Fisheries and Oceans Canada Pêches et Océans Canada

BRINE BALLAST WATER TREATMENT STUDY – SHIPBOARD TESTING

FISCAL YEAR 2008-09

FINAL REPORT

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DISCLAIMER

This report summarizes work completed to date on on-going projects to meet fiscal year-end reporting requirements. **All results are preliminary and must not be distributed without authorization of the authors.**

INTRODUCTION

Ballast water associated with commercial shipping activities within the Laurentian Great Lakes has been identified as the most important vector for nonindigenous species (NIS) since the St. Lawrence Seaway opened in 1959 (Holeck et al. 2004; Ricciardi 2006). Human mediated dispersal of NIS has been in progress for thousands of years, but with technological advances, the signing of free trade agreements and expanding global economies, the number of invaders is steadily on the rise (e.g. Work et al. 2005). NIS are defined as species that have no previous evolutionary history in the Great Lakes and have been introduced since the arrival of European colonists (Ricciardi 2006). Introduction of NIS is becoming increasingly recognised as one of the most important threats to the biodiversity of aquatic ecosystems (Sala et al. 2000; Millennium Ecosystem Assessment 2005; Lawler et al. 2006). In addition to their detrimental ecological and environmental effects, 20-30% of NIS have negative economic impacts (Pimentel et al. 2001).

Models seeking to identify determinants of invasion success have recently focused on the importance of propagule pressure (e.g. Veltman et al. 1996; Lockwood et al. 2005; Colautti et al. 2007). Propagule pressure is the combined introduction effort exerted on a community by nonindigenous species. It encompasses both the number of introduction events and the number of propagules released per event. Physiological status of propagules upon release may also have a bearing on invasion success.

Ballast is used by vessels without cargo shipments for stabilization, and to permit safe movement between ports by weighing down the ship, and allowing it to travel through the water instead of floating on it (Aquatic Sciences 1996). Before the use of liquid ballast around 1900, ships utilized solid ballast - mainly soil, gravel, or rubble found in proximity to the port. Solid ballast was often contaminated with plants, insects and other organisms (Lindroth 1957; Mills et al. 1993).

Voluntary regulations requiring deep ocean ballast water exchange (BWE) were put in place by Transport Canada in 1989 and made mandatory by the United States Coast Guard in 1993, however this requirement only applied to vessels with 'declarable water on board'. BWE is intended to flush organisms from ballast tanks, thus dramatically lowering potential 'propagule pressure' (Colautti et al. 2007). Organisms contained within unpumpable ballast following emptying of the tanks would subsequently be killed by emersion in salt water loaded to replace discharged freshwater as extant salinity levels would exceed physiological tolerances of most freshwater species (Ricciardi 2006). Ballast water is not the only vector associated with cargo vessels, but it is of primary concern due to the sheer volume of water being moved as well as the number of potential invaders found within it (Mills et al. 1993; Ricciardi 2001).

Oceanic vessels import approximately 5,000,000 tonnes of ballast water per year (Aquatic Sciences 1996), and are attributed with ~65% of all recorded aquatic invasions since the opening of the St. Lawrence Seaway in 1959 (Ricciardi 2006). Saltie vessels lacking cargo and carrying ballast constitute only a small fraction of trade into the Great Lakes (Colautti et al 2003). Far more vessels arrive with cargo in their holds, and thus carry only residual ballast water (Colautti et al 2003). The breakdown of ballast tanks loaded with water to those that contain only residual water and sediment was ca. 10%:90%, respectively, in 2006 (US Coast Guard, unpubl. data).

In 2005, Transport Canada amended the Ballast Water Control and Management Regulations to require saltwater exchange or flushing of foreign ballast water to minimize the risk of introduction of aquatic invasive species via commercial shipping activities. While these strategies certainly reduce the total abundance and diversity of taxa carried in ballast water, there are conditions under which these practices cannot be performed effectively. In addition, saltwater exchange and flushing will not meet the ballast water discharge standard proposed by the International Maritime Organization, thus alternative treatment methods (which are both biologically- and cost-effective) are needed.

The addition of NaCl brine has been proposed as a treatment solution for management of both residual (NOBOB), and incompletely-exchanged (BOB), ballast water. As marine seawater (30 ppt salinity) used in flushing and exchange practices can effectively reduce viability of fresh- and brackish-water taxa, brine (230 ppt full-strength) is expected to provide complete protection against low-salinity taxa, as well as against marine taxa. A feasibility study indicated that liquid brine is a cost-effective and readily-available strategy for management of ballast water on the Great Lakes that would pose little interference to current shipping operations. Preliminary laboratory studies indicate that brine is biologically effective on both freshwater and marine taxa, given sufficient concentrations and/or exposure time. The primary objective of this study is to evaluate the efficacy of brine treatment of ballast water under normal operational conditions at full ship scale. Two hypotheses will be tested: 1) brine/road salt can be uniformly distributed in ballast tanks and 2) at least 95% of organisms will be exterminated. A secondary objective of this study is to develop more robust methods for inspection and analysis of ballast water samples.

METHODS

A.1. Shipboard Trials: Ballast On Board (BOB)

Twenty-three percent sodium chloride brine will be added through main deck or void space hatches, or through tank vents to partially ballasted tanks in a proportion that should result in a homogeneous residual ballast of at least 40ppt salinity. Vertical salinity profiles will be conducted through forward and aft hatches, and additional water samples will be collected through sounding tubes to test the salinity of the water at different locations of the tank. If possible, NOAA/U. Michigan instrumentation will be installed prior to ballast uptake to achieve additional salinity/temperature measurements in the tanks. Measurements will be taken from the experimental tank and a paired control tank, before and after treatment.

Zooplankton samples will be collected to examine the biological efficacy of brine treatment. Samples will be collected from both treatment and paired control tanks. Consecutive plankton net tows (30 cm diameter, 40 μm mesh) will be conducted to filter approximately 1000L of ballast water through both forward and aft hatches. The depth of zooplankton net tows will be recorded so that the volume of water sampled and the density of animals in the tanks can be calculated when samples are returned to the laboratory. As it is possible that net tows will be restricted to the upper portion of the tank (due to horizontal platforms inside tanks), additional bottom samples will be collected through the sounding tube by manually pumping ballast to the ship deck using standard methods (i.e., tubing with foot valve). Salinity and zooplankton samples will be collected at time 0 (pre-treatment), 1h, 6h, 12h, 24h, 48h, and 72h after treatment. To achieve this temporal sampling, it may be necessary for two scientists to remain on board the ship as it transits the Great Lakes. A secondary option would be to meet the ship at the following ports – but this may limit the extent of temporal sampling.

A.2. Shipboard Trials: No Ballast Tanks On Board (NOBOB)

Liquid NaCl brine will be used to treat NOBOB tanks. Brine will be added directly to one tank for each experiment through sounding tubes. The quantity of brine added will be calculated based on the residual volume of ballast and initial water salinity, to achieve a desired salinity of 115 ppt. To test for uniformity of brine distribution, small water samples will be collected at various locations in the tank before and after addition of brine. Tank diagrams will be consulted to select locations of sample collection in relation to brine application point. A sample of brine will be collected during each experiment for later elemental analysis.

Zooplankton samples will be collected to examine the biological efficacy of brine treatment. Samples will be collected from both treatment and paired control tanks. Tank entry will be conducted to collect water samples using methods previously employed on the NOBOB project (i.e., hand operated bilge pump). The volume of water collected will be measured in a calibrated carboy prior to filtration through a plankton net inside the tank. It will be desirable to collect 20-50L water samples from three locations in the tank: forward, mid- and aft. Later calculations of animal density per sample will need to account for sample dilution as a result of the addition of brine into tanks, and possibly also due to return of filtrate from sample collection at previous timepoint(s). Salinity and zooplankton samples will be collected at time 0 (pre-treatment), 1h, 2h, (hourly for up to 4h???). Experimentation with NOBOB tanks is expected to be completed at a single port.

Sample Analysis

All samples collected from BOB and NOBOB tanks will be rinsed into collection jars with water of appropriate salinity (i.e., same salinity as the water being collected). Instant Ocean water (or fresh water) will be brought on ship to ensure that water void of zooplankton is available for this purpose. Samples will be assessed for viability immediately after collection as follows. Each sample (or a subsample of each) will be washed into a counting tray with appropriate water of matching salinity for counts of the percentage of live individuals using microscopes. After this count, the entire sample will be rinsed back into water at the initial ballast salinity for one hour before reassessment (i.e., a recovery period will be given at the original salinity level following treatment with salt or brine). After one hour, a second assessment of percentage viable will be made. Vital stains may be incorporated into this assessment; however, as vital stains can be unreliable, traditional optical assessments will always be conducted. Major groups of taxa will also be identified on site. After viability assessment, the samples will be preserved in 95% ethanol for transportation to the laboratory and future examination of total zooplankton density and diversity. Any individuals found to be alive after treatment will be bottled separately from dead individuals in each sample. This will allow for later evaluation of the type of taxa able to survive – possibly through genetic testing.

Statistical Analysis

T-tests, and possibly repeated measures analysis of variance (RM-ANOVA) may be used to examine salinity and zooplankton data. Ship will be the replicated measure of variability within treatment. Data from control tanks, and from 'before' samples will be contrasted against treated data to measure efficacy of the treatment. This may be done using paired T-tests (before vs. after), or more complicated RM-ANOVA may be used to incorporate temporal measures. Data obtained from multiple tank locations within a time point will be examined to assess the uniformity of salt/brine application. If there is found to be a significant difference, the above evaluation of efficacy may need to be conducted separately for the different locations in the tank.

A.3. Development of Inspection Tools and Methodology

Partial funding for this project was used to purchase, and to development methodology for, a FlowCAM (Flow Cytometry and Microscope) tool for automated assessment of ballast water samples. Much progress has been made in the development of standard operating procedures for analysis of ballast water samples (attached as Appendix 1). In addition, funds from this project were used in combination with funding provided by Fisheries and Oceans Canada to purchase a Laser Optical Plankton Counter (LOPC). Methodology development for use of the LOPC for analysis of ballast water samples will begin in fiscal year 2009-10.

RESULTS TO DATE

Instrumentation

A major issue that had to be resolved was whether we could instrument ballast tanks on the type of NOBOB ship that was selected for the experiment. It turned out that chemical tankers were an ideal type of vessel to work with for the NOBOB ships because of their tank configuration, easy access, and consistent trading routes, however, tankers will not permit instruments to be placed in tanks unless they can be certified to be intrinsically safe from any possibility of electrical spark. The instrumentation that we have does not meet this standard, but the team collectively chose to proceed with using these ships for the experiments given their other advantages. Consequently we will only be able to add the instrumentation component of the studies to the experiments on the ballasted ships.

Instrument Maintenance and Calibration

Four YSI 6600 EDS multi-parameter sondes and two In Situ 9500 Troll multi-parameter sondes were set-up for use on the project. The addition of the two In Situ 9500 sondes provided for greater spatial coverage in the tanks and also reduced the number of new optical dissolved sensors that needed to be purchased as they were already equipped with these sensors. Project funds have been used to partially update the sondes with new optical DO sensors and new conductivity and temperature sensors. The optical DO provided accurate continuous dissolved oxygen in the tanks during shipboard experiments which helped to confirm that biological results were related to the experimental application of the brine solution and not due to natural deteriorating water quality conditions.

Prior to deployment for the first shipboard experiment, all of the conductivity sensors were calibrated with certified conductivity standards purchased from the sonde manufacturer. Dissolved oxygen sensors were tested against expected saturation levels given defined temperature and barometric conditions. During the calibration process we found one of the YSI sondes was not operational and the sonde was returned to the manufacturer for repair and re-calibration, as well as, adding a new optical DO sensor. Second, we created calibration curves for each sonde covering a range of NaCl brine solutions including 0, 30, 60, 120 ppt at three temperatures each including 8, 20, and 30 ^{o}C. These empirical calibration curves are necessary for determining actual concentrations of brine present in the tanks during experiments because the sondes are not calibrated specifically to NaCl and the reported salinity values from the sondes at conductivities above 60 mS/cm are not accurate or within the manufacturer specifications.

Shipboard Experiments

Six instruments were set-up for inclusion in the first ballasted shipboard brine experiment aboard the MV Gadwall which took place from Dec 1 – 6, 2008. Instruments were programmed to record water depth, conductivity, salinity, temperature, and dissolved oxygen at 5 minute intervals. Instruments were delivered to the University of Windsor scientific team for installation on the ship at the ballasting port of Toronto. Instructions and all required materials for installation were provided. Five instruments were distributed throughout the designated 'treatment' tank and one instrument was placed in the control tank. NaCl brine was added to the treatment tank just prior to entry into the Welland Canal a day after the tanks were initially filled with freshwater from the port in Toronto. Two Canadian scientists stayed onboard the ship to conduct a time-series of biological sampling while the ship was underway to its discharge port in Thunder Bay, Ontario. Two CILER scientists meet the ship in Thunder Bay and completed the instrument retrieval and returned all samples, equipment and personnel to Windsor.

All of the data files have been retrieved from the sondes and provided in an accompanying Excel spreadsheet. All of the sondes appeared to have functioned properly and delivered complete data sets were retrieved for each of the available sensors. Some additional calibration of the conductivity cells may be needed to confirm differences seen throughout the tank.

Details of the experimental process can be examined in the time-series of water depth and salinity recorded at 5 minute intervals over the duration of the experiment (Figure 1). The time series captures the initial filling with freshwater on Dec 1 around 15:00, the addition of approximately 0.5 m of NaCl brine on Dec 3 around 17:30, and a final discharge of the tank on Dec 6 at 16:00. A detailed time series plot of the brine addition processes in shown in figure 2. From these data you can verify the exact timing and duration of the brine addition from 17:30 to around 18:50. Comparing multiple sensors located in different positions throughout the tank indicates that it took approximately 6 hours to distribute the salinity throughout the tank (Figure 3). Interestingly there were distinct differences in the distribution of the brine within the tank itself both horizontally and vertically. There appeared to be a permanent density stratification and the salinity recorded by the sonde 2 m high in the tank was only 40 ppt versus nearly 60 ppt near the very bottom of the tank. In addition the water did not appear to mix uniformly across the tank as sondes 81-AC recorded a salinity of only 48 ppt. While the vertical stratification remained throughout the entire deployment the horizontal gradients did change while the ship was under transit (Figure 4). High salinity water appeared to seep into the lower position where sonde 81-AC was mounted, probably through lower draining holes in the vertical flooring structures.

Time series of dissolved oxygen were recorded in both treatment and control tanks to assure that general water quality conditions were similar between the two tanks and that observed zooplankton mortalities were not due to deteriorating conditions such as development of hypoxia. As expected we observed a slight decline in the dissolved oxygen concentration over time within the control tank (Figure 5). However, the cold temperatures limited the rate of this decline and concentrations remained sufficiently high such that they should have had no additional influence on any observed mortality rates of the zooplankton. A small, but immediate decline in oxygen concentration was observed with the addition of the brine in the treatment tank, but again the concentration remained well above any levels that may have impacted the biological results.

Biological Results

Replicated experiments were conducted on each BOB and NOBOB voyage. Samples were collected over a 6 day timespan for the BOB experiment. Preliminary results indicate that all taxa sampled were dead by the end of the voyage. For the NOBOB experiment, all taxa collected were exterminated at one hour after brine exposure. Taxonomic identification of samples is ongoing, and statistical analyses will be completed after completion of more ship trials.

CONCLUSION

Preliminary results look promising, but efficacy of brine treatment cannot be fully evaluated until additional shipboard trials have been completed. We will continue working with Algoma tankers for NOBOB experiments, and anticipate commencing additional trials in May 2009. We also have agreement from two companies (SCL and Canfornav) to conduct BOB trials on board operational vessels that meet our requirements in 2009.

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P.T. Jenkins (P.T. Jenkins & Associates Ltd) was contracted to plan and manage application of brine to ballast tanks during experiments. T. Johengen (U. Michigan) and D. Reid (U.S. NOAA) were contracted to instrument tanks and analyse data recovered. T. Wang (GLIER) conducted biological analyses of invertebrate samples. M. Deneau (DFO) and H. MacIsaac (GLIER) assisted with field work.

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APPENDIX 1

STANDARD OPERATING PROCEDURES FOR BALLAST WATER SAMPLE ANALYSIS

USING THE FLOWCAM

STANDARD OPERATING PROCEDURE

SAMPLE PREPARATION FOR ZOOPLANKTON AND PHYTOPLANKTON ANALYSIS

- For each sample collected employ size exclusion step i.e., pass the sample through Nitex or similar sieve having dimension of 300 um. Collect the sample passing through the sieve and the pass part of the sample through another sieve having the dimension of 50 um. The operation will remove excessively large particles that may clog the flow cell. The original sample should be gently stirred before and during this separation step. This will ensure 'homogeneity' and reduce the settling and sticking of the organism on the sides of the funnel and container.
- The zooplankton analysis will be conducted with 4x Objective and Flow Cell having depth of 300 um. The sample will be 300 um size fractionated.
- Take a known sub sample such as 10/20/50 ml from 300 um size separated ballast water sample.
- For phytoplankton, analysis will be conducted with 20x Objective and Flow Cell having depth of 50 um, with 50 um size fractionated sample.
- Take a known sub sample such as 10/20/50 ml from 50 um size separated ballast water sample.
- For 20x Objective, you may require a collimator.
- If a collimator(light focuser) or diffuser (light diminisher/spreader) is required for the chosen objective, thread it into appropriate slot(see Figure: 3 I)
- Install the Objective by threading it into appropriate cube (see Figure: 3 E)

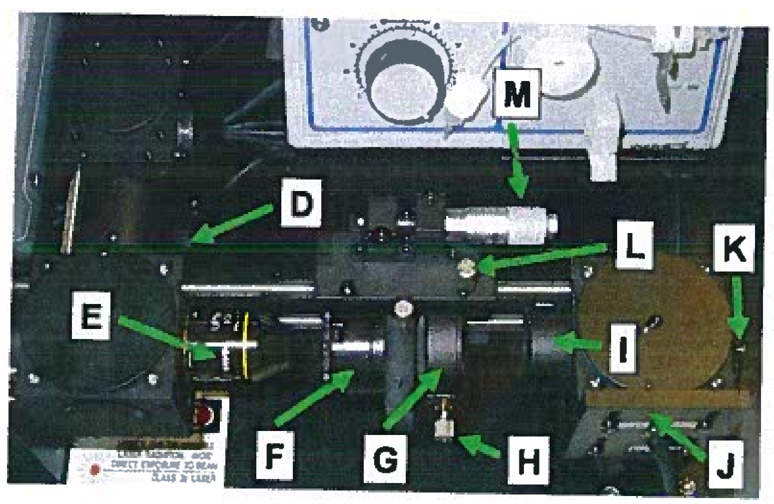

Figure 3. Components of a Bench Top FlowCAM (**Inset 2** from Figure 1)

- **D.** **532 nm Laser**
- **E.** **Microscope Objective**
- **F.** **Flow Cell Holder**
- **G.** **Flow Cell Holder Mount**
- **H.** **Flow Cell Horizontal Positioning Knob**
- **I.** **LED Collimation or Diffuser Lens**
- **J.** **Scatter Detector Adjustment Plate**
- **K.** **Flash LED**
- **L.** **Course Focus Adjustment Knob**
- **M.** **Fine Focus Adjustment Knob**

INSTALL FLOW CELL

- Take out the Flow Cell Holder from the flow cell Holder Mount Assembly (Figure: 3 F or Figure: 7 a) by loosening thumb screw by turning counter clockwise.
- Flow Cell Holder cap should be opened by turning the cap counter clockwise.
- Place the Flow Cell in the Flow Cell Holder and tightened the Flow cell Holder cap by turning it clockwise. Ensure that the inlet tube (tube to be connected to the funnel), attached to the Flow Cell is at the same end as the thumb screw on the Flow Cell Holder. During this operation one has to gently hold the Flow Cell straight as the tightening the cap tends to twist the Flow Cell. Care must also be taken not to over tighten the cap which may damage the Flow Cell.
- Reinstall the Flow Cell Holder with the Flow Cell on the Focussing Collar and tightened the thumb screw (Figure: 7 a) on the Flow Cell Holder Assembly.

Figure 7. Installation of Flow Cell

INSTALL FLOW CELL TUBING

- Cut the silicone tubing attached to the upstream side of the of the Flow Cell (this is the side through which the sample should be entering the Flow Cell), to a length of approximately 3-4 inches or whatever length is feasible, which in turn will be connected to sample funnel, to be attached to funnel stand. Minimum practical length of the tubing and straighter the tubing, better for the Flow Cell.
- Lower the supplied funnel holder ring along with the funnel, and attach inlet tubing to the funnel outlet. Raise the funnel holder ring along with the connected funnel to the point where the tube is fully extended and attach the funnel ring to the stand. This tubing connection should be set up in such a way that it is slanted a little bit to the left from vertical axis of the funnel stand.
- The pump tubing should be approximately the same diameter as the tubing that comes attached to the Flow Cell.
- Connect the output line from the Flow Cell that comes with the Flow Cell to a barb fitting with a slotted tube lock. The slotted locked fitting is wrapped around the round head (white) of the peristaltic pump (Inlet of the pump).

INSTALL WASTE TUBING

- Attach tubing from the outlet of the pump through barb connection to a waste container or measuring flask/cylinder.

ENGAGE PERISTALTIC PUMP TO FORWARD PRIME

- Put the sample in the in the funnel.
- Start the pump on **FORWARD** and use **PRIME (PURGE)** speed to draw the sample through the Flow Cell and entire length of the tubing or at least to the entry point of the pump.
- The **PRIME (PURGE)** setting on the pump will draw the sample through the Flow Cell at high rate and fill the Flow Cell completely and ensure there are no air bubbles that will interfere with data acquisition.

REMOVE BUBBLES (if there is any) FROM FLOW CELL AND ASSOCIATED TUBING ASSEMBLY

- Bubbles can be removed from smaller Flow Cells (50,100,300 um) and associated tubing by pinching the tubing between the Flow Cell and the pump (Inlet of pump) tubing. (Squeezing for 1-3 seconds, release, and repeat as necessary. Care should be taken that Flow Cells, specially the 100um Flow Cell is not damaged in the process.)
- When one is using the 20x Objective, and a collimator, either the collimator or Objectve should be screwed into the appropriate threaded slot of the FlowCAM one after another. The Flow Cell Holder with Flow Cell should be installed last on the focussing collar; care should be taken that the Objective lens does neither come into contact with finger tip nor with the Flow Cell Holder. Since there is limited space, the Flow Cell Holder Assembly should be introduced in angular position and tightened the thumb screw (Figure: 7 a). During dismantling the above configuration the principle of LIFO should be used (i.e., Last In First Out), i.e., the Flow Cell Holder should be taken out first following above precaution followed by either collimator or Objective.
- Power the monitor. The monitor is usually powered first.
- Locate and activate the power toggle marked "P" on the toggle platform of the FlowCAM(Figure:6, I= On, O= Out)

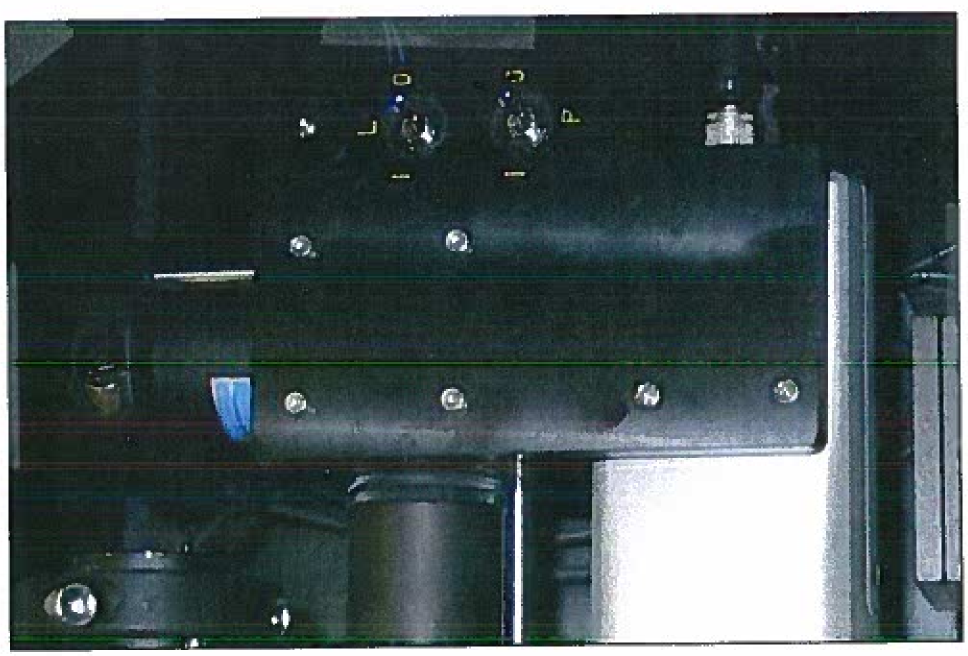

Figure 6. Location of FlowCAM's power (P) and laser (L) switches. (I = on, O = off)

OPEN THE SETUP AND FOCUS WINDOW

Main Window: SETUP > SETUP and FOCUS

This will enable to view the live footage of the Flow Cell in the SETUP and FOCUS MODE WINDOW. This window is used for the following purposes:

a) Focussing particles:

b) Positioning the Flow Cell horizontally and vertically, with X and Y positioning screw (Figure: 3G)

c) Inspecting the flow cell for imperfection: scratches, dirt etc.

d) Live viewing of particle

For focussing the following steps should be followed:

A) Rough Focus:

- To roughly focus the field of view, loosen the rail lock thumb screw (figure: 3 L). Slide the entire Flow Cell Holder Assembly to bring any part of the Flow Cell into focus. When the Flow Cell has been located, tightened the rail rock thumb screw. Extra care should betaken with 20x Objective, to prevent it from coming in contact with the Flow Cell assembly (potentially breaking the Flow Cell).

B) Fine Movement/Focussing of the Flow Cell:

- Fine positioning/focussing of the Flow Cell is done by manipulating X and Y positioning screw located at Flow Cell Holder Mount (Figure: 3G)
- For fine horizontal movement of the Flow Cell, turn the X- positioning screw (.Figure: 3G). For fine vertical movement of the Flow Cell, turn the Y- positioning screw.
- The Y- positioning screw is not shown in Figure: 3G. It is located at 90 degrees from the X- positioning screw.
- The above maneuver will locate and move scratches/cavities in the glass wall of the Flow Cell to a position beyond the field of view so that it does not interfere with the actual cell/organism count in the sample. The scratches/cavities will be perceived as particles and imaged, processed and added to various calculated measurements/parameters by the FlowCAM.
- At this point the foreign particles on the surface of the Flow Cell will be clearly visible. Clean the surface of the Flow Cell with cotton swab or similar tool, if required. Also at large magnifications, scratches and other imperfection may appear as foreign particles which will interfere with data acquisition. If these can not be placed beyond the field of view by manipulation of X and Y positioning screw, discard the Flow Cell.

TO CREATE WHITE/GREY COLOURED BACKGROUND IN THE SETUP AND FOCUS MODE WINDOW:

- MAIN WINDOW: SETUP > SET UP and FOCUS > SETUP and FOCUS MODE WINDOW
- SETUP and FOCUS MODE WINDOW: SETUP > CAMERA > CAMERA 1 SET UP WINDOW
- CAMERA 1 SET UP WINDOW: CAMERA SETTING
- To make background of SETUP and FOCUS MODE WINDOW grey or white (grey is the optimal), use the colour coordinate U and V.

IF NECESSARY ADJUST THE FIELD OF VIEW ILLUMINATION:

- MAIN WINDOW: SETUP > SETUP and FOCUS > SETUP and FOCUS MODE WINDOW
- SETUP and FOCUS MODE WINDOW: SETUP > CAMERA > CAMERA 1 SET UP WINDOW
- CAMERA 1 SET UP WINDOW: CAMERA SETTING >Adjust Flash and Gain

Or,

- MAIN WINDOW: SETUP > CONTEXT> CAMERA

The values for the camera Gain and Flash Duration is adjusted to optimize the intensity of the field of view illumination for best images all around the field of view.

First Adjust the Gain:

- Higher the value of Gain, the brighter is the field of view. Too high a Gain will result in the loss of contrast between the particles and background. Image brightness can also be controlled by LED (Light Emitting Diode) screw adjustment. So the brightness/illumination should be optimized in conjunction with Gain and by manipulating the LED screws. When using LED screw you should use small turns to gauge the effect of each turn on illumination.
- The default value for camera Gain for 4x, 20x Objectives are 1 and 3 respectively. Increasing camera Gain at higher magnification (20X) may increase brightness, quality and focus, whereas lower optical setups (2x, 4x) may be able to operate at a lower camera Gain. Increase in camera Gain also increases background electronic noise.

Then Adjust the Flash Duration

- The default value for Flash Duration for 4x, 20x Objectives are 40. Here again, higher the value, the brighter will be the field of view.
- The intensity value is given as minimum, maximum and mean. For optimal illumination the supplier of the FlowCAM has suggested the following intensity values:

  Maximum=190;
  Minimum=180;
  Mean=170;

  Intensity is the function of Flash Duration and Flash/Camera Delay. These values can be manipulated by Flash Duration and Flash/Camera Delay as follows:

- MAIN WINDOW: SETUP > SETUP and FOCUS > SETUP and FOCUS MODE WINDOW
- SETUP and FOCUS MODE WINDOW: SETUP > CAMERA > CAMERA 1 SET UP WINDOW
- CAMERA 1 SET UP WINDOW: CAMERA > Adjust Flash/Camera Delay and Flash Duration

Or
MAIN WINDOW: SETUP > CONTEXT> CAMERA > Adjust Flash/Camera Delay and Flash Duration.

The default value for Flash Duration and Flash/Camera Delay for 4x, 20x Objectives are 40 and 50 respectively.

C) FINE FOCUS ON PARTICLES USING MICROMETER:

- Switch the peristaltic pump from **FORWARD** to **OFF** position.
- Perform fine focus by adjusting the micrometer adjustment knob (Figure 3: M)

CLOSE THE SETUP and FOCUS WINDOW

OPEN THE CONTEXT WINDOW AND REVISE AND REVIEW SETTING

- Large number of parameters in the Context Window has to be set, before running experiments to fulfill objectives of the experiments. Every time parameters are selected in the Context Sub Windows the "OK" button has to be clicked before navigating to other Sub Window.

CLOSE THE CONTEXT WINDOW

CONFIGURE THE MAIN WINDOW

- MAIN WINDOW: PREFERENCES > MAIN WINDOW LAYOUT > LAY OUT > Select the number and type of graph and click the other tabs from various Sub Windows. (Main Window Layout)

PROCEDURE FOR RUNNING SAMPLE IN AUTOIMAGE MODE

AutoImage mode takes images of moving particles in the field of view at a regular, user defined interval. (This is done by adjusting AutoImage Rate). Images of counted particles are stored on a disk along with count information and particle size as measured from the images. The volume analysed is determined from flow cell dimensions and the concentration of the particles is reported at the end of the run. This mode is useful when counting the cells with high particle concentration, where fluorescence is not important or not present.

- Determine and set your initial "Context" setting
- Introduce sample to the FlowCAM.
- Click on the SETUP menu in the Main Window and select "Set Up and Focus". Focus and adjust illumination of your FlowCAM as discussed previously.
- Note "Particle per Used Image "at the left down corner of SETUP and FOCUS MODE WINDOW.
- Determine if the sample is too concentrated and dilute as needed.
- Click on the SETUP menu in the Main Window and select "AutoImage Mode (No Save)"
- Change the context setting as necessary. Be sure to click "OK" every time you

change context setting in every Context Sub Window before exiting that Sub Window.

- Determine if your focus is correct and if the sample is too concentrated, dilute if necessary. This is the good time to test the effects of using different context setting.
- Close "AutoImage Mode (No Save)" once concentration and focus are both deemed to be appropriate.
- Click on the "Imaging" menu option on the Main Window.
- Select "AutoImage Mode" under Imaging Menu in the Main Window.
- Select your stop criteria in the Stop Sub Window and click "OK".
- Save the run in an appropriate location with any file name you wish and click "OK". If you don't give the file name, the system will give file name as follows: JJJ-hhmmss.xxx where JJJ is 001 on January 1st and 365 on December 31. hh = hour of the day. mm = minute of the hour and ss = seconds after the minute. Finally .xxx = is the file name extension for the stored file. The automatically generated prefix for data files can be overridden. Whenever an experiment is performed the user is prompted for experiment name and the default is used only when an alternative is not entered.
- Allow the run to finish.
- Repeat the above procedure for each sample.

OPEN THE TRIGGER MODE SET UP

WARNING:
DO NOT LOOK AT THE LASER DIRECTLY

- Locate the laser toggle marked "L" on the toggle platform on the FlowCAM and activate it (Figure: 6; I= On, O= Out). Allow the laser to warm up for about fifteen minutes.
- The optimal dilute solution with respect to particle image capture should have one "Particle per Used Image". This means that only one trigger able particle should be present per field of view.
- The "Particles per Used Image" can be accessed during Set Up operation as follows:

  MAIN WINDOW: SETUP > SETUP and FOCUS > SETUP and FOCUS MODE WINDOW
- At the left down corner SETUP and FOCUS MODE WINDOW will give the value of the "Particles per Used Image" parameter. The value of this parameter should be between 1 to 1.1. If the value is greater than 1.1, you should dilute your sample.
- MAIN WINDOW: SET UP > CONTEXT> PMT/Scatter Tab
- Adjust the Threshold value of PMT (Photomultiplier Tube)
- Channel 1 is the > 650 nm PMT (Red fluorescence)
- Channel 2 is the > 575 nm PMT(Green fluorescence)
- Channel 3 is the scatter detector.

The Threshold controls the scatter or fluorescence level at which the camera will be triggered to take picture. Setting threshold value lower than 400 will capture background noise as particle.

PROCEDURE FOR RUNNING SAMPLE IN TRIGGERED MODE

The diffuser must be removed when running in trigger mode, otherwise the sensitivity of the scatter detector will be significantly diminished.

- Determine and set your initial "Context" setting
- Introduce sample to the FlowCAM.
- Click on the SETUP menu in the Main Window and select "Set Up and Focus". Focus and adjust illumination of your FlowCAM as discussed previously.
- Note "Particle per Used Image "at the left down corner of SETUP and FOCUS MODE WINDOW.
- Determine if the sample is too concentrated and dilute as needed.
- Click on the SETUP menu in the Main Window and select "Trigger Mode Setup"
- Change the context setting as necessary. Be sure to click "OK" every time you change context setting in every Context Sub Window before exiting that Sub Window.
- Close "Trigger Mode Setup"
- Click on the Set Up menu on the Main Window and select "Trigger Mode (No Save).
- Determine if your focus is correct and if the sample is too concentrated, dilute if necessary. This is the good time to test the effects of using different context setting.
- Close "Trigger Mode (No Save)" once concentration and focus are both deemed to be appropriate.
- Click on the "Imaging" menu option on the Main Window.
- Select "Trigger Mode" under Imaging Menu in the Main Window.
- Select your stop criteria in the Stop Sub Window and click "OK".
- Save the run in an appropriate location with any file name you wish and click "OK". If you don't give the file name, the system will give file name as follows: JJJ-hhmmss.xxx where JJJ is 001 on January 1st and 365 on December 31. hh = hour of the day. mm = minute of the hour and ss = seconds after the minute. Finally .xxx = is the file name extension for the stored file. The automatically generated prefix for data files can be overridden. Whenever an experiment is performed the user is prompted for experiment name and the default is used only when an alternative is not entered.
- Allow the run to finish.
- Repeat the above procedure for each sample.

SHUTTING DOWN THE FLOWCAM

- Immediately after use, flush the Flow Cell with deionized or distilled or sea water.
- If necessary, precede this with rinse with appropriate solvent or surfactant in order remove particles. This will keep Flow Cells clear free from contamination from day to day use.

- For longer storage periods, the Flow Cells can be filled with a weak bleach solution (2 – 5%).
- The tubing can be sealed off on both ends to keep the solution from leaking out or use a double ended connector to create a closed loop.

RELEASE PUMP TUBING

- To extend the life of pump tubing, release one end from the peristaltic rollers when instrument is not in use.

TURN OFF FLOWCAM

- Turn off the laser.
- Close VisualSpreadsheet.
- Go to START menu and select "Shut Down." The computer is shut down once the screen goes black.
- Power off the monitor if desired.

If the instrument is not going to be used for a fairly long time (greater than 8 hours), it is recommended that the FlowCAM be shut down by switching the "P" button.