of the United States in a number of ways including incidental transport by ships. Discharge of ballast water is a major vector for introductions, and state, federal, and international regulations have attempted to regulate ballast water discharge to reduce the introductions and potential harm. The most common practice currently in use to reduce introductions is mid-ocean ballast water exchange (BWE) during which ballast water taken up in ports or coastal areas is exchanged for open-ocean water. While this method may be effective, safety considerations and routes often make BWE impossible. Thus BWE is considered an interim solution until viable ballast water treatment (BWT) systems can be developed and approved.

To be successful, BWT systems must be safe, practical, and cost effective for shipboard use. To be approved by the United States Coast Guard (USCG), they must also be effective in reducing the number of live organisms within the ballast tanks. Testing the systems for approval must be rigorous, unbiased, and repeatable. The USCG has partnered with the Environmental Protection Agency's Environmental Technology Verification (ETV) Program to develop a test protocol that meets these criteria for full-scale BWT systems. The protocol includes the use of surrogate organisms in a closely controlled and characterized challenge water. The surrogates are intended to be non-toxic, culturable organisms which can be used to assess the technology's efficacy over a broad range of taxa. The use of surrogates also provides a measure of consistency among test facilities in various geographic locations.

The test protocol developed in partnership with the ETV Program calls for organism (surrogates) injection and sampling from treatment systems operating at or near full-scale (nominal 300 m^3/hr, or ~1300 gallons per minute). Surrogates are injected upstream of the treatment system and then sampled from the flow stream after mixing has been achieved but before entering the treatment system, immediately after the treatment, and again from a storage tank after a specified holding time. Samples are generally acquired in triplicate and are 1 cubic meter in size. Samples are analyzed for organism viability and enumeration. This is a substantial requirement since the intention is to acquire a sufficiently large and representative sample to insure statistical rigor of measurements and conclusions.

The current project was designed to evaluate a variety of methods to inject and sample surrogates without causing damage to the organisms. Samples were drawn from an 8-inch diameter pipe having a water flow rate of approximately 300 m^3/hr. Samples were characterized on the basis of impact on organism viability (or conversely, mortality) and on how well the sample represented the overall population of the test organisms. Prior to this work, no data existed regarding methods of injecting organisms into flowing pipes or for obtaining accurate biological samples from flowing pipes. Viability is important since organism death caused by injection or sampling would cause an inaccurate evaluation of the treatment system's efficacy. Sample representativeness is an indicator that the sample genuinely represents the entire volume of water flowing through the BWT system and that organisms were not lost in the injection assembly. This latter part is important in determining how many surrogates must be available for injection to reach the desired concentration in the challenge water.

The fluid mechanics of pipe flow was at the core of both the injection and sampling studies, and previous work by Naval Research Laboratory had shown that the impact of these fluid dynamics was directly related to organism size. As a result, all experiments in this study were conducted with the zooplankton *Artemia* as the basis of viability, recovery and representative analyses, since *Artemia* are relatively large (~ 400 microns) compared to phytoplankton or bacteria. The *Artemia* size class provided a "worst case scenario" system evaluation in terms of fluid dynamics on large organisms. The use of *Artemia* as a surrogate had the added advantage that they are relatively easy to culture in mass quantities, are relatively hardy, and are not a risk for invasion of local waters should a spill have occurred. It was assumed that the results of this study would be used to set the methods for smaller surrogates to be injected and sampled in the system.

The surrogate injection experiments tested three mechanical elements: a diaphragm pump, a positive displacement pump (typically used in environmental water sampling) and a pressurized vessel assembly. Results of these mechanical systems were compared to the direct addition of *Artemia* to the pipe without any injection assembly. The pumps were evaluated relative to percent recovery, residual volume, effect on organism viability, and engineering considerations. Overall, the pressure vessel assembly provided the best compromise between operational/engineering concerns and organism viability and representativeness.

Sampling experiments were broken into two distinct elements: components for collecting the sample and assemblies for obtaining the sample from the flow. Experiments with the sample recovery tank, plankton net, and discharge piping were conducted first to determine which arrangement of the discharge pipe had the least effect on organism mortality. The best arrangement consisted of a plankton net within a filled sample tank with the discharge pipe fitted with a diffuser head immersed below the waterline and interior to the plankton net. This arrangement was used for all subsequent experiments.

Four sampling wand assemblies were tested for acquisition of a representative sample from the eight-inch diameter pipe. Configurations included a simple tee fitted to the pipe and three sample wands that protruded into the center of the eight-inch pipe. The wands sampled approximately two percent of the total pipe flow, and the sample was split into three replicate recovery tanks. While results indicated no significant differences among the four geometries, the simple tee configuration provided a slightly more representative sample.

Based on the results of this experimental effort, it is recommended that a pressure vessel be used to inject surrogate organisms into the challenge water flow. Representative samples can be obtained from a simple tee fixture rather than using a wand protruding into the flow. Samples can be drawn off from the sample port by means of a flexible hose and manifold without injuring organisms. A diffuser head should be used at the discharge end of the sampling hose, and it should be submerged within the sample tank to avoid injury. Samples should be analyzed within two hours to obtain good viability data. Since only about 2 percent of the total flow is sampled, it is necessary to determine the representativeness of the sample in order to judge the efficacy of a treatment system.

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List of Acronyms and Abbreviations

ANOVA = analysis of variance
APHA = American Public Health Association
bar = barometric pressure
BWE = ballast water exchange
BWT = ballast water treatment
BWTE = ballast water treatment equipment
BWTF = ballast water treatment facility
CO_2 = Carbon dioxide
CFU = colony forming units
Chl_a = Chlorophyll_a
df = degrees of freedom
DO = dissolved oxygen
EPA = Environmental Protection Agency
ETV = Environmental Technology Verification
ESS = Environmental Source Samplers Inc.
F = Calculated random-effects analysis of variance
F crit = Critical random-effects analysis of variance values
FIU = Florida International University
gal = gallon
gph = gallons per hour
gpm = gallons per minute
IC = inorganic carbon
ID = identification
IMO = International Maritime Organisation
JD = Julian day
L = liter
m = meter
mean = measure of central tendency
m/s = meters per second
mg = milligram

mg/L = milligrams per liter

mL = milliliter

mm = millimeter

MS = mean square

NA = not available

NISA = National Invasive Species Act

NDIR = non-dispersive infra-red

NOBOB = no ballast on board

NRLKW = Naval Research Laboratory in Key West, Florida

NRL = Naval Research laboratory

ntu = nephlometric unit

PC = personal computer

pH = potential of Hydrogen

POC = purgeable organic carbon

POM = particulate organic matter

ppm = parts per million

psig = pounds per square inch (gauge)

p-value = the probability of observing a test statistic that is as extreme or more extreme than currently observed (assuming that the null hypothesis is true).

PVC = polyvinyl chloride

sig = significant

Slr = Solar light radiation

SPSS = Statistical Package for Social Sciences

SS = sum of squares

ssw = surface saltwater

TOC = total organic carbon

TSS = total suspended solids

UK = United Kingdom

USCG = United States Coast Guard

YSI = YSI Environmental/ Son Tek Company

^{o}C = degrees Celcius

µm = micron

= number

® = registered sign
α = alpha at 95% confidence interval

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1 Introduction

Ballast water is a significant pathway for the introduction of non-indigenous species into U.S. coastal waters. Recognizing that alternatives to ballast water exchange are necessary, the National Invasive Species Act (NISA) of 1996 and its reauthorization language include provisions for a mandatory ballast water management plan including either ballast water exchange or ballast water treatment. Ballast water exchange is considered an interim method for controlling species migration; both safety and route issues limit the practicality of mid-ocean exchange. Ballast water treatment (BWT) holds significant promise in achieving the level of treatment that is currently required in the international arena under the recent diplomatic convention adopted by the International Maritime Organization (IMO, 2004).

As standards become adopted and ratified, it is important to consider how ballast water treatment systems ought to be tested, validated and qualified for use. There is much to consider since testing may or may not include fitness-for-service, biological effectiveness, safety, maintenance, and manning requirements, among other factors. While the development of treatment technologies is important, the methods used to proof-test them are equally important. At present, there is little uniformity in testing facilities and testing approaches globally (Voigt and Gollasch, 2000), (Mountfort et al, 2003), (Herwig et al, 2003), (Waite et al, 2003), (Holdo, 2001).

Considerable efforts throughout academia and industry are currently focused on technologies to reduce or eliminate the translocation of organisms in ships' ballast water. A pilot-scale facility to test candidate technologies has been constructed with the intention of providing a means to test shipboard equipment as close to full-scale as possible. The test facility will allow increased control of various parameters affecting performance, which are typically unknown or uncontrolled in the ship's environment. For example, input water properties are controlled by the surface waters in which the ship typically operates, whereas the waters used at the test facility may be supplemented to adjust organism densities, salinity and suspended solids. Treatment technology tests should be standardized such that comparable results are achieved at varying geographical locations and testing facilities (land-based or ship-based) and that afford a high degree of scientific and statistical rigor. The latter points are critical to protect all interested parties including regulatory agencies, technology vendors, and the customers (industry).

As a direct result of the need for a standardization of test methods, the Naval Research Laboratory in Key West, FL (NRLKW) has been tasked by the United States Coast Guard (USCG), the Environmental Protection Agency, and NSF International (as part of the EPA's Environmental Technology Verification (ETV) program) to conduct a pilot-scale beta test of the draft protocol for the BWT technologies (Tanis and Hunt, 2003). The intent of such testing is to provide the shipping industry, resource managers, and regulators (via the USCG, EPA and NSF International) with reliable information about the effectiveness, costs, and environmental risks associated with individual technologies.

The USCG Research & Development Center commissioned NRLKW to investigate both the injection and sampling of organisms from pipe flow streams. While the current ETV Protocol and many other testing protocols require the use of surrogate species, not a great deal of

information is available on the effects of injection and sampling on organisms. Surrogates are defined as organisms that have been introduced or augmented in the ambient feedwater at controlled concentrations. Under the current protocol, these will be separated into bacteria, protists/phytoplankton and zooplankton. The surrogates plus organic and inorganic particles will be used to establish standardized challenge water at shipboard flow rates across all applicable testing and temporal conditions. To date, little effort has been spent on the mechanism to introduce surrogates into the flow stream prior to entering into the test tank or the treatment technology. The challenge of organism injection is insuring that the mechanism or apparatus utilized for injection does not result in significant organism mortality or loss. For sample acquisition, there are several requirements within the current ETV protocol and in current and proposed shipboard sampling programs which require sampling ballast water intake or effluent water. The ETV program requires large (~ 1 m^3) triplicate samples for enumeration and viability analysis of a variety of organisms. It is important, as with the injection apparatus, that the methods and apparatus used for sampling do not result in mortality, organism loss or loss of sample representativeness. This requirement is somewhat unique in that few industries require continuous sampling of large flow streams at high flow rates for generally sparse organisms.

The information from these experiments will be critical to standardized testing of BWT equipment (BWTE) in programs such as the ETV efforts. To date, little information is available on the effects of various mechanical systems on marine organisms. This research has two central objectives:

- The development, optimization and demonstration of methods for injecting live organisms ranging from viruses to macrozooplankton into water flowing in a pipe approximately eight inches in diameter.

- The development of methods for taking representative discrete samples of organisms from water flowing in pipes, approximately eight inches in diameter.

2 Experimental Procedure

2.1 Ambient Water Characterization

Water chemistry and bio-assays were performed on the ambient waters around Fleming Key, FL to obtain baseline data and temporal scales of variability for consideration when creating and testing various challenge water conditions. All field sampling began on 13 April 2004 and was continued on a weekly basis from a land-based station at NRLKW Pier (Figures 1 and 2) in close proximity to an *in situ*, moored, YSI 6600 Environmental Data Sonde. The Sonde measurements of dissolved oxygen, pH, salinity, turbidity, chlorophyll, temperature and water level were obtained as a high-resolution, continuous time-series of water quality parameters, taken every 15 minutes. Concurrent meteorological parameter measurements from a local Campbell Scientific Meteorological Station were obtained hourly for the entire sampling period. Water chemistry measurments and indigenous bio-assays were obtained weekly on the 13th, 19th , and 27th of April, and the 3rd and 10th of May. The site has semi-diurnal tides and sampling always occurred on a rising tide and occurred at approximately high tide for days 19 and 27 April, and 3 and 10 May as shown in Figure 3.

Figure 1. Research Laboratory, located on Fleming Key (Key West in the background).

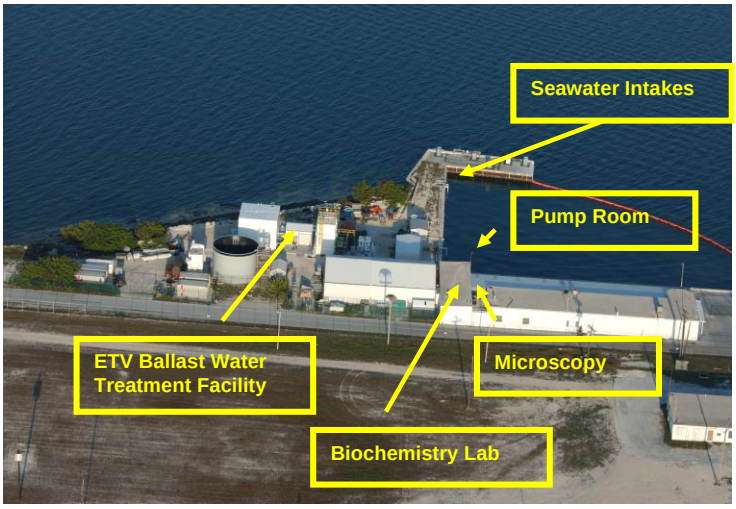

Figure 2. Naval Research Laboratory.

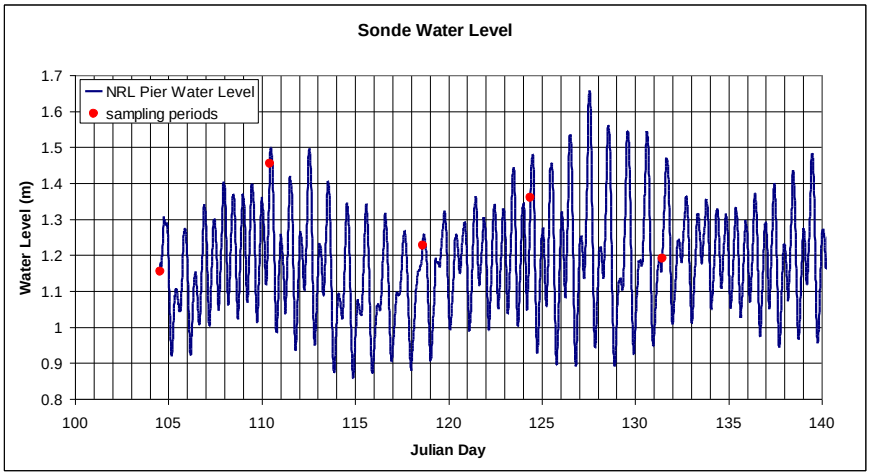

Figure 3. NRLKW Pier water levels in meters (m) over time (Julian Day, JD) taken by *in situ* data Sonde. Red dots mark day of discrete water sampling. All Sampling occurred on a rising tide.

An Analysis of Variance (ANOVA) statistical test was used to measure the variability between sampling times for total organic carbon (TOC), total suspended solids (TSS) and Chlorophyll_a (Chl_a).

Sampling on the 13th and 19th of April (JD 104 and 110) consisted of three replicate surface water samples, 15 gallons total, that were obtained with a plastic, five-gallon bucket. Each replicate was sub-sampled for analysis of TOC, TSS, Chl_a, and enumeration of bacteria, protists and zooplankton.

Phytoplankton and zooplankton samples were collected on April 27th (JD 118), May 3rd (JD 124), and May 10th (JD 131). A submersible pump located at mid-depth was used to pump approximately 200 gallons of *in situ* seawater through four plankton nets to retain organisms in size classes greater than 120 µm, between 120 µm and 80 µm, between 80 µm and 50 µm, and between 50 µm and 25 µm. In addition to this, a total of three 5-gallon replicates were pumped into amber Nalgene bottles for water quality analysis. Two liters per replicate were analyzed for TSS, and two half-gallon (1.89 L) samples per replicate were obtained for Chl_a. Finally, a 50-mL sample vial per replicate was obtained for analysis of TOC and another three 50-mL sample vials for bacterial enumeration.

Total Suspended Solids: The methods for sample preparation and analysis of TSS were performed as outlined in the American Public Health Association (APHA) Standard Method 2540D (Clesceri, et al, 1998A). A gravimetric analysis was used by filtering seawater through Millipore 47-mm RWO3 membrane filters (and, later, through glass fiber filters for comparison).

Two liters per sample were filtered onto Whatman 47-mm glass fiber filters. Filters with sample were then dried and weighed (mg/L).

Total Organic Carbon: The method used for the determination of TOC is published under EPA Method 415.1. This method was used in conjunction with the manuals for a SHIMADZU TOC-V ANALYZER (Shimadzu, 2001), which is an instrument that determines TOC via combustion and non-dispersive infra-red (NDIR), carbon dioxide (CO_2) analyzer. Three 50-mL replicate samples were collected for analysis using a submersible pump. In this type of analysis, acid is added to the sample and the solution is sparged until the purgeable organic carbon (POC) and inorganic carbon (IC) are removed and TOC is then measured in parts per million (ppm).

Chlorophyll_a: The Environmental Source Samplers Inc. (ESS) Method 150.1 (endorsed by the EPA), (Arar, 1997), for spectrophotometric analysis of Chlorophyll_a was performed using a Hitachi U-34010 Spectrophotometer. Three replicate samples of 1.89 L were collected, filtered, analyzed and recorded in mg/L.

Bacteria: Bacteria were plated at 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions on 2216 marine agar plates and incubated for four days at 25 ^oC under 24-hour fluorescent light in a Percival Incubator (Straube and Robbins, 2004A). Colony forming units (CFU's) were counted on each plate twice, once after 24 hours, next, after 168 hours (7 days). Dilutions that yielded between 30 and 300 CFU's were used for the final count.

Phytoplankton: APHA Methods 10200C, 10200F, 10200G, were used to process plankton samples (Clesceri, et al, 1998B,C,D). Protists and zooplankton samples were preserved with four percent Lugols iodine solution, settled through graduated cylinders with the settled volume being recorded for each sampling date. Supplemental samples from 4 May were also enumerated with FLOW-CAM® (flow-cytometry). Sample volumes of approximately 200 gallons were pumped through nested plankton nets which size-selected organisms into four categories: (> 120 microns); (120-80 microns); (80-50 microns); (50-25 microns). All samples were placed in amber Nalgene containers and preserved with four percent Lugols Solution. Both gravimetric and grab sample techniques were used to obtain samples for direct count analysis. Samples were placed in gravimetric settling chambers and the settled volume was recorded for each sampling date. Gravimetric samples were analyzed for identification (ID) and enumeration using a Nikon inverted microscope. Discrete grabs of 1-mL aliquots were taken from the fixed, well-mixed sample and placed on a Sedgwick-Rafter cell. These cells were then analyzed under a microscope for identification and enumeration. Because this technique was found to be quite time intensive, a switch to an automated counting method of (number of individuals/mL) was made. For automated counting methods, fixed, well-mixed samples were run at 50-mL volumes at a flow rate of 1 mL per minute through FLOW-CAM® for identification and enumeration analyses.

2.2 Surrogate Selection

The experiments of this work were intended to evaluate the impact of various mechanical operations on the representativeness and viability of organisms. In order to simplify that process, it was determined that it was desirable to use surrogate organisms to supplement the indigenous population. The initial plan had been to add at least one organism from each of the following categories: bacteria, phytoplankton and zooplankton. Further consideration resulted in the

conclusion that only zooplankton would be required for this work. The fundamental rationale for this choice was that for both the injection and sampling experiments, organism viability would be a function of various hydrodynamic effects such as turbulent boundary layer wall shear forces and hydrostatic forces such as pressure differential operating on the organism. It has been shown (Lemieux et al., 2004) that susceptibility to these forces will increase with an organism's body diameter/size, thus theoretically zooplankton will be more sensitive, relatively, to the impact of injection and sampling than bacteria or protists.

The surrogate selected was *Artemia*, a brine shrimp, phylum Arthropoda, class Crustacea. This zooplankton can tolerate both fresh and marine water conditions, beginning its life cycle as a dormant cyst which houses a metabolically dormant embryo. The *Artemia* embryo can be held in this condition for years as long as the cyst is kept dry. *Artemia* suppliers estimate a 90-percent hatch rate with densities of 2.2×10^5 individuals per one gram of cysts. Hatching begins after 15-24 hours at 25 ^{o}C (77 ^{o}F). The *Artemia* larvae emerges in the Instar I phase and ranges between 400-500 microns in body size. *Artemia* are commonly used for a multitude of aquaculture applications. Both the surrogate injection and surrogate sampling experimentation used *Artemia* that were 24 hours old. The target organism density after injection was 100 organisms per liter. These were delivered, as will be discussed subsequently, from a variety of sources during the injection experiments so starting densities in the delivery vessel were variable. However, the sampling experiments utilized a 40-gallon pressure vessel with a target density of $3x10^7$ organisms *Artemia* (corrected for 90-percent hatch rate).

The vendor's protocol for hatching cysts was used to culture new batches of *Artemia*. Each incubation vessel was filled with artificial seawater and equipped with sufficient aeration to keep the cysts suspended. Vessels were exposed to continuous, bright, warm light (approx 30 ^{o}C (86 ^{o}F)) for 24 hours. Both population and mortality were used as the criteria to assess the health of newly hatched *Artemia* batches. Initial population was defined as the number of hatched individuals per vessel. Mortality was defined as the number of individuals devoid of any visual movement. This analysis was performed by collecting three, 1-mL replicates per vessel which were dispensed onto glass Sedgwick-Rafter microscope slides. The slides were viewed at both 4X and 10X magnification using a phase-contrast microscope. First, dead *Artemia* were tallied. Next, the slide was treated with seltzer water to narcotize the *Artemia* to obtain the total number per mL. The number of viable individuals was acquired by subtracting the number of dead individuals from the total individuals present. Estimates of the hatched population were obtained by multiplying the density (number of individuals/mL) by the vessel volume. Each population was then placed in aerated, graduated beakers to await injection.

2.3 Surrogate Injection

Since it will be necessary in the evaluation of ballast water treatment equipment to inject surrogate organisms at prescribed concentrations, various methods by which injection could occur were tested and compared. Most injection systems will consist of a number of parts including: a) the surrogate containment vessel, 2) a mechanical device to provide the requisite energy to the system for injection, and 3) the injection wand and associated plumbing. The specifics of the test assembly utilized in these experiments is discussed in another section, however, the central question is which injection system (vessel, wand, and mechanical device) provides the optimum injection of organisms. The resolution of the question must be answered by evaluating the effect of the injection system on both organism viability (number living or

percent viability) and the percent representativeness (quantity of organisms or conservation of mass). Obviously, an injection device which severely or moderately decreases the viability of organisms as a result of the injection process will not allow for a consistent and repeatable concentration of organisms to be delivered to the ballast water treatment equipment. Therefore Percent Viability is calculated by dividing the number of living *Artemia* per liter in the sample by the original living concentration per liter. Percent Representativeness (conservation of mass) is equally important since it is a measure of organism loss. That is, the optimum injection system will minimize the residual quantity of organisms remaining in the injection vessel and plumbing. Percent Representativeness is calculated by dividing the number of *Artemia* collected in the sample tank by the number of *Artemia* injected into the system. The optimal approach will ensure that during actual testing of ballast water equipment both the intended concentration and viability of organisms will be maintained following surrogate injection.

2.3.1 Injection Setup

The injection experiments used clear, smooth, polyvinyl chloride (PVC) piping to deliver organisms from the injection vessel to the mainstream flow via a nominal 1" Schedule-40 injection pipe fitted with a 90º elbow to direct the flow in parallel with the mainstream. The main system pipe was a nominal 6" Schedule-40 PVC pipe. The system layout is shown in Figure 4.

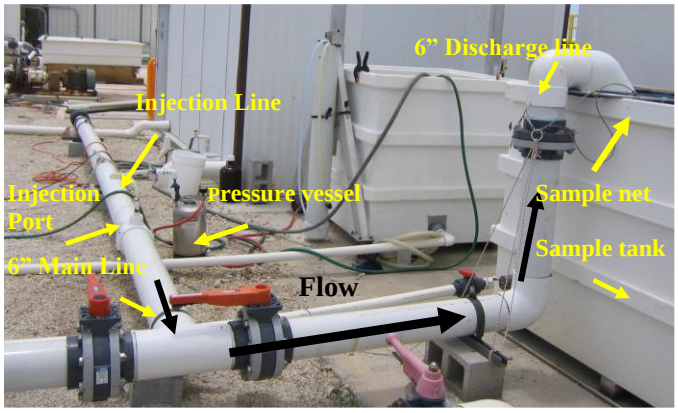

Figure 4. Surrogate injection: Annotated setup of 6-inch pipeline system components and setup.

The discharge tank, partially shown in Figure 5, was 78" long x 33" wide x 48" high, fiberglass coated, with an approximate capacity of 2 m^3. The effluent was discharged into a conical, 25-micron mesh net which ended in a detachable, PVC, cod-end with 80-micron screen.[1] The discharge water passed through the net thereby collecting the *Artemia* for viability and concentration assessments. The same tank, net, and cod-end configuration was used for all test runs.

[1] It was found that substituting the 25-µm cod-end with an 80-µm cod-end decreased the amount of suspended solids collected in the sample thereby improving the sample's water quality. Furthermore, it is felt that this increase in micron size did not affect the net retention of the surrogate which is 400-microns in size.

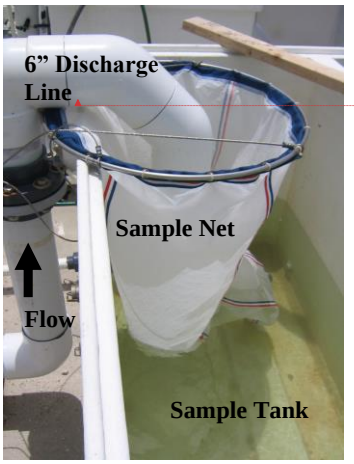

Figure 5. Conical, 25-micron mesh net configuration for surrogate collection at effluent end of main 6-inch discharge line. Large black arrow indicates flow direction.

2.3.2 Control Experiment

In order to evaluate the impact of the test apparatus shown in Figure 4, a control experiment was conducted. The purpose of this exercise was to test the dependent variables (Percent Viability and Percent Representativeness) without the presence of a peripheral injection system. *Artemia* densities of at least 100,000 individuals per liter were poured through a funnel directly into the main pipe via the injection portal as demonstrated in Figure 6. Water was then added to the main pipeline to displace any air trapped in the line. Flow was initiated at 500 gpm into the line leading to the discharge tank. The *Artemia* and water in the line were then discharged into the collection tank until the 528-gallon (2 m^3) vessel was full, with time being recorded. Sampling and analysis of samples were performed as outlined in later sections of this report.

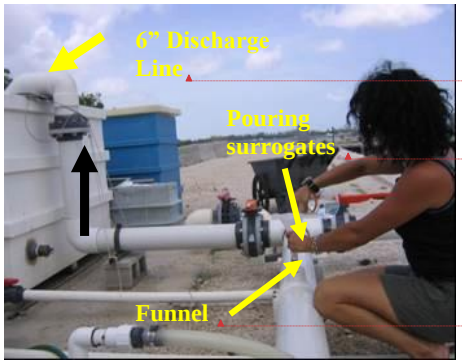

Figure 6. Funneling of surrogates through the injection port and into the 6-inch pipeline. Large, black arrow indicates flow.

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2.3.3 Injection Mechanical Devices Experiments:

The three experimental mechanical injection elements included an auto-reclaimer pump, a pressure vessel, and a diaphragm pump.

Auto-reclaimer Pump

The auto-reclaimer, shown in Figure 7, is an automatic, positive displacement, pneumatically actuated pump. An operational schematic is shown in Figure 8. This device essentially draws the fluid mixture from the bottom of the pump. Additionally, the pump is marketed as functioning at 1 psi above the static head and capable of handling fluids with large particles (<1.59 mm diameter) (Geotech, 2003). *Artemia* at a known density were added to an injection vessel which had an effluent hose connected to the injection port.

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Figure 7. Surrogate injection: Auto-reclaimer pump (left-hand side) with adjacent surrogate vessel (right-hand side). Pump is placed inside the vessel containing surrogates where it pumps the surrogates up through the pump and out the hose at the top.

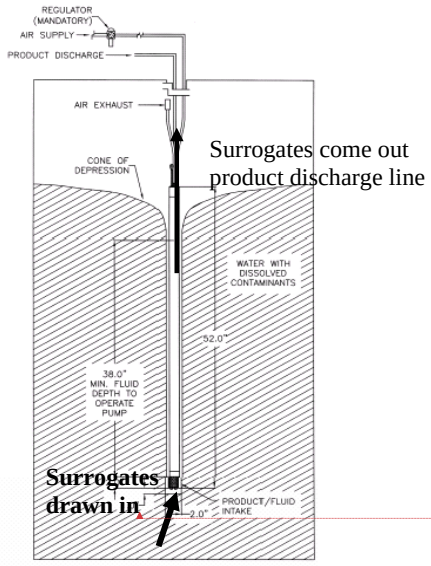

Figure 8. Surrogate Injection: Operational schematic of the bottom-fill auto-reclaimer pump.

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Pressure Vessel

A slightly more elegant, yet simplistic approach, was the pressure-vessel. These devices are used throughout industry for a variety of applications. For example, these units are used in industrial coating applications as a transfer and delivery system for the highly viscous components of coating systems. In the present work, a 3.0-gal pressure vessel was utilized. The pressure vessel used compressed air to physically displace the water, thereby causing it to move out of the vessel through the injection line and finally through the injection port (Figure 9).

Diaphragm Pump

Diaphragm pumps are designed for high-flow applications at high pressures. The direction of fluid flow is controlled by check valves. Diaphragm pumps are self-priming, submersible, and can be run dry without damage; further, they lack seals or packing, so there are fewer moving parts to wear out. Diaphragm pumps are often cited for use for pump sampling marine invertebrates (Harris, et al, 2000). A disadvantage of this type of system is that the supplied flow is generally intermittent in concert with compressions of the diaphragm. As a result, organisms are not evenly distributed in the discharge.

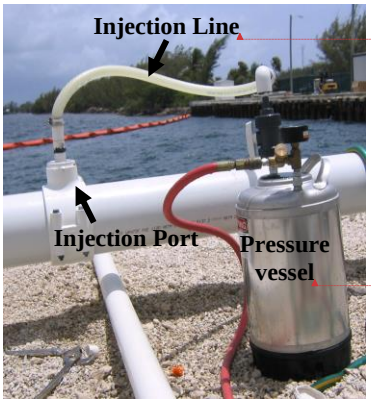

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Figure 9. Surrogate Injection: 3.0-gallon, Pressure Vessel attached to the injection port by the injection line.

2.3.4 Transfer and Discharge

In order to evaluate the Percent Viability and Percent Representativeness of organisms at injection, it was necessary to collect and enumerate them. Sampling configurations used to perform these tasks are described in section 2.3.3. Early runs of the control experiments revealed problems with the original design that resulted in either high mortality or a low recovery rate. A total of three experimental configurations were tested to evaluate fluid transfer and discharge effects on surrogate species viability. These included:

- Method 1: Discharge of water through an exposed and open-ended nominal 8-inch diameter Schedule 40 pipe into a 25-µm net, with a submerged cod-end, in a two-cubic meter collection tank as shown in Figure 10.
- Method 2: Discharge of water through exposed and open-ended nominal 8-inch diameter Schedule 40 pipe into a two-cubic-meter collection tank which was then gravity drained through a 25-µm mesh net as shown in Figure 10.
- Method 3: Discharge of water through nominal 8-inch diameter Schedule 40 pipe that extended down three-quarters of the way into the two-cubic-meter tank and was capped with a diffuser. All discharge water passed through a 25-µm net with a submerged cod-end (Figure 10).

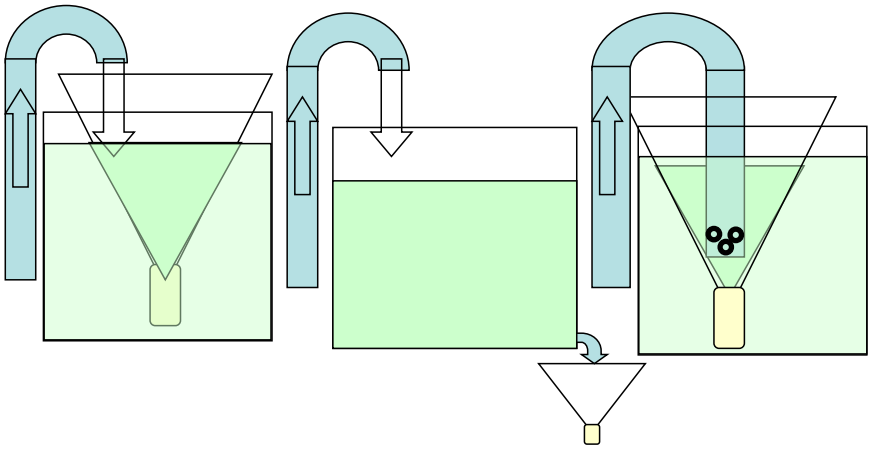

Figure 10. Surrogate Injection: Three Discharge Methods. From left to right: Method 1, Method 2 and Method 3.

To compare these three methods and their effects on surrogate viability, the first null and alternate hypotheses (H_o and H_a respectively) evaluated were:

- H_o: There is no significant difference in *Artemia* Percent Viability among the three discharge methods.
- H_a: There is a significant difference in *Artemia* Percent Viability among the three discharge methods.

To compare these three methods and their effects on surrogate recovery the second hypotheses evaluated were:

- H_o: There is no significant difference in *Artemia* Percent Representativeness among the three discharge methods.
- H_a: There is a significant difference in *Artemia* Percent Representativeness among the three discharge methods.

2.3.5 Surrogate Injection: Sampling and Analysis Methods

The control experiment and the experiments utilizing the three different injection system mechanical elements were conducted to determine the most effective injection technique for surrogate species into the pipeline flow stream. The effectiveness was judged on the basis of *Artemia* conservation of mass injection (Percent Representativeness) and post injection survival (Percent Viability). The effects on surrogate representativeness and viability were to be evaluated using an analysis of variance test (ANOVA).

The third pair of hypotheses evaluating surrogate presence were:

- H_o: There is no significant difference in *Artemia* Percent Representativeness among the four injection configurations.
- H_a: There is a significant difference in Artemia Percent Representativeness among the four injection configurations.

The fourth hypotheses evaluating surrogate viability were:

- H_o: There is no significant difference in *Artemia* Percent Viability among the four injection configurations.
- H_a: There is a significant difference in *Artemia* Percent Viability among the four injection configurations.

The following procedure was used throughout each of the experiments for the injection of organisms from the cultures through the collection of samples for analyses:

1. The discharge tank contained the 25-µm plankton net complete with 80-µm cod end that was secured around the discharge end of the main pipe and its corresponding diffuser head cap (Figure 5).
2. The discharge tank was filled with seawater to a depth that covered the entire diffuser head and the sample net's cod-end.
3. The tank depth was then measured in inches.
4. The injection vessel was filled with pumped sea water and *Artemia* (except for the control run in which *Artemia* were added directly to the main line by funnel)(Figure 6).
5. The hose of the injection vessel was then connected to the main pipe injection port. (Example in Figure 9).
6. Water was supplied to the system by turning on the 500-gpm pump. The recording of tank fill time (in minutes) was started when the seawater reached the discharge line.
7. The tank was filled until the water level was approximately 5 inches from the tank lip. At this time, main pipe flow was terminated and the total fill time was recorded.
8. The sample tank depth was measured (in inches) after the fill was complete. Once the tank depth measurements were recorded, the tank was drained to obtain better access to the sample net.
9. A seawater hose was used to spray the net sides and concentrate the *Artemia* into the cod-end. The cod-end was detached and taken into the lab (Figure 11).

Figure 11. Collection and concentration of injected surrogate (*Artemia*) in the detachable cod-end.

10. The organisms in the cod-end were rinsed into a 4000-mL graduated flask using artificial seawater and a funnel with tubing running down into the flask. Once all surrogates were rinsed into the flask, additional artificial seawater was added until the sample water reached the 4000-mL mark. The flask was labeled *Artemia*: LIVE/DEAD.
11. Each flask was sampled and evaluated for *Artemia* mortality and total number present.
    a. The 4000-mL flask was capped and gently homogenized by inverting and righting the flask 10 times.
    b. A graduated 10-mL pipette complete with automatic pipette helper was used to draw and dispense 1-mL replicates into counting wells. (The use of 12 multiwell plates over a Sedgwick-Rafter Counting Cell was found to decrease processing and analysis time while maintaining accuracy).
    c. Once each plate held ten 1-mL samples, they were analyzed using a stereo dissection microscope for mortality and total number.
12. Mortality was determined by counting the dead individuals, defined as those not showing any visible movement after ten seconds of passive observation. The total number of dead organisms was then recorded.
13. Two milliliters of carbonated water were subsequently added to each well to narcotize the organisms.
14. Once organisms had stopped moving, total count for each well was obtained and recorded as # individuals/mL. All ten replicates were then averaged for mean # individuals/mL. This mean was then multiplied by the original flask volume to estimate the total number of individuals injected.
15. The number of living Artemia, Number Alive, for each well was calculated by subtracting the number dead from the total number present (Live = Total – Dead).
16. The residual *Artemia* in the injection vessel were collected by flushing both the injection vessel and the injection line with seawater through an 80-micron mesh net and cod-end.

17. The post-injected residual cod-end sample was transferred into a flask from which a 1-liter sample was obtained and fixed with 4-percent Lugols solution.
18. All *Artemia* total counts were then added together to obtain conservation of mass (Percent Representativeness) (number of individuals injected = number of individuals recovered). The equation used was:

    # individuals (pre-injection) = # Live + # Dead + # residual (post-injection).

2.4 Pipeline Biological Sampling - Sample Wand Design

Four different sampling wand configurations were tested to determine an effective technique for biologically sampling a pipe flow stream of greater than 1000 gpm. The system devised for organism injection in Section 2.3 was utilized to provide a consistent and known concentration to the flow stream. Triplicate 1-m^3 samples were analyzed to determine Percent Representativeness and Percent Viability of acquired samples, and these data were compared to the known concentrations and viability of input organisms.

2.4.1 Pipeline Biological Sampling Setup

For these experiments, a more rigorous and industrial version of the pressure vessel system was constructed, as shown in Figure 12, to deliver the surrogate organisms at a rate of 0.5 gpm to the mainline seawater flowing at approximately 1100 gpm.

Figure 12. Thirty-gallon pressurized injection vessels for surrogates.

Each experimental sampling wand was fitted into an 8-inch PVC saddle which was then fastened to the 8-inch pipe, an example of which is shown in Figure 13. Externally, each wand was fabricated to accept a removable 2-inch PVC pipe that extended well out from the sampling port saddle as shown in Figure 14. The external pipe cap contained three identical holes arranged linearly. Each was fitted with nominal 0.75-in vinyl tubing extending into one of three discharge tanks that had been utilized for the injection experiments previously described. All sampling efforts used Method 3 for sample collection and filtering as described in Section 2.3.4. A 25-, 50- or 80- micron mesh conical net was secured within each of the three tanks so that the

sampling line extended down into it as shown in Figure 15. Each net was equipped with a detachable cod-end with the same micron mesh size as the net. This compared the effects of different mesh sizes among pseudoreplicates on Percent Representativeness and Percent Viability. The nets and cod-ends screened the water being sampled and collected the *Artemia* for analysis. A 40-gallon pressure vessel was used as the injection vessel with a tank supplying ultra pure grade air for an air source. The same tanks, conical nets, and pressure vessel configurations were used for all test runs. When tested, all four sampling wand openings were positioned facing into the flow stream to collect the challenge water from the center of the 8-inch main pipe.

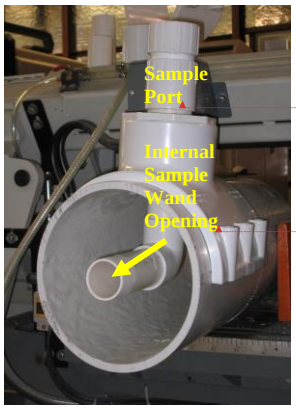

Figure 13. Cross-section of 8-inch PVC pipeline with sampling wand and external sample port.

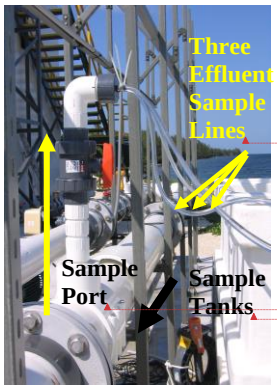

Figure 14. Side view of sample port with capped line and three effluent sample lines. Large black arrow indicates direction of flow. Sample wand opening inside the pipe faces into the flow stream. Long yellow arrow indicates flow direction of sample collected into sample tanks.

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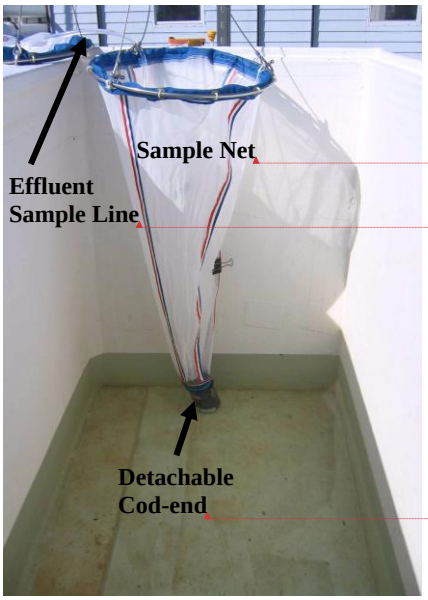

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Figure 15. View inside one of the three sampling tanks complete with corresponding net for collecting and screening challenge water.

2.4.2 Control & Wand Design Experiments

The four designs of wand openings were as follows: a dead end (control), a 90-degree elbow with an 8-inch extension, a straight wand with a 60-degree angle cut, and a 90-degree elbow with a 4-inch extension. The diagram in Figure 16 displays the four experimental wand designs corresponding with the order of text descriptions below.

Dead-end (Control)

The purpose of this run was to determine what the effect of having no sample wand extending into the flow stream would be. This experimental design was essentially a tee-fitting on the main pipe with a Nominal 2-inch Schedule 40-PVC fitting as shown in Figure 16 (# 1).

90-Degree Elbow 8-inch Length

This experimental design, shown in Figure 16 (# 2), was based on an L design intended to provide a nominal 2-inch Schedule 40-PVC pipe opening to the center line of the main pipe, eight inches upstream from the sample head. This would reduce any hydrodynamic forces on the sample.

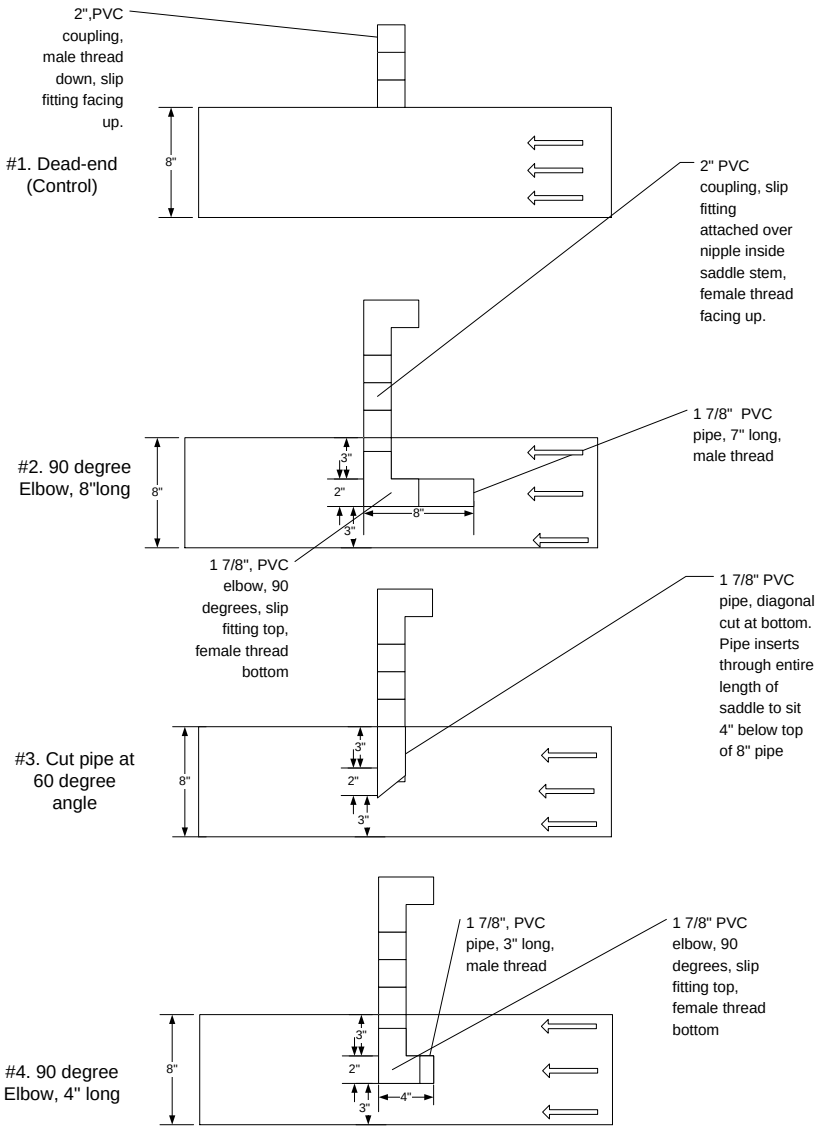

Figure 16. Surrogate Sampling: Experimental wand configurations. Arrows indicate flow direction.

60-Degree Angle

This experimental design (#3 in Figure 16) resembled a hypodermic needle in that a nominal 2-inch Schedule 40-PVC pipe was extended from the saddle opening into the center of the main pipe. A sixty-degree angle cut at the exposed base of the pipe created an exposed opening to the flow. Cuts at other angles were not tested.

90-Degree Elbow 4-inch Length

This experimental design, shown in Figure 16 (# 4), was essentially the same as the previous L design except that the upstream pipe extension was only 4-inches long. Like the 8-inch version, this wand was intended to evaluate the effects of upstream distance on sample wand performance.

2.4.3 Pipe-Line Biological Sampling: Sampling and Analysis Methods

To compare the different sampling wands in terms of surrogate Percent Representativeness and Percent Viability, the fifth hypotheses evaluated were:

- H_o: There is no significant difference in *Artemia* Percent Representativeness among the four sampling wand configurations.
- H_a: There is a significant difference in *Artemia* Percent Representativeness among the four sampling wand configurations.

The sixth hypotheses evaluated were:

- H_o: There is no significant difference in *Artemia* Percent Viability among the four sampling wand configurations.
- H_a: There is a significant difference in *Artemia* Percent Viability among the four sampling wand configurations.

The experimental procedures below were followed for all experiments.

1. Pre-run setup:
    a. All three sample tanks were positioned equidistant from the sample wand head.
    b. A net complete with cod-end was secured in each sample tank. Each sample line from the sample wand head was secured into the net such that the discharge of the sample line delivered all effluent into the net without restricting flow.
    c. Each sample tank was filled with enough seawater to cover each cod-end.
    d. The pre-run water level in each sample tank was recorded in inches.
    e. Surrogate injection vessel line was secured to the injection port. If pressurized, desired pressure was obtained before injection.
2. The full-scale system flow was started through the 8-inch main line.
3. Once the system reached a flow of 1100 gpm, the lines for the surrogate injection vessels and downstream sampling wand were opened simultaneously which represented the beginning of the one hour injection time. Water not captured by the sampling wand flowed into the ballast (storage) tank.

4. The flow rate of the injection vessel was recorded using a flow meter capable of recording total volume injected in both gph and gpm. Time was measured in minutes using a digital stopwatch.
5. At the conclusion of one hour, both the surrogate injection vessel line and sample wand line were closed simultaneously. End time was recorded in minutes.
6. The post-run, challenge-water depth in each sample tank was measured in inches to calculate the total volume sampled from the main water stream.
7. Each net sample was concentrated into the corresponding cod-end by rinsing down the sides of the net with seawater. The cod-end was then detached and taken into the lab (Figure 11).
8. Organisms were rinsed into 4000-mL flasks using artificial seawater and a funnel with tubing running down into the flask. Once all the *Artemia* were rinsed into the flask, sample volume was equalized by filling the flask to the 4000-mL mark with artificial seawater.
    d. A graduated 10-mL pipette complete with automatic pipette helper was used to draw a 5-mL subsample and then dispense it in 1 mL aliquots into counting wells. Three replicate subsamples (15 mL total) per sample tank were dispensed. The use of 12 multi-well plates over Sedgwick-Rafter Counting Cell was found to cut down on processing and analysis time while maintaining accuracy.
    e. Once 15 mLs of sample was dispensed into wells, they were analyzed using a stereo dissection microscope for mortality and total number.
9. Mortality count was performed by counting the dead individuals, as previously defined. The total number dead was then recorded.
10. Two milliliters of carbonated water were added to each well to narcotize the organisms.
11. Once organisms had stopped moving, total count for each well was obtained and recorded as number of individuals/mL. The 15 mLs were then averaged for mean number of individuals/mL.
    a. These procedures were performed for each sample tank resulting in three means for the number of individuals per mL. Each mean was then multiplied by the corresponding 4000 mL flask volume to estimate the total number of individuals collected in each sample tank.
    b. The total number for each sample tank was then divided by the sample tank seawater volume to obtain the number of individuals per liter.
12. The number of live *Artemia* for each tank was calculated by subtracting the number dead from the total count (Total # – # Dead = # Live (Viable)). Percent Viability was calculated by dividing the number of Live by the Total number counted .
13. For each sample tank, a 1-Liter sample of the 4000-mL flask volume was archived by being placed in an amber Nalgene bottle and fixed with 4-percent Lugols.
14. The injection vessel *Artemia* residual was collected by flushing both the injection vessel and line with seawater through an 80-micron mesh net and cod-end.

15. The post-injected residual cod-end sample was rinsed with seawater into a flask from which a 1-Liter sample was obtained and fixed with 4-percent Lugols.
16. Total counts (# individuals/mLs) were conducted on 15 mLs (three 5-mL replicates) of the injection vessel residual. The total count was then multiplied by the residual volume to obtain total number left in pressure vessel (i.e., organisms not injected).
17. The population not injected was subtracted from the initial population to calculate the injected population.
18. The total injected population was divided by the volume in the ballast tank to obtain an estimate of the number of individuals/L in the ballast tank. This represented the overall concentration of organisms injected. Percent Representativeness was calculated by dividing the sample tank density from step 11b by the tank density of the ballast tank. This was done for the three sample tanks.

3 Results and Discussion

3.1 Ambient Water Characterization

The water quality parameters recorded by the YSI 6600 Sonde over the course of the experiments and evaluation period are shown in Figure 17. Metorological parameters recorded by the NRLKW weather station over the course of the experiments and evaluation period are shown in Figure 18 and Figure 19. Briefly, the sharp decrease in salinity and water temperature, as shown in Figure 17, and air temperature, as shown in Figure 18 around Julian day (JD) 106 was due to the large rain event and frontal passage on JD 104 where winds shifted from south to the north, northeast as shown in Figure 19. The incident solar radiation was strong and rainfall was scarce for the entire sampling period as can be seen in Figure 18. The sharp increase in turbidity from JD 120 to 125, as shown in Figure 17, corresponded to a strong South-East (SE) wind event, as shown in Figure 19. Continued elevated turbidity levels for the remainder of the sampling period corresponded with winds out of the East (E)which would tend to "trap" particulates against the eastern facing shoreline at NRLKW. Further, this time period corresponded with an increase in Chlorophyll_a, which is to be expected because organisms that contain chlorophyll will appear as "turbidity" to the turbidity sensor. It appears that wind stress from the east, with a corresponding lack of wind stress from the north as shown in Figure 19, can be correlated with an increase in turbidity and Chlorophyll_a (Figure 17). It is important to be cautious with regard to the interpretation of the increase in turbidity during this period because the overall trend could also be due to biofouling. However, the return of the chlorophyll sensor to levels that were found at the beginning of the deployment argues against bio-fouling for the optics associated with the turbidity and chlorophyll sensors. In general, although the data sonde was equipped with an automated anti-fouling system that consists of a physical cleaning of the sensors before each sampling event, care needs to be taken during sampling so that the sensors do not foul. Likewise, post-sampling care must be taken to remove trends due to bio-fouling. This is especially apparent with the dissolved oxygen sensor; the trend must be removed before analysis.

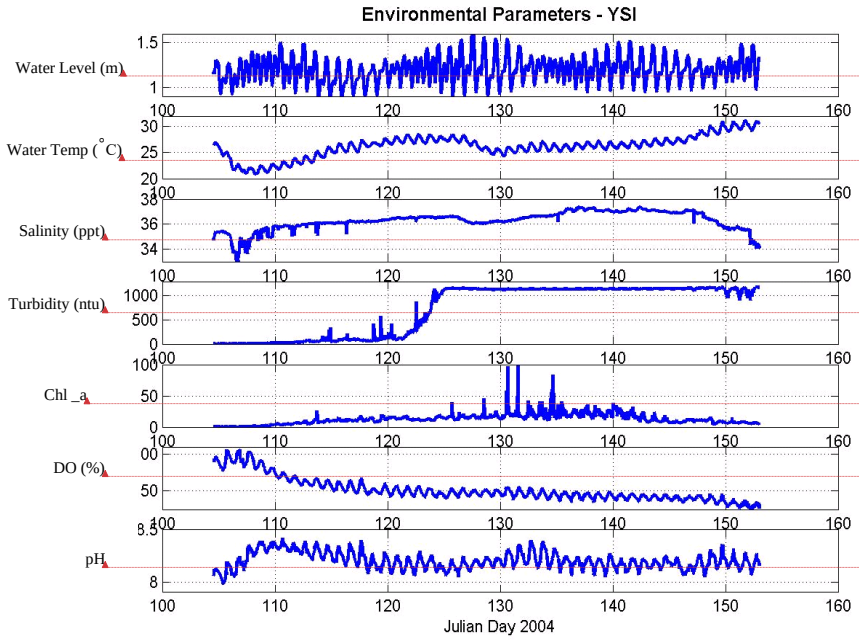

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Figure 17. NRLKW Pier Environmental Water Quality Parameters over time (Julian Day, JD) taken by *in situ* data Sonde YSI 6600. Sampling for NRLKW laboratory water chemistry and organism enumeration occurred on Julian days 104, 110, 118, 124, and 131. Measured parameters from top to bottom; Water Level (meters), Water Temp (^{o}C), Salinity (ppt), Turbidity (ntu), Chl_a, DO (%), pH.

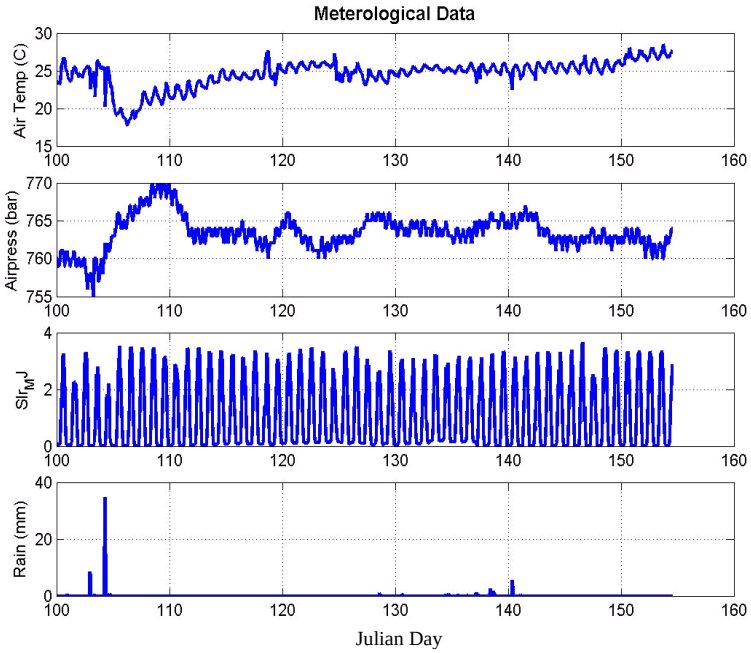

Figure 18. Meteorological data from NRLKW *in situ* Weather Station. Parameters from Top to bottom; Air temp (^{o}C), Air pressure (bar), Solar light radiation (Slr) in mega joules (M^{J}), Rain fall (mm). Sampling for NRLKW laboratory water chemistry and organism enumeration occurred on Julian days (JD) 104, 110, 118, 124, and 131.

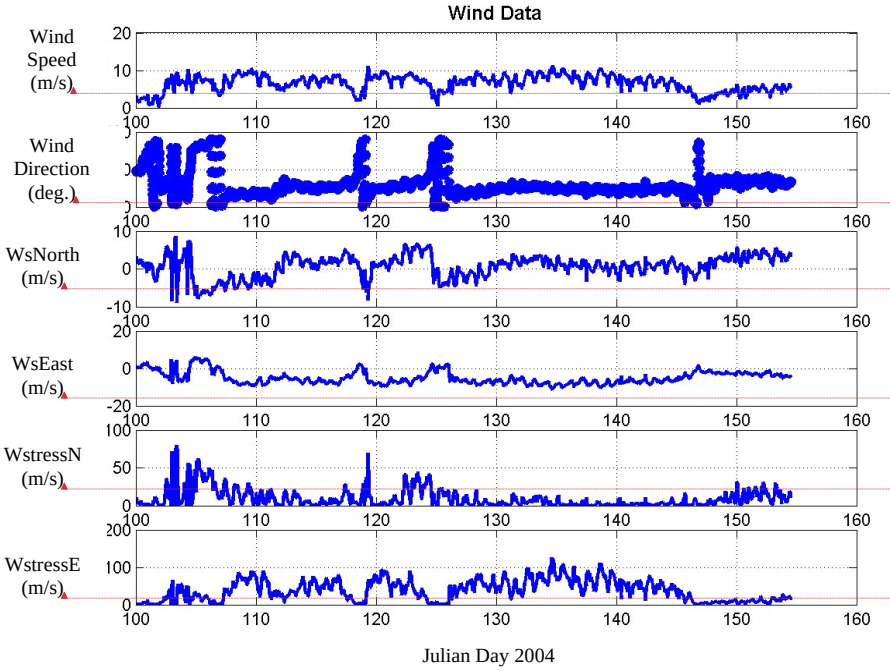

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Figure 19. Measured parameters as recorded by the NRLKW *in situ* Weather Station. From top to bottom; Wind Speed (m/s), Wind Direction (deg.), and Wind Speed-North (WsNorth) in meters per second (m/s), Wind Speed-East (WsEast) (m/s), Wind Stress-North (Wstress N) (m/s), and Wind Stress-East (Wstress E) (m/s). Sampling for NRLKW laboratory water chemistry and organism enumeration occurred on Julian days 104, 110, 118, 124, and 131.

Seasonal measurements taken at Garrison Bight (24 34.500N, -81 47.300E) by Florida International University (FIU) are presented for TOC and Chl_a in Figure 20 and Figure 21 to provide *in situ* comparisons to the NRLKW Pier data. The FIU seasonal TOC measurements show that TOC is approximately 250 micro-molar, or 3 ppm (Figure 20). The NRLKW Pier TOC levels measured in the laboratory, as shown in Table 1 and Figure 22, varied between 2.1 and 2.9 ppm and exhibited biweekly oscillations which are consistent with FIU measurements. The ANOVA that tested the NRLKW feedwater TOC levels across the five sampling days is outlined in Table 2 and shows a significant difference (95% significance) in TOC levels ($\alpha = 0.05$, $p = 0.0001$). This may be due to the fortnightly oscillations of the spring and neap tide. Regardless of this difference, the levels consistently held at around 2-to-3 ppm and are approximately 4 to 6 times below the 16 to 24 ppm TOC levels specified for the challenge water in the ETV draft protocol for Ballast Water Treatment tests.

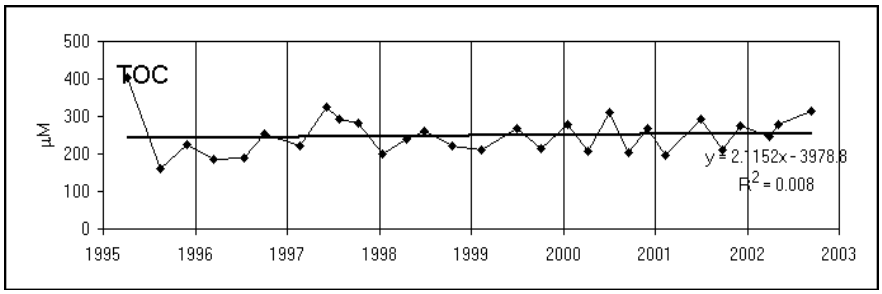

Figure 20. Seasonal TOC measurements conducted by the Florida International University (1995-2003).

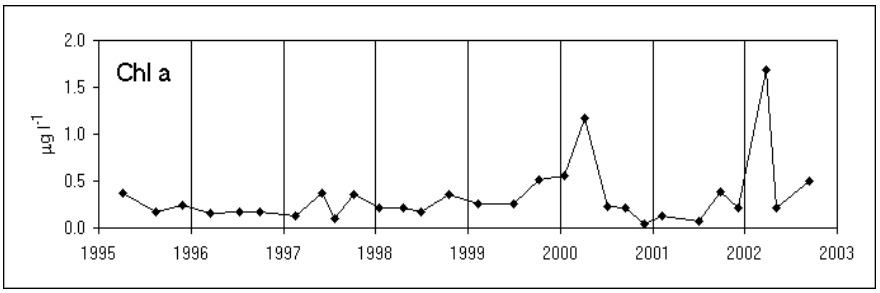

Figure 21. Seasonal Chl_*a* measurements conducted by the Florida International University (1995-2003).

Table 1. Water Chemistry: Total Organic Carbon - surface saltwater (ssw) samples 1-3 taken off the NRLKW Pier. Mean +/- standard deviations for each sample day are displayed in far right columns.

DATE	Sample ID	TOC *mg/L*			*mg/L*
13-Apr	ssw1	2.68		**mean**	**2.66**
	ssw2	2.61		***std***	***0.04***
	ssw3	2.70			
19-Apr	ssw1	2.53		**mean**	**2.52**
	ssw2	2.53		***std***	***0.02***
	ssw3	2.50			
27-Apr	ssw1	2.87		**mean**	**2.84**
	ssw2	2.87		***std***	***0.06***
	ssw3	2.77			
3-May	ssw1	2.26		**mean**	**2.17**
	ssw2	2.13		***std***	***0.08***
	ssw3	2.11			
10-May	ssw1	2.33		**mean**	**2.33**
	ssw2	2.32		***std***	***0.01***
	ssw3	2.32			

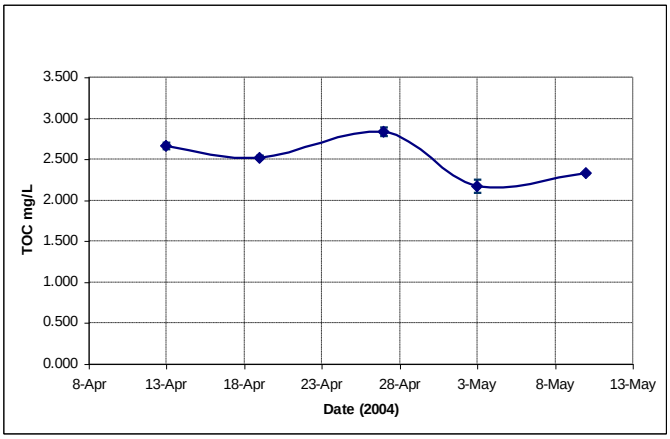

Figure 22. Water Chemistry: Total Organic Carbon- NRLKW samples displayed as mean values +/- standard deviations in milligrams per liter (mg/L) for measured Total Organic Carbon for sampling periods of April and May, 2004.

Table 2. Water Chemistry: Total Organic Carbon - Analysis of variation about the mean at 95% significance (ANOVA) TOC levels from water sampled off the NRLKW Pier.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
4/13/2004	3	7.988	2.667	0.001982
4/19/2004	3	7.557	2.519	0.000399
4/27/2004	3	8.514	2.838	0.003097
5/3/2004	3	6.501	2.167	0.006799
5/10/2004	3	6.975	2.325	3.7E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.847657	4	0.211914	86.0437	1.05E-07	3.47805
Within Groups	0.024629	10	0.002463			
Total	0.872285	14				

The measured Chlorophyll_a, from the NRLKW Pier varied between 0.7 μg/L and 4.8 μg/L for the time period measured (Figure 21, Table 3). Although these measurements are higher than the seasonal chlorophyll measurements made by FLU (Figure 19), it is important to note that studies conducted in the UK found that biweekly and diurnal variability in chlorophyll were on the same order of magnitude as the seasonal variation (Hartman, 2003). The ANOVA that tested the Chl_a levels in the NRLKW feedwater across the five sampling days (Table 4) shows there was no significant difference in Chl_a levels ($\alpha = 0.05$, $p = 0.173$). Overall, it appears that Chl_a for ambient NRLKW feedwater is typical for oligotrophic tropical regions and is consistent with long-term measurements conducted in the region.

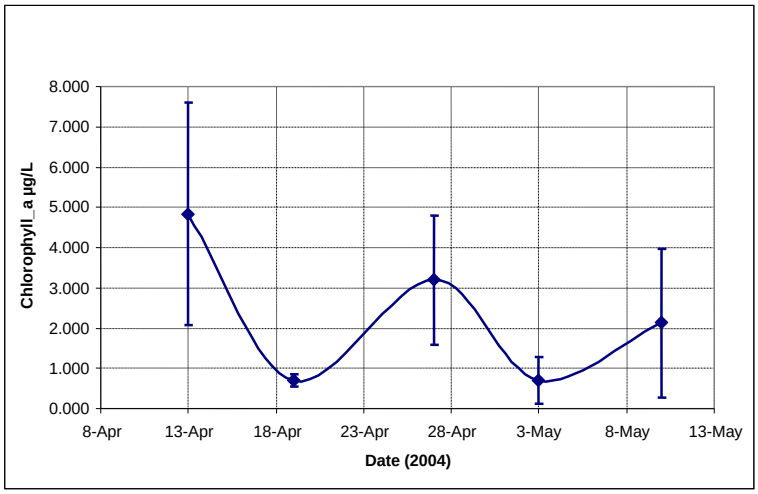

Figure 23. Water Chemistry: NRLKW samples of measured Chlorophyll_a levels for sampling periods during April and May, 2004 displayed as means +/- standard deviations.

Table 3. Water Chemistry: Chlorophyll_a – NRLKW Pier surface seawater (ssw) samples. Mean +/- standard deviations for each sample day are displayed in far right columns. (NA = Not Available, std = +/- standard deviation)

DATE	Sample ID	Chl_a µg/L			µg/L
13-Apr	ssw1	6.78		**mean**	**4.83**
	ssw2	NA		***std***	***2.76***
	ssw3	2.87			
19-Apr	ssw1	0.59		**mean**	**0.70**
	ssw2	0.81		***std***	***0.16***
	ssw3	NA			
27-Apr	ssw1	4.02		**mean**	**3.19**
	ssw2	1.36		***std***	***1.59***
	ssw3	4.20			
3-May	ssw1	0.30		**mean**	**0.70**
	ssw2	NA		***std***	***0.57***
	ssw3	1.11			
10-May	ssw1	NA		**mean**	**2.13**
	ssw2	3.44		***std***	***1.85***
	ssw3	0.82			

Table 4. Water Chemistry: Chlorophyll_a. The Analysis of variation about the mean (ANOVA) of Chl_a levels from water sampled off the NRLKW Pier.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
4/13/2004	2	9.65	4.825	7.636
4/19/2004	2	1.400	0.700	0.025
4/27/2004	3	9.574	3.191	2.531
5/3/2004	2	1.408	0.704	0.323
5/10/2004	2	4.253	2.126	3.433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	25.31758	4	6.329395	2.304288	0.172687	4.533677
Within Groups	16.48074	6	2.74679			
Total	41.79832	10				

The Naval Research Laboratory measurements of total suspended solids (TSS) were on the order of 1 mg/L as shown in Table 5. The ANOVA that tested the analysis of variation about the means of TSS levels in the NRLKW feedwater across the four sampling days (Table 6, and Figure 22) shows a significant difference in TSS levels ($\alpha = 0.05$, $p = 0.002$). This may be due to the fortnightly oscillations of the spring and neap tide. Regardless of this difference, the levels were consistently around 1 to 2 ppm and are approximately an order of magnitude lower than the 24 to 34 ppm TSS levels specified for the challenge water in the ETV test protocol for ballast water treatment tests.

Similarly, DO measurements from the YSI Sonde were clearly affected by biofouling, and as with the turbidity sensor, frequent calibration of the DO sensor (weekly) will be necessary to obtain high-quality results. Measurements of TOC were relatively constant between 2 and 3 mg/L for the entire sampling duration (Table 1 & Table 2), while TSS varied significantly. This suggests that TOC is relatively insensitive to TSS, which would in turn indicate that most of the suspended solids are, in fact, not organic substances that would contribute to the total mg/L of TOC.

Table 5. Total Suspended Solids: Surface seawater (ssw) samples, 1-3 taken at NRLKW Pier. Mean +/- standard deviations for each sample day are displayed in far right columns.

DATE	Sample ID	TSS *mg/L*			*mg/L*
13-Apr	ssw1	1.50		**mean**	**1.42**
	ssw2	1.45		***std***	***0.10***
	ssw3	1.30			
19-Apr	ssw1	1.05		**mean**	**0.83**
	ssw2	0.60		***std***	***0.32***
	ssw3	NA			
27-Apr	ssw1	1.67		**mean**	**1.73**
	ssw2	1.23		***std***	***0.53***
	ssw3	2.28			
3-May	ssw1	0.13		**mean**	**0.12**
	ssw2	0.17		***std***	***0.06***
	ssw3	0.05			
10-May	ssw1	0.94		**mean**	**1.45**
	ssw2	1.82		***std***	***0.46***
	ssw3	1.60			

Table 6. Total Suspended Solids: The Analysis of variation about the mean (ANOVA) of TSS levels from seawater sampled off the NRLKW Pier.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
4/13/2004	3	4.25	1.4166	0.01083
4/27/2004	3	5.175	1.725	0.2775
5/3/2004	3	0.35	0.11666	0.00395
5/10/2004	3	4.36	1.45333	0.20973

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.675	3	1.558	12.416	0.0022	4.06
Within Groups	1.004	8	0.1255			
Total	5.6792	11				

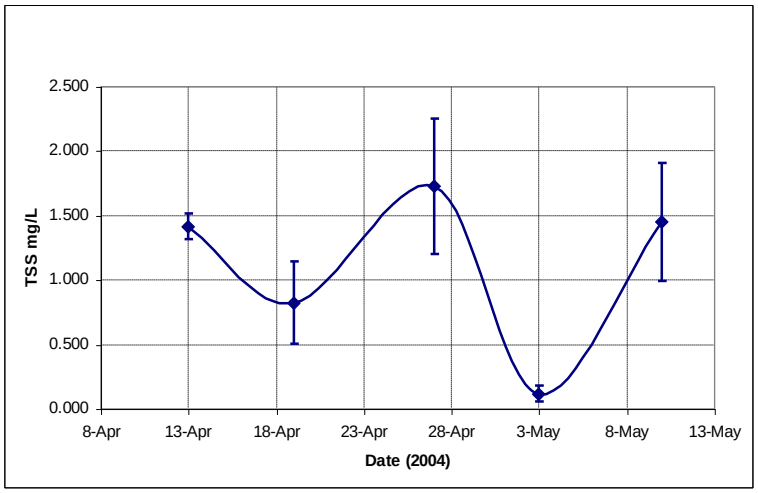

Figure 24. Mean TSS values (mg/L) from NRLKW samples for sampling periods in April and May 2004. Bars indicate standard deviations at 95% significance.

Biological Parameters:

Total counts of bacteria measured using epi-fluorescent microscopy on NRLKW Pier samples showed that bacteria were at densities of 10^6/mL, which is typical for coastal ocean waters (Straube – 1/28/03, Rogerson – 5/12/04; personal communication). Bacterial counts resulting from the serial dilutions were on the order of 10^2 CFU per mL (Table 7). It should be noted that the difference between the direct counts and the plating counts is consistent with the low number of culturable bacteria in the marine environment (Straube – 1/28/03, Rogerson – 5/12/04; personal communication).

The FlowCam® phytoplankton counts estimated 30 individuals per mL and a total density of 10^4 individuals per liter. Figure 25 displays the seven most abundant organism shapes obtained from the digital image output. Because specific taxonomic distinctions were not made, each organism ID potentially represented several genera. Although this technique indicates that pennate diatoms were the dominant organism, equaling 1375 of 1501 individuals counted (92 %), the densities are indicative of organisms size-selected by body dimensions greater than 120, 80, 50 and 25 microns. This means that organisms less than 25 microns in size were not accounted for. The size selective bias created by screening samples is a significant factor in characterizing population dynamics. It is recommended that whole water samples be analyzed to preserve somatic distinctions when determining ambient phytoplankton densities. However, if sampling logistics call for screening phytoplankton samples, it is recommended that the smallest micron mesh that will sustain adequate sample flow be used.

Table 7. Bacterial Enumeration - NRLKW Surface seawater (ssw) samples (1-3). Mean +/- standard deviations (# individuals/mL) for each sample day are displayed in far right columns.

DATE	Sample ID	Bacterial Count *indiv/-mL*		*indiv/L*
13-Apr	ssw1	200	**mean**	**173.3**
	ssw2	200	***std***	***46.2***
	ssw3	120		
19-Apr	ssw1	NA	**mean**	**NA**
	ssw2	NA	***std***	***NA***
	ssw3	NA		
27-Apr	ssw1	110	**mean**	**300.0**
	ssw2	600	***std***	***262.9***
	ssw3	190		
3-May	ssw1	110	**mean**	**100.0**
	ssw2	100	***std***	***10.0***
	ssw3	90		
10-May	ssw1	20	**mean**	**76.7**
	ssw2	120	***std***	***51.3***
	ssw3	90		

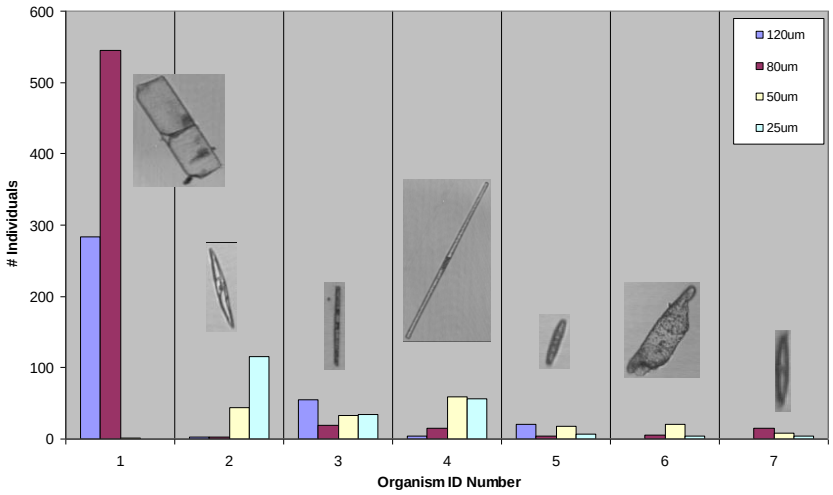

Figure 25. FlowCam® imaging output displaying (120-, 80-, 50- and 25-micron) size class. distribution of phytoplankton in NRLKW feedwater. Counts and images obtained from the analysis of a 50-mL sample. Images are not to scale.

3.2 Surrogate Injection

3.2.1 Transfer and Discharge

3.2.1.1 Organism Percent Viability

A total of nine injection experiments were conducted between June 28th and July 21st, 2004, during daylight hours. Three discharge configuration experiments were performed first. Methods 1, 2, and 3 were tested as described previously. The Percent Viability and Percent Representativeness data and ANOVA statistics are given in Table 8. These results indicate that both Method 1 and 2 resulted in a substantial decrease in viability relative to Method 3 which had a Percent Viability of 96.3 percent. The relatively high mortality in Method 1 suggests that discharging zooplankton surrogates through an open pipe and down into a net housed in an empty tank results in substantial organism death. Note that this method also resulted in substantial loss in organism representativeness. Additionally with Method 2, when the surrogate was discharged out an open pipe and down into an empty tank and later screened through a collection net, approximately 36.2 % mortality occurred. The addition of pipe extensions along with a diffuser cap reduced mortality by at least an order of magnitude, to 3.7 % with Method 3. While the specific mechanism of mortality involved in Method 1 and 2 are not clear, these are likely to be 1) interaction with air entrainment and the associated pressure differentials, 2) the impact of the water and organisms on the water/air interface or 3) the effective force on the organism due to water velocity on the net. Since immersing the discharge head and providing for a larger surface area (diffuse pipe head) resulted in relatively no mortality, it is likely that this approach remedies all three degradation mechanisms. It is recommended that for future sampling efforts of relatively large flow rates (> 200 gpm) that samples be acquired such that the sample discharge point is both immersed and as diffuse as design permits.

The ANOVA test of the between-subjects factor Percent Viability indicated that there were significant differences in the survival of surrogates between the three methods ($\alpha = 0.05$, $p = 0.0001$). Results are shown in Table 9. The Tukey post hoc test for multiple comparisons indicated specific significant differences between Method 3 and both Method 1 ($\alpha = 0.05$, $p = 0.0001$) and Method 2 ($\alpha = 0.05$, $p = 0.0001$) (shown in Table 10, bottom panel).

Table 8. Surrogate Injection: Descriptive statistics on the dependent variables "Viability" (PERVIAB) and "Representativeness" (PERRECOV) as measured per sample methods (Methods 1, 2 and 3) of the three injection runs testing the three experimental discharge methods. Displayed as mean +/- standard deviation. Generated by SPSS statistical software package.

Descriptive Statistics

	METHOD	Mean	Std. Deviation	Number
PERVIAB	method1	46.73	6.54	3
	method2	63.87	4.87	3
	method3	96.27	0.97	3
	Total	68.96	22.17	9
PERRECOV	method1	53.37	8.57	3
	method2	110.27	22.07	3
	method3	101.27	5.40	3
	Total	88.30	29.14	9

Table 9. Surrogate Injection: Results from ANOVA univariate test performed on the dependent variables "Percent Viability" (PERVIAB) and "Percent Representativeness" (PERRECOV). Three methods (Methods 1, 2 and 3) were tested, each with a different discharge configuration on the effluent end of the main discharge line. Data output generated by SPSS statistical software package.

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Multiple Comparisons

Tukey HSD

Dependent Variable	(I) METHOD	(J) METHOD	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PERVIAB	method1	method2	-17.1333*	3.8715	.011	-29.0124	-5.2543
		method3	-49.5333*	3.8715	.000	-61.4124	-37.6543
	method2	method1	17.1333*	3.8715	.011	5.2543	29.0124
		method3	-32.4000*	3.8715	.000	-44.2790	-20.5210
	method3	method1	49.5333*	3.8715	.000	37.6543	61.4124
		method2	32.4000*	3.8715	.000	20.5210	44.2790
PERRECOV	method1	method2	-56.9000*	11.4472	.006	-92.0232	-21.7768
		method3	-47.9000*	11.4472	.014	-83.0232	-12.7768
	method2	method1	56.9000*	11.4472	.006	21.7768	92.0232
		method3	9.0000	11.4472	.724	-26.1232	44.1232
	method3	method1	47.9000*	11.4472	.014	12.7768	83.0232
		method2	-9.0000	11.4472	.724	-44.1232	26.1232

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 10. Tukey Post Hoc test of multiple comparisons from the Multivariate ANOVA performed on the three discharge configurations (Method 1, Method 2 and Method 3).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	PERVIAB	3796.862[a]	2	1898.431	84.437	.000
	PERRECOV	5613.020[b]	2	2806.510	14.278	.005
Intercept	PERVIAB	42793.818	1	42793.818	1903.357	.000
	PERRECOV	70172.010	1	70172.010	357.007	.000
METHOD	PERVIAB	3796.862	2	1898.431	84.437	.000
	PERRECOV	5613.020	2	2806.510	14.278	.005
Error	PERVIAB	134.900	6	22.483		
	PERRECOV	1179.340	6	196.557		
Total	PERVIAB	46725.580	9			
	PERRECOV	76964.370	9			
Corrected Total	PERVIAB	3931.762	8			
	PERRECOV	6792.360	8			

a. R Squared = .966 (Adjusted R Squared = .954)

b. R Squared = .826 (Adjusted R Squared = .768)

3.2.1.2 Organism Percent Representativeness

As reported in Table 8, the mean Percent Representativeness of the surrogate for Methods 1, 2 and 3 were respectively, 53.4 %, 110.3 % and 101.3 %. This indicated that discharging zooplankton surrogates through an open pipe and down into a net housed in an empty tank will result in a 50-percent loss of surrogate individuals. Meanwhile, both Method 2 and Method 3

had 100 Percent Representativeness of the individuals initially injected (Figure 10). As discussed earlier, it is evident that the mechanism effecting the viability and representativeness of the organisms in Method 1 is the physical degradation of organisms such that they passed through the net or simply disintegrated. This could be caused by air entrainment or by forces created as moving water forced organisms against and through the plankton net.

The ANOVA test of the between-subjects factor Percent Representativeness indicated that there were significant differences in the recovery of the surrogate between the three methods ($\alpha = 0.05$, $p = 0.005$) (Table 9, first row, second variable). The Tukey post hoc test for multiple comparisons indicated that Method 1 was significantly different from Method 3 ($\alpha = 0.05$, $p = 0.014$) and from Method 2 ($\alpha = 0.05$, $p = 0.006$) (Table 10, bottom panel). There was no significant difference between Method 2 and 3 ($\alpha = 0.05$, $p = 0.724$).

3.2.1.3 Evaluation Criterion for Surrogate Injection/Sampling

Though the data showed that Method 3 was clearly the most effective in viability and representativeness, discerning why this method was the most successful raises the importance of considering both Percent Viability and Percent Representativeness together. If Percent Viability were the only variable analyzed for each method, it could be inferred that there was a 17 percent difference between Method 1 and 2. However, when recovery is factored in as well, the results show that Method 1 lost 50 percent of its individuals. This significant loss is not apparent when calculating Percent Viability and therefore yields an inaccurate calculation of Percent Viability given that the organisms need to be recovered in order to be labeled alive or dead. Taking Percent Representativeness into account shows that Method 2 is significantly better at recovering organisms than is Method 1. Given these considerations, it could be concluded that the water discharged from the open-ended pipe exerted large pressure differentials and bombardment by air entrapment upon the surrogate. Additionally the presence of the net below the open-ended pipe allowed the surrogates to be smashed against the netting resulting in a large portion of the bodies being damaged beyond recognition. The testing scenarios just discussed indicate that the relationship between Percent Mortality and Percent Representativeness will remain an important consideration when testing the effects of different technologies on surrogates.

When evaluating the results of the discharge Method 3 configuration, given the 400-micron average body size of the *Artemia* tested, it is rational to conclude that smaller organisms such as phytoplankton and bacteria will be able to withstand similar hydraulic forces using the pipe extension and diffuser cap method. Therefore, the Method 3 discharge configuration was put into place for the remaining six injection runs.

3.2.2 Surrogate Injection Mechanical Systems

3.2.2.1 Organism Representativeness

The Percent Representativeness means and standard deviations for the four injection configurations are shown in Table 11 and compared in Figure 26. The pressure vessel had the highest mean representativeness (116.5 % +/- 13.0%) followed by the auto-reclaimer pump (103.4% +/- 38.1%), Control run (101.3% +/- 5.4%) and the diaphragm pump (100.1% +/- 9.4%). Despite this, the overlap of standard deviations would indicate that any difference between the injection techniques is minimal. The ANOVA test output in Table 12 supports the

findings that no significant differences in Percent Representativeness occurred in regards to the four injection configurations ($\alpha = 0.05$, $p = 0.519$). Since 100.1% was the lowest mean representativeness rate, it is apparent that the three mechanical injection vessels did not physically mutilate the surrogates thereby validating the recovered population as representative of the population initially injected. Given this finding, the corresponding estimates of Percent Viability should be representative as well.

Table 11. Surrogate injection: Comparison of Percent Representativeness (% Representativeness) and Percent Viability (% Live) between the four injection configurations. Gallons per minute = gpm and meters per second = m/s.

	Control		Auto-reclaimer		Pressure vessel		Diaphragm	
	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
# individuals in incubator	322,200	61,024	273,933	25,445	302,355	28,602	228,275	13,470
Flow Rate (gpm)	505		601		604		601	
Velocity in Pipe (m/s)	1.75		2.08		2.09		2.08	
# individuals recovered in Live/Dead flask	326,267	17,386	64,200	2,163	252,978	14,607	173,410	115,244
% LIVE	96.3	0.97	77.7	7	81.6	6.2	86.1	3.8
% DEAD	3.7	0.97	22.3	7	18.4	6.2	13.9	3.8
# individuals in Residual	0	0	204,622	29,445	79,972	37,377	64,556	56,317
Total # Injected	326,267	17,386	69,311	29,445	252,978	14,607	173,410	115,244
% Representativeness	101.3	5.4	92.6	38.1	114	13.0	106	9.4

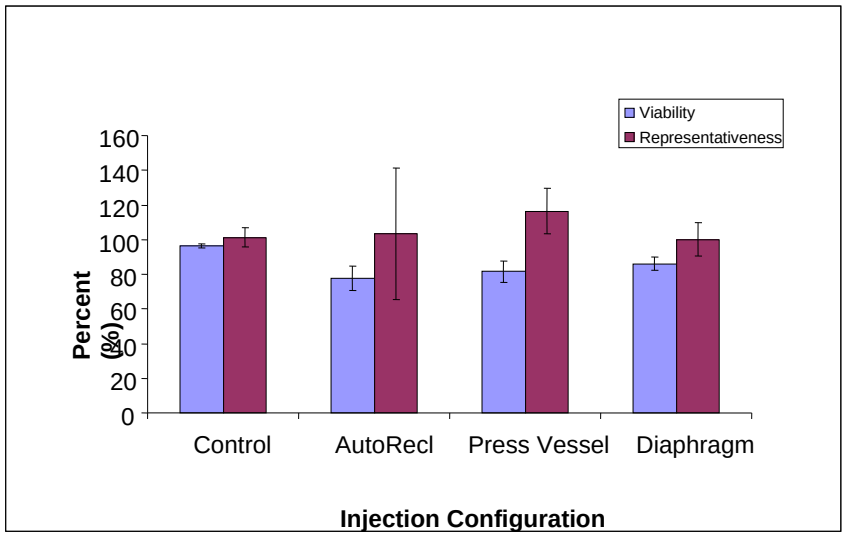

Figure 26. Surrogate Injection: A comparison of the mean and standard deviation for the four experimental injection configurations of the variable Percent Representativeness and Percent Viability. From left to right; Control run, Auto-reclaimer pump run (AutoRecl), Pressure vessel run (Press Vessel) and Diaphragm pump run (Diaphragm).

Table 12. Surrogate Injection: Results from the ANOVA univariate test. The between subjects factor (INJ_TYPE) represents the test statistic comparing the four injection configurations performed on the dependent variable Percent Representativeness (PER_RECV). Data output generated by SPSS statistical software package.

Tests of Between-Subjects Effects

Dependent Variable: PER_RECV

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	497.747[a]	3	165.916	.784	.519
Intercept	181439.155	1	181439.155	857.273	.000
INJ_TYPE	497.747	3	165.916	.784	.519
Error	3597.996	17	211.647		
Total	238401.040	21			
Corrected Total	4095.743	20			

a. R Squared = .122 (Adjusted R Squared = -.033)

3.2.2.2 Percent Viability

The Percent Viability (% Live) means and standard deviations for the four injection configurations are also shown in Table 11 and compared in Figure 26. The control run had the highest mean Percent Viability (96.3 % +/- 0.97%) followed by the diaphragm pump (86.1% +/- 3.8%), pressure vessel (81.6% +/- 6.2%), and the auto-reclaimer pump (77.7% +/- 7.0%). The control injection run (i.e., *Artemia* poured directly into the main line) was designed to minimize contact with mechanical devices other than the pipe itself and the discharge device (Method 3). It is logical then that Percent Viability was highest in the control run. The kinetic forces generated from traveling through the system affected only a small percentage of the injected population in terms of mortality. It was concluded from these results that the piping and sampling configuration utilized for these experiments was not a source of organism mortality in subsequent tests of mechanical elements and any mortality found was independent of the experimental apparatus. This is particularly significant from the larger perspective that the organisms encountered rather large flows (> 500 gpm) and substantial fluid turbulence as a result of pipe fittings and valves prior to sampling. Thus as previously predicted theoretically (Lemieux et al., 2004), the effect on organism viability of hydrodynamic shear forces of these pipe diameters and fluid velocities was insignificant.

The comparison of the standard deviations and relative means in Figure 26 indicates a significantly higher rate of survival in the control run as compared to the remaining three runs. This is supported by the ANOVA test of the between-subjects factor for Percent Viability ($\alpha = 0.05$, $p = 0.002$), which is shown in Table 13. Specifically, the Tukey post hoc test for multiple comparisons, shown in Table 14, showed significant differences between the control run and both the auto-reclaimer ($\alpha = 0.05$, $p = 0.003$) and the pressure vessel ($\alpha = 0.05$, $p = 0.004$). These same statistics indicate a marginally significant difference between the control and diaphragm pump ($\alpha = 0.05$, $p = 0.065$). Despite this, when the three mechanical elements are compared (without the control), there was no significant difference between them.

Table 13. Results from ANOVA univariate test performed on the between-subjects factor (INJ_TYPE) represent the test statistic comparing the four injection configurations performed on the dependent variable Percent Viability (PER_VIAB).

Tests of Between-Subjects Effects

Dependent Variable: PER_VIAB

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	645.242[a]	3	215.081	7.584	.002
Intercept	123582.844	1	123582.844	4357.430	.000
INJ_TYPE	645.242	3	215.081	7.584	.002
Error	482.144	17	28.361		
Total	150768.590	21			
Corrected Total	1127.386	20			

a. R Squared = .572 (Adjusted R Squared = .497)

Table 14. Surrogate Injection: Tukey Post Hoc test of multiple comparisons from the Multivariate ANOVA performed on the four injection configurations (INJ_TYPE): Autoreclaimer, Control, Diaphragm and Pressure Vessel.

Dependent Variable: PER_VIAB

Tukey HSD

					95 % Confidence Interval	
(I) INJ_TYPE	(J) INJ_TYPE	Mean Difference (I-J)	Std. Error	Significance (Sig)	Lower Bound	Upper Bound
Control	auto-rec	18.57*	4.35	0.003	6.21	30.93
	diaph	10.18	3.76	0.065	-0.52	20.89
	pressv	14.68*	3.55	0.004	4.58	24.77
Auto Reclaimer Pump	control	-18.57*	4.35	0.003	-30.93	-6.21
	diaph	-8.38	3.76	0.156	-19.09	2.32
	pressv	-3.89	3.55	0.697	-13.98	6.2
Diaphragm Pump	auto-rec	8.38	3.76	0.156	-2.32	19.09
	control	-10.18	3.76	0.065	-20.89	0.52
	pressv	4.49	2.81	0.404	-3.48	12.47
Pressure Vessel	auto-rec	3.89	3.55	0.697	-6.2	13.98
	control	-14.68*	3.55	0.004	-24.77	-4.58
	diaph	-4.49	2.81	0.404	12.47	3.48

Based on observed means

*. The mean difference is significant at the 0.05 level.

3.2.2.3 Injection Assemblies Discussion

The preceeding findings suggest that in terms of organism viability, other engineering considerations such as injection volume and control of injection rate will be significant. The approach for the ETV BW Test Protocol was a known concentration of organisms injected into a flowing pipe over a specified time to achieve a specific concentration of organisms. A comparison of the physical attributes of the injection methodologies investigated indicates that the diaphragm pump operates at a significantly reduced flow rate compared to the pressure vessel and the other pumps (e.g. centrifugal), but it is capable of injection at substantially higher pressures (> 100 psig) than centrifugal or peristaltic pumps. The auto-reclaimer pump lacks flow rate and pressure controls which resulted in a large residual volume. Thus although the Percent Representativeness of the injected organisms was substantial, the residual concentrations of organisms in the injection flask were an order of magnitude greater than for all other injection methodologies. Thus the concentrations in the challenge water and control tank were lower than expected. The pressure vessel injection system provided relatively low residual organism concentration, highly variable flow rates and pressure compensation, and a continuous stream of organisms into the injection pipe. Thus although there was no significant difference between any of the injection methodologies in terms of organism representativeness or viability, the pressure vessel's physical characteristics, reliability, and consistency of providing a steady flow of organisms at the desired concentration made it the best choice.

3.3 Pipeline Biological Sampling (Zooplankton)

3.3.1 Control and Sample Wand Experiments

A total of thirteen experiments took place between October 29 and November 12, 2004 during daylight hours. The first run was used as a trial while the remaining twelve where divided into three runs per experimental sample wand (3 runs x 4 wand types = 12 experimental runs total). Three sample tanks simultaneously collected water from the same wand during each run and thus are not independent of one another. This results in an n = 3 rather then an n = 9 when conducting statistical tests on the four sampling wands.

A summary of means and standard deviations for each sampling wand is shown in Table 15. For each wand, three runs were averaged to provide a single mean and one standard deviation for each operational and biological parameter collected. The values listed in the (% Live), and (% Dead) rows were obtained from Table 16 which displays the results of the ANOVA testing viability. The Percent Representativeness column reflects data obtained from the descriptive tables of the ANOVA testing recovery (Table 17). This variable was calculated by taking the number of *Artemia* injected into the system and dividing it by the ballast test tank volume (liters) to obtain the estimated concentration per liter in the test tank. The objective was to see if the in-line sampling wands collected a "representative" concentration of *Artemia* per liter as compared to the estimated ballast test tank concentration per liter. The system's flow rates averaged 1,095 gpm with a typical static pressure of 14.9 psig on average. The injection vessel flow rate was 0.65 gpm with a static vessel pressure of 24.2 psig. The three sample tanks averaged 441 gal each of screened water. The target sample volume was 264.2 gallons (1 m^3) per tank, thus the minimum sample volume was achieved.

Table 15. Surrogate Sampling. Summary of run logistics along with a comparison of Percent Representativeness and Viability between the four sampling wand configurations. Gallons per minute are expressed as gpm, minutes as min, pounds per square inch as psig, and gallons as gal.

	Dead End (Control)		8" Elbow		60 degree angle		4" Elbow	
	Mean	Std. Dev (+/-)	Mean	Std. Dev (+/-)	Mean	Std. Dev (+/-)	Mean	Std. Dev (+/-)
Artemia hatched	23450700	4172295	30191700	3385758	26174400	1929018	37293900	4476966
System Flow Rate (gpm)	1094.0	1.0	1091.7	2.9	1100.2	3.9	1094.5	5.1
System Pressure (psi)	15.0	0.2	14.7	0.3	14.9	0.1	14.9	0.2
Total *Artemia* Injected	22779083	4014296	27306367	1531608	25646928	2112532	36782138	5097153
Ballast Tank Volume (gal)	61986	5430	66724	7340	66010	233	65670	304
Injection Flow Rate (gpm)	0.68	0.10	0.63	0.12	0.59	0.04	0.69	0.24
Injection pressure (psi)	24.3	0.6	22.3	2.9	25	0	25	0
Injection time (min)	56.7	4.9	61.1	6.8	60	0	60	0
Artemia recovered in flask	95992	16118	100809	9146	75891	32612	125262	36933
Sample Tank Volume (gal)	426	26	429	35	454	5	456	6
% LIVE	85.8	6.4	77.2	7.8	79.2	9.4	67.4	17.8
% DEAD	14.2	6.4	22.8	7.8	20.8	9.4	32.6	17.8
% Representativeness	61.7	6.9	57.6	7.2	42.3	15.3	49.1	12.5

Table 16. Surrogate Sampling: Percent Live. Mean +/- standard deviation descriptive statistics on the between subjects factor (S_WAND) which represents four injection configurations in relation to Percent Viability (PER_VIAB) of injected organisms.

Dependent Variable: PER_VIAB

S_WAND	Mean	Std. Deviation	Number
Control	85.77	6.35	3
8_elbow	77.23	7.85	3
60_angle	79.20	9.44	3
4_elbow	67.37	17.75	3
Total	77.39	11.81	12

Table 17. Surrogate Sampling: Percent Representativeness. Mean +/- standard deviation descriptive statistics on the between subjects factor (S_WAND) which represents the four injection configurations in relation to Percent Representativeness (PER_REP) of injected organisms.

Dependent Variable: PER_REP

S_WAND	Mean	Std. Deviation	Number
Control	61.67	6.80	3
8_elbow	57.60	7.18	3
60_angle	42.27	15.25	3
4_elbow	49.13	12.46	3
Total	52.67	12.24	12

3.3.2 Representativeness

The dead-end configuration (Control, Figure 14, # 1) was run to determine what the lack of an extension into the main flow stream would do to the Percent Viability and Percent Representativeness of *Artemia*. As outlined in Table 15, approximately 2.3 x 10^7 *Artemia* were initially estimated to be within the injection vessel. Following the experimental run, 2.27 x 10^7 organisms were found within the ballast tank for a concentration of 97.2 organisms per liter. The sample tanks averaged a concentration of 59.5 organisms per liter. The Percent Representativeness for the Control (61.2%) was found as described in Step 18 of Section 2.4.3. The same procedure was followed for the other three wand configurations, and the results are provided in Table 15.

The 8-inch elbow sample wand experiment had approximately 3.0 x 10^7 *Artemia* initially estimated within the injection vessel. There were 100,809 *Artemia* recovered for a representative rate of 57.6%.

It was estimated that the 4-inch elbow sample wand experiment had approximately 3.7 x 10^7 *Artemia* within the injection vessel. There were 125,262 *Artemia* recovered for a representative rate of 49.1%.

The 60-degree angle sample wand experiment resulted in approximately 2.6 x 10^7 *Artemia* within the injection vessel. There were 75,891 *Artemia* recovered for a representative rate of 42.3%.

The above data for Percent Representativeness are illustrated in Figure 27 for individual experiments (Note that the error bars are for the standard deviations among sample tanks). The results from the 60-degree angle and 4-inch Elbow sample wand experiments exhibited a relative inconsistency amongst replicates as compared to the dead-end (control) and 8-inch Elbow samples wands. The reason for this was unknown, but the variability gives a relative indication of consistency in the samples acquisition.

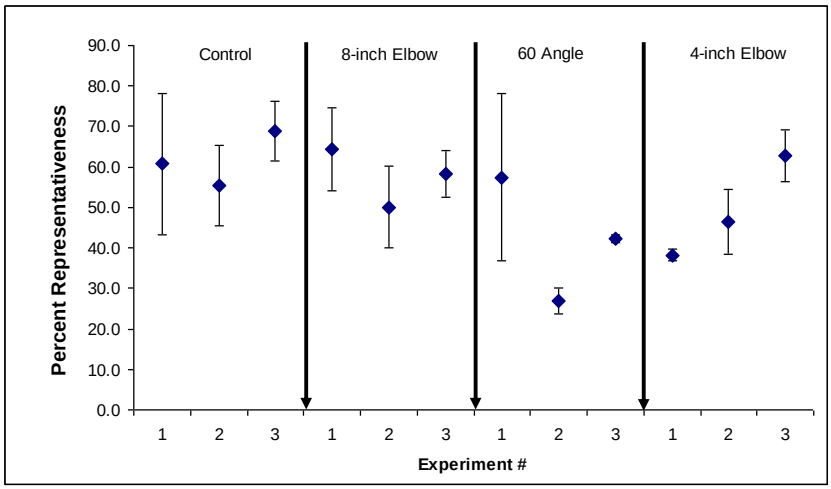

Figure 27. Surrogate Sampling: Comparison of Percent Representativeness (%), mean and standard deviation for each sample wand's experimental run.

The results for Percent Representativeness of each sample wand are presented in Figure 28. Here, each of the three runs per sample wand were averaged and displayed as one mean +/- standard deviation. Table 17 outlines these values with the Control having the highest mean Percent Representativeness (61.7% +/- 6.8%), followed by the 8-inch Elbow (57.6% +/- 7.2%), then the 4-inch Elbow (49.1% +/- 12.5%), and finally the 60 degree angle (42.2% +/- 15.3%). Though the Control has the highest Percent Representativeness, the overlap of the standard deviations indicates that any difference between the sampling wands in relation to Percent Representativeness of *Artemia* was minimal. The ANOVA test of the between-subjects factor, Percent Representativeness, (Table 18) supports the trend shown in Figure 28 that there were no significant differences in the recovery of *Artemia* among the four wands ($\alpha = 0.05$, $p = 0.214$).

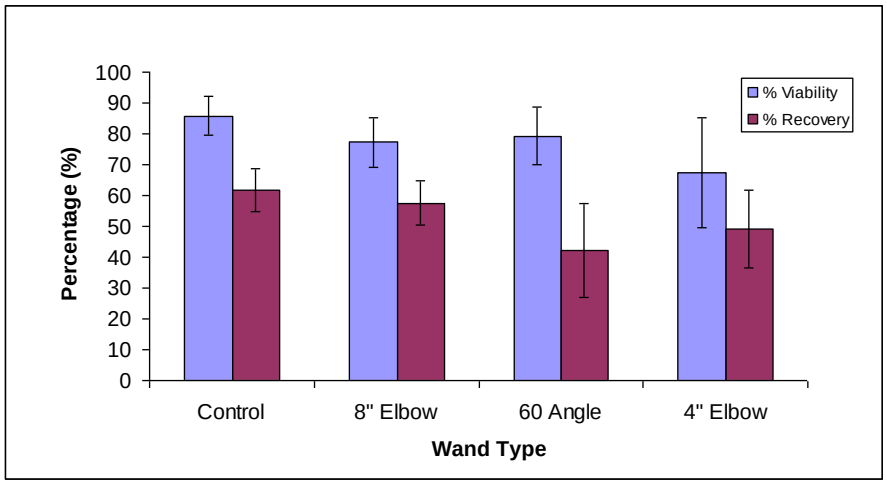

Figure 28. Surrogate Sampling: A comparison of the mean and standard deviation for the four experimental wand configurations of the variable Percent Representativeness and Percent Viability.

Table 18. Surrogate Sampling: Percent Representativeness. Results from ANOVA univariate test performed on the between subjects factor (S_WAND) represents the test statistic comparing the four injection configurations performed on the dependent variable Percent Representativeness (PER_REP). ANOVA output generated by SPSS statistical software package.

Tests of Between-Subjects Effects

Dependent Variable: PER_REP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	677.947[a]	3	225.982	1.862	.214
Intercept	33285.333	1	33285.333	274.190	.000
S_WAND	677.947	3	225.982	1.862	.214
Error	971.160	8	121.395		
Total	34934.440	12			
Corrected Total	1649.107	11			

a. R Squared = .411 (Adjusted R Squared = .190)

The premise of Percent Representativeness was to determine how the *Artemia* densities collected by the sampling wands compare to the actual or intended ballast tank concentration. In other words, was the acquired sample representative of the entire concentration in the ballast tank? Note that only 2% of the entire volume of transferred fluid was actually sampled. The relatively

low recoveries discussed above are worrisome since they are indicative of the representativeness of the sample, which will be critical in the future evaluation of ballast water treatment equipment. This disparity is potentially (though not validated) a result of inaccuracy in the measurement of main stream flow rates and volume estimations. Currently the flow meters utilized for measurement of flow are paddle-wheel meters. These are typically chosen because they are easy to maintain and are economical. However, the flow rates are only accurate to within 5% of the measured value; at 1100 gpm, 5% is a significant variation of total volume in 2 hours of flow. Furthermore, the flow rate signal is highly averaged by the receiver and data display to filter out the high frequency variations in the flow rate measurement. Sample representativeness is strongly correlated to the measured and/or calculated flow rate and ballast tank volume, thus errors in these measurements are more likely to affect Percent Representativeness than are sampling wand geometries. However, given the uniform procedures during the runs, the trends observed in the data are relevant and useful in determining an effective sample wand for future applications. More accurate means of measuring relevant flows and volumes would be a valuable addition to any test system.

3.3.3 Organism Viability

The mean and standard deviation of Percent Viability for all four wand configurations are compared in Figure 29. Due to time constraints, only one out of three runs conducted for each wand was processed for Percent Viability. The comparison shows that the control had the highest mean Percent Viability while the 4-inch Elbow had the lowest. No significant differences appear to exist between the Control, 8-inch Elbow, and 60 degree angle.

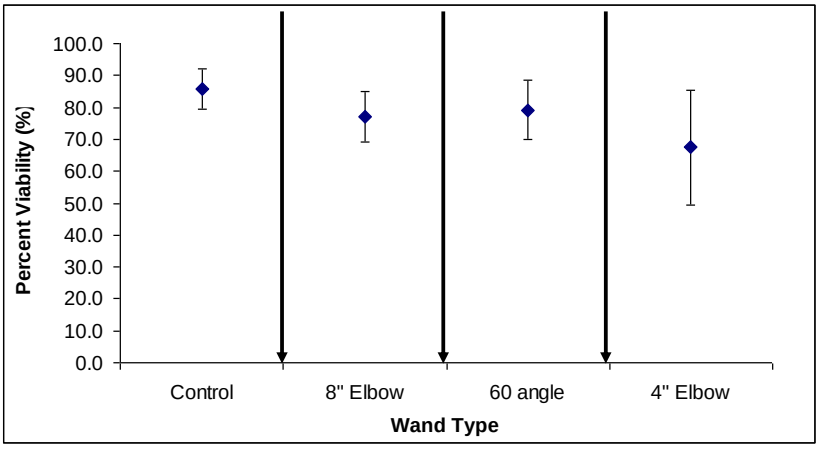

Figure 29. Surrogate Sampling: Comparison of mean +/- standard deviation for Percent Viability in each experiment run per sample wand. (Note: Viability analysis performed on one out of three experiments for each wand (n = 1)).

The ANOVA test of the between-subjects factor, Percent Viability, shown in Table 19, supports the findings from Figure 29 that there is no significant difference in the survivability of *Artemia* among the four sample wands ($\alpha = 0.05$, $p = 0.319$). It was peculiar that viability was the highest in the method which drew *Artemia* out of the flow stream through the laminar boundary layer and not from the center of the pipe.

Table 19. Surrogate Sampling: Percent Viability. Results of ANOVA univariate test performed on the between subjects factor (S_WAND) representing the four injection configurations. The dependent variable *Percent Viability* (PER_VIAB) was used for the tests. ANOVA output generated by SPSS statistical software package.

Tests of Between-Subjects Effects

Dependent Variable: PER_VIAB

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	521.809[a]	3	173.936	1.375	.319
Intercept	71873.641	1	71873.641	567.991	.000
S_WAND	521.809	3	173.936	1.375	.319
Error	1012.320	8	126.540		
Total	73407.770	12			
Corrected Total	1534.129	11			

a. R Squared = .340 (Adjusted R Squared = .093)

3.3.4 Sampling Discussion

3.3.4.1 Overall Representativeness & Viability

The conclusion from this work was that while only marginal differences were noted between sampling configurations, the Control and 8-inch wand configurations resulted in the highest mean viability and representativeness as demonstrated in Figure 28. Again, this was rather surprising and indicates that sampling wands should not be necessary for shipboard sampling from pipes either, which will significantly aid in the acquisition of samples. Future work should include an examination of the dependency of both configurations on pipe and wand diameter. There are scenarios in which it is advantageous to have a wand inserted into the flow stream. This is particularly the case when the internal pipe pressure is under suction and acquiring a sample is more difficult without including a pump or other mechanical device. Note that opening a valve in the tee configuration would likely result in a Venturri effect and subsequently air entrainment and pump loss of prime. However, use of a wand takes advantage of the incident momentum of the flow stream (if it is directed upstream) such that the loss of prime is delayed until the momentum and hydrostatic pressures are balanced by the suction pressure.

The relatively low recoveries were likely the result of poor or inadequate flow rate measurement and volume estimations rather than an indicator of poor sampling methodologies. Regardless, the mean mortality rate for all sampling wands combined was 23% (+/- 10%) which corresponds with the mortality rate generated previously during the pressure vessel injection experiment (18.4% +/- 6%). Thus, no effect on organism viability was found due to the sampling apparatus.

3.3.4.2 Sample Tank and Net Size Effects on Sampling

A concern during experimentation was the potential effects the 25-, 50- and 80-µm mesh collection screens and the orientation of sample tanks 1, 2 and 3 would have on *Artemia* Percent Viability and Percent Representativeness. The placement of the screens was randomly chosen during each run. The tanks were stationary for the entire duration of the testing and were placed as equidistant from the external sampling port as possible. To address these questions the following sets of hypothesis were tested. The first was:

- H_o: There is no significant difference in *Artemia* Viability among the three sample tanks.
- H_a: There is a significant difference in *Artemia* Viability among the three sample tanks.

The second hypotheses tested were:

- H_o: There is no significant difference in of *Artemia* Viability among the three screen sizes.
- H_a: There is a significant difference in *Artemia* Viability among the three screen sizes.

The third hypotheses tested were:

- H_o: There is no significant difference in *Artemia* Representativeness among the three sampling tanks.
- H_a: There is a significant difference in *Artemia* Representativeness among the three sampling tanks.

The fourth hypotheses tested were:

- H_o: There is no significant difference in *Artemia* Representativeness among the three screen sizes.
- H_a: There is a significant difference in *Artemia* Representativeness among the three screen sizes.

Data analysis was performed using four, single-factor ANOVA's generated in Microsoft® Excel's® statistical package. The ANOVA outputs for all four tests are outlined in Table 20.

Table 20. Surrogate Sampling: Summary of single factor ANOVA outputs that tested the between subject factor effects of sample tank location 1, 2, & 3 (tanks) and screen size 25-, 50- and 80-µm (screens), on the dependent variables Percent Viability and Percent Representativeness.

		Source of Variation	*SS*	*df*	*MS*	*F*	*P-value (signif)*	*F crit*
Percent Viability	tanks	Among Groups	560.6	2	280.3	2.6	0.13	4.3
Percent Viability	screens	Among Groups	359.3	2	179.7	1.4	0.30	4.3
Percent Represent.	tanks	Among Groups	333.0	2	166.5	0.8	0.44	3.3
% Representativeness	screens	Between Groups	148.0	2	74.0	0.4	0.70	3.3

The tests on Percent Viability found no significant differences in relation to sample tanks ($\alpha = 0.05$, $p = 0.13$) or screen size ($\alpha = 0.05$, $p = 0.30$). Similarly, the tests on Percent Representativeness showed no significant differences in relation to sample tanks ($\alpha = 0.05$, $p = 0.44$) or screen size ($\alpha = 0.05$, $p = 0.70$). These results indicate a variety of things with regards to the sampling apparatus. For *Artemia* and organisms having a similar body size (400-um), little effect on representativeness or viability is anticipated with screen size (25-, 50- or 80-micron mesh). However, mesh size should be a consideration in future tests in which particulates such as mineral matter and particulate organic matter (POM) are used to asses the correlation between small mesh sizes and a potential decrease in water quality.

Secondly, organism viability and representativeness were unaffected by the hose manifold on the discharge end of the sampling wand, and an even distribution of organisms amongst the sample tanks was realized. Tank dimensions and orientation are important considerations in sampling designs that rely on pipe pressure and gravity feeds. Uniformity for each sample tank assembly should be a priority. Furthermore, from a statistical perspective, the apparatus used for this experiment included three sample tanks fed from a single wand with attached tubing manifold for water distribution. The result of this type of setup is that the three tanks cannot be considered independent. The alternative is to place individual sampling wands to feed individual sample replicate tanks. In the alternative scenario, an additional measure would be required to achieve truly independent samples. Specifically, the requisite three wands in the latter would result in a hydrodynamic "shielding" of successive wands and thus an uneven organism distribution within the pipe. The remedy for this problem would be to allow for the flow stream after each sampling wand to return to fully-developed flow at which point an even distribution is likely. This becomes restrictive, since, as a rule-of-thumb, 28 pipe diameters are required to achieve fully-developed flow. Therefore with 8-inch pipe a spacing of roughly 18 feet would be required or a total of 54 feet for all three. In the single wand scenario utilized in this work, only 18 feet was required and used. In the opinion of these authors, there is substantial risk and physical limitations involved in the placement of truly independent replicate sampling assemblies, especially when a total of 15 such assemblies would be required for the proposed ETV protocol.

Finally, if a single wand is used for a set of replicates, and the three sample tanks are not considered independent of one another, the statistical benefit of three 1-m^3 sampling tanks in lieu of a single 3-m^3 is non-existent. From a physical or operational perspective, the former also increases the probability for human error due to increased handling. As will be discussed in a later section, these constraints become increasingly important when the effects of sample degradation with time are considered.

3.4 Surrogate Sample Degradation

Through the course of the experiments presented above, particularly during the injection work, it was noted while running control samples that there was high degree of variability in organism representativeness and viability amongst replicates and sets of replicates (one control run to the next). After substantial investigation into a variety of factors, both physical setup and operational, the impact of elapsed time from the collection of the cod end to microscopic analyses was examined. While a formal experiment was not completed to investigate this specifically, available data was considered. Figures 30 and 31, represent Percent Representativeness as a function of time (unit-less in this case) and Percent Viability and

Live/Dead Ratio as a function of time respectively. Two series of replicates C1 and C2 are shown which correspond to two different surrogate injection control experiments. In the case of C1, "Time 1, 2 and 3" are elapsed times of approximately 1, 14 and 15 hours, respectively. For the C2 replicate set, these numbers correspond to approximately 0.75, 1.25 and 2 hours, respectively. In other words, the C1 data set was analyzed over approximately 15 hours and the C2 data set in less than 2 hours. The data in Figure 30 indicate that for C1 a noticeable drop in Percent Representativeness between ostensibly identical replicates occurred between 1 and 14 hours of elapsed time. However, when each replicate is analyzed quickly as in C2, little to no drop in Percent Representativeness is noted. It is possible that this is the result of deterioration of dead organisms being held for analyses. Death could be caused by successive bombardment with entrained air that is used to oxygenate the sample during hold time. If dead organisms are mechanically degraded and broken apart to the point of disintegration, this would have the added effect of artificially increasing the Percent Viability and the live-to-dead ratio, which is exactly what is demonstrated in Figure 31 for the C1 series. From these data it was concluded that for the viability analyses of *Artemia*, samples must be examined within a 2 hour period.

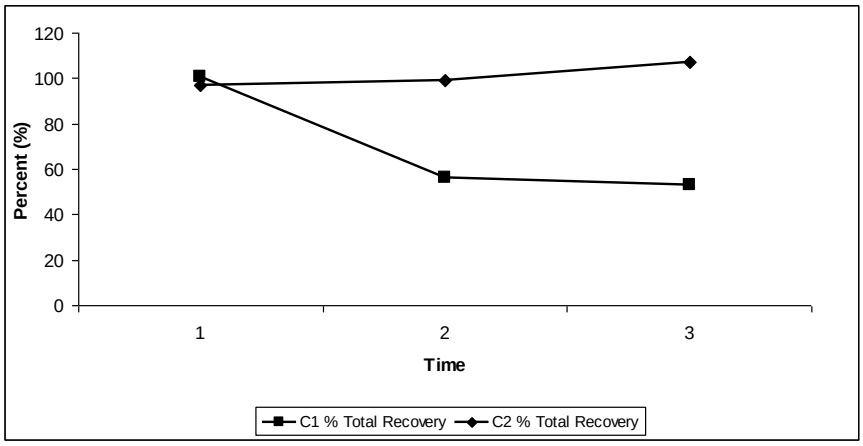

Figure 30. Impact of sample holding elapsed time on Percent Representativeness. Series C1 was analyzed over a 15 hour period and C2 in less than 2 hours.

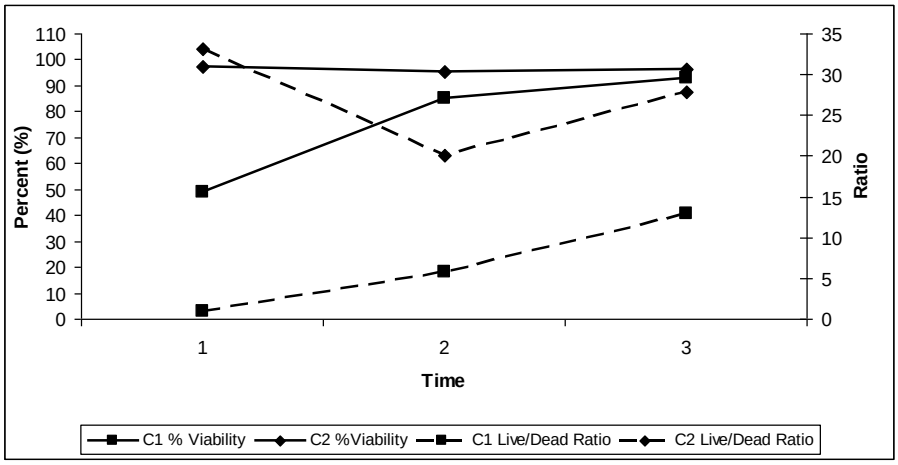

Figure 31. Impact of sample holding elapsed time on Percent Viability and Live/Dead Ratios. Series C1 was analyzed over a 15 hour period and C2 in less than 2 hours.

Note this is a rather significant finding when much larger volumes and sample quantities are considered (recall Section 3.3.4.2). It presents a rather large obstacle to test facilities for Ballast Water Treatment equipment as proposed by the ETV protocol. As a result of this finding, the following recommendations are made:

- sampling schedules should be reduced as much as necessary to provide a balance between statistical significance and the spatial and time-related constraints which significantly alter the accuracy of measurements,
- significant resources should be committed to the pursuit of tools and techniques which reduce or eliminate the necessity for immediate sample analysis and
- efforts which address the selection of surrogate organisms and their analyses for viability should consider the effect of sample degradation.

4 Conclusions

The experiments conducted during this study resulted in a substantial increase of the understanding of how organisms may be injected or sampled from flowing pipes, and furthermore, how these methods or systems may be optimized.

Overall

- The validation of injection methodologies for surrogate organisms in the testing of BWTE requires the consideration of organism representativeness (continuity of mass) and organism mortality/viability, in order to adequately define the injection performance.

- A zooplankton organism was chosen as the surrogate for this work since it was determined that mechanical effects (due to turbulent flows, pumps, etc.) on organisms are a function of organism size with increasing sensitivity at increasing sizes.
- When conducting viability analyses on acquired samples, dead organisms may be mechanically degraded and broken apart to the point of disintegration, and have the effect of artificially increasing the Percent Viability and the live-to-dead ratio. Using data from these experiments, it was concluded that assessing the viability of *Artemia* requires that samples must be examined within a 2 hour period.

Organism Injection

- The injection experiments indicated that in terms of representativeness, no significant differences were exhibited amongst the pressure-vessel, auto-reclaimer pump or diaphragm pump. In addition, none of these resulted in a significant loss of organisms above the control after consideration of residual volumes (~100%).
- The injection control experiment validated that the sampling test assembly, used subsequently for the Sampling portion of this investigation, resulted in only a 3.7% mortality.
- Use of the positive displacement auto-reclaimer pump resulted in a large residual volume, thus providing inadequate injection of surrogate organisms.
- While the control experiment had the highest mean Percent Viability (96.2%), the viability of organisms injected using the diaphragm pump, pressure vessel, and the auto-reclaimer pump were 86.1%, 81.6% and 77.7%. Statistical analyses indicate that there was no significant difference between the control and the diaphragm pump.
- Both the pressure vessel and auto-reclaimer result in a 78-82% organism viability.

Organism Sampling

- Sampling piping, tanks and nets for large volumes and flow rates may have a large effect on organism viability and representativeness.
- A substantial decrease (~50%) in viability was shown when discharging zooplankton surrogates through an open pipe and down into a net housed in an empty tank.
- Surrogates discharged out an open pipe and down into an empty tank and later screened through a collection net suffered approximately 36.2 % mortality.
- While the specific mechanism of mortality involved in Method 1 and 2 was not clear, it is likely to be either a) interaction with air entrainment and the associated pressure differentials, b) the impact of the water and organisms on the water/surface interface or c) the effective force on the organism due to water velocity on the net. Since immersing the discharge head and providing for a larger surface area (diffuse pipe head) resulted in relatively no mortality, it is likely that this approach remedies all three degradation mechanisms.
- A discharge pipe with a diffuser head situated within a plankton net and immersed in water in the sample recovery tank resulted in the least damage to organisms and a mortality of 3.7 percent.
- An insignificant difference was exhibited by the various sample wand geometries with the control or classic tee fitting having the highest mean Percent Representativeness of 61.7% (+/- 6.8%) which was followed consecutively by the 8-inch Elbow (57.6%), the 4-inch extended elbow (49.1%), and the 60 degree angle (42.2%).

- While the recovery and representativeness of the samples were lower than anticipated, the inaccuracy in measurement of main stream flow (1100 gpm +/- 50-100 gpm) and total flow volumes is likely the cause, thus actual recovery percentages and therefore sample representativeness are anticipated to be higher than observed.
- In terms of viability, the dead end or classic tee fitting experiment had the highest mean Percent Viability (85.8 %), followed by the 60 degree angle (79.2%), the 8-inch elbow (77.2%), and the 4-inch elbow (67.4%).

5 Recommendations

The results from testing a variety of injection and sampling methods disclosed a number of points and recommendations to be borne in mind when planning full-scale tests of BWT systems.

The in-line sampling configuration was designed to obtain continuous representative samples over a one-hour period. During that time, only 2% of the total flow was diverted into the sample tanks. Because of the continuous sampling, a consistent concentration of organisms was required over the full sample period. It is recommended that a pressure vessel be used for delivering organisms into the main flow due to its ease of use, reliability, low residual, and continuous stream of organisms. Accurate measurement of flow rates and volumes are required to obtain good values for Percent Representativeness.

Organisms retained within the delivery system or those disintegrated by sampling are not counted and thus bias the Percent Live and Percent Representativeness calculations. It is therefore necessary to consider the conservation of mass and reduce aspects that cause damage or loss. The pressure vessel proved to be the best means of injecting organisms. The conventional piping tee without any extension proved to sample representatively from the turbulent flow. This is important not only for designing shore-based tests but also for shipboard tests and enforcement inspections.

A submerged diffusion head interior to a plankton net of appropriate mesh size is recommended as the best means for discharging the sample. The piping manifold that distributed the sample to the three separate tanks did not affect viability, but since the tanks are not true replicates, it should not be necessary to use three tanks. In order to obtain true replicate samples, individual sampling ports placed a minimum of twenty-eight pipe diameters apart would be necessary. This would greatly increase the space required at test facilities as it would require approximately sixty feet for just the three sample ports. A single, larger tank that allowed sub-sampling could replace the three smaller tanks and the distribution manifold.

Viability of organisms is of key importance in determining the efficacy of BWT systems. Data from control samples from this study indicated that there can be significant degradation of samples with increases in elapsed time from the collection from the cod end to the end of microscopic evaluation of viability. The larger the sample to be evaluated, the greater this problem can become. Thus sampling schedules should consider these constraints as well as

statistical requirements. Likewise organism degradation should be considered during the selection of surrogate organisms and their viability analyses. Options such as live stains and/or automated analysis should be pursued to reduce the necessity for immediate analysis by trained technicians.

These experiments were conducted with a large (~400 μm) surrogate. Plankton nets used for concentrating the organisms ranged from 25 μm to 80 μm. The larger mesh size proved useful in separating the organisms from finer particles in the water, thus making the microscopic examinations easier. During actual tests of BWT systems, it must be remembered that the smallest mesh size used will govern the size of the smallest organisms retained. Whole water samples will be required for anything smaller.

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