Microcosm Evaluation of Community Level Physiological Profiling in Oil Sands Process Affected Water

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Alberta Innovates – Technology Futures

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Oil Sands Research and Information Network

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Citation

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REPORT SUMMARY

A microcosm-based experiment was conducted to investigate the ability of community level physiological profiling (CLPP) to detect changes in an aquatic microbial community resulting from exposure to oil sands process affected water (OSPW). Detection of the microbial response was done by using the Biolog EcoPlate system, a commercially-available system originally developed for the assessment of rhizosphere microbial communities. The Biolog system consists of a 96-well microtitre plate. Each well on the plate contains both a pure organic compound (the substrate), and a tetrazolium dye. When a microorganism metabolizes the substrate, the dye is reduced into a purple formazan product. The purple colour of each well is characterized using a spectrophotometer measuring optical density (OD) at 590 nm. In this study, we used the EcoPlate version of the Biolog System.

Reductions in metabolic activity and inoculum density were detected in the high OSPW group. Overall, indicators of microbial metabolic activity decreased over time. One of these indicators, the sum of substrate means (SSM), showed a dramatic response to weekly water changes. Low cyclicity naphthenic acids demonstrated a reduction over the first and last weeks of the exposure period. Higher cyclicity naphthenic acids demonstrated reductions in the first but not the last week of exposure. The total naphthenic acid (TNA) content of the microcosms appeared to increase over the last week of the exposure period, which may reflect the accumulation of products of microbial metabolism.

Our results suggest that inoculum density remains a source of variability for CLPP results. Furthermore, the biological context under which the microbial community forms has a strong influence on its metabolic characteristics. The changes in naphthenic acid concentration (total and speciated) likely reflect adsorption and/or microbial metabolism. Our observation of increased phytoplankton in the presence of OSPW is consistent with the available literature. Additional research will be required to determine if this finding can be developed into an indicator of toxic effect, rather than just the presence/concentration of OSPW.

ACKNOWLEDGEMENTS

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1 BACKGROUND

The development of oil sands resources in northern Alberta impacts aquatic ecosystems (Grant et al. 2008), sometimes through the introduction of oil sands process affected water (OSPW) (Barr 2009, Legg 2009, Leonhardt 2003, Whelly 1999). Monitoring aquatic ecosystems for disturbance associated with the presence of OSPW is a difficult task. The traditional approach often involves a great deal of labour, time, and expense associated with surveys of various chemical, physical, and biological parameters. To make this process more efficient, Alberta Innovates – Technology Futures (AITF) has been investigating the use of microbial community level physiological profiling (CLPP) as an ecological monitoring tool. The potential of CLPP as a monitoring technology was recently summarized in a review document for OSRIN (Davies and Eaton 2011).

In this project we investigated the ability to detect a microbial response to the presence of OSPW using the Biolog EcoPlate system, a commercially-available system originally developed for the assessment of rhizosphere microbial communities. The Biolog system consists of a 96-well microtitre plate; each well contains both an organic substrate and a tetrazolium dye. When a microorganism metabolizes the substrate, the dye is reduced into a purple formazan product. The purple colour of each well is characterized using a spectrophotometer measuring optical density (OD) at 590 nm. In this format, the 96-well plate contains 31 carbon compounds plus a control well replicated 3 times across the plate.

One of the toxic components of OSPW is a collection of compounds known as naphthenic acids (Frank et al. 2008, MacKinnon and Boerger 1986, Nero et al. 2006). The microbial degradation of these, and similar, compounds seems to be a community-based aerobic phenomenon (Del Rio et al. 2006, Tian et al. 2008). Since our assay is based on the aerobic heterotrophic portion of the microbial community, we hoped to find some metabolic indicator of this capacity to degrade naphthenic acids within the EcoPlate array. We also wished to investigate whether this microcosm approach could be used to select for a microbial community well adapted to the degradation of these compounds. To this end water samples were collected during the first and last weeks of the exposure period and were used to measure total oil sands acid extractable organics (OSAEO), total naphthenic acids (TNA), and speciated naphthenic acids.

2 MATERIALS AND METHODS

2.1 Microcosm Preparation and Maintenance

Ten-litre glass aquaria (25 cm x 25 cm x 20 cm) were purchased from Aquarium Illusions (Edmonton, Alberta, Canada) for the study. The aquaria were washed with soap and water, rinsed with tap water, rinsed with 5% bleach, rinsed again in sterile deionized water and covered in plastic film prior to filling. Once the experiment began, the tanks remained open to ambient atmosphere. Full spectrum fluorescent lights (Fluval PCL 13, Rolf C. Hagen Inc., Baie d'Urfé, Quebec, Canada) were attached and centered on each tank. On the first day of experimentation, the microcosms (aquaria) were filled with water collected from a marsh just west of the AITF Vegreville main building (427107 m E, 5928769 m N, zone 12U). Water was collected in new

plastic carboys on August 16, 2011 and stored at -20°C until processing. Processing consisted of passing the marsh water sequentially through a standard aquarium net to remove large debris, 4 to 5 coffee filters to remove large particles and a 0.45 µm pore size capsule filter (Polycap GW, Whatman) to remove finer particulates and the bulk of the microbial population. After processing, the filtered marsh water was stored in disinfected plastic carboys and frozen at -20°C.

Raw OSPW was received from two oil sands companies. This OSPW was filtered and stored in the same manner as the marsh water. Samples from each company were mixed in equal volumes to produce a composite OSPW. This composite OSPW was used to 'dose' the microcosms (from here on the term *OSPW* refers to the composite OSPW).

At weekly intervals, 50% of aquarium fluids (i.e., either pure marsh water or the mixture of OSPW diluted in marsh water) was removed and replaced with similar fresh material. After an initial colonization period of 3 weeks, OSPW was introduced to the tanks. Natural wetlands in the Athabasca oil sands region have been documented to contain 0 to 2 mg/L total naphthenic acids, while the wetlands most severely affected by OSPW may contain in excess of 60 mg/L (Hadwin et al. 2006, Leonhardt 2003). The microcosms in this experiment were assigned to four levels of naphthenic acid concentration with four replicates per level (total of 16 aquaria; Table 1) to bracket much of the range of NA concentration encountered in northeastern Alberta.

Table 1. Experimental Groups.
Experimental treatments were distributed equally across blocks of tanks (microcosms), such that each set of four contained one tank at each treatment level.

Group	Microcosm Tank #	Oil Sands Process Affected Water Content (% by volume)	Targeted Total Naphthenic Acids Content (approximate) (mg/L)
Control	1, 5, 9, 13	0	0
Low	2, 6, 10, 14	0.25	0.25
Mid	3, 7, 11, 15	2.5	2.5
High	4, 8, 12, 16	25	25

2.2 OSPW Toxicity Assessment

Our initial intent was to dose the microcosms based on the results of a *Daphnia magna* toxicity assay which would determine the LC50 for OSPW in our microcosms. Accordingly, 1 L samples of the filtered marsh water and composite OSPW were sent to Maxxam Analytics (Edmonton, Alberta, Canada) for the toxicity assay. The assay was run for 48 hours at

concentrations of 0, 6.25, 12.5, 25, 50% (v/v) OSPW in marsh water as well as 100% (i.e., pure) OSPW.

2.3 Analytical Chemistry

Microcosm water samples were collected at the start and end of the first and fourth weeks of OSPW exposure. Water samples were collected between water changes such that any chemical changes were the result of the processes occurring within the microcosms, and not confounded by the water change.

Chemical analysis of the microcosm water was undertaken to determine the oil sands acid extractable organic (OSAEO) and TNA content, and to see how those values changed over time. Water samples (1 L) were adjusted to pH ~10 with 2 M sodium hydroxide, and then extracted with hexane to remove the base/neutral fractions. Sample pH was then adjusted to <2 with phosphoric acid and extracted with dichloromethane. The extract was dried through acidified sodium sulfate and concentrated to 500 µL with nitrogen.

An 'authentic' oil sands standard, prepared as per Rogers et al. (2002), was obtained from Environment Canada and both the OSAEO and a TNA value were determined using this standard. This method attempts to isolate the classic naphthenic acids from the OSAEO fraction but does not restrict the TNA value from containing compounds that are non-classic such as dicarboxylic or hydroxylated compounds or those which incorporate nitrogen and sulfur.

An underivatized portion of the sample extract was run on a Varian GC/MS-ion trap using a mass range of 40 to 550 m/z and data were collected for the full chromatographic time window. This process gives a value for acid extractable compounds[1].

Naphthenic acid concentration and speciation methods are based on those of Holowenko et al. (2002). After extraction, an aliquot of the samples and standards was derivatized with N-methyl-N-(tert-butyldimethylsiyl) trifluoroacetamide containing 1% tert-butyldimethylsilylchloride. This procedure converts the carboxylic acid group to an ester; when analyzed on a GC/MS-ion trap, the molecular ion of each of the classic naphthenic acids can be identified and speciated. The naphthenic acids for the range of $C_6Z_0O_2$ to $C_{30}Z_6O_2$ were determined. This series gives a mass range of 173 to 509 m/z (with derivatization this corresponds to M+57). The mass spectrometer is then set to collect data only for a mass range of 170 to 510. This narrow mass range, as well as a restricted chromatographic time window, helps isolate the naphthenic acid component from the other compounds in the OSAEO fraction. The method detection limit was 0.10 and 0.02 mg/L for OSAEO and TNA respectively.

[1] For further information on analytical methods for naphthenic acids see Zhao, B., R. Currie and H. Mian, 2012. *Catalogue of Analytical Methods for Naphthenic Acids Related to Oil Sands Operations*. OSRIN Report No. TR-21. 65 pp.

2.4 Biofilm Generation, Harvest and Processing

Biofilms formed on cylindrical acrylic rods approximately 5 cm in length and 1 cm in diameter. These rods were suspended to a depth of approximately 8 cm, which allowed the rods to remain submerged during water changes. Rods were installed in each aquarium according to the schedule described in Table 2. Pre-exposure rods were installed at the beginning of the experiment (study day -21) and harvested on study days -1, 0 (the day OSPW was added to the microcosms), 1, 7, 14, 21, 28, 35, 42 and 49. OSPW was removed from the microcosms on study day 28, after all samples had been collected, by a 100% change in microcosm water. Post-exposure rods were installed at study day 0 (after the water change), and harvested on days 7, 14, 21, 28, 35, 42 and 49. Water samples for analytical chemistry were taken just after water changes on days 0 and 21, and just before water changes on days 7 and 28. This arrangement allowed us to determine the effect(s) of microbial metabolism on OSAEO, TNA, and speciated naphthenic acids over 1-week intervals.

Rods were collected and stored in small volumes (20 to 40 mL) of microcosm water until they could be processed. Rods were transferred from their storage water into 20 mL of room temperature sterile pH 7.2 phosphate buffered saline (PBS). Rods in PBS were then sonicated using a Bransonic 220 sonicating bath for 15 minutes. After sonication, the 20 mL suspension was passed through a sterile syringe filter (5 µm pore-size nylon membrane) to remove the bulk of the phytoplankton and filamentous fungi from the sample. It has been well established that inoculum density (i.e., the concentration of actively respiring aerobic microbes) can have a dramatic effect on the results of CLPP investigations (Christian and Lind 2006, Garland and Mills 1991, Konopka et al. 1998, Preston-Mafham et al. 2002). We attempted to normalize inoculum density by using total ATP as an indicator of microbial metabolic activity. The filtered suspension was assayed for total ATP using a Hygiena SystemSURE plus ATP bioluminometer and an Aquasnap Total sampling device. The filtrate was diluted with sterile PBS to achieve a calculated total ATP value of 10 relative light units (RLU). On study day 49, an additional set of EcoPlates was inoculated with a uniform dilution (1:20) of the filtrate produced from the last pre-exposure rods; this was done in response to some unexpected results from earlier inoculations. Apart from using a pipetter to apply samples to the Aquasnap, ATP measurement was conducted according to the manufacturer's instructions.

The concentration of colony forming units (CFU/mL) was used as an indicator of inoculum and filtrate density. Colony counts were done on 10 µL samples spread over duplicate R2A agar plates, which were incubated aerobically in darkness at 20°C for 6 days. All colony counts were done under magnification, and mean CFU calculated from each pair of R2A plates.

Biolog EcoPlates were inoculated with 100 µL of ATP-normalized inoculum per well. EcoPlates were incubated aerobically in darkness at 20°C for 3 days. Ecoplates were read for optical density at 590 nm using a Biolog Microstation running under automatic mode.

Table 2. Study Schedule.

The timing of water changes, water chemistry sampling, rod (biofilm substrate) installation, and rod harvest is described in the table below. Water changes were generally 50% (i.e., 5 L of water was changed in a 10 L tank), with the exception of the study day 28 water change, which was 100%.

	Study Day												
	-21	**-14**	**-7**	**-1**	**0**	**1**	**7**	**14**	**21**	**28**	**35**	**42**	**49**
Study Period	**Pre-Exposure**				**Exposure**						**Recovery**		
Water Change	X	X	X		X		X	X	X	X	X	X	X
Water Chemistry					X		X		X	X			
Rods Installed	Pre-Exposure				Post-Exposure								
Harvest Pre-Exposure Rods				X	X	X	X	X	X	X	X	X	X
Harvest Post-Exposure Rods							X	X	X	X	X	X	X

2.5 Data Collection and Analysis

Each carbon substrate is replicated three times on the 96-well EcoPlate, for a total of 31 unique carbon substrates and one water blank control (Table 3). Optical density (OD) for each substrate was calculated as the mean of all three replicates. The substrate was considered "used" if the mean OD was greater than the upper threshold value, and "unused" if less than or equal to this value. Threshold values are determined by the Biolog software installed on the Microstation computer. From this information we calculated metabolic richness as the sum of all "used" substrates (e.g., a plate on which 12 substrates were coded as "used" would have a metabolic richness of 12). A second metabolic measure, the sum of substrate means (SSM), was calculated by adding the mean ODs from all substrates found on the EcoPlate, regardless of whether they were classified as "used" or not. This measure can be obtained with any suitable spectrophotometer, and provides an index of overall catabolic activity irrespective of which compounds are being metabolized.

Statistical analyses were performed using SAS 9.3 for Windows x64-based systems. Statistical significance was defined by $p < 0.05$.

Filtrate density, inoculum density, SSM, metabolic richness, OSAEO concentration, and TNA concentration were analyzed separately using a repeated measures analysis of variance. Data were normalized prior to analysis using a log-transformation of the value plus a constant. Post-hoc testing for significant differences between study days, groups, and groups by day interaction was carried out using Tukey-Kramer adjusted comparisons. Microbial parameters were analyzed separately for pre- and post-exposure rods.

For the pre-exposure rods, log regressions of the SSM response to inoculum density were used to investigate the metabolic effects of OSPW. A separate regression was run for rods collected from each group within the exposure period (i.e., study days 0, 1, 7, 14, 21, and 28). The slopes of the regression lines were compared between groups by using an indicator variable in the combined regressions and then testing for the significance of parameter estimates in the model fit. This analysis could not be done for the post-exposure rods, as metabolic activity in these rods dropped off too precipitously.

Table 3. Substrates Present on the Biolog Ecoplate

Carbon Compound	Category
A1 - blank	blank control
A2 - β-Methyl-D-glucoside	carbohydrate
A3 - D-Galactonic Acid γ-lactone	carbohydrate
A4 - L-Arginine	amino acid
B1 - Pyruvic acid methyl ester	carboxylic acid
B2 - D-Xylose	carbohydrate
B3 - D-Galacturonic acid	carboxylic acid
B4 - L-Asparagine	amino acid
C1 - Tween 40	polymer
C2 - i-Erythritol	carbohydrate
C3 - 2-Hydroxy benzoic acid	carboxylic acid
C4 - L-Phenylalanine	amino acid
D1 - Tween 80	polymer
D2 - D-Mannitol	carbohydrate
D3 - 4-Hydroxy benzoic acid	carboxylic acid
D4- L-Serine	amino acid
E1 - α-Cyclodextrin	polymer
E2 - N-acetyl -D-Glucosamine	carbohydrate
E3 - γ-Hydroxybutyric acid	carboxylic acid
E4 - L-threonine	amino acid
F1 - Glycogen	polymer
F2 - D-Glucosaminic Acid	carboxylic acid
F3 - Itaconic acid	carboxylic acid
F4 - Glycly - L-Glutamic Acid	amino acid
G1 - D-cellobiose	carbohydrate
G2 - Glucose-1-Phosphate	carbohydrate
G3 - α-Ketobutyric acid	carboxylic acid
G4 – Phenylethylamine	amine
H1 - α-D-Lactose	carbohydrate
H2 - D,L -α- glycerol phosphate	carbohydrate
H3 - D - Malic Acid	carboxylic acid
H4 – Putrescine	amine

The naphthenic acid speciation results quantify a range of acidic organic compounds from both OSPW and non-OSPW sources (i.e., marsh water and microbial production). These compounds are categorized into a set of sub-ranges, each of which is defined by the mass of a classical naphthenic acid of a certain size and cyclicity. For example, all organic acid compounds which have the same mass as the classical naphthenic acid composed of 12 carbon atoms and one ring structure would be found under the C12Z2 mass sub-range. We selected 17 specific mass sub-ranges as indicators of OSPW-sourced naphthenic acids (Bataineh et al. 2006, Martin et al. 2008). We expected Z-family, as an indicator of cyclicity, to have a greater effect on level of degradation than did carbon number (Han et al. 2008). As such, the sub-ranges were categorized according to Z-family (Table 4) and the chromatographic area, summed for each Z-family. Values for chromatographic area, an approximation of quantity, were normalized using a square root transformation, and then analyzed using a repeated measures analysis to evaluate differences between groups, study days, and group by study day interaction.

Table 4. Mass Sub-Ranges Included in Statistical Analyses.

	Z-Family			
	-2	-4	-6	-8
Carbon Number	12, 13, 14, 15	12, 13, 14, 15, 16	12, 13, 14, 15, 16	14, 15, 16

3 RESULTS

3.1 *Daphnia magna* Toxicity

The 48-hour *Daphnia magna* toxicity assay resulted in no mortality or signs of morbidity at any of the concentrations tested, including pure (100%) OSPW. This suggests the OSPW had aged to some degree, as the toxicity of OSPW is known to decrease with age (MacKinnon and Boerger 1986).

3.2 Gross Observations

Over time, visible films of material accumulated on the rods, walls, and floors of the microcosms. This film was variably composed of green, brown, and filamentous gray/white material. While all of the microcosms had a similar appearance prior to OSPW exposure, the high concentration group seemed to accumulate green material more rapidly than did the others. By the fourth week of exposure, two of the high concentration microcosms (#12 and #16 – 25% OSPW v/v) were considerably greener than the others (Figures 1 and 2). In addition, all of the high concentration microcosms (#'s 4, 8, 12, and 16) produced dark brown spots on their floors. Similar but more diffuse spots were also seen in some of the mid-concentration

microcosms. Both of these characteristics disappeared almost completely over the 3-week recovery period (Figures 3 and 4).

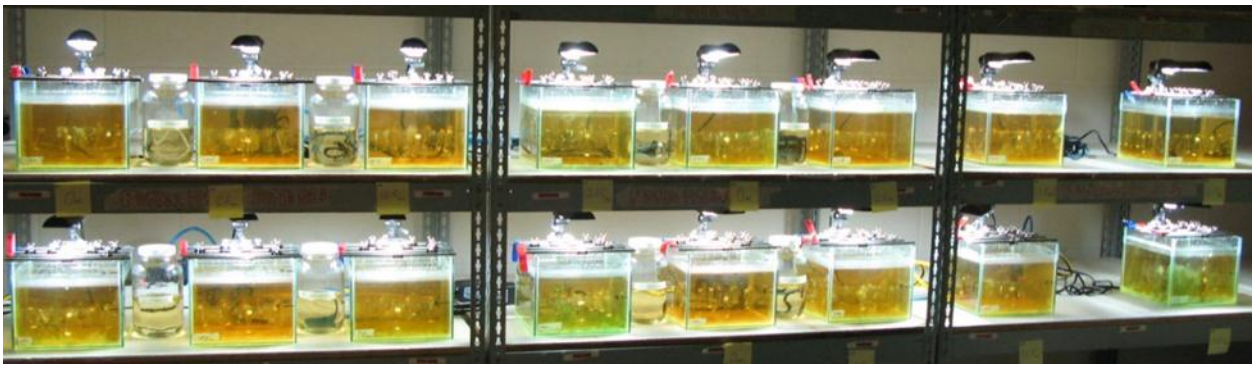

Figure 1. Microcosms on Study Day 28.
Microcosms #1 to #16 are arranged from top left to bottom right. Note that the high concentration microcosms, particularly #12 and #16, appear distinctly greener than the other treatments.

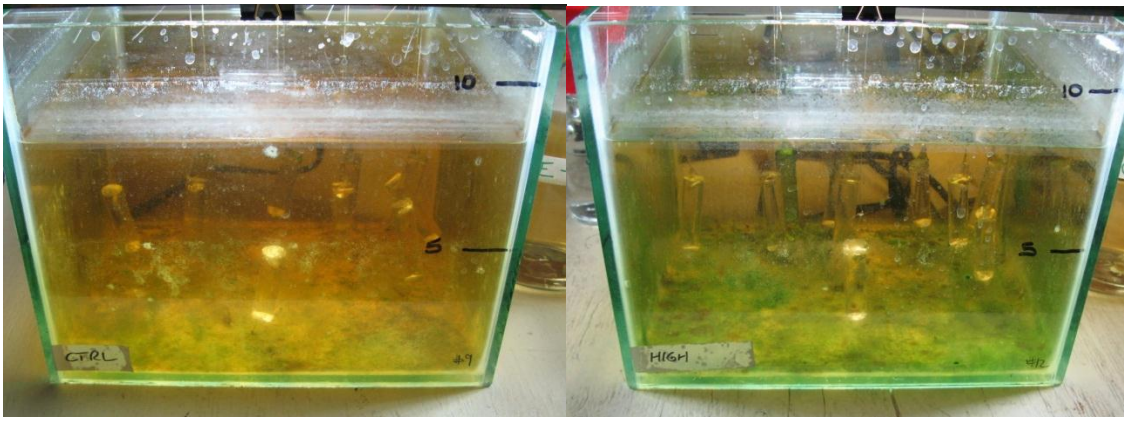

Figure 2. Microcosm #9 and #12 on Study Day 28.
Microcosm #12 (right), from the high concentration group appeared much greener than microcosm #9 (left) from the control group. Well demarcated dark spots are present on the floor of Microcosm #12.

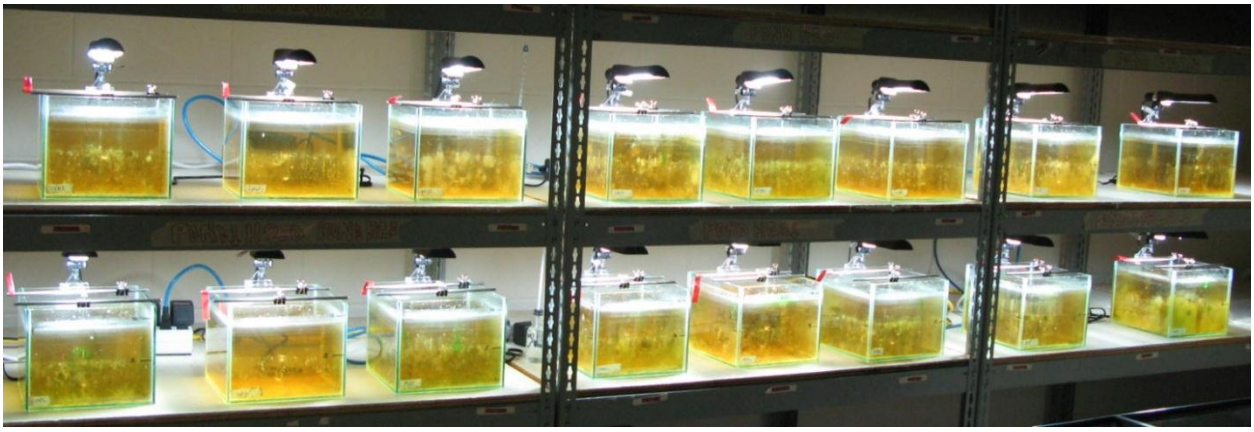

Figure 3. Microcosms on Study Day 49.
No group-wise variation in appearance was observed by the end of the recovery period.

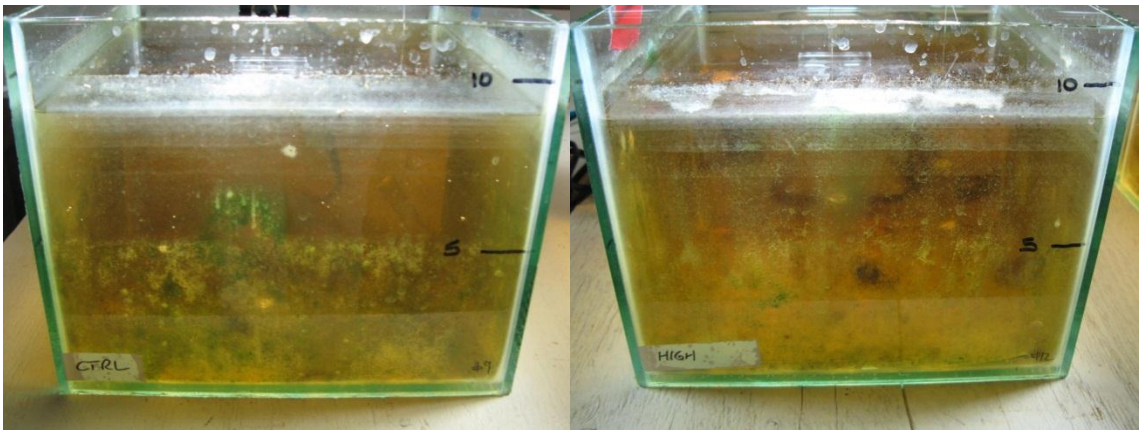

Figure 4. Microcosms #9 and #12 on Study Day 49.
While both microcosms demonstrate more advanced biofilm development, the distinctive green appearance of microcosm #12 has disappeared. Brown spots were often obscured by biofilms and/or reflections from the water's surface. However, where visible, these spots appeared much less distinct by study day 49.

3.3 Pre-Exposure Rods

In general, filtrate density remained between 10^5 and 10^6 CFU/mL. There was, however, a significant increase in filtrate density at study day 1, roughly 24 hours after a water change, compared to pre-exposure values (study day -1; $p<0.0001$). There was a gradual increase in filtrate density after study day 21 (Figure 5). Inoculum density, which was subject to an ATP normalization procedure, fell within the range of 10^3 to 10^4 CFU/mL over the course of the experiment. However, the study day 1 value for inoculum density was not significantly different from the pre-exposure values ($p=1.0000$). Inoculum density appeared to decrease until study day 21, at which point its changes largely mirrored those of filtrate density.

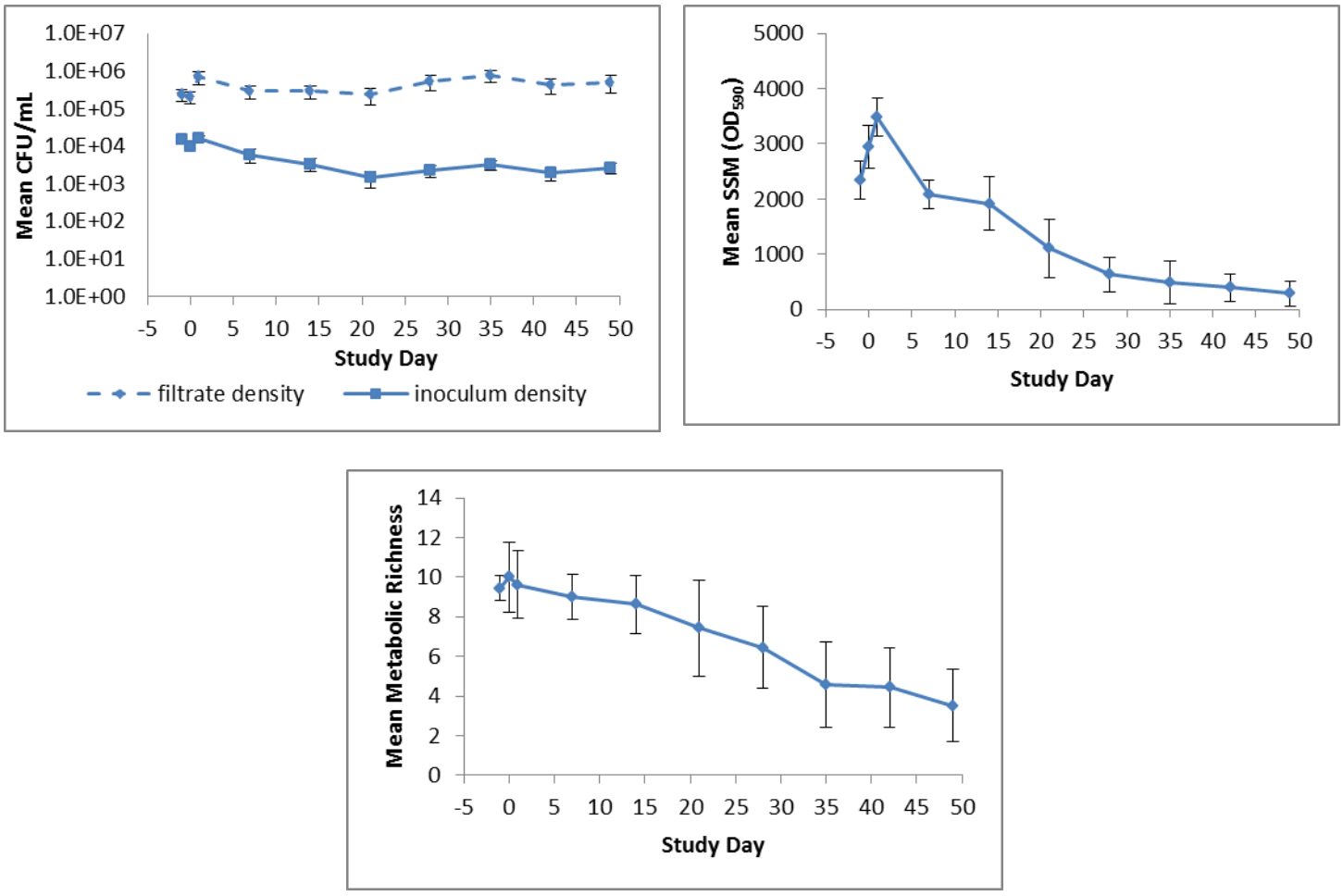

Figure 5. Mean Microbial Values (+/- Standard Deviation) for Pre-Exposure Rods. Inoculum and filtrate density (top left), SSM (top right), and metabolic richness (bottom) are plotted against study day. Note the sharp increase in SSM over study days 0 and 1 is not reflected in inoculum density or metabolic richness.

Plate-wide optical density, as measured by SSM, showed statistically significant increases on study days 0 and 1, as compared to the pre-exposure values ($p=0.0410$ and 0.0023 for days 0 and 1, respectively). SSM dropped until becoming relatively stable on study day 28. In contrast, metabolic richness showed no significant changes over study days -1, 0, and 1. Over the course of the experiment, metabolic richness tended to decrease until stabilizing somewhat at day 35.

The response of pre-exposure rod SSM and metabolic richness to inoculum density is shown in Figure 6. A dramatic shift in response is seen at approximately 4,000 CFU/mL. Above this threshold, the SSM value seldom drops below approximately 1,600. Below 4,000 CFU/mL, SSM and metabolic richness show similar patterns, demonstrating a 'turn' in the scatterplot at approximately 4,000 CFU/mL.

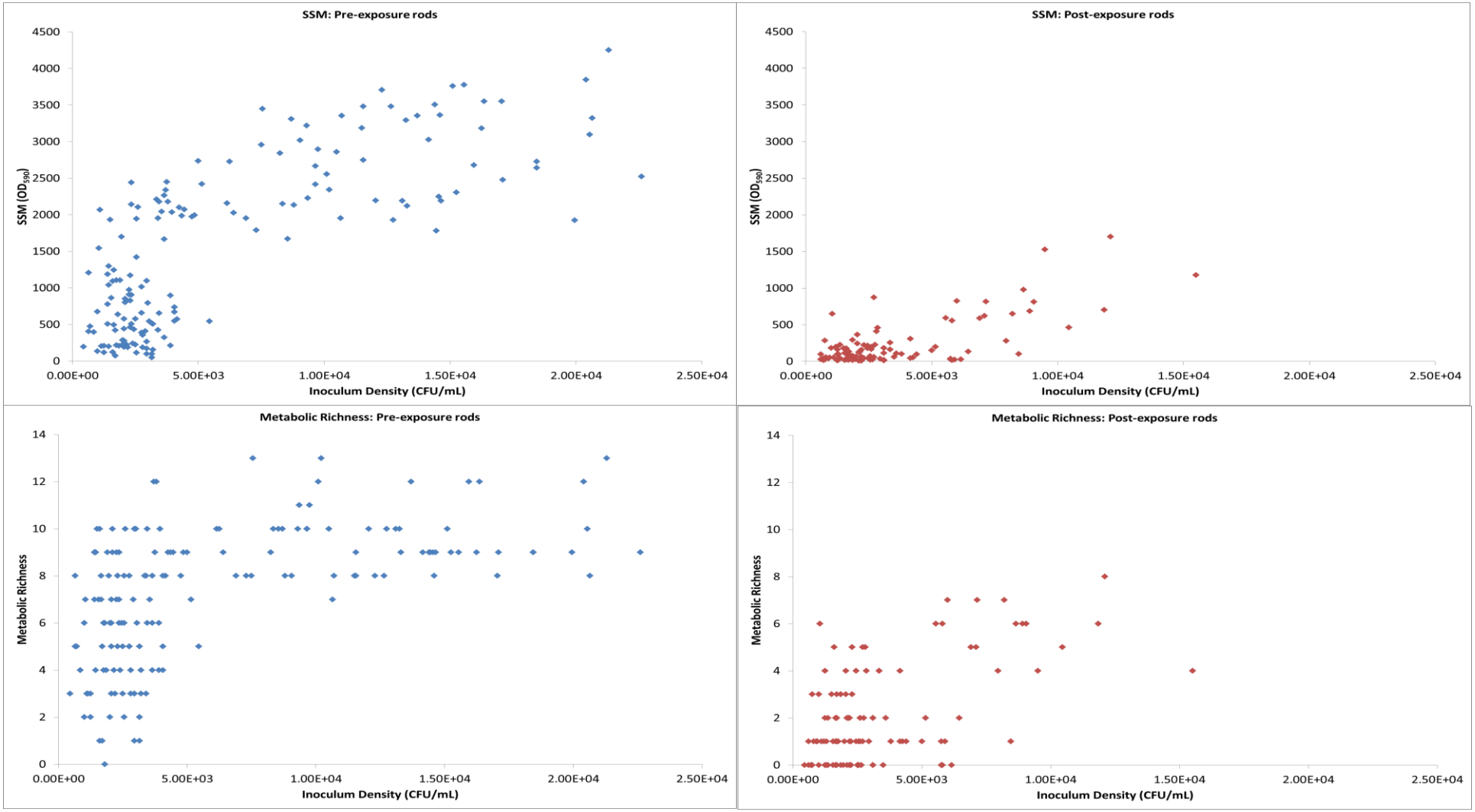

Figure 6. Scatter plots of SSM and Metabolic Richness against Inoculum Density for Pre- and Post-Exposure Rods.

SSM appeared to respond to inoculum density in a logarithmic manner when plots were constructed using data collected during the OSPW exposure period (study days 1, 7, 14, 21, and 28). Moreover, this log curve appeared to 'flatten' as OSPW concentration increased. Intersecting but divergent logarithmic regression curves explain the turn identified in the previous pre-exposure scatterplots. Figure 7 displays the data with log regression lines for each experimental group. Table 5 provides the regression equation for each group and the corresponding coefficients of determination (R^2). The slope of the SSM response to inoculum density curve for the high concentration group differed significantly from the slopes of the curves for all other experimental groups (p= 0.0025, 0.0481 and 0.0425 for the control, low, and mid concentration groups respectively). No significant differences were detected between the control, low, and mid concentration groups.

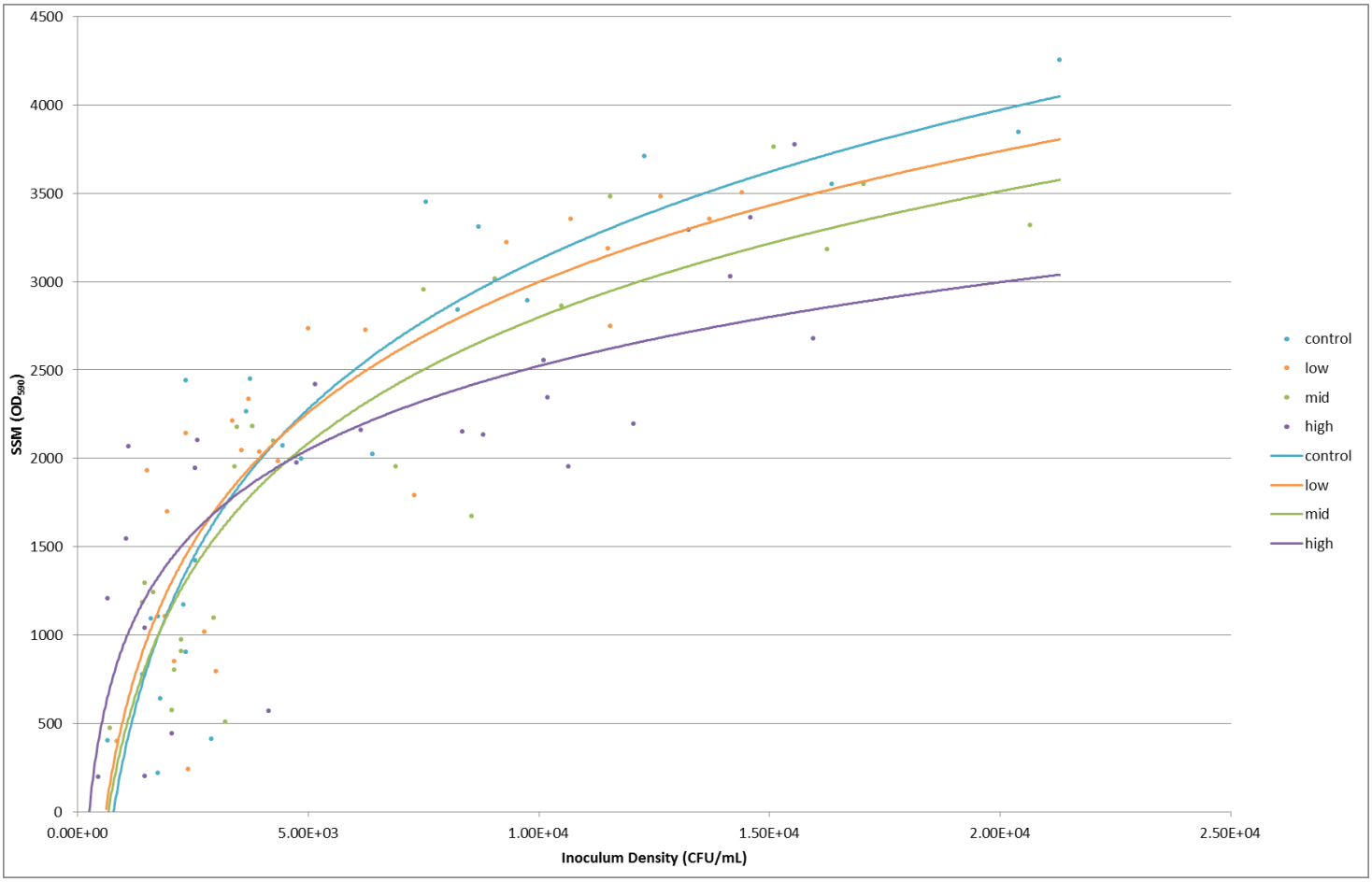

Figure 7. Response of SSM to Inoculum Density by Group
For display purposes, the response curves of the low, mid, and high concentration groups have been extended beyond the limits of their data to match the length of the control curve. Prior to extension the low, mid, and high concentration response curves terminated at 1.44, 2.07, and 1.60 x 10^4 CFU/mL. Data presented here represent the exposure period only.

Table 5. SSM Response Curve Equations and Coefficients of Determination.

Group	Response Curve	Coefficient of Determination (R^2)
Control	1220.5ln(ID) - 8114.7	0.8386
Low	1066.9ln(ID) - 6827.8	0.7215
Mid	1029.1ln(ID) - 6679.6	0.7951
High	683.4ln(ID) - 3771.3	0.6038

ID = Inoculum Density

On study day 49, an additional set of EcoPlates was inoculated with a uniform dilution (1:20) of the filtrate produced from the last pre-exposure rods. It was thought that this dilution would produce an inoculum density similar to that produced by the pre-exposure rods on study day -1. The results are displayed in Figure 8.

3.4 Post-Exposure Rods

For both SSM and metabolic richness, study day 7 demonstrated significantly higher values than all other study days. Study day 49 exhibited significantly higher SSM values ($p<0.05$) than study days 28, 35, and 42 and significantly higher metabolic richness scores than study days 28 and 42. There was some minor increase in filtrate density until study day 35, when these values levelled off. Inoculum density demonstrated a reduction in value followed by a levelling off at study day 28. In contrast, SSM and metabolic richness both showed a precipitous drop at study day 14. No recovery was evident until study day 49. Also, unlike the pre-exposure rods, there was no obvious 'threshold value' in terms of CFU per well for either SSM or metabolic richness. However, it is difficult to describe the relationship accurately given the large proportion of very low SSM and metabolic richness values (Figure 9).

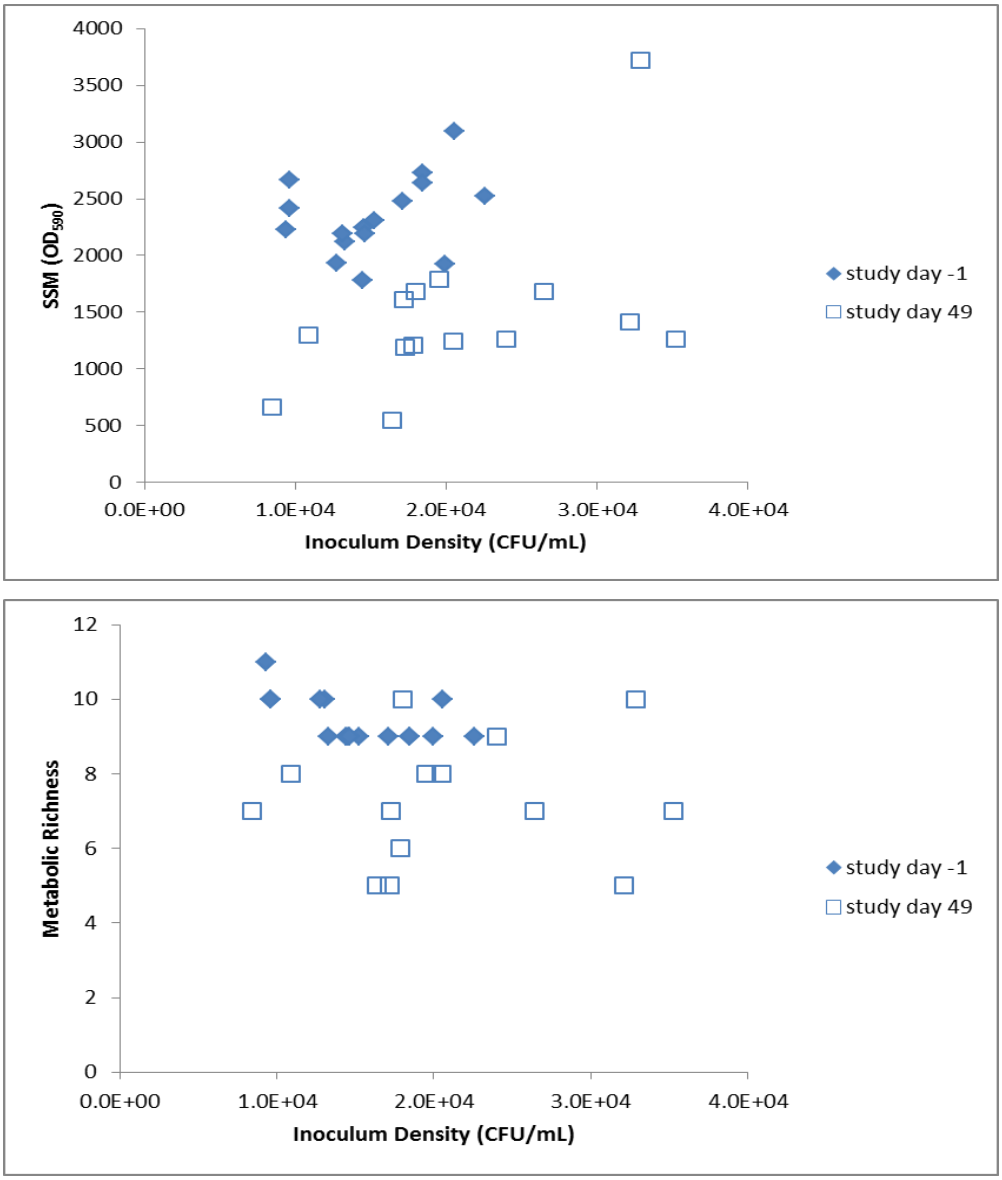

Figure 8. Scatter plots of SSM (top) and Metabolic Richness (bottom) against Inoculum Density.
Note that values for study day 49 from microcosms #6 and #11 have been omitted. Smearing/spreading colonies rendered CFU counts, and therefore estimates of inoculum density, unreliable for those microcosms.

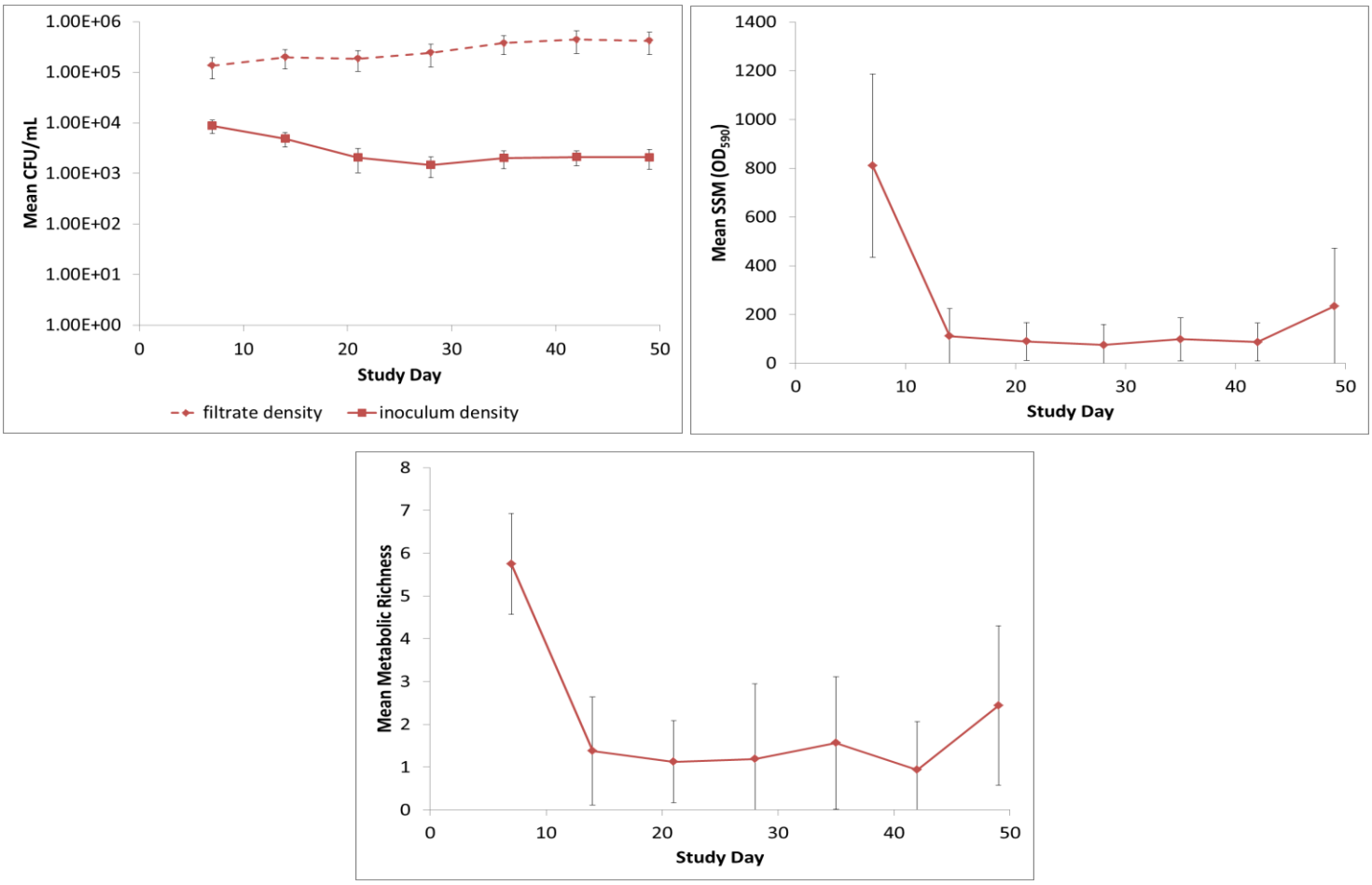

Figure 9. Mean Microbial Values (+/- Standard Deviation) for Post-Exposure Rods. While inoculum and filtrate density trends (top left) are similar to those of the pre-exposure rods, SSM (top right) and metabolic richness (bottom) appear qualitatively different.

Significant group-wise differences in inoculum density started to appear after 21 days of OSPW exposure. The high concentration group demonstrated statistically lower inoculum density than the low and mid concentration group ($p=0.0023$ and $p=0.0371$, respectively). By study day 28, inoculum density in the high concentration group was significantly lower than in the control group ($p = 0.0176$) and lower than in the low and mid concentration groups, with the differences approaching statistical significance ($p=0.0894$ and $p=0.0534$, respectively) (Figure 10). No group-wise differences in filtrate density were observed. No significant day by group effects were observed in SSM or metabolic richness.

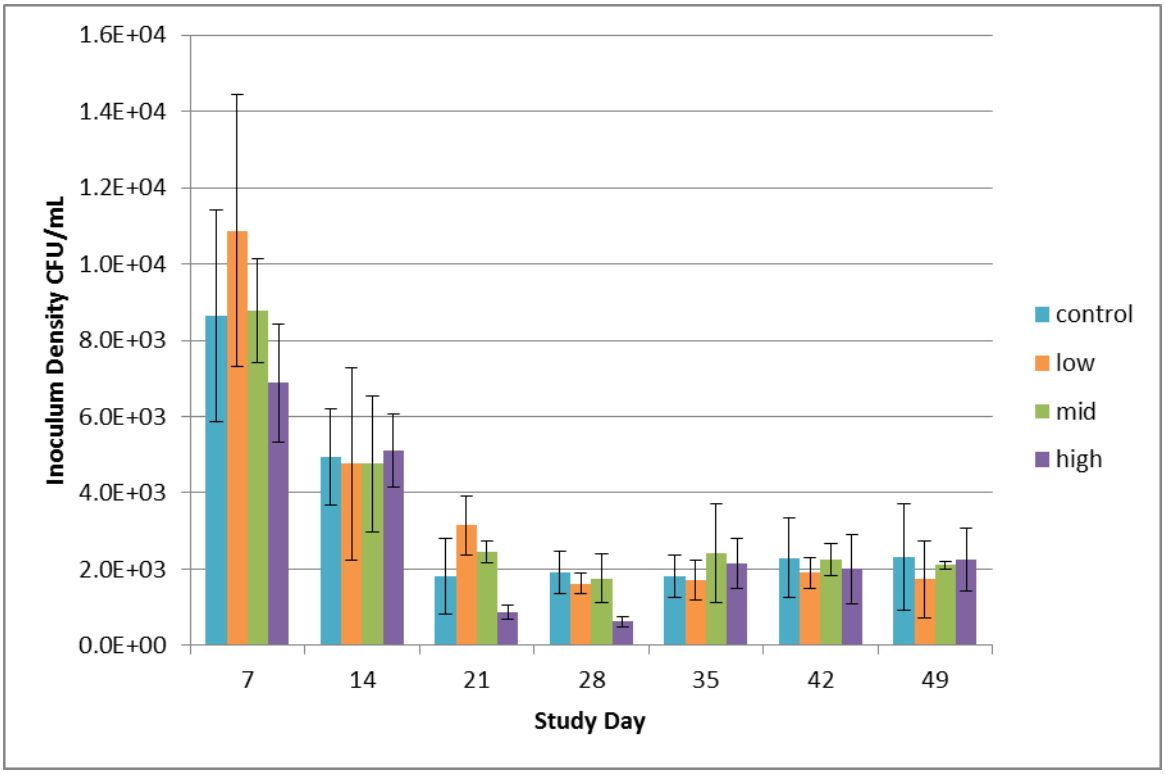

Figure 10. Mean Inoculum Density (+/- Standard Deviation) for Post-Exposure Rods by Group. Note the lower inoculum densities for the high concentration group than the other groups on study days 21 and 28. This difference disappears after OSPW has been removed from the microcosms on study day 28.

Figure 11 presents inoculum density, SSM, and metabolic richness data from age-matched pre- and post-exposure rods collected from the control (untreated) microcosms. While inoculum density appears comparable between the rod types, SSM and metabolic richness values are typically lower in post-exposure rods compared to pre-exposure rods of the same age.

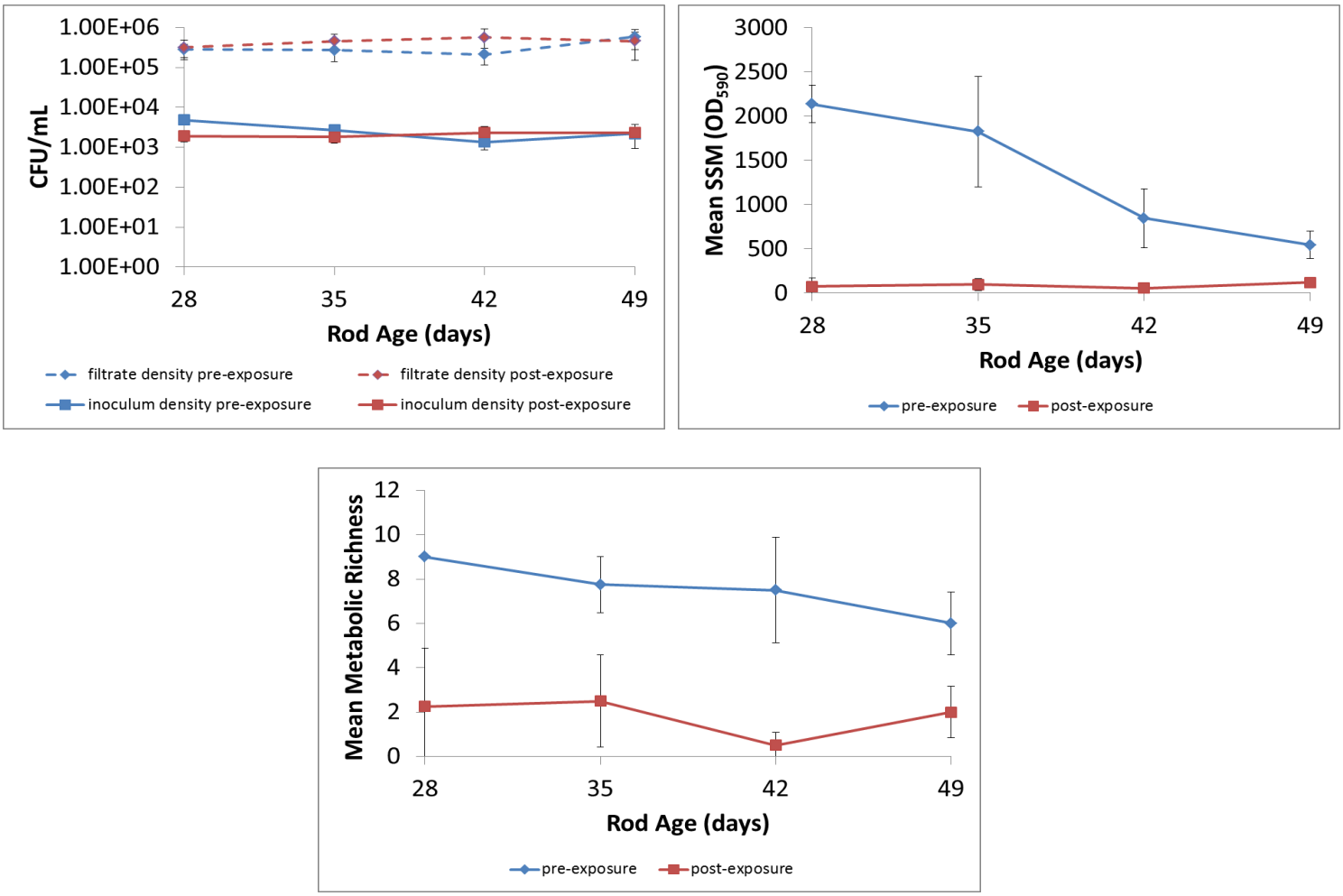

Figure 11. Mean Microbial Values (+/- Standard Deviation) for Age Matched Pre- and Post-Exposure Rods from the Control Group.
Note that while the filtrate and inoculum densities are similar (top left), the SSM (top right) and metabolic richness (bottom) of the pre- and post-exposure rods differ substantially. The terms pre- and post-exposure refer only to the timing of rod deployment. All of the data represented here were collected from the control microcosms, which were never exposed to OSPW.

3.5 Chemistry

The results of the TNA and OSAEO analyses are presented in Table 6. TNA increased in the last week of the experiment ($p=0.0150$); though this change was not specific to any particular group. No similar effect was seen in the first week of exposure. No significant changes were observed in OSAEO over either week.

Table 6. Mean TNA and OSAEO Concentrations (± Standard Deviation) by Group and Study Day.
Note the control tanks sometimes produce values well above the method detection limits (0.1 mg/L and 0.02 mg/L for OSAEO and TNA, respectively), suggesting that some natural constituents of marsh water are co-extracted with oil sands organic compounds.

		Fraction							
		OSAEO (mg/L)				**TNA (mg/L)**			
	Study Day	**0**	**7**	**21**	**28**	**0**	**7**	**21**	**28**
Exposure Group	**Control**	1.76 ±2.22	0.05*	1.45 ±1.78	0.20 ±0.30	0.12 ±0.21	0.14 ±0.26	0.08 ±0.11	0.19 ±0.15
	Low	1.99 ±2.36	0.05*	0.91 ±1.72	0.05*	0.12 ±0.15	0.12 ±0.22	0.03 ±0.04	0.15 ±0.12
	Mid	6.38 ±5.05	6.50 ±1.12	5.24 ±0.78	7.05 ±0.47	1.84 ±1.25	2.06 ±1.14	1.03 ±0.41	1.99 ±0.20
	High	55.97 ±24.03	34.55 ±8.03	30.32 ±5.50	38.30 ±1.81	25.01 ±3.69	24.36 ±6.84	23.88 ±5.22	28.07 ±3.95

*A value of ½ the method detection limit was assigned to samples which contained no detectable analyte.

The results of naphthenic acid speciation analyses are presented in Figure 12. For all Z-families, chromatographic area demonstrated statistically significant reductions over the first week ($p = <0.0001, 0.0095, 0.0046$, and 0.0006 for Z -2, -4, -6, and -8 respectively). However, over the last week, only the Z-2 family experienced a statistically significant reduction in amount ($p = <0.0001, 1.0000, 0.9998$, and 0.9979 for Z -2, -4, -6, and -8 respectively). This pattern was similar across all groups.

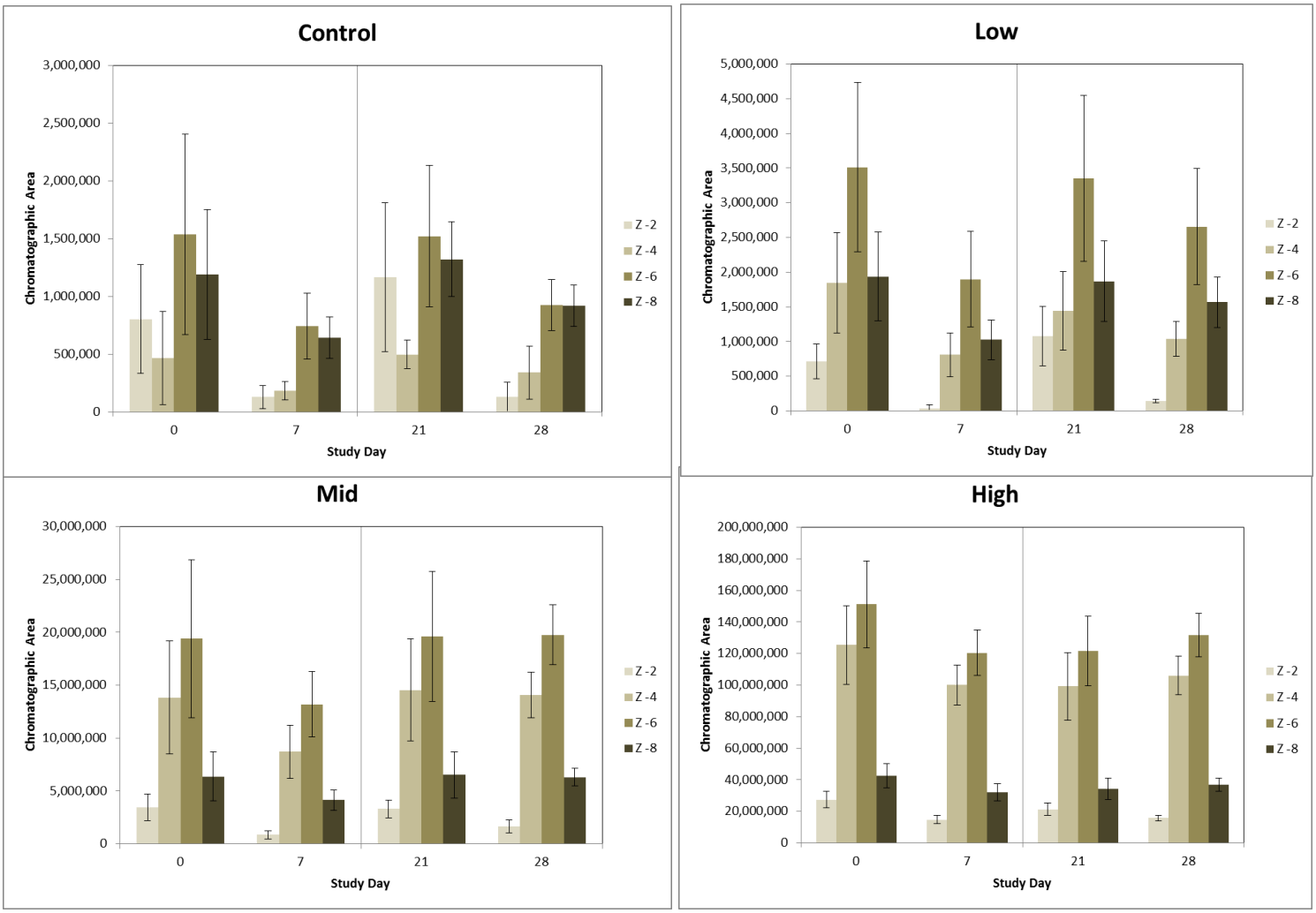

Figure 12. Mean Chromatographic Area (+/- Standard Deviation) for Naphthenic Acid Speciation Mass Sub-Ranges Across Groups and Shaded by Z- Family. Note the group-wise changes in vertical scale. The central line in each graph separates the first week of exposure (on the left) from the last week of exposure (on the right).

4 DISCUSSION

Grossly, the green films and brown spots present in the high concentration microcosms suggest a shift in the phytoplankton community. A number of studies have noted an alteration, and sometimes expansion, of the phytoplankton community in aquatic systems affected by OSPW (EVS Environment Consultants 1996, Leung et al. 2001, 2003, Quagraine et al. 2005).

The exposure period was characterized by a reduction in inoculum density without any concordant decrease in filtrate density. This difference may be explained by the presence of large amounts of free ATP (ATP outside of cells) in both the microcosm water and the filtrate. Though somewhat imprecise, bioluminometer results suggest that the free component accounted

for up to 50% of the ATP found in the microcosm water, and almost all of the ATP found in the filtrate. Unfortunately, because the variability in ATP measurement was quite high, we could not reliably subtract free from total ATP to determine the intracellular component. In addition, the free ATP did not appear to diminish appreciably over sample processing, a procedure which can exceed thirty minutes in length. We had expected that any free ATP liberated by our sample processing would be rapidly consumed by the heterotrophic community present in the filtrate/inoculum. However, even when ATP was measured in the final inoculum, the difference between free and total ATP was generally within the bioluminometer's margin of error (Buffington, pers. comm.).

While we cannot be certain of the source of free ATP, the coincidence of reduced inoculum density with increased phytoplankton in the high concentration microcosms suggests a photoautotrophic source. Moreover, the degree to which bacterial ATP appears to be overwhelmed by free ATP suggests a very rich (i.e., eukaryotic) source.

Both SSM and metabolic richness responded to inoculum density. However, the relationship was not the same for the pre- and post-exposure rods (Figure 6). Importantly, the pre-exposure rods seemed to show a qualitative change in the relationship between SSM and inoculum density at approximately 4,000 CFU/mL. This appears to be the result of a reduction in microbial metabolism associated with the presence of OSPW. The response of SSM to inoculum density is attenuated by OSPW in a dose-dependent manner. This results in divergent SSM response curves which intersect near the 4,000 CFU/mL threshold. The reasons for that change, and the absence of a similar change in the post-exposure rods, are unknown.

Metabolic measures did not consistently reflect changes in inoculum density over time. This was especially evident in the post-exposure rods where changes in SSM and metabolic richness seemed to have little correlation with changes in inoculum density (Figure 9). The data displayed in Figure 8 imply a reduction in metabolic activity that is associated with age rather than inoculum density.

SSM showed a strong response to a 50% water change which was not paralleled by a similar response in inoculum density. Presumably, the increase in SSM which occurred between study days 0 and 1 was the result of the introduction of nutrients and/or the removal of metabolic wastes. Other authors have described an EcoPlate bias towards microorganisms adapted to high nutrient environments (Garland 1997). A 50% change in microcosm water could plausibly promote metabolic activity from this component of the microbial community without increasing the number of culturable cells or changing metabolic richness.

The timing of rod deployment appears to be an important variable in determining the metabolic profile. The microbial community which developed in the pre-exposure period behaves differently from the community which developed during the exposure period. Besides the absence of OSPW, there was a relative abundance of resources (nutrients, area available for colonization) and a dearth of competitors at the beginning of the experiment compared to the same microcosms later in the study. Even if one considers only the control microcosms, age-

matched[2] pre- and post-exposure rods produce dramatically different profiles (Figure 11), likely reflecting the ecological and physiological constraints imposed by microcosms of different ages. The interaction between allogenic and autogenic factors in the development of microbial communities has been noted by others (Paule et al. 2009). While variation in inoculum density impairs our ability to make comparisons, it appears that the presence of OSPW has less effect on the development of biofilm communities than does the age of the microcosm.

OSPW appears to cause a dose dependent reduction in metabolic activity as measured by SSM. This effect only achieved statistical significance when OSPW concentration reached 25%. However, Figure 7 suggests that some effect may occur at lower levels. The data used to construct Figure 7 came from rods of various ages (from 3 to 7 weeks) and included data from rods collected shortly after a water change. If these variables had been held constant or had there been better control over inoculum density, finer resolution of the effect may have been possible. In future experiments, it may be desirable to generate a series of dilutions from each biofilm sample and apply each dilution to a separate EcoPlate as a means of eliminating inoculum density as a confounding factor. At 25% concentration, OSPW had a statistically significant effect on both the metabolic characteristics of the pre-exposure biofilms and the inoculum density of post-exposure biofilms. This latter effect is likely mediated by a relative increase in phytoplankton numbers. Both of these effects occurred at OSPW levels well below that required to cause lethality in *D. magna*, suggesting that CLPP may be a highly sensitive technique for the detection of environmental toxicity.

The lack of *D. magna* toxicity suggested that our composite OSPW was quite old, and that the most toxic constituents had likely already been degraded *in situ*. We were somewhat surprised to see non-zero values for TNA in the control microcosms, as well as an increase in TNA in the final week of the exposure period. The microcosms are largely closed systems with evaporation held constant. Therefore, we have attributed these findings to microbial metabolism. We cannot determine which component(s) of the microbial community would be responsible for the production of compounds detected under the TNA extraction protocol. This production capacity may have developed over time, as there was no similar increase in TNA over the first week of the exposure period. It is interesting to note that some of the microbial species known to be present in OSPW-contaminated wetlands are also known to be a rich source of hydrocarbons (Schenk et al. 2008).

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[2] Paule et al. (2009) have demonstrated that the age of a microbial community has a strong influence on its characteristics. Since the pre-exposure biofilms have a 3-week 'head start' on the post-exposure biofilms, rods collected on different study days must be compared in order to eliminate the confounding effects of age. Since only the rods collected from control tanks are being included in this comparison, any difference detected between age-matched pre- and post-exposure rods can be reasonably attributed to differences in the conditions under which the biofilms formed. For example, 28-day old pre-exposure biofilms (harvested on study day 7) had grown under conditions of high nutrients and low competition when compared to 28-day old post-exposure biofilms (harvested on study day 28). The different conditions under which the biofilms formed seem to have a large effect on the metabolic characteristics of the community and little to no effect on cell density (Figure 11).

While no specific indicator of OSPW biodegradation was identified, we did see some reductions in naphthenic acids which varied according to Z-family. The reduction of low cyclicity (Z -2) compounds in both the first and last weeks of OSPW exposure was, to some degree, expected. Other investigators have demonstrated that low cyclicity naphthenic acids are more amenable to biodegradation than larger more complex molecules (Han et al. 2008). The reduction in higher cyclicity compounds (Z -4, -6, and -8) over the first week of the exposure period was surprising, but might be explained on the basis of adsorption. The organic fraction of OSPW may have adsorbed to the biotic and abiotic surfaces present in the microcosm. By the fourth week of exposure, these surfaces may have become saturated, resulting in more or less stable concentrations of the less degradable organics. In contrast, the free portion of low cyclicity compounds would continue to be metabolized, and show a decrease in concentration across the first and last weeks of exposure. An adsorption process may have also contributed to the increase in TNA over the last week, although it does not explain the relative stability of the OSAEO fraction over the first week. Others have observed the ability of naphthenic acids to cause the aggregation of phytoplankton (Fahlman, pers. comm.), suggesting that these molecules may in fact stick to cell surfaces. The potential adsorption of naphthenic acids to either microbial cell walls or biofilm matrix suggests a relatively simple mitigation process. Biofilms could be grown on inert materials floating on or near the water surface, then physically removed (i.e., scraped off) and incinerated, thereby removing recalcitrant naphthenic acids entirely.

Changes to the microbial community over time could also explain cyclicity-based differences in naphthenic acid concentration in the fourth (last) week of exposure. Early in the exposure period, the microbial communities appeared to be quite metabolically active, with a fairly small phytoplanktonic and fungal component. Over time, however, bacterial heterotrophic metabolic activity waned, while fungal and phytoplanktonic numbers appeared to expand. The fungi and phytoplankton may have become sources of metabolically available carbon for the heterotrophic bacteria. Whatever its origin, the increase in TNA over the last week of OSPW exposure suggests that new carbon sources were becoming available. New carbon sources, if metabolically available, would tend to retard the consumption of difficult-to-assimilate polycyclic organic molecules, without impairing the consumption of smaller molecules.

Obviously, the inoculum normalization procedure needs improvement. A new normalization procedure is being designed to take into account the presence of large amounts of free ATP. If inoculum density cannot be adequately controlled, the inoculation of multiple EcoPlates with serial dilutions of a single biofilm sample would allow analysis based on the regression techniques used in this study. In the meantime, however, the oil sands industry may wish to consider a phytoplankton-based toxicity monitoring system. Our results suggest that phytoplankton will respond to the presence of OSPW at levels well below that needed to induce acute toxicity. While analytical chemistry and genetic tools are available, simpler and more expedient methods may be possible.

5 CONCLUSIONS AND RECOMMENDATIONS

The presence of 25% OSPW was associated with a statistically significant reduction in ATP-normalized inoculum density produced from post-exposure rods and SSM values for the pre-exposure rods. The CLPP profiles produced by the Biolog EcoPlates responded to microcosm age, timing of rod deployment, and water change events. Some analyses were hampered by excessive variation in inoculum density. This variation was the result of an unexpectedly high concentration of free ATP in the microcosm water and processed inocula. There were no reductions in the oil sands extractable organic fraction or TNA. In fact, an increase in TNA was observed across all groups in the final week of the exposure period, and was likely the result of the microbial production of compounds co-extracted with oil sands naphthenic acids. Both low and high cyclicity naphthenic acids demonstrated reductions in concentration over the first week of exposure. However, only the low cyclicity compounds continued to show a reduction over the last week of exposure. Adsorption and/or changes in the microbial community may be responsible for this difference.

The following recommendations are made:

- An improved inoculum normalization procedure should be developed to better evaluate the capabilities of CLPP as an ecological monitoring tool with a minimum of data processing. Future studies with the technique should incorporate reasonably stable inoculum densities well in excess of 4,000 CFU/mL, constant rod residence times, and a rod harvest schedule which maintains a constant temporal relationship with water change events. In the meantime, analytical methods based on serial dilution and regression analysis can be used, albeit at the cost of increased time and materials.
- An ecological monitoring tool based on phytoplankton should be considered. The results of this work, and other studies, suggest that phytoplankton communities respond to the presence and concentration of OSPW.

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7 GLOSSARY

7.1 Terms

Adenosine Triphosphate (ATP)

The molecule which is the source of energy for most metabolic processes in living organisms.

Aerobic

Living or occurring only in the presence of oxygen. This in contrast to the term anaerobic, meaning living or occurring only in the absence of oxygen.

Allogenic

Caused by outside factors.

Autogenic

Produced from within.

Biofilm

Collections of microorganisms that exist in a multicellular community form in an exopolysaccharide extracellular matrix, adherent to each other or a surface. The biofilms discussed in this document are related to, but much less developed than, the benthic microbial mats with which some readers may be familiar.

Bioluminometer

A device which measures very low light levels produced through a biochemical process. In this study the bioluminometer uses the luciferin/luciferase reaction to measure levels of ATP.

Catabolic

The metabolic breakdown of complex molecules into simpler ones, often resulting in a release of energy.

Colony Forming Unit (CFU)

A measure of viable and replicating bacterial or fungal numbers. Colony forming units (CFU) refer to the number of colonies which form on solid growth media. CFU is indicative of, but not necessarily proportional to, live microbial numbers. Factors like growth conditions, degree of cellular dispersion within the inoculum, and culturability of the cells all influence the relationship between CFU and actual numbers of living cells.

Daphnia magna

A species of freshwater water flea which is native to northern and western North America. It is commonly used to test the toxicity of chemicals.

Eukaryote

An organism whose cell(s) contain a distinct membrane-bound nucleus among other distinctive features. In this study, the eukaryotic cells of significance are fungi and algae. In general, eukaryotic cells will contain more ATP than prokaryotic cells.

Filtrate

A liquid that has passed through a filter. In this study, filtrate refers to the microbial suspension which is produced by passing the sonicated biofilms through a 5 µm filter. The filtrate is expected to be devoid of intact eukaryotic microorganisms.

Filtrate Density

The concentration of microorganisms present within the filtrate. In this study, filtrate density was defined in colony forming units (CFU) rather than individual cells.

Heterotroph

An organism that depends on complex organic substances for energy.

Inoculum

A substance or organism that is introduced into surroundings suited to cell growth. In this study, inoculum refers to the microbial suspension introduced into the wells of the Biolog EcoPlate. Generally, inocula had undergone an ATP-normalization step.

Inoculum Density

The concentration of microorganisms present within the inoculum. In this study, inoculum density was defined in colony forming units (CFU) rather than individual cells.

LC50

Lethal Concentration 50%. The concentration of a substance which will result in the death of 50% of exposed test organisms. This value varies both with the substance and the test organism. In this document the substance was OSPW and the test organism was *Daphnia magna*.

Metabolism

The set of chemical reactions which occur within a living organism to maintain life.

Microbe

A microorganism.

Microcosm

A community, place, or situation regarded as encapsulating in miniature the characteristic features of something much larger. In this study, the microcosms were small glass aquaria containing water collected from a local marsh, the microorganisms inhabiting that water, and often OSPW.

Naphthenic Acids (NA)

Classically, naphthenic acids are described by the general formula $C_nH_{2n+z}O_2$ where n is the number of carbon atoms, z is a negative even integer that specifies hydrogen deficiency and therefore degree of cyclicity. The O_2 signifies a monocarboxylic molecule. Typical ranges are n=5 to 33 and Z=0 to -12 with the majority of components in the n=12 to 22 and z=0 to -8 range. However, the term has become more loosely used to describe the range of organic acids found in crude oil. The crude oil acids are known to be composed of not only classical NA but significant levels of other organic acids that contain nitrogen and/or sulfur atoms and impurities with various levels of saturation and aromaticity.

Photoautotroph

An organism capable of synthesizing its own food from inorganic substances using light as an energy source.

Phytoplankton

Microscopic, potentially free-floating, photosynthetic organisms in aquatic systems.

Prokaryote

A typically unicellular organism that lacks a cell nucleus or any other membrane-bound organelles. Prokaryotes are typically much smaller than eukaryotes and thus can be separated through filtration.

Rhizosphere

The soil zone that surrounds and is influenced by the roots of plants.

Sonicate

To disrupt with (ultra)sound waves.

Sum of Substrate Means (SSM)

A term coined for use in this study to describe the overall level of metabolic activity in the EcoPlate. The sum of substrate means is calculated by adding the mean optical density for all substrates found on the EcoPlate.

7.2 Acronyms

AITF	Alberta Innovates – Technology Futures
ATP	Adenosine triphosphate
CFU	Colony Forming Unit
CLPP	Community Level Physiological Profiling
OD	Optical Density
OSAEO	Oil Sands Acid Extractable Organics
OSPW	Oil Sands Process Affected Water
OSRIN	Oil Sands Research and Information Network
PBS	Phosphate Buffered Saline
RLU	Relative Light Unit
SEE	School of Energy and the Environment
SSM	Sum of Substrate Means
TNA	Total Naphthenic Acids

7.3 Measurement Units

°C	Degrees Celsius
L	Litre
mL	Millilitre
m/z	mass-to-charge ratio
nm	Nanometre
µm	Micrometre

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