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MICROBIAL COMMUNITY COMPOSITION OF MARINE BIOFILMS IN A NORTHERN TEMPERATE

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MICROBIAL COMMUNITY COMPOSITION OF MARINE BIOFILMS IN A NORTHERN TEMPERATE ESTUARY

BY

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ABSTRACT

Marine biofilms are microbial aggregates that ubiquitously develop on substrates in seawater. Biofilms are not simple layers of microorganisms adhering to a surface followed by new organisms growing on top, but they instead have a complex developmental process making biofilms dynamic, diverse and functional communities. The negative effects of biofilms on ships, underwater cables and pipelines have spawned research in antifouling approaches; however, little is known about their development in a northern temperate estuary, such as Narragansett Bay. The goal of this study was to investigate the first steps in biofouling in this area by assessing biofilm biomass through chlorophyll, carbon, nitrogen, total DNA extractions and percent biomass coverage, as well as bacterial biofilm community composition through the use of a molecular technique, the automated ribosomal intergenic spacer analysis (ARISA). Comparisons were made between biofilms on control surfaces and surfaces treated with a commercial foul-release coating, between biofilms grown in the summer and winter seasons, as well as over a 90-day immersion time. Biofilm biomass data revealed no overall significant differences between seasons or across surface types; however, immersion time had a significant effect as biomass tended to accumulate over time. Bacterial community composition obtained from ARISA profiles was influenced by immersion time, as communities separated out into 'early,' 'mid,' and 'late' groupings. It was also influenced by season as well as surface type.

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PREFACE

This thesis contains one manuscript and has been formatted to meet the requirements for the journal *Biofouling: The Journal of Bioadhesion and Biofilm Research*, but has not yet been submitted for publication. The author did not and does not have a financial relationship with the coating manufacturer.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGMENTS iii

PREFACE v

TABLE OF CONTENTS vi

LIST OF TABLES vii

LIST OF FIGURES viii

MICROBIAL COMMUNITY COMPOSITION OF MARINE BIOFILMS IN A NORTHERN TEMPERATE ESTUARY 1

REFERENCES 45

APPENDIX A: RAW DATA 54

APPENDIX B: SUMMARY OF STATISTICS 68

LIST OF TABLES

TABLE **PAGE**

Table 1. Sampling breakdown for the number of samples processed for each step of investigating bacterial community composition using ARISA 14

Table 2. The range of the environmental conditions for the length of immersion time for each experiment, as taken from Narragansett Bay, and not the experimental tanks directly.. 17

LIST OF FIGURES

FIGURE **PAGE**

Figure 1. Experiment set-up. 10

Figure 2. Biomass data plotted against immersion time for control and treated surfaces in two seasons.. 27-28

Figure 3. Venn Diagrams displaying the number of bacterial OTUs from biofilm communities on control and treated surfaces for all sampling days in the summer only, the winter only and those that were present during both seasons. 29

Figure 4. Species richness data plotted against immersion time for control and treated surfaces in two seasons.. 30

Figure 5. ARISA results for both summer and winter biofilm communities on control and treated surfaces, as visualized in non-metric multi-dimensional scaling. Numbers indicate immersion time. 31

Figure 6. MDS plot showing ARISA results for summer biofilm communities with 3 distinct clusters. Numbers indicate immersion time. 32

Figure 7. MDS plot showing ARISA results for winter biofilm communities with 3 distinct clusters. Numbers indicate immersion time. 33

MICROBIAL COMMUNITY COMPOSITION OF MARINE BIOFILMS IN A NORTHERN TEMPERATE ESTUARY

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INTRODUCTION

Marine biofilms are microbial aggregates that ubiquitously develop on substrates in seawater. Biofilms can significantly influence the productivity of coastal ecosystems by being key contributors in the production and breakdown of organic matter, the degradation of pollutants and the cycling of nitrogen (Davey and O'Toole 2000; Egan et al. 2008), however, biofilms are more commonly known for their detrimental impacts. Biofilm accumulation negatively influences the efficiency of ships by reducing speed and increasing fuel needs, while also negatively impacting navigational buoys by encouraging significant macrofouling which leads to buoys sinking, creating blockages in pipelines and compromising the stability of oil and gas platforms (Railkin 2004). Furthermore, optically clear surfaces, such as periscope head windows and environmental sensors, provide an additional challenge when considering anti-fouling practices due to the fact that those clear surfaces and sensors cannot be blocked or hindered by opaque paints and coatings. The aforementioned negative impacts due to biofilm growth are far reaching, from coastal to oceanic environments.

Currently, two of the methods being used to reduce or hinder biofilm growth in the marine setting include biocidal antifouling paints and non-toxic foul-release coatings, both of which are applied directly to an existing surface where biofilms are undesirable. Biocides in antifouling paints can result in negative environmental impacts by accumulating in sediment and shellfish, as well as being toxic to some

marine algae (Burgess et al. 2003). Finding a biocide with a low toxicity that targets a broad range of microorganisms and is easy to produce has been difficult. Research costs and regulations on toxins released in the environment hamper the development and commercial use of antifouling paints (Finnie and Williams 2010; Lejars et al. 2012). Foul-release coatings, on the other hand, are environmentally friendly biocide-free coatings that have a dual mode of action in that they reduce adhesion of microorganisms by altering the energetics at the biofilm-surface interface, as well as use hydrodynamic stress to remove fouling (Lejars et al. 2012).

Basic knowledge of the microbial community composition and formation of the biofilm in specific geographic areas could lead to more accurate and effective methods of biofilm control and antifouling practice. During initial development of a biofilm, dissolved organic matter in seawater is adsorbed and a conditioning film is formed on the submerged surface almost immediately (Bakker et al. 2003; Garg et al. 2009). Biofilm development appears to be influenced by the carbohydrate polymers adsorbed from the surrounding water as part of the conditioning film (Garg et al. 2009). The composition of these carbohydrates, along with uronic acids and proteins also found in the conditioning film, has been observed to vary seasonally and temporally (Garg et al. 2009). Once the conditioning film is in place, gel-like extracellular polymeric substances (EPS) develop from the first microorganisms to colonize, promoting further microbial colonization, as well as settlement by marine invertebrates and algae (Dobretsov and Qian 2006; Huggett et al. 2009; Dobretsov 2010; Hadfield 2011; Mieszkin et al. 2012). Biopolymers of the EPS form a matrix, which immobilizes the

bacteria keeping the biofilm together, traps nutrients and hydrates the biofilm, while also determining the immediate environment of the biofilm by influencing factors such as porosity, density and mechanical stability (Flemming et al. 2007; McDougald et al. 2012).

The concept of bacteria living as part of a biofilm community is now well accepted, and biofilm formation is a feature common to most microorganisms (McDougald et al. 2012). Biofilms are no longer viewed as uniform layers of matrix materials, as they once were, but instead as diverse and functional communities that mature over time through a complex developmental process (Stoodley et al. 2002). The composition of a marine biofilm, such as specific species of bacteria and diatoms, often dictates the subsequent settlement of many invertebrates and algae (Huggett et al. 2009, and references therein). The process of biofilm development, including the order the various components settle in, may be variable with location and time (Jenkins and Martins 2010). Seasonal changes further influence the species composition of early biofilms (Dobretsov 2010), as the physical and chemical conditions of the substrate and environment vary between winter and summer. Furthermore, physical and biological disturbances can change the order of colonization (Jenkins and Martins 2010), and different phases of settlement have the potential to overlap or even develop in parallel (Dobretsov 2010). Since microorganisms live in such close proximity in the biofilm, intercellular interactions can create complex and highly differentiated communities in this competitive environment (Egan et al. 2008). In addition to space constraints, the settlement and colonization of microorganisms can be selected by

nutrient limitations and water turbulence, or by chemical attractants and repellants released by already settled microorganisms (Egan et al. 2008; Dobretsov 2010).

Molecular methods such as automated ribosomal intergenic spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), have all been used to successfully study marine microorganism community composition. Since the development of ARISA (Fisher and Triplett 1999), microbial communities can be quickly analyzed for their composition, species richness, as well as diversity in specific environments. ARISA has covered a wide array of applications including, determining microbial diversity across marine and lagoon sediments, coastal seawater and groundwater (Danovaro et al. 2006), as well as describing spatial and temporal patterns of bacterial community distribution in the marine water column (Acinas et al. 1999; Fuhrman et al. 2006; Mapelli et al. 2013), and comparing bacterial biofilm communities in streams and freshwater runoff (Lear and Lewis 2009; Ancion et al. 2010). Mapelli et al. (2013) utilized ARISA on a research cruise across the Mediterranean Sea to investigate planktonic bacterial communities at three depths, and identified distinctly different populations in the eastern and western basins. Both DGGE and T-RFLP have been used to specifically compare marine bacterial biofilms (Lee et al. 2008; Kriwy and Uthicke 2011; Bellou et al. 2012; Briand et al. 2012). Briand et al. (2012) developed marine biofilms for two weeks on the French Mediterranean coast and compared growth on various artificial substrata using DGGE, finding that although bacterial communities were controlled by the type of substrata, about 25% of species were

common to both geographical locations. Lee et al. (2008) focused on studying very early biofilm growth (up to 36 h after deployment of surfaces) across different substrata (acryl, glass and steel) in Sacheon harbor, Korea, and found, through the use of T-RFLP, slight differences in bacterial communities across substrata, but dramatic changes in bacterial community structure on all substrata between 9 and 24 hours after deployment of surfaces.

The focus of this study was on characterizing the bacterial community composition of initial biofilm development in substrates placed in waters from a northern temperate estuary, using ARISA.

The goal of this study was to understand the first steps in biofouling in a temperate northern estuary during the winter and summer seasons on two different glass surfaces by answering the following questions:

- Are there differences in biofilm biomass and composition between control surfaces and surfaces treated with a foul-release coating?
- Are there differences in biomass and composition between summer and winter biofilms?
- Are there differences in biofilm biomass and composition over immersion time?

Developing a basic knowledge of the bacterial biofilm communities could help lead to the development of more effective methods to control the detrimental effects of

biofilms in northern temperate estuaries by knowing which early settlers to target. Biofilm growth on immersed optically clear surfaces yields additional challenges when considering vision and proper operation of sensors, therefore plain glass surfaces were chosen as a surrogate to periscope head windows, underwater cameras and environmental sensors. Lastly, since biofilm development can be influenced by numerous biological, physical and chemical factors, this study aims to address comparisons between seasons, treated and control surfaces, and biomass accumulation over time, in an effort to create a sizable pool of information for marine biofilms in a northern temperate estuary.

MATERIALS AND METHODS

Cleaning slides

All of the substrates used in this study were glass microscope slides (Fisher Scientific, cat # 12-544-3) that were cleaned by soaking in a 50% methanol / 50% concentrated hydrochloric acid mixture for two hours, transferred to 100% concentrated hydrochloric acid for two hours, then rinsed in a continuous flow of deionized water for 30 minutes (Finlay et al. 2002). Slides were individually dried with KimWipes® tissues and stored in their original boxes until use.

Treating slides

Clean slides were treated on both sides with two coats of a commercial anti-fouling coating (Hullkote, Team McLube®) or remained untreated as control slides. The coating is comprised of a bonded polytetrafluoroethylene (PTFE) suspension system with a citrus-based high gloss polish, a silane polymer, aluminum-based particles as cleaning abrasives, and a biodegradable detergent (Snyder 2013). The coating is bound to the surface of the substrate through a chemical reaction that occurs upon application (Snyder 2013), and dries clear, which allowed optically clear and hard substrates to be tested. Foul-release coatings normally create hydrophobic surfaces (Finnie and Williams 2010), so a contact angle goniometer (ramé-hart Instrument Co., model 200) was used in conjunction with DROP Image software v2.4 to measure contact angles and determine the types of surfaces, hydrophobic or hydrophilic, characterizing control and treated slides before initial immersion in seawater (Appendix Table A.1).

Slide deployment and retrieval

Slides were deployed for one experiment in the summer (June – Sept. 2011) and one experiment in the winter (Dec. 2011 – March 2012). Environmental data were collected daily, using a YSI 6600 multiprobe sonde (YSI, Inc.) located at a distance of 4 meters from the intake valve supplying water to the experimental tanks, by the Marine Ecosystems Research Laboratory (MERL) at the Gradate School of Oceanography (GSO) in Narragansett, Rhode Island, USA (RIDEM-OWR, 2007).

Nutrient data, also collected by MERL, were recorded on a weekly basis from water samples collected off the GSO dock (Krumholz, 2012).

For each experiment, control and treated slides were suspended vertically, and maintained at a constant depth (10 cm below the water surface) in plastic slide holders, in two separate outdoor flow-through tanks (diameter of 1.2 m) (Figure 1). Two separate tanks were used to eliminate any possible contamination of the control slides by the foul-release coating being released from the treatment slides into the surrounding waters. Raw seawater from Narragansett Bay, RI (41° 29.5' N, 71° 25' W) flowed into the tanks via a shared pipe, and each tank experienced approximately 14 complete water turnovers per day during both seasons. Slides immersed in seawater were retrieved after 3, 7, 15, 30 and 90 days; they were vertically submerged in sterile filtered seawater (0.45 μm) during transport in clean plastic containers. Prior to processing for analyses of biofilm biomass and composition, all slides were rinsed in sterile filtered (0.45 μm) seawater to remove any organisms or particles not attached as a true component of the biofilm.

Figure 1. Both summer and winter experiments were set-up with (A) control surfaces and (B) treated surfaces in two separate tanks. Water from Narragansett Bay entered the tanks via the shared pipe located between the tanks. Surfaces were suspended vertically in slide holders hanging by strings in the tanks.

The methods that follow apply to both control and treated surfaces, and for both winter and summer experiments:

Biomass analysis

Chlorophyll

Upon each harvest, triplicate samples (14 to 56 cm^2) were removed from three different slides by scraping the biofilm onto 25-mm Whatman GF/F filters with sterile razors. To measure chlorophyll-a concentration as an index for photosynthetic biomass, the samples were transferred to 95% ethanol for 24 hours at -20°C and analyzed on a Turner 10AU fluorometer (Parsons et al. 1984; Nagarkar and Williams 1997). A 10-AU Solid Secondary Standard (P/N 10-AU-904, Turner Designs) was used at both low and high orientations before each use to check for instrument drift.

Carbon and Nitrogen

For particulate organic carbon and nitrogen, biomass was removed (14 to 70 cm^2) from triplicate slides with sterile razors into tin capsules (Costech, 9 x 10 mm) and frozen. Samples were dried at 50 ºC for 48 hours and stored over desiccant until analyzed with a carbon and nitrogen analyzer (Costech 4010 Elemental Analyzer), following the methods of Verardo, et al. (1990). The instrument was calibrated using standards between 0.6 and 4.0 μmol N for nitrogen, and standards between 3.6 and 24 μmol C for carbon.

Percent of Surface Covered in Biomass

Slides containing biofilms that were not harvested were fixed for 30 min in glutaraldehyde (4% in seawater), followed by 10-min rinses in sterile seawater, sterile seawater:deionized water (1:1), and twice in sterile deionized water, dried at room temperature, and then stored at -20 ºC. Triplicate slides were stained with SYBR® Green I Nucleic Acid Gel Stain according to manufacturer's instructions (Lonza), rinsed in sterile and filtered (0.22 μm) deionized water, preserved with the anti-fade mounting gel Fluoro-Gel (Microscopy Sciences 17985-10), covered with a 22 x 22-mm confocal cover slip, sealed with clear nail polish and stored at 4 ºC in the dark until further analysis.

The percentage of surfaces covered in biomass was determined using light and epifluorescence microscopy (Nikon Eclipse 80*i* equipped with a QImaging Retiga-2000R digital camera) in conjunction with the object count feature in NIS-Elements

AR 3.0 imaging software (Nikon Instruments Inc.). For each replicate slide, five random fields of view (0.065 mm^2) were photographed at 40X. The software object count feature identified fluorescently labeled cells and provided a value indicating the percentage of the surface that was covered with biomass. Data from all five fields of view from a given slide were averaged. Triplicate samples for each immersion time, season and surface type were averaged to determine the overall percent of biomass covering the surface.

Biofilm composition

A molecular approach, the automated ribosomal intergenic spacer analysis (ARISA), was used to obtain a detailed look at changes in marine biofilm bacterial community composition between treatments and controls over time and between seasons. ARISA is a rapid and repeatable whole-assemblage genetic fingerprinting method that characterizes bacterial genotype diversity by differentiating operational taxonomic units (OTUs). This very sensitive method can effectively estimate community composition shifts and relative diversity, as well as identify similarities in and the taxonomic organization of bacterial communities, making this method extremely useful as a tool for comparison of microbial populations (Fisher and Triplett 1999; Ancion et al. 2010).

Upon each harvest, biomass was removed (28 to 168 cm^2) from triplicate slides with sterile razors and immediately extracted for total DNA using the MO BIO PowerBiofilm™ DNA isolation kit according to the manufacturer's instruction, with

the biofilm being scraped directly into the bead-beating tubes. Total DNA concentration (ng/μL) in the extracts was measured on a NanoDrop 1000 Spectrophotometer in conjunction with v.3.6.0 measurement software (Thermo Scientific). DNA was stored at -80 ºC until amplification. The 16S-23S intergenic spacer region was amplified on a Mastercycler® pro (Eppendorf) from duplicate 8 ng of DNA template through polymerase chain reaction (PCR) (95 ºC for 5 min; 27 cycles of 94 ºC for 30 s, 56 ºC for 30 s, 72 ºC for 45 s, and then 72 ºC for 8 min). PCR parameters for the amplification of marine bacterial biofilm DNA from Narragansett Bay waters were optimized for this study using the following reaction mix: 2.0 μL of 10X buffer, 0.8 μL of 50 mM $MgCl_2$, 2.0 μL of 10 mM dNTP mix, 1.0 μL of HiSpec Additive, 2.0 μL of 10 μM forward primer, 2.0 μL of 10 μM reverse primer, 0.4 μL of bioXact short polymerase (4 units per μL), 3.2 μL of DNA template (2.5 ng/μL), and 6.6 μL water for a total volume of 20 μL. Universal primer 16S – 1392F (5′-G[C/T]ACACACCGCCCGT-3′) was used as the forward primer, and bacterial primer 23S – 125R FAM – (5′-GGGTT[C/G/T]CCCCATTC[A/G]G-3′), was labeled at the 5′ end with a 6-carboxyfluorescein fluorochrome (FAM, Integrated DNA Technologies, United States), and used as the reverse primer (Fisher and Triplett 1999; Hewson and Fuhrman 2004; Danovaro et al. 2006). These primers were selected and used for amplification of the intergenic spacer region. 30 ng of cleaned (QIAquick PCR Purification Kit, Qiagen, United States) PCR product were submitted to the Genomics and Sequencing Center (University of Rhode Island, Kingston, RI) in Hi-Di™ Formamide (Applied Biosystems) in the same tube as the size standard GeneScan™ 1200 LIZ® (Applied Biosystems). The fluorescent FAM-labeled primer enables

detection during fragment analysis by laser after separation of the various sized fragments by capillary electrophoresis in a genetic analyzer (*3130xl* Applied Biosystems).

Peaks of fluorescence were analyzed with GeneMapper® v4.0 software (Applied Biosystems), with sample binning set to 1 basepair (bp), and all samples normalized by sum of signal for subsequent analysis in Microsoft Excel. DNA fragment sizes less than 300 bp and greater than 1200 bp were excluded from the analysis, and background fluorescence was removed by excluding peaks with weak signals (< 50 relative fluorescence units). DNA fragment sizes and the area under each peak were rounded to the nearest whole number. Two separate PCRs were analyzed for each sample: only fragment sizes present in both runs were included and the areas under the peaks were averaged (Table 1). Each resulting fragment size representing a different length of intergenic spacer sequences present in the communities, was then reported as an operational taxonomic unit (OTU).

Table 1: Sampling breakdown for the number of samples processed for each step of investigating bacterial community composition using ARISA. There were 3 true replicates for all sampling days on each surface and in both seasons. Pseudo-replicates were submitted for ARISA as a quality control check.

		For each sampling day *(3, 7, 15, 30 and 90 days after immersion)*	Total # of samples per surface	Total # of samples per season	Total # of overall samples
1.	DNA extracted from biofilm	3 extracts per surface type (control and treated)	15	30	60
2.	Polymerase Chain Reaction	2 PCR per DNA extract = 6 per surface type	30	60	120
3.	Submitted for ARISA	All 6 PCR from step 2	30	60	120

Statistical Analysis

Before statistical analysis using an analysis of variance (ANOVA) for factorial design in the software package JMP® v10.0.2 (SAS Institute Inc), all biofilm biomass data (Appendix Tables A.2 to A.7) were log-transformed. Unless otherwise noted, averages are reported with one standard error about the mean. Similarities in marine bacterial community composition, between seasons, over immersion time and between control surfaces and those treated with a foul-release coating, were determined using the Bray-Curtis similarity index in the statistical software package PRIMER® (Plymouth Routines in Multivariate Ecological Research) v6.1.5 (Clarke and Warwick 2001). Data used for these analyses were peak areas from the electropherograms output from ARISA. All ARISA data were square-root transformed before resemblance matrices were created in PRIMER®. Hierarchical cluster analyses were then performed to build dendrograms for ARISA data using the resemblance matrices, which allowed the similarity of communities to be visualized as a tree-structured graph. The same resemblance matrices were also used to derive non-metric multi-dimensional scaling (MDS) plots to further visualize the data. On these MDS plots, points located close together represent communities that have very similar bacterial composition and points located far apart represent communities that have highly dissimilar bacterial composition (Clarke and Warwick 2001). Data were also plotted without transformation and with a log transformation (not shown). All three types of MDS plots (not transformed, log transformed and square-root transformed) were fairly identical, with the square-root transformation providing the lowest 2D stress. On the MDS plots, contour lines display clusters of similar communities, and were chosen

based on the branches determined by the hierarchical cluster analysis and displayed on their respective dendrogram. R was calculated in PRIMER®, using the analysis of similarities (ANOSIM), to determine whether or not the separation of data points representing communities was significant. R scales from -1 to +1, with a perfect separation among groups indicated by +1, and total similarity by 0. Null hypotheses were that no differences in composition between the bacterial communities existed across surface type, season or immersion time (Appendix B).

RESULTS

Contact Angle

Contact angle measurements (Appendix Table A.1) revealed a significant difference between control surfaces and surfaces treated with HullKote ($F(1, 96) = 1156.9$, $p < 0.0001$) confirming that the two starting surfaces were different. The averaged low contact angle of control slides for summer (36° ± 1.7°) and winter (31° ± 1.1°) experiments were characteristic of hydrophilic surfaces, as they were well below the 90° cutoff at which surfaces are considered hydrophobic. The higher contact angle of treated slides for summer (83°± 1.8°) and winter (81°± 0.9°) experiments were much closer to 90°, suggesting that they had nearly hydrophobic surfaces.

Environmental Data

The range of environmental conditions present during the time of each immersion

(Narragansett Bay Fixed-Site Monitoring Network (NBFSMN), 2011 & 2012), as well as nutrient data (MERL, 2011 & 2012), are summarized in Table 2. These environmental data came from Narragansett Bay, as opposed to directly from the tanks where experiments were conducted. The MERL dataset, taken from near the intake valve supplying water to the experimental tanks, is more complete than the periodic temperature and salinity measurements taken directly in the experimental tanks. With only a 3ºC temperature difference and 1 psu salinity difference between MERL and tank measurements, the complete MERL dataset provided detailed environmental conditions during the length of immersion.

Table 2. The range of the environmental conditions for the length of immersion time for each experiment, as taken from Narragansett Bay, and not the experimental tanks directly.

Season	Temp (ºC)	Salinity (psu)	Day length (h:min)	PO_4 (µM)	SiO_2 (µM)	$NO_3 + NO_2$ (µM)
Summer	17 – 24	29 – 31	12:55 – 15:08	0.5 – 1.3	4.3 – 38.1	0.6 – 5.4
Winter	3 – 12	28 – 33	9:23 – 11:38	1.0 – 1.1	18.4 – 25.9	6.8 – 8.6

Biofilm Biomass

The greatest factor affecting biofilm biomass was immersion time (Figures 2A to 2E), as biomass tended to accumulate with length of immersion. The effects of season and surface type tended to only be significant in the older and more developed biofilms. All biomass data were analyzed using an ANOVA for factorial design.

Chlorophyll

Chlorophyll concentrations remained low in both summer and winter biofilms until a dramatic peak in concentration on day 90 in the summer, and earlier on day 30 in the winter (Figure 2A). Significant effects for accumulated chlorophyll in biofilms included immersion time ($F(4, 40) = 227.86$, $p < 0.0001$) and the interaction between immersion time and season ($F(4, 40) = 210.35$, $p < 0.0001$). Tukey's Honestly Significant Difference (HSD) test showed chlorophyll values from day-30 biofilms in the winter were significantly higher than those in all other days from both seasons. Day-90 summer biofilms had chlorophyll values significantly higher than day-90 biofilms in the winter, whereas both had concentrations significantly higher than biofilms from all remaining days in both seasons. All remaining effects from the ANOVA were not significant, including the main effect for season ($F(1, 40) = 0.02$, $p = 0.90$) and the main effect for surface type ($F(1, 40) = 0.01$, $p = 0.94$).

Chlorophyll results were further analyzed using a two-way ANOVA, where summer and winter biofilm data were separated and the main effect of surface type could be examined more closely within each season. Immersion time was significant for summer biofilms ($F(4, 20) = 54.14$, $p < 0.0001$), which was due solely to the very high chlorophyll values of 90-day biofilms, as revealed by Tukey's HSD test, $p < 0.0001$. There was no interaction between surface type and immersion time ($F(4, 20) = 0.09$, $p = 0.99$), nor was the biomass on the summer biofilms different between controls and treatments.

Unlike the summer, the interaction between surface type and immersion time was significant for winter biofilms ($F(4, 20) = 2.94$, $p = 0.046$). Chlorophyll concentrations were significantly greater on treatment slides than control slides on day-30 biofilms only. Day-30 biofilms on both surfaces had significantly higher chlorophyll values than day-90 biofilms, which in turn were significantly higher than 3-, 7-, and 15-day biofilm concentrations.

Carbon

Carbon concentrations significantly increased with immersion time ($F(4, 39) = 217.67$, $p < 0.0001$) (Figure 2B), and all three interactions that included the effect of immersion time were significant. These interactions were between (1) season and immersion time ($F(4, 39) = 32.74$, $p < 0.0001$); (2) surface type and immersion time ($F(4, 39) = 13.13$, $p < 0.0001$); and (3) season, surface type and immersion time ($F(4, 39) = 16.90$, $p < 0.0001$).

Overall, the main effect of season was not significant ($F(1, 39) = 1.43$, $p = 0.24$), nor was the main effect of surface type ($F(1, 39) = 0.17$, $p = 0.68$). Carbon concentrations from biofilms developed on treated surfaces were higher compared to control surfaces on some days, however, this was not true for all days. Summer and winter biofilm data were analyzed separately using a two-way ANOVA to look more closely at the main effect of surface type. In the summer, Tukey's HSD test revealed significantly more carbon on treated surfaces than control surfaces on day 90. In the winter there was significantly more carbon in biofilms from control surfaces on day 15, yet by day

30 there was significantly more carbon in biofilms developed on treated surfaces.

Nitrogen

Not surprisingly, the results for nitrogen were very similar to those for carbon. Nitrogen concentrations significantly increased with immersion time ($F(4, 39) = 58.24$, $p < 0.0001$) (Figure 2C). All three interactions that included the effect of immersion time were significant, as was the case with carbon. These interactions were between (1) season and immersion time ($F(4, 39) = 12.25$, $p < 0.0001$); (2) surface type and immersion time ($F(4, 39) = 11.48$, $p < 0.0001$); and (3) season, surface type and immersion time ($F(4, 39) = 18.42$, $p < 0.0001$).

Nitrogen in the biofilms further reflected that of carbon, with the main effect of season not being significant ($F(1, 39) = 0.56$, $p = 0.46$), nor was the main effect of surface type ($F(1, 39) = 0.10$, $p = 0.75$). Nitrogen concentrations from biofilms developed on treated surfaces were higher compared to control surfaces on some days, but not all. When summer and winter biofilm data were analyzed separately using a two-way ANOVA to look more closely at the main effect of surface type, the results were identical to that of carbon: summer biofilms had significantly more nitrogen on treated surfaces than control surfaces on day 90, and winter biofilms had significantly more nitrogen from biofilms on control surfaces than treated surfaces on day 15, yet by day 30 there was more nitrogen on treated surfaces.

Total DNA

Immersion time was a significant effect for the total amount of DNA extracted from the biofilm ($F(4, 40) = 293.44$, $p < 0.0001$), and an increase in total DNA over time was evident in both seasons (Figure 2D). The interaction between season and immersion time was also a significant effect on the total amount of DNA extracted from the biofilm ($F(4, 40) = 15.80$, $p < 0.0001$).

Overall, the main effect of season was not significant ($F(4, 40) = 0.73$, $p = 0.40$), nor was the main effect of surface type ($F(4, 40) = 0.03$, $p = 0.85$). The amount of DNA extracted from the biofilms for summer and winter was analyzed separately using a two-way ANOVA, which revealed significant difference across surface types only in the winter. Biofilms from day 30 and day 90 had significantly more DNA extracted from control surfaces than treated surfaces.

Percent of Surface Covered in Biomass

The percent of surface covered by biofilm biomass was the only data set that showed a significant effect by season alone ($F(1, 40) = 15.22$, $p < 0.0004$). Tukey's HSD test revealed summer surfaces had an overall greater percent coverage of biomass than surfaces during the winter season, which was driven by the significant differences in percent coverage across seasons on days 3 and 7 (Figure 2E).

Not surprisingly, the main effect of immersion time was also significant for the percent of surface covered by the biomass ($F(4, 40) = 134.25$, $p < 0.0001$). The

amount of biomass covering the control and treated surfaces increased significantly over time. Three-day biofilms covered the least area (0.02-3.48%), whereas 7- and 15- day biofilms covered significantly more (0.14-18.74%), and 30- and 90-day biofilms covered the most (7.15-92.3%) (Figure 2E). The percent coverage for summer and winter biofilms were analyzed separately using a two-way ANOVA, but no significant differences between surface types were observed at any immersion time during either season. Many surfaces had a patchy deposition of biomass, as evidenced by the wide range of area coverage.

Biofilm Composition

Species Richness

Overall, 338 different bacterial OTUs were detected, which ranged from 375 to 1196 bp. The number of fragments in summer samples ranged from 41 (day 7, treatment) to 80 (day 30, treatment), and winter samples had a minimum of 56 fragments (day 3, control) to a maximum of 76 (day 90, treatment) (Appendix Table A.7). The variation in the number of fragments with immersion time, season and surface type was analyzed using an ANOVA for factorial design (Figure 4). The effects of season and surface type, as well as the interactions, were not significant. Immersion time was the only significant effect overall ($F(4, 39) = 13.08$, $p < 0.0001$). The number of OTUs was analyzed separately for winter and summer communities using a two-way ANOVA, which revealed immersion time was a significant effect in both summer ($F(4, 19) = 11.42$, $p < 0.0001$) and winter ($F(4, 20) = 3.09$, $p < 0.039$), as the number of OTUs increased over immersion time.

Of the 338 fragments detected, 73 OTUs (22%) were specific to summer communities and 52 OTUs (15%) were detected only in winter communities. A 63% overlap of bacterial OTUs between summer and winter communities was detected, as 213 of the 338 OTUs were common to both seasons. The overlap of bacterial OTUs between summer and winter communities was apparent at all sampling days and on both surface types (Figure 3). For bacterial communities on control surfaces, there was a 13 – 17% overlap of OTUs between the summer and winter, while bacterial communities on treated surfaces overlapped between the two seasons by 12 – 23%. Some bacterial OTUs persisted in biofilms throughout all five sampling days (3, 7, 15, 30 and 90) for given surface types and seasons. In the summer, control surfaces consistently had three OTUs (640, 741, and 856 bp), while treated surfaces had two (640 and 736 bp) for all sampling days. In the winter, four OTUs (623, 868, 892, and 1070 bp) persisted on control surfaces for all sampling days, while treated surfaces had three (623, 785, and 878 bp). There was one OTU in each season, 640 bp in the summer and 623 bp in the winter, which persisted on both control and treated surfaces for all five sampling days.

Automated Ribosomal Intergenic Spacer Analysis

One MDS plot of all biofilm bacterial samples provides an overall view of communities from both surface types, all immersion times and both seasons (Figure 5). Three clusters are revealed at the 18% similarity level, which starts to show differences in the biofilm communities for immersion time and season. A cluster of summer communities from days 3 and 7 do not overlap with the winter cluster of

communities from days 3, 7 and 15, indicating a separation of early communities by season. The third cluster of biofilms contains communities from both seasons during later stages of immersion (days 30 and 90). At a similarity level of 36%, more clusters are revealed and they show communities further separated by season and immersion time. Within these summer and winter clusters, data points representing control and treated surfaces within given immersion times, are very close together, indicating strong similarities in the bacterial community composition between the surface types. Since stress increases with reducing dimensionality of the ordination, the high 2D stress of 0.21 for this MDS plot indicates the 2-dimensional plot is useful, but reliance should not be placed on the details of the clusters (Clarke and Warwick 2001). Therefore, to gain a clearer understanding of the biofilm communities within each season, two additional MDS plots were constructed to examine summer and winter communities separately.

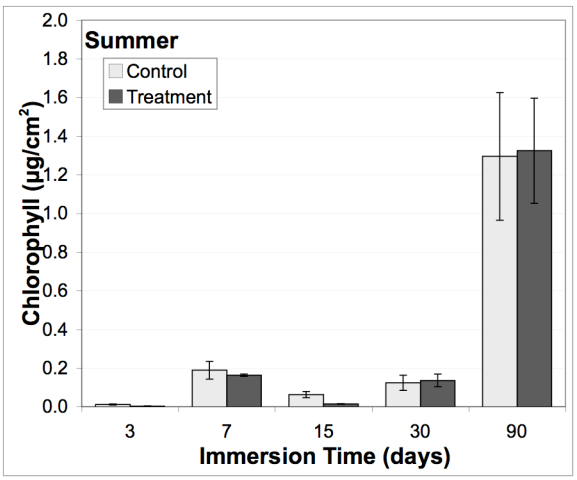
The MDS plot (2D stress = 0.08) for the summer biofilm communities reveals three distinct clusters at the 30% similarity level (Figure 6). The first cluster contains early biofilm communities, including 3- and 7-day biofilms. Within this cluster, the 3-day communities are at least 60% similar to each other and do not separate out by surface type. The 7-day communities are also at least 60% similar to each other; however, their composition on control surfaces is differentiated from that of treated surfaces at the 80% similarity level (contour line not shown). The second cluster within the 30% similarity level comprises communities immersed for 15 and 30 days. Within this cluster, the 15-day communities on the control and treated surfaces are less than 40%

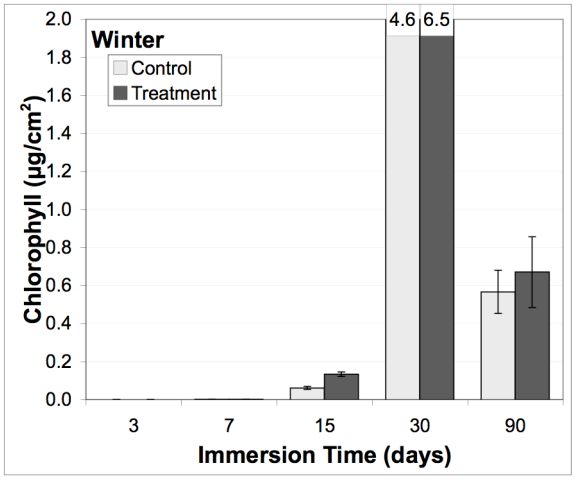
similar to each other, whereas the 30-day communities are less than 60% similar across control and treated surfaces. The third summer cluster is made up exclusively of 90-day biofilm communities. This cluster of only the most developed biofilms indicates a strong difference in biofilm composition from the first 30 days of immersion. Day-90 biofilm communities on control surfaces are at least 40% similar to each other, whereas communities on treated surfaces are at least 60% similar to each other. Day-90 biofilm communities on control and treated surfaces are different at the 60% similarity level. A two-way crossed with replicates design was used for ANOSIM for summer bacterial communities; it indicated significant separation in communities for both immersion time ($R = 0.989$, $p < 0.001$) and surface type ($R = 0.865$, $p < 0.001$).

The MDS plot (2D stress = 0.07) for the winter biofilm communities also reveals three distinct clusters at the 30% similarity level (Figure 7). The first cluster contains early biofilm communities, including 3-, 7-, and 15-day biofilms. Within this cluster, communities from 3- and 7-day biofilms are 40% similar to one another, however biofilm communities from all three days (3, 7, and 15) separate out into three distinct clusters at the 60% similarity level. The composition of day-3 communities on control surfaces are differentiated from that of treated surfaces only at the 80% similarity level (contour line not shown), day-7 communities on the control surfaces are differentiated from that of the treated surfaces at the 70% similarity level (contour line not shown), and day-15 communities are not observed to separate out by surface type. The second cluster in the winter is made up solely by day-30 communities, which are at least 40%

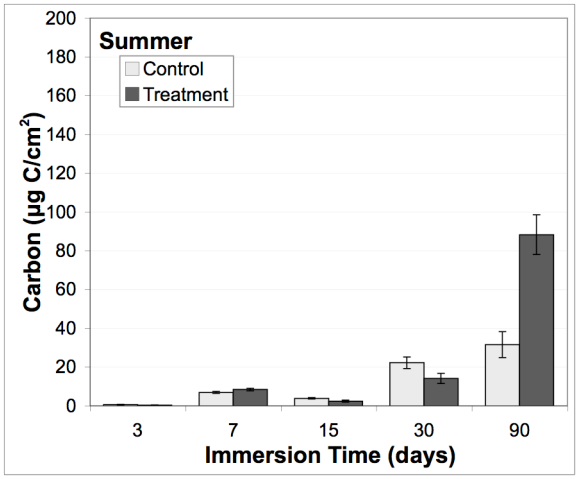
similar to one another. A separation of day-30 communities by surface type is evident at the 60% similarity level. The third cluster in the winter contains day-90 communities, which are also at least 40% similar to one another and separate out by surface type at the 60% similarity level. A two-way crossed with replicates design was used for ANOSIM for winter bacterial communities, which revealed significant separation in communities for both immersion time ($R = 0.998$, $p < 0.001$) and surface type ($R = 0.985$, $p < 0.001$).

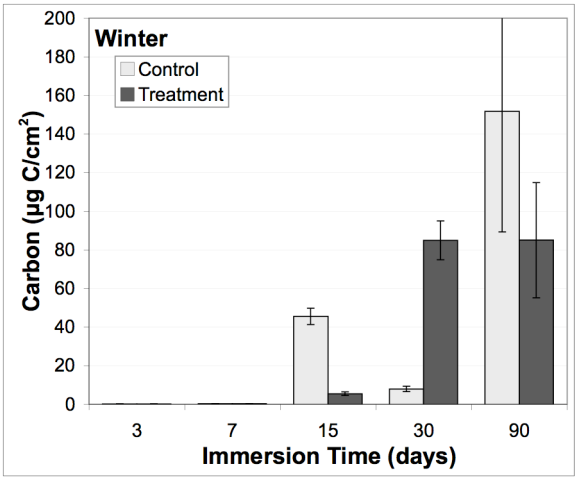
A


Summer
Control
Treatment
Chlorophyll (µg/cm²)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
7
15
30
90
Immersion Time (days)

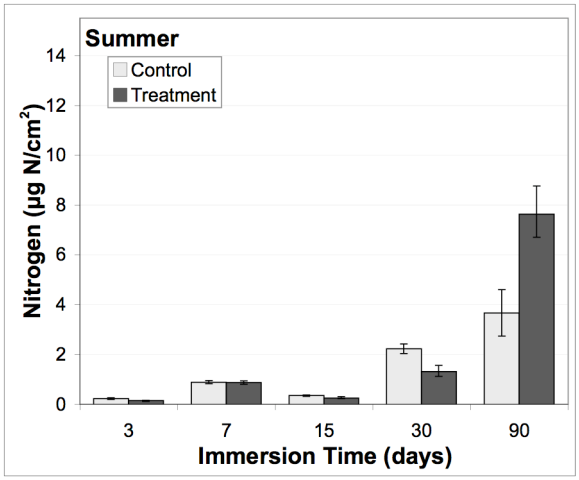

Winter
Control
Treatment
4.6
6.5
Chlorophyll (µg/cm²)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
7
15
30
90
Immersion Time (days)

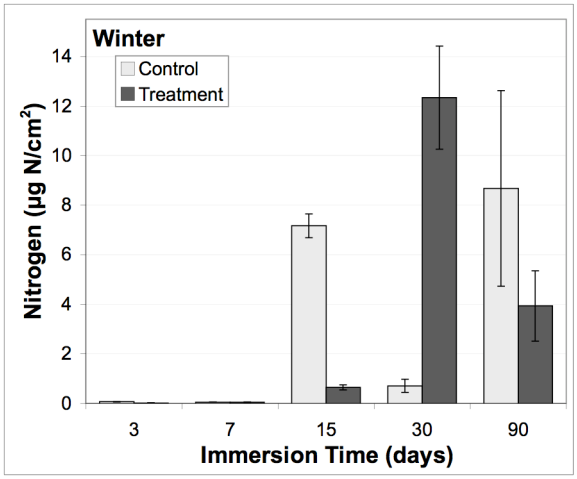
B


Summer
Control
Treatment
Carbon (µg C/cm²)
200
180
160
140
120
100
80
60
40
20
0
3
7
15
30
90
Immersion Time (days)


Winter
Control
Treatment
Carbon (µg C/cm²)
200
180
160
140
120
100
80
60
40
20
0
3
7
15
30
90
Immersion Time (days)

C


Summer
Control
Treatment
Nitrogen (µg N/cm²)
14
12
10
8
6
4
2
0
3
7
15
30
90
Immersion Time (days)


Winter
Control
Treatment
Nitrogen (µg N/cm²)
14
12
10
8
6
4
2
0
3
7
15
30
90
Immersion Time (days)

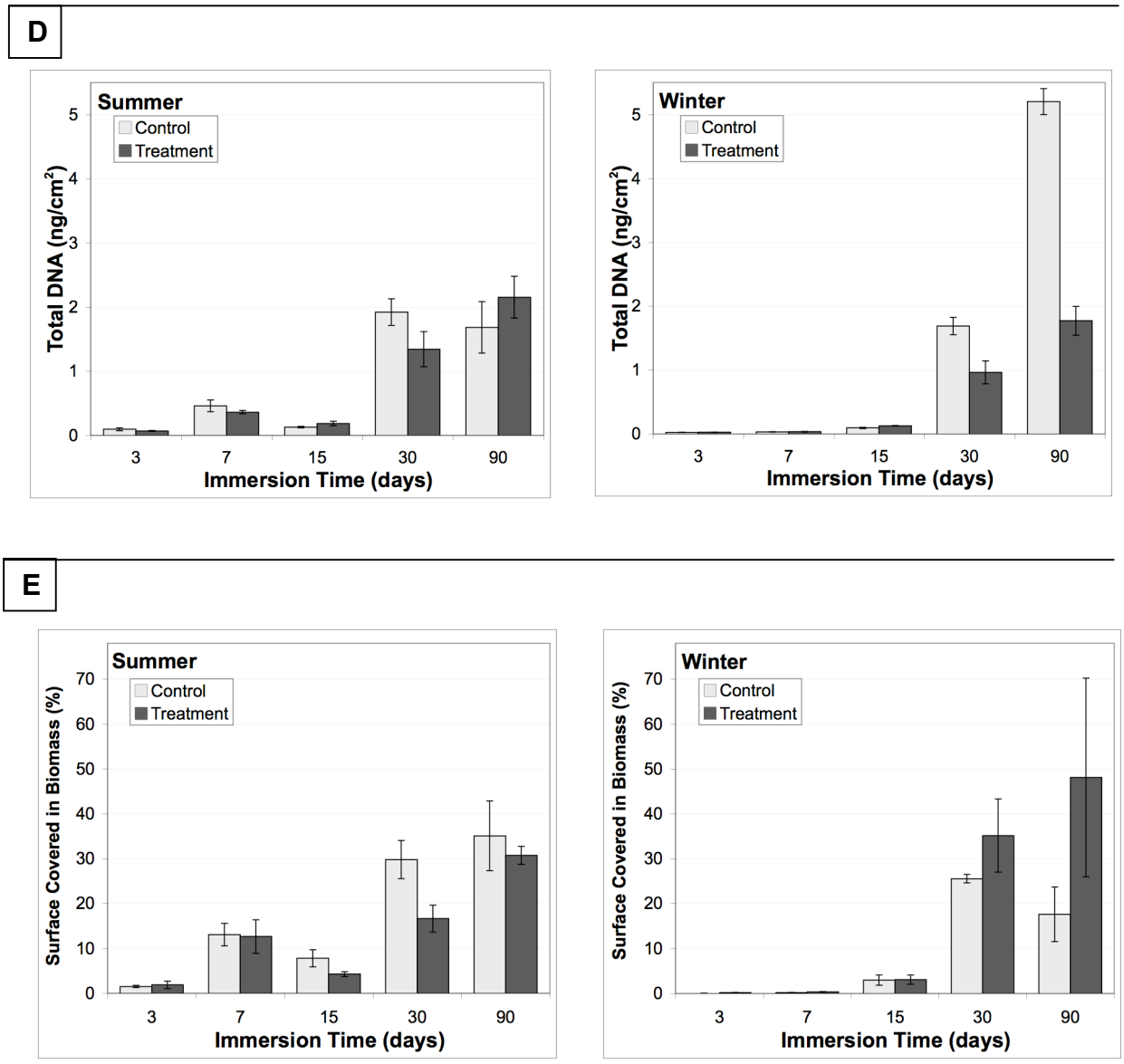


Figure 2. Biomass data plotted against immersion time for control and treated surfaces in two seasons. Community biomass was determined by: (A) chlorophyll (μg/cm^2), (B) carbon (μg C/cm^2), (C) nitrogen (μg N/cm^2), (D) total DNA (ng/cm^2), and (E) percent surface coverage (%). The error bars are standard error about the mean biomass obtained from three different surfaces. All plots have data for all five days of immersion, although measurements may be too low to visualize clearly on some.

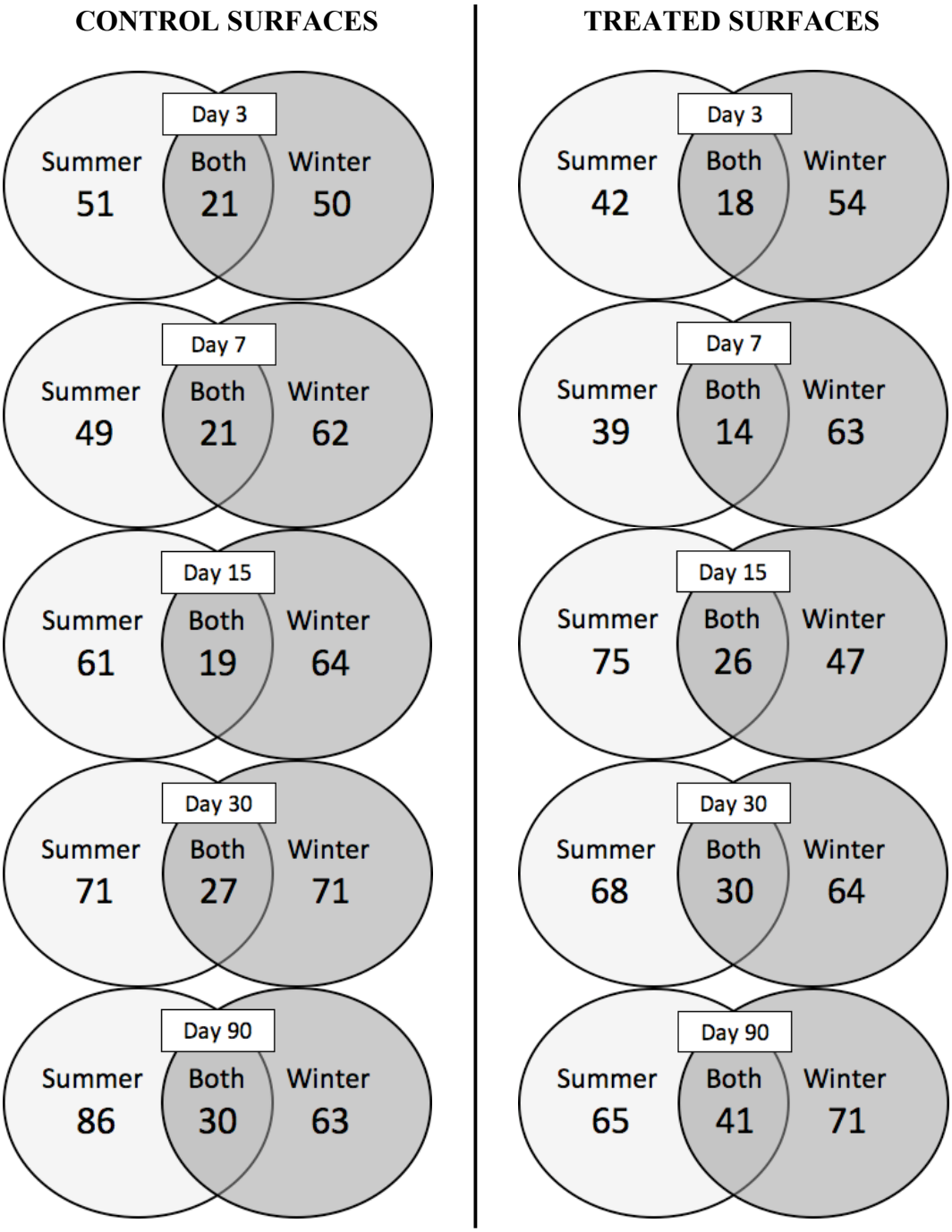


Figure 3. Venn Diagrams displaying the number of bacterial OTUs from biofilm communities on control and treated surfaces for all sampling days in the summer only, the winter only, and those that were present during both seasons.

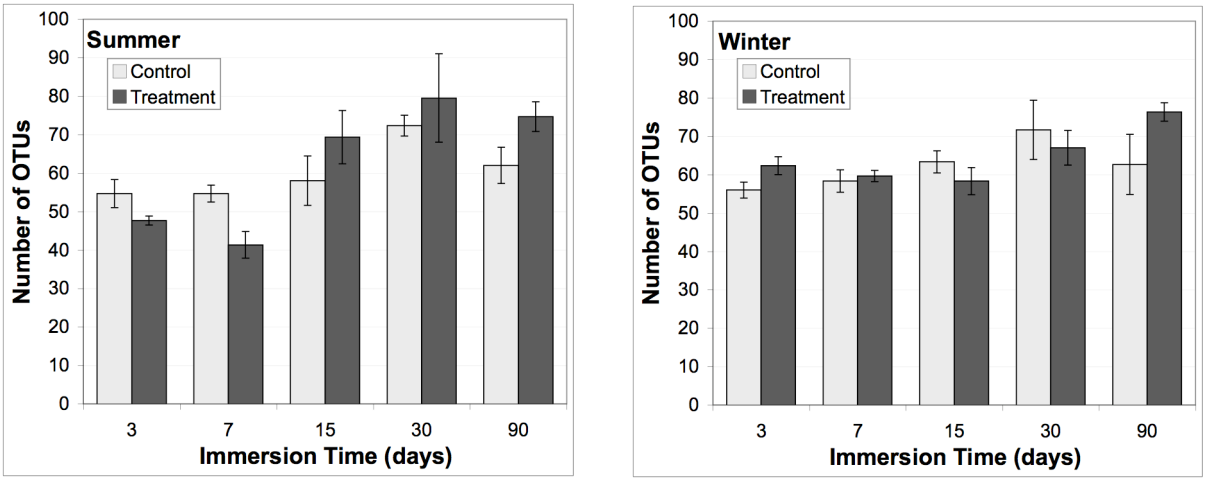


Figure 4. Species richness data plotted against immersion time for control and treated surfaces in two seasons. Species richness was determined by the number of OTUs (fragments) detected by ARISA. The error bars are standard error about the mean number of OTUs obtained from three different samples.

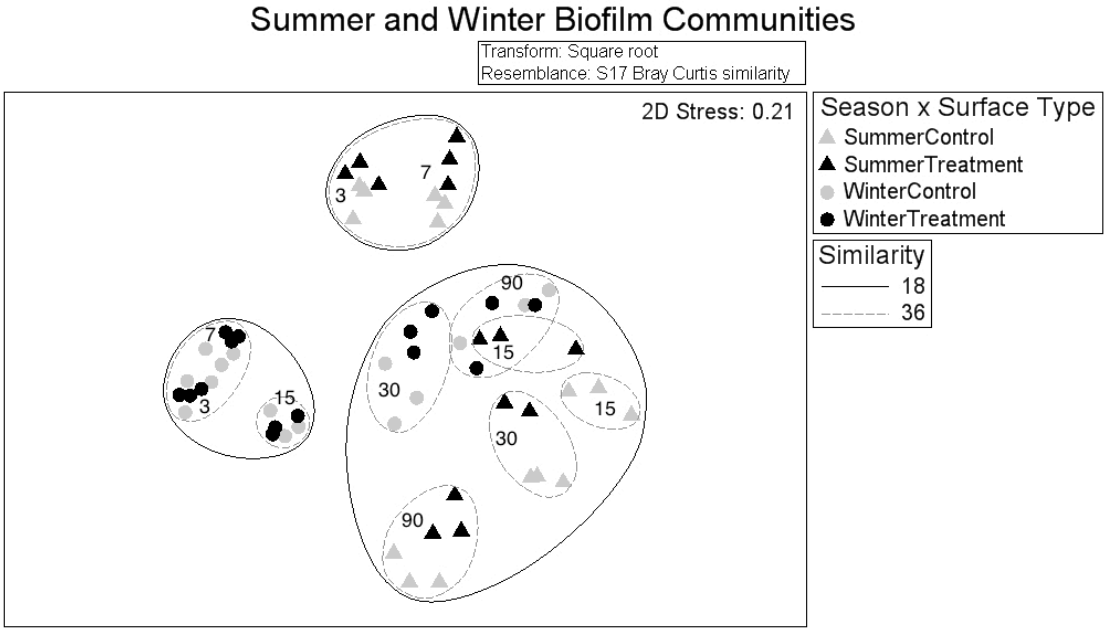


Figure 5. ARISA results for both summer and winter biofilm communities on control and treated surfaces, as visualized in non-metric multi-dimensional scaling, where points located close together represent communities that have very similar bacterial composition and points located far apart represent communities that have highly dissimilar bacterial composition. Numbers indicate immersion time.

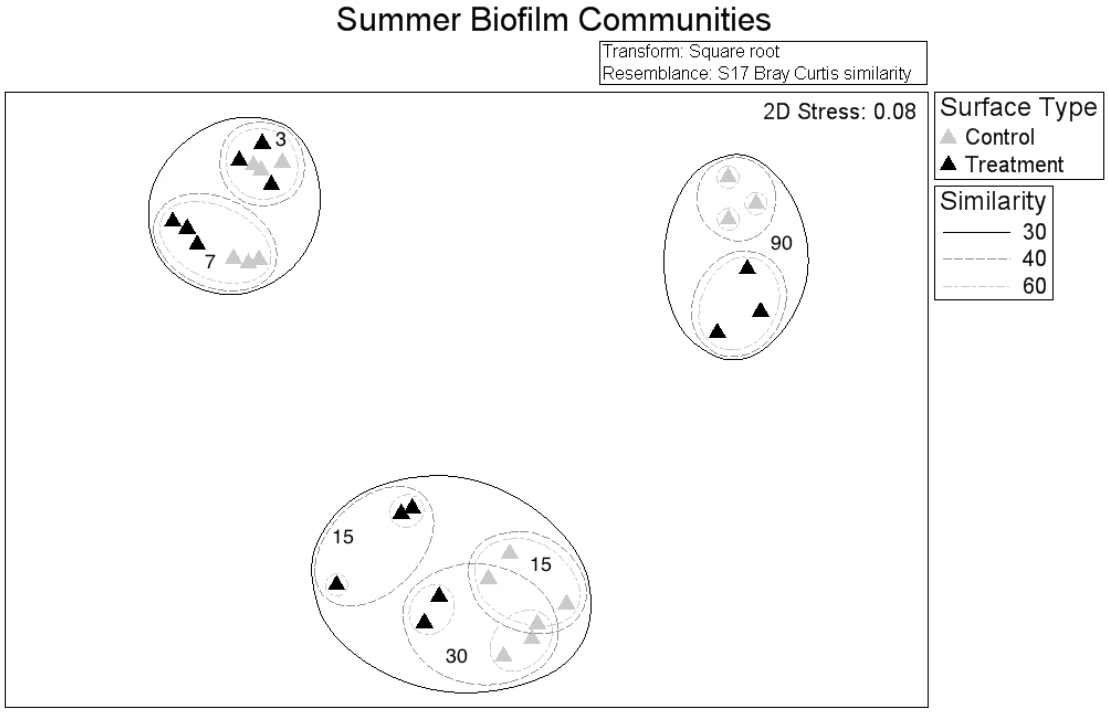


Figure 6. MDS plot showing ARISA results for summer biofilm communities with 3 distinct clusters. Numbers indicate immersion time.

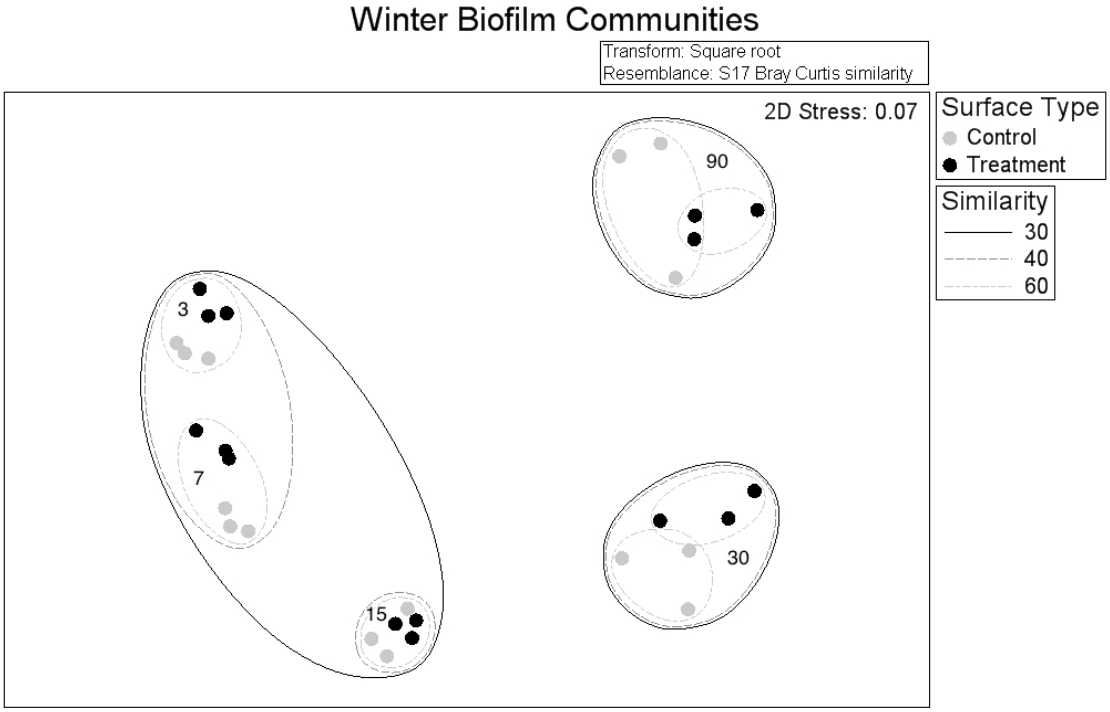


Figure 7. MDS plot showing ARISA results for winter biofilm communities with 3 distinct clusters. Numbers indicate immersion time.

DISCUSSION

In order to understand the first steps of marine biofouling in Narragansett Bay, this study assessed the evolution of biofilm community composition and biomass over immersion time and on two different surfaces. The use of the molecular method ARISA allowed for the detailed investigation of the initial bacterial community composition in biofilms, whereas biofilm biomass measurements took into account all the members of the biofilms, which could include both bacteria and settled eukaryotes. Biofilm biomass data revealed no overall significant differences between seasons or across surface types; however, immersion time had a significant effect as biomass tended to accumulate over time. Bacterial community composition was also influenced by immersion time, as communities separated out into 'early,' 'mid,' and 'late' groupings. Differences in bacterial community composition were observed between seasons, as well as surface types.

Comparing control and treated surfaces

The comparison of biofilm biomass between control glass surfaces and surfaces treated with a foul-release coating resulted in no clear difference between the surface types. Significant differences in biomass values from biofilms on control and treated surfaces were observed after certain days of immersion for some measurements, however no clear pattern could be discerned between the two surface types. Even when separated by seasons, no consistent difference between control and treated surfaces was revealed. For example, control surfaces from day-15 biofilms in the winter had greater carbon and nitrogen concentrations than biofilms from treated

surfaces, followed by higher carbon and nitrogen concentrations on treated surfaces compared to control surfaces just 15 days later.

The fluctuating biomass data on the two different surfaces is consistent with the mechanism of action of the foul-release coating used in this study. HullKote is an environmentally friendly coating, i.e., one that does minimal to no harm to the ecosystem and environment and does not contain biocides, by instead using a PTFE suspension system with a citrus-based high gloss polish, a silane polymer, aluminum-based particles, and a biodegradable detergent that work together to limit biofilm growth and also release accumulated growth during movement of the substrate (McLube, personal communication). The foul-release capacity of HullKote was not fully tested in this study since there was no water turbulence in the experimental tanks and movement of both control and treated surfaces during transport and rinsing was limited. Our results confirm that HullKote did not negatively impact the developing biofilms as a coating containing biocides would, since biomass values fluctuated between control and treated surfaces and were not drastically reduced on treated surfaces. Furthermore, similar numbers of OTUs were observed on both surfaces, with treated surfaces having an overall average of just 21 more OTUs than control surfaces, indicating no apparent negative impacts of the foul-release coating on settled organisms. Indeed biocides, such as copper and zinc used in antifouling paints, are known to affect bacterial communities. Using ARISA, Ancion et al. (2010) studied the effects of heavy metals (zinc, copper and lead) on the community structure and composition of stream biofilms over 21 days of exposure and at different

concentrations of metals. They found that bacterial biofilm communities began to shift in their composition as soon as 3 days into the exposure to metals, and they determined that both low and high concentrations of metals shifted communities (Ancion et al. 2010). In future studies using a foul-release coating, surfaces with biofilms would need to undergo movement in the water of at least 10 knots, such as that of a moving ship, in order to test the release of accumulated biofilm and fully determine the benefits from this type of antifouling practice (Buskens et al. 2013).

Comparing summer and winter biofilms

The comparison of biofilm biomass between summer and winter biofilms revealed no overall significant differences between the two seasons, irrespective of treatment or immersion time. Biomass data fluctuated between the two seasons and discrepancies in pattern between the different measured variables were apparent. Since biomass measures encompassed both bacterial and eukaryotic biomass, both types of populations are addressed below.

Bacterial activity in the water column is controlled by substrate availability and temperature (del Giorgio and Cole 1998), and in Narragansett Bay, the abundance and production of bacteria indeed vary significantly with temperature (Staroscik and Smith 2004). Bacterial production and abundance generally peak during June to early July (temperatures > 18ºC), and are at their lowest in colder temperatures (September through January) (Staroscik and Smith 2004). The bacterial abundance could increase 5-fold from winter to summer (Staroscik and Smith 2004). Given that the bacteria in

the water column are the source of what is available to settle into biofilms, it would have been expected that summer biofilms in this study would reflect a greater amount of biomass than winter biofilms. Although there may be less bacteria in the overlying water column during the winter season, it is still possible for winter biofilms to produce more biomass, as biofilm bacterial populations tend to be more active than those in the waters above as long as nutrients and carbon are available, encouraging growth (Araya et al. 2003). Based on the MERL environmental dataset, nutrients concentrations were high in early winter in Narragansett Bay.

In addition to the settled bacteria, eukaryotes were also part of the biofilm in this study in Narragansett Bay waters and could contribute greatly to all of the biomass measures, which included chlorophyll, carbon, nitrogen, total DNA and percent of surfaces covered. Chlorophyll concentrations can be used as an indicator of the relative contribution of phototrophic eukaryotes to biomass, since the presence of chlorophyll is a feature not found in bacteria other than cyanobacteria (Mulkidjanian et al. 2006). Although there was no significant difference in chlorophyll values across the seasons when all data are taken together, the highest chlorophyll concentration of all biofilms came from the winter season on day 30. This increased concentration of chlorophyll may be explained by the presence of a carpet of healthy naviculoid pennates and some unidentified tube-forming diatoms as observed by L. Maranda (personal communication). When compared to the MERL environmental dataset, chlorophyll concentrations in the biofilms from this study follow the patterns of the chlorophyll concentrations of the water in Narragansett Bay, except that the increase

in chlorophyll on day-30 biofilms preceded by two weeks the start of the winter-spring bloom in bay water. Just as in the water column and despite low water temperatures, the availability of nutrients and the increase in light levels favor diatom growth in the biofilm at this time of year.

Carbon and nitrogen concentrations, as well as the percent of the surface covered in biomass, from day 30 in the winter, were also all greater on this day compared to others. This increase in carbon and nitrogen biomass measures also reflects a large presence of eukaryotic autotrophs, as chlorophyll did, although the increase is more apparent on treated surfaces than control surfaces. A study by Montagnes and Franklin (2001) found that the cell size of several diatom species increases as temperatures decrease, which may relate to both the carbon and nitrogen concentrations in the biofilms as well as the surface covered. Larger cells would indicate a greater carbon and nitrogen content, and may also result in a greater percent area of the surface covered by biomass. Given the variability observed within replicates of given samples, confirmation of these seasonal trends will benefit from an increase in replication in future studies.

Lastly, total DNA extracted from the biofilms were also erratic across seasons. Variability between replicates prevented uncovering season patterns, if present. Only an increase in DNA concentrations with immersion time was evident, as was observed with the other measured biomass variables.

Comparing biofilms over immersion time

Of the three factors (season, surface type and immersion time) influencing biofilm biomass, the effect of immersion time was the dominating factor, as biomass tended to accumulate over immersion time in both seasons and on both surface types. Not all biomass measures, however, continued their increase from day 3 to day 90. In the summer, chlorophyll concentrations, along with carbon and nitrogen concentrations in the biofilms increased over immersion time, with biomass values peaking on day 90. This was not the case for chlorophyll or nitrogen concentrations in winter biofilms, as values peaked in day-30 biofilms before drastically decreasing in day-90 biofilms. A decrease in biofilm biomass may be explained through the dynamic nature of biofilms, as sloughing and detachment of parts of the biofilm are common; this opens spaces for new colonizers to attach or gives existing microorganisms room to grow (Railkin 2004; Dobretsov 2010). Another naturally occurring and frequent phenomenon on biofilms that could further explain the upset in steady accumulation of biomass is the effect of grazing. Micrograzers, such as heterotrophic flagellates and ciliates, have the potential to control biomass and diversity in biofilms, as well as large grazers (Dobretsov 2010). The sea snail *Crepidula fornicata* was observed on some summer and winter sample surfaces from day 90 during this study in Narragansett Bay water, and the activity of these grazers could definitely explain the impact on biofilm biomass. Although the natural processes of sloughing, detachment and grazing can potentially affect all biofilms, regardless of season, the late winter biofilms in this study appear to have experienced a greater grazing pressure than summer biofilms did.

Changes in community composition

Obvious differences in bacterial biofilm community composition were observed between summer and winter, whereas differences in biofilm communities between control surfaces and surfaces treated with foul-release coating were not as obvious, nevertheless, showed significant separation based on R of the ANOSIM function. The greatest visual change in community composition was observed over immersion time.

Three clusters of biofilm communities were observed in MDS plots for both seasons; however, the groupings varied between summer and winter. In the summer, the first cluster included biofilm communities from days 3 and 7, whereas the first winter cluster included biofilm communities from days 3, 7 and 15. The diversity of bacteria in each community, and the rate at which they colonize, may explain the differences shown in the MDS clusters; this is combined with bacteria most likely being added to communities at different times across season.

Microorganisms of many species express behaviors that allow them to select the site on which they are going to settle, taking biotic and abiotic factors into account (Prendergast 2010), therefore, the early biofilm communities are going to encompass the first microorganisms that found the conditions and surfaces favorable. Once initial settlers adhere to the surface, subsequent recruitment and settling take place and develop the biofilm over immersion time. Studies investigating the settlement of bacteria on hard substrates have identified various groups of bacteria dominating the biofilm communities at different stages of immersion (Dobretsov 2010); however, this

study in Narragansett Bay waters revealed 10 OTUs that persisted throughout the entire length of immersion, indicating an overall presence opposed to dominance of groups at different stages of immersion. From the work of others, some examples of the variation of bacterial settlers include the *Roseobacter* subgroup of Alphaproteobacteria dominating very early (24-72 h) biofilms in a salt marsh system in South Carolina (Dang and Lovell 2000), whereas Gammaproteobacteria dominated mature marine biofilms from the South China Sea, Caribbean and North Sea (Dobretsov 2010). Elifantz et al. (2013) were able to differentiate between early and late biofilms, finding that Alphaproteobacteria dominated initial biofilms, but decreased in abundance as the concentration of Bacteroidetes increased over the first 2 weeks of biofilm development. This study in Narragansett Bay water did reveal changes in bacterial community composition over immersion time, as visualized through MDS plots of the ITS; however, the identity of the all groups of bacteria making up the biofilms was not pursued.

Overall, there was a greater percentage of OTUs found only in the summer (22%) compared to the winter (15%), but the greatest percent of bacterial OTUs detected in biofilms was common to both seasons (63%). This indicates an interesting dynamic of the Narragansett Bay bacteria that are settling into biofilms, in that most are settling out of the water column in both seasons, while only a small percentage are season specific. However, when pairing community composition from surfaces sampled after the same immersion time, the number of bacterial OTUs specific to each season was greater than the number common to both seasons. This trend was observed on both

control and treated surfaces, thus, not all of the common OTUs were present at every sampling point throughout the experiment. This disconnect could be explained by the natural processes of sloughing, detachment and grazing, followed by new settlement of opportunistic bacteria and those common bacteria found in both seasons. Furthermore, it is possible that some of the OTUs in summer and winter are not the same bacteria; the fragments may have been of the same length without having similar sequences.

Limitations in the study

Primers were carefully chosen to reflect those of the most commonly used with marine species when employing the ARISA technique. Multiple primer sets have been utilized successfully for this type of work (Fisher and Triplett 1999; Fuhrman et al. 2006; Ancion et al 2010). Based on the primer sets used, different biases may arise and influence comparison of studies. Bias associated with the amplification of the 16S-23S intergenic spacer region can include possible preferential amplification of shorter templates and secondary structures or DNA flanking the template region (Fisher and Triplett 1999). In this study, such biases could lead to an underestimation of the diversity of the bacterial community compositions, and relative abundances of specific populations would have to be made carefully.

Fragments larger than 1200 bp were excluded from this study, which may eliminate important information. The largest fragment identified in this study was 1196 bp, which was determined by setting the fragment upper limit to 1200 bp with the

GeneScan™ 1200 LIZ® (Applied Biosystems) size standard. Raw data from ARISA electropherograms did include fragments above 1200 bp, but they were not included in this analysis. A search on Genbank (http://www.ncbi.nlm.nih.gov/) for different lengths of intergenic spacer regions results in few fragments above 1200 bp. Although this study includes the size range of most ITS regions (400-1000 bp), as identified in Genbank, excluding fragments about 1200 bp may ignore fragments of importance.

Future considerations

The use of a foul-release coating as a treatment did not result in significant differences in regards to biofilm biomass; however, separation of bacterial communities was evident when compared to untreated surfaces. It is expected that similar studies using an antifouling coating containing a biocide would produce different biomass results from those based on environmentally friendly coatings. The same molecular analytical techniques, however, could be used. ARISA proved to be an effective and efficient way to assess overall community composition of the biofilms, resulting in visually engaging MDS plots for analysis of similarity. Significant results for both bacterial community composition and biofilm biomass over immersion time, as well as changes in community composition throughout the seasons, indicate the need to take season, environmental conditions and length of immersion into account when studying marine biofilms in environments where conditions are not constant. Future work in this area should consider the identification of specific bacteria making up the biofilm communities in Narragansett Bay. Having a detailed understanding of the biofilm bacteria and their functions in a northern temperate estuary, opposed to just

knowing the groups of bacteria, may lead to even more effective and accurate methods to control unwanted biofilm growth by being able to target specific species.

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APPENDIX A

Raw Data

Table A.1a. Contact angle measurements of control and treated slides taken before immersion in the summer. The reported water drop average (°) comes from three consecutive measurements on one drop of water (5 μL). There were five different drops of water measured across the length of each slide. All 5 drops of water per slide measured are taken into the overall average contact angle for the surface type and season.

Slide	Season	Surface Type	Water Drop Average (°)	Surface Type	Water Drop Average (°)
1	summer	control	48.8	treatment	99.7
1	summer	control	31.9	treatment	102.4
1	summer	control	31.8	treatment	100.7
1	summer	control	35.0	treatment	85.0
1	summer	control	28.4	treatment	81.1
2	summer	control	54.9	treatment	91.5
2	summer	control	45.3	treatment	80.4
2	summer	control	35.2	treatment	77.9
2	summer	control	28.7	treatment	77.2
2	summer	control	27.2	treatment	78.8
3	summer	control	46.0	treatment	88.0
3	summer	control	36.6	treatment	74.6
3	summer	control	34.5	treatment	71.5
3	summer	control	30.6	treatment	78.9
3	summer	control	31.1	treatment	68.5
4	summer	control	48.2	treatment	82.3
4	summer	control	33.4	treatment	86.4
4	summer	control	25.6	treatment	73.9
4	summer	control	24.6	treatment	79.0
4	summer	control	25.4	treatment	76.4
5	summer	control	49.9	treatment	79.3
5	summer	control	43.7	treatment	83.1
5	summer	control	36.9	treatment	81.6
5	summer	control	33.0	treatment	87.8
5	summer	control	33.9	treatment	92.4
		Average	36°	Average	83°
		Standard Error	1.7°	Standard Error	1.8°

Table A.1b. Contact angle measurements of control and treated slides taken before immersion in the winter. The reported water drop average (°) comes from three consecutive measurements on one drop of water (5 μL). There were five different drops of water measured across the length of each slide. All 5 drops of water per slide measured are taken into the overall average contact angle for the surface type and season.

Slide	Season	Surface Type	Water Drop Average (°)	Surface Type	Water Drop Average (°)
1	winter	control	38.5	treatment	83.7
1	winter	control	38.6	treatment	85.4
1	winter	control	30.8	treatment	79.2
1	winter	control	33.8	treatment	74.0
1	winter	control	30.8	treatment	76.5
2	winter	control	28.6	treatment	78.5
2	winter	control	23.3	treatment	76.8
2	winter	control	24.1	treatment	74.8
2	winter	control	22.9	treatment	73.3
2	winter	control	25.7	treatment	76.0
3	winter	control	42.9	treatment	83.3
3	winter	control	30.9	treatment	81.6
3	winter	control	29.8	treatment	78.0
3	winter	control	25.5	treatment	81.0
3	winter	control	27.3	treatment	78.5
4	winter	control	39.6	treatment	90.6
4	winter	control	29.1	treatment	84.1
4	winter	control	28.8	treatment	82.2
4	winter	control	30.4	treatment	82.5
4	winter	control	33.5	treatment	79.2
5	winter	control	41.6	treatment	89.3
5	winter	control	32.5	treatment	80.8
5	winter	control	28.7	treatment	78.5
5	winter	control	32.5	treatment	80.9
5	winter	control	32.6	treatment	84.4
		Average	31°	Average	81°
		Standard Error	1.1°	Standard Error	0.9°

Table A.2a. Chlorophyll concentrations (μg/cm^2) for control and treated surfaces in the summer.

Season	Surface	Immersion Time	Chlorophyll (μg/cm^2)	Average Chlorophyll (μg/cm2)	Standard Error
summer	control	3	1.7E-02		
summer	control	3	7.8E-03		
summer	control	3	1.1E-02	1.2E-02	2.60E-03
summer	control	7	2.8E-01		
summer	control	7	1.6E-01		
summer	control	7	1.3E-01	1.9E-01	4.60E-02
summer	control	15	7.9E-02		
summer	control	15	3.1E-02		
summer	control	15	7.7E-02	6.2E-02	1.60E-02
summer	control	30	1.9E-01		
summer	control	30	1.3E-01		
summer	control	30	5.2E-02	1.2E-01	4.00E-02
summer	control	90	6.4E-01		
summer	control	90	1.6E+00		
summer	control	90	1.7E+00	1.3E+00	3.30E-01
summer	treatment	3	2.9E-03		
summer	treatment	3	5.3E-03		
summer	treatment	3	8.4E-04	3.0E-03	1.30E-03
summer	treatment	7	1.7E-01		
summer	treatment	7	1.7E-01		
summer	treatment	7	1.5E-01	1.6E-01	6.10E-03
summer	treatment	15	1.7E-02		
summer	treatment	15	1.3E-02		
summer	treatment	15	1.4E-02	1.5E-02	1.30E-03
summer	treatment	30	2.0E-01		
summer	treatment	30	9.1E-02		
summer	treatment	30	1.2E-01	1.4E-01	3.30E-02
summer	treatment	90	9.4E-01		
summer	treatment	90	1.8E+00		
summer	treatment	90	1.2E+00	1.3E+00	2.70E-01

Table A.2b. Chlorophyll concentrations (µg/cm^2) for control and treated surfaces in the winter.

Season	Surface	Immersion Time	Chlorophyll (µg/cm^2)	Average Chlorophyll (µg/cm^2)	Standard Error
winter	control	3	0.0E+00		
winter	control	3	6.4E-06		
winter	control	3	0.0E+00	2.1E-06	2.10E-06
winter	control	7	1.1E-03		
winter	control	7	9.4E-04		
winter	control	7	8.0E-04	9.5E-04	9.60E-05
winter	control	15	4.5E-02		
winter	control	15	7.2E-02		
winter	control	15	6.6E-02	6.1E-02	8.20E-03
winter	control	30	5.7E+00		
winter	control	30	3.9E+00		
winter	control	30	4.2E+00	4.6E+00	5.50E-01
winter	control	90	5.9E-01		
winter	control	90	3.6E-01		
winter	control	90	7.5E-01	5.7E-01	1.10E-01
winter	treatment	3	2.4E-05		
winter	treatment	3	6.6E-05		
winter	treatment	3	2.7E-05	3.9E-05	1.40E-05
winter	treatment	7	7.9E-04		
winter	treatment	7	1.4E-03		
winter	treatment	7	1.1E-03	1.1E-03	1.80E-04
winter	treatment	15	1.2E-01		
winter	treatment	15	1.6E-01		
winter	treatment	15	1.3E-01	1.3E-01	1.20E-02
winter	treatment	30	6.8E+00		
winter	treatment	30	6.2E+00		
winter	treatment	30	6.5E+00	6.5E+00	1.60E-01
winter	treatment	90	4.9E-01		
winter	treatment	90	4.7E-01		
winter	treatment	90	1.0E+00	6.7E-01	1.90E-01

Table A.3a. Carbon concentrations (µg C/cm^2) for control and treated surfaces in the summer.

Season	Surface	Immersion Time	Carbon (µg C/cm^2)	Average Carbon (µg C/cm^2)	Standard Error
summer	control	3	0.9		
summer	control	3	0.2		
summer	control	3	0.6	0.6	0.2
summer	control	7	7.1		
summer	control	7	6.0		
summer	control	7	7.6	6.9	0.5
summer	control	15	4.4		
summer	control	15	3.8		
summer	control	15	3.2	3.8	0.4
summer	control	30	28.1		
summer	control	30	19.9		
summer	control	30	18.7	22.2	3.0
summer	control	90	22.1		
summer	control	90	28.2		
summer	control	90	44.5	31.6	6.7
summer	treatment	3	0.3		
summer	treatment	3	0.5		
summer	treatment	3	0.2	0.4	0.1
summer	treatment	7	7.7		
summer	treatment	7	9.0	8.4	0.7
summer	treatment	15	3.5		
summer	treatment	15	1.9		
summer	treatment	15	1.8	2.4	0.5
summer	treatment	30	11.9		
summer	treatment	30	19.2		
summer	treatment	30	11.2	14.1	2.6
summer	treatment	90	102.3		
summer	treatment	90	94.1		
summer	treatment	90	68.4	88.3	10.2

Table A.3b. Carbon concentrations (µg C/cm^2) for control and treated surfaces in the winter.

Season	Surface	Immersion Time	Carbon (µg C/cm^2)	Average Carbon (µg C/cm^2)	Standard Error
winter	control	3	0.1		
winter	control	3	0.2		
winter	control	3	0.1	0.1	0.0
winter	control	7	0.2		
winter	control	7	0.2		
winter	control	7	0.2	0.2	0.0
winter	control	15	52.5		
winter	control	15	37.8		
winter	control	15	46.2	45.5	4.3
winter	control	30	10.7		
winter	control	30	6.8		
winter	control	30	6.3	7.9	1.4
winter	control	90	27.6		
winter	control	90	225.6		
winter	control	90	201.9	151.7	62.4
winter	treatment	3	0.1		
winter	treatment	3	0.1		
winter	treatment	3	0.1	0.1	0.0
winter	treatment	7	0.3		
winter	treatment	7	0.4		
winter	treatment	7	0.3	0.3	0.0
winter	treatment	15	3.7		
winter	treatment	15	6.5		
winter	treatment	15	6.2	5.5	0.9
winter	treatment	30	105.0		
winter	treatment	30	73.2		
winter	treatment	30	76.5	84.9	10.1
winter	treatment	90	92.7		
winter	treatment	90	30.0		
winter	treatment	90	132.4	85.0	29.8

Table A.4a. Nitrogen concentrations (μg N/cm^2) for control and treated surfaces in the winter.

Season	Surface	Immersion Time	Nitrogen (μg N /cm^2)	Average Nitrogen (μg N/cm^2)	Standard Error
summer	control	3	0.3		
summer	control	3	0.2		
summer	control	3	0.2	0.2	0.03
summer	control	7	0.9		
summer	control	7	0.8		
summer	control	7	0.9	0.9	0.06
summer	control	15	0.4		
summer	control	15	0.3		
summer	control	15	0.3	0.3	0.03
summer	control	30	2.6		
summer	control	30	2.1		
summer	control	30	2.0	2.2	0.19
summer	control	90	2.2		
summer	control	90	3.4		
summer	control	90	5.4	3.7	0.93
summer	treatment	3	0.1		
summer	treatment	3	0.2		
summer	treatment	3	0.1	0.1	0.01
summer	treatment	7	0.8		
summer	treatment	7	0.9	0.9	0.07
summer	treatment	15	0.4		
summer	treatment	15	0.2		
summer	treatment	15	0.2	0.3	0.05
summer	treatment	30	1.1		
summer	treatment	30	1.8		
summer	treatment	30	1.0	1.3	0.25
summer	treatment	90	9.7		
summer	treatment	90	7.4		
summer	treatment	90	5.8	7.6	1.13

Table A.4b. Nitrogen concentrations (µg N/cm^2) for control and treated surfaces in the winter.

Season	Surface	Immersion Time	Nitrogen (µg N /cm^2)	Average Nitrogen (µg N/cm^2)	Standard Error
winter	control	3	0.1		
winter	control	3	0.1		
winter	control	3	0.1	0.1	0.01
winter	control	7	0.0		
winter	control	7	0.0		
winter	control	7	0.1	0.0	0.01
winter	control	15	8.1		
winter	control	15	6.5		
winter	control	15	6.9	7.2	0.48
winter	control	30	1.2		
winter	control	30	0.4		
winter	control	30	0.5	0.7	0.27
winter	control	90	1.0		
winter	control	90	14.2		
winter	control	90	10.8	8.7	3.94
winter	treatment	3	0.0		
winter	treatment	3	0.0		
winter	treatment	3	0.0	0.0	0.01
winter	treatment	7	0.0		
winter	treatment	7	0.1		
winter	treatment	7	0.0	0.0	0.01
winter	treatment	15	0.4		
winter	treatment	15	0.7		
winter	treatment	15	0.8	0.6	0.10
winter	treatment	30	16.4		
winter	treatment	30	11.2		
winter	treatment	30	9.5	12.3	2.08
winter	treatment	90	3.5		
winter	treatment	90	1.7		
winter	treatment	90	6.6	3.9	1.42

Table A.5a. Total DNA (ng/cm^2) extracted from biofilms developed on both surface types in the summer.

Season	Surface	Immersion Time	Total DNA (ng/cm^2)	Average DNA (ng/cm^2)	Standard Error
summer	control	3	0.14		
summer	control	3	0.09		
summer	control	3	0.06	0.09	0.02
summer	control	7	0.62		
summer	control	7	0.46		
summer	control	7	0.30	0.46	0.09
summer	control	15	0.13		
summer	control	15	0.11		
summer	control	15	0.15	0.13	0.01
summer	control	30	2.15		
summer	control	30	2.11		
summer	control	30	1.51	1.92	0.21
summer	control	90	2.47		
summer	control	90	1.15		
summer	control	90	1.43	1.68	0.4
summer	treatment	3	0.08		
summer	treatment	3	0.08		
summer	treatment	3	0.05	0.07	0.01
summer	treatment	7	0.31		
summer	treatment	7	0.40		
summer	treatment	7	0.37	0.36	0.03
summer	treatment	15	0.18		
summer	treatment	15	0.13		
summer	treatment	15	0.25	0.18	0.03
summer	treatment	30	1.61		
summer	treatment	30	1.63		
summer	treatment	30	0.79	1.34	0.27
summer	treatment	90	2.64		
summer	treatment	90	1.54		
summer	treatment	90	2.29	2.15	0.33

Table A.5b. Total DNA (ng/cm^2) extracted from biofilms developed on both surface types in the winter.

Season	Surface	Immersion Time	Total DNA (ng/cm^2)	Average DNA (ng/cm^2)	Standard Error
winter	control	3	0.03		
winter	control	3	0.02		
winter	control	3	0.03	0.03	0
winter	control	7	0.03		
winter	control	7	0.04		
winter	control	7	0.04	0.03	0
winter	control	15	0.08		
winter	control	15	0.10		
winter	control	15	0.11	0.09	0.01
winter	control	30	1.47		
winter	control	30	1.93		
winter	control	30	1.67	1.69	0.13
winter	control	90	4.81		
winter	control	90	5.49		
winter	control	90	5.31	5.20	0.2
winter	treatment	3	0.03		
winter	treatment	3	0.03		
winter	treatment	3	0.03	0.03	0
winter	treatment	7	0.03		
winter	treatment	7	0.02		
winter	treatment	7	0.04	0.03	0.01
winter	treatment	15	0.12		
winter	treatment	15	0.13		
winter	treatment	15	0.14	0.13	0.01
winter	treatment	30	1.30		
winter	treatment	30	0.90		
winter	treatment	30	0.69	0.96	0.18
winter	treatment	90	2.21		
winter	treatment	90	1.47		
winter	treatment	90	1.63	1.77	0.22

Table A.6a. Percent (%) of surface covered by biomass on control and treated slides in the summer.

Season	Surface	Immersion Time	Percent Coverage	Average (%)	Standard Error
summer	control	3	1.94		
summer	control	3	1.04		
summer	control	3	1.49	1.49	0.26
summer	control	7	8.06		
summer	control	7	15.95		
summer	control	7	15.16	13.06	2.51
summer	control	15	3.93		
summer	control	15	9.70		
summer	control	15	9.73	7.79	1.93
summer	control	30	32.23		
summer	control	30	21.50		
summer	control	30	35.60	29.77	4.25
summer	control	90	46.67		
summer	control	90	38.28		
summer	control	90	20.28	35.08	7.78
summer	treatment	3	3.48		
summer	treatment	3	1.46		
summer	treatment	3	0.63	1.86	0.85
summer	treatment	7	13.33		
summer	treatment	7	5.84		
summer	treatment	7	18.74	12.64	3.74
summer	treatment	15	3.21		
summer	treatment	15	4.64		
summer	treatment	15	4.97	4.27	0.54
summer	treatment	30	21.93		
summer	treatment	30	16.38		
summer	treatment	30	11.56	16.62	2.99
summer	treatment	90	30.32		
summer	treatment	90	34.36		
summer	treatment	90	27.51	30.73	1.99

Table A.6b. Percent (%) of surface covered by biomass on control and treated slides in the winter.

Season	Surface	Immersion Time	Percent Coverage	Average (%)	Standard Error
winter	control	3	0.02		
winter	control	3	0.04		
winter	control	3	0.03	0.03	0.01
winter	control	7	0.25		
winter	control	7	0.15		
winter	control	7	0.14	0.18	0.04
winter	control	15	1.62		
winter	control	15	2.00		
winter	control	15	5.26	2.96	1.15
winter	control	30	23.73		
winter	control	30	25.95		
winter	control	30	26.97	25.55	0.96
winter	control	90	7.15		
winter	control	90	17.52		
winter	control	90	28.26	17.64	6.09
winter	treatment	3	0.10		
winter	treatment	3	0.13		
winter	treatment	3	0.29	0.17	0.06
winter	treatment	7	0.37		
winter	treatment	7	0.21		
winter	treatment	7	0.42	0.33	0.06
winter	treatment	15	1.83		
winter	treatment	15	5.07		
winter	treatment	15	2.23	3.04	1.02
winter	treatment	30	23.00		
winter	treatment	30	31.83		
winter	treatment	30	50.65	35.16	8.15
winter	treatment	90	92.32		
winter	treatment	90	25.06		
winter	treatment	90	26.87	48.08	22.12

Table A.7a. Species Richness data represented as operational taxonomic units (OTUs) for the summer season, as determined by the number of fragments from ARISA.

Season	Surface Type	Immersion Time	Number of OTUs	Average	Standard Error
summer	control	3	62		
summer	control	3	51		
summer	control	3	51	55	4
summer	control	7	52		
summer	control	7	59		
summer	control	7	53	55	2
summer	control	15	56		
summer	control	15	70		
summer	control	15	48	58	6
summer	control	30	74		
summer	control	30	76		
summer	control	30	67	72	3
summer	control	90	69		
summer	control	90	53		
summer	control	90	64	62	5
summer	treatment	3	50		
summer	treatment	3	47		
summer	treatment	3	46	48	1
summer	treatment	7	47		
summer	treatment	7	42		
summer	treatment	7	35	41	3
summer	treatment	15	70		
summer	treatment	15	57		
summer	treatment	15	81	69	7
summer	treatment	30	91		
summer	treatment	30	68	80	12
summer	treatment	90	79		
summer	treatment	90	67		
summer	treatment	90	78	75	4

Table A.7b. Species Richness data represented as operational taxonomic units (OTUs) for the winter season, as determined by the number of fragments from ARISA.

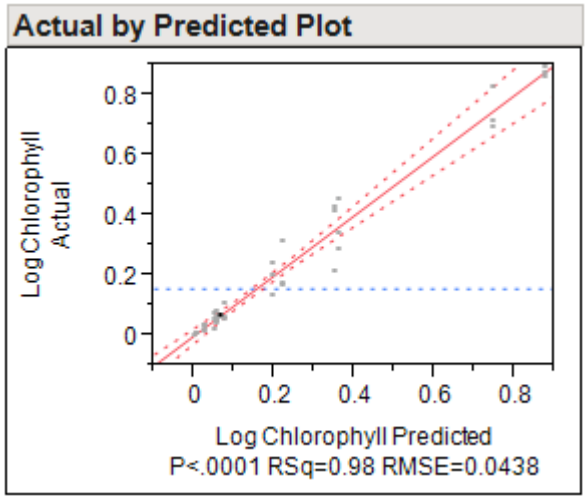
Season	Surface Type	Immersion Time	Number of OTUs	Average	Standard Error
winter	control	3	60		
winter	control	3	53		
winter	control	3	55	56	2
winter	control	7	63		
winter	control	7	53		
winter	control	7	59	58	3
winter	control	15	68		
winter	control	15	64		
winter	control	15	58	63	3
winter	control	30	83		
winter	control	30	75		
winter	control	30	57	72	8
winter	control	90	61		
winter	control	90	77		
winter	control	90	50	63	8
winter	treatment	3	66		
winter	treatment	3	63		
winter	treatment	3	58	62	2
winter	treatment	7	57		
winter	treatment	7	62		
winter	treatment	7	60	60	1
winter	treatment	15	65		
winter	treatment	15	53		
winter	treatment	15	57	58	4
winter	treatment	30	76		
winter	treatment	30	62		
winter	treatment	30	63	67	5
winter	treatment	90	73		
winter	treatment	90	81		
winter	treatment	90	75	76	2

APPENDIX B

Summary of Statistical Test Results

RESPONSE LOG CHLOROPHYLL

Whole Model



Summary of Fit

RSquare	0.978999
RSquare Adj	0.969023
Root Mean Square Error	0.043792
Mean of Response	0.155216
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	3.5759378	0.188207	98.1385
Error	40	0.0767109	0.001918	Prob > F
C. Total	59	3.6526487		<.0001*

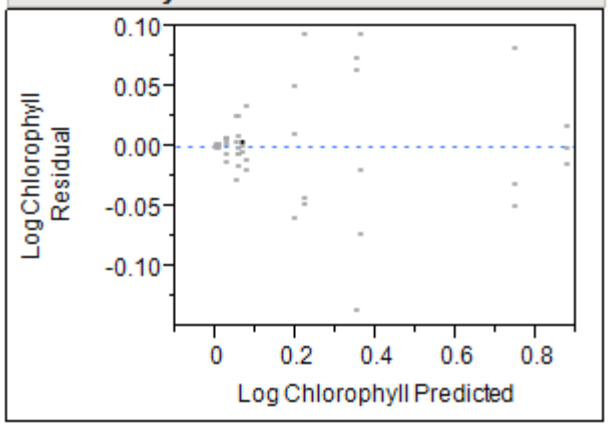
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0016155	0.012642	0.13	0.8990
Season[summer]	0.0016065	0.012642	0.13	0.8995
Surface[control]	0.0009584	0.012642	0.08	0.9399
Immersion Time[7-3]	0.0336685	0.017878	1.88	0.0670
Immersion Time[15-7]	-0.007149	0.017878	-0.40	0.6914
Immersion Time[30-15]	0.4025164	0.017878	22.51	<.0001*
Immersion Time[90-30]	-0.150256	0.017878	-8.40	<.0001*
Season[summer]*Surface[control]	0.0009664	0.012642	0.08	0.9394
Season[summer]*Immersion Time[7-3]	0.0332288	0.017878	1.86	0.0704
Season[summer]*Immersion Time[15-7]	-0.046693	0.017878	-2.61	0.0126*
Season[summer]*Immersion Time[30-15]	-0.366209	0.017878	-20.48	<.0001*
Season[summer]*Immersion Time[90-30]	0.4533169	0.017878	25.36	<.0001*
Surface[control]*Immersion Time[7-3]	0.0012597	0.017878	0.07	0.9442
Surface[control]*Immersion Time[15-7]	-0.004455	0.017878	-0.25	0.8045
Surface[control]*Immersion Time[30-15]	-0.031544	0.017878	-1.76	0.0853
Surface[control]*Immersion Time[90-30]	0.02502	0.017878	1.40	0.1694
Season[summer]*Surface[control]*Immersion Time[7-3]	0.0012859	0.017878	0.07	0.9430
Season[summer]*Surface[control]*Immersion Time[15-7]	0.009882	0.017878	0.55	0.5835
Season[summer]*Surface[control]*Immersion Time[30-15]	0.0192202	0.017878	1.08	0.2888
Season[summer]*Surface[control]*Immersion Time[90-30]	-0.027543	0.017878	-1.54	0.1313

Effect Tests

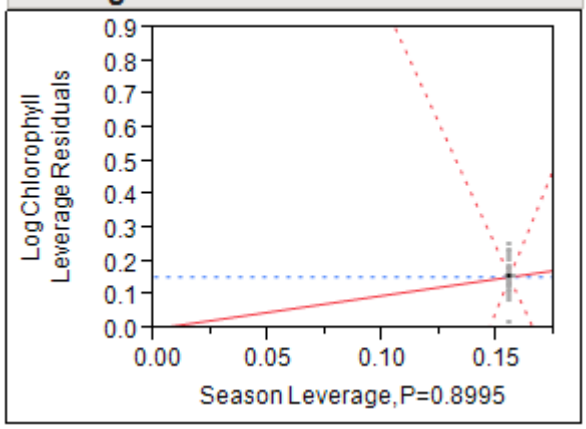
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	0.0000310	0.0161	0.8995
Surface	1	1	0.0000110	0.0057	0.9399
Immersion Time	4	4	1.7479328	227.8598	<.0001*
Season*Surface	1	1	0.0000112	0.0058	0.9394
Season*Immersion Time	4	4	1.6136551	210.3554	<.0001*
Surface*Immersion Time	4	4	0.0105914	1.3807	0.2579
Season*Surface*Immersion Time	4	4	0.0076853	1.0019	0.4179

Residual by Predicted Plot

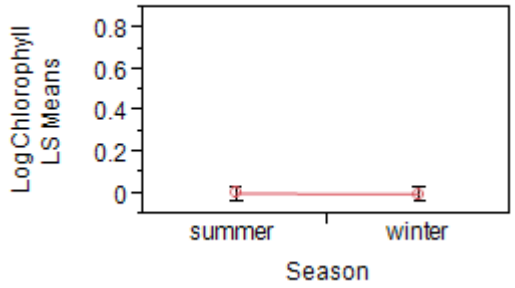


Season

Leverage Plot



LS Means Plot



LSMeans Differences Student's t

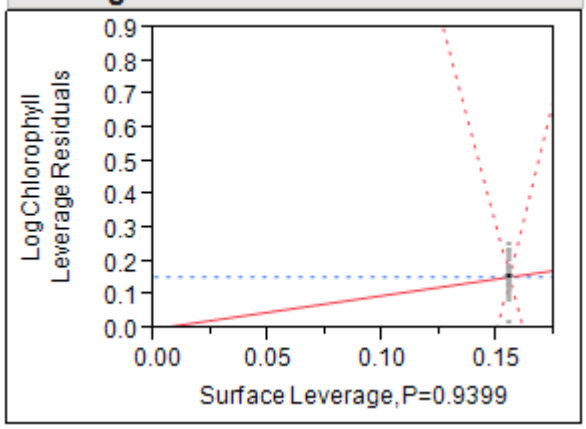
α= 0.050 t= 2.02108

Level		Least Sq Mean
summer	A	0.00322207
winter	A	0.00000898

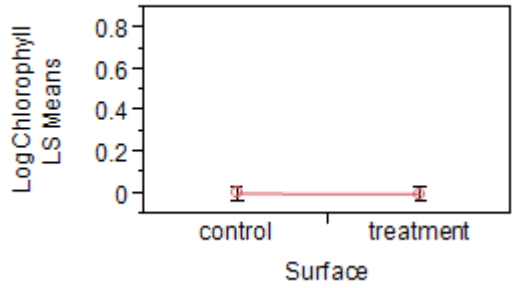
Levels not connected by same letter are significantly different.

Surface Type

Leverage Plot



LS Means Plot



LSMeans Differences Student's t

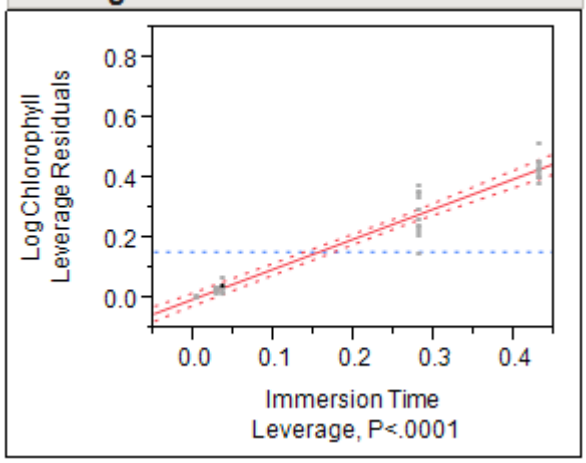
α= 0.050 t= 2.02108

Level		Least Sq Mean
control	A	0.00257392
treatment	A	0.00065713

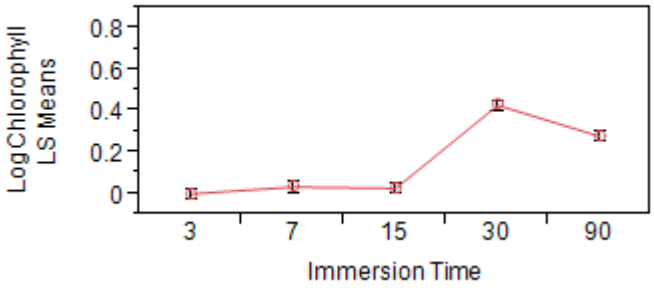
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



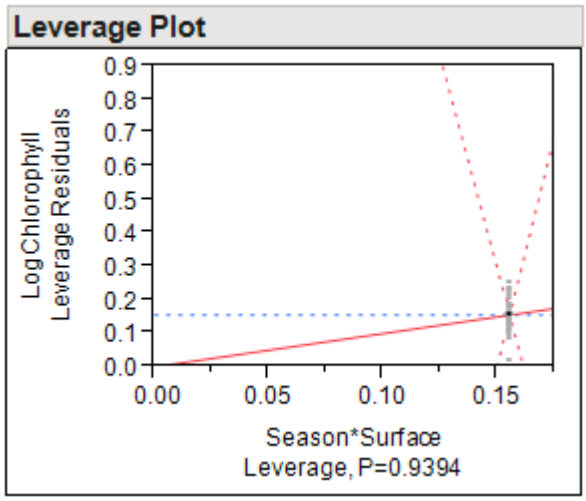
LSMeans Differences Tukey HSD

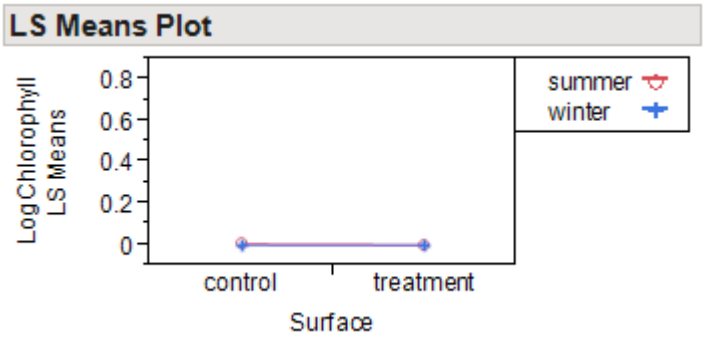
α= 0.050 Q= 2.85609

Level			Least Sq Mean
30	A		0.43065171
90		B	0.28039563
7		C	0.03528398
15		C	0.02813531
3		C	0.00161553

Levels not connected by same letter are significantly different.

Season*Surface Type





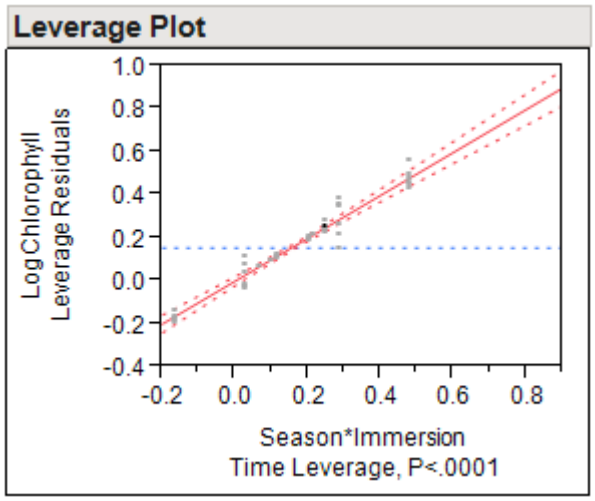
LSMeans Differences Tukey HSD

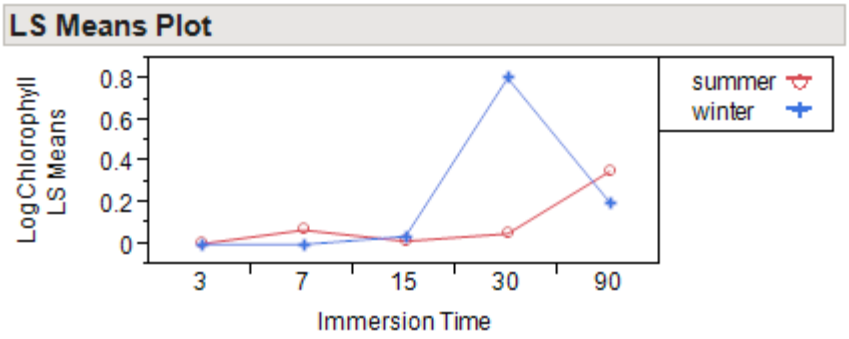
α= 0.050 Q= 2.68042

Level		Least Sq Mean
summer,control	A	0.00514691
summer,treatment	A	0.00129724
winter,treatment	A	0.00001702
winter,control	A	0.00000093

Levels not connected by same letter are significantly different.

Season*Immersion Time





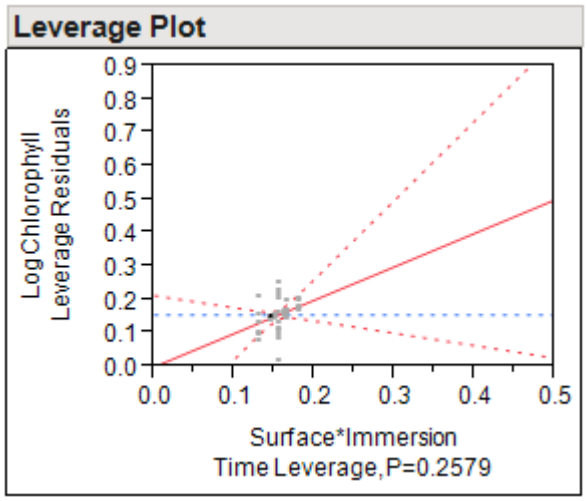
LSMeans Differences Tukey HSD

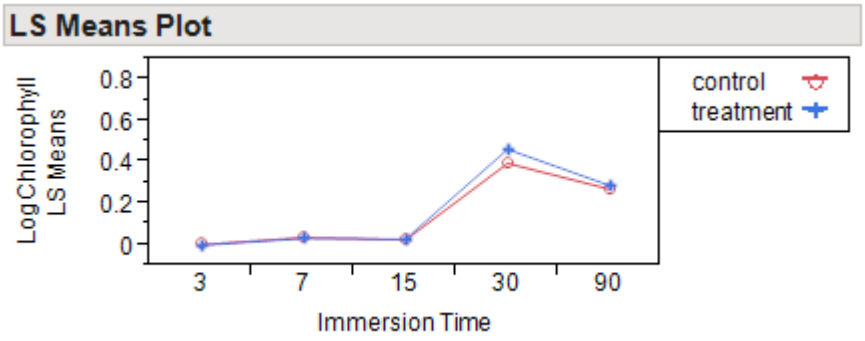
α= 0.050 Q= 3.34782

Level			Least Sq Mean
winter,30	A		0.80871901
summer,90		B	0.35564519
winter,90		C	0.20514607
summer,7		D	0.07011934
summer,30		D	0.05258442
winter,15		D	0.03999335
summer,15		D	0.01627727
summer,3		D	0.00322207
winter,7		D	0.00044863
winter,3		D	0.00000898

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





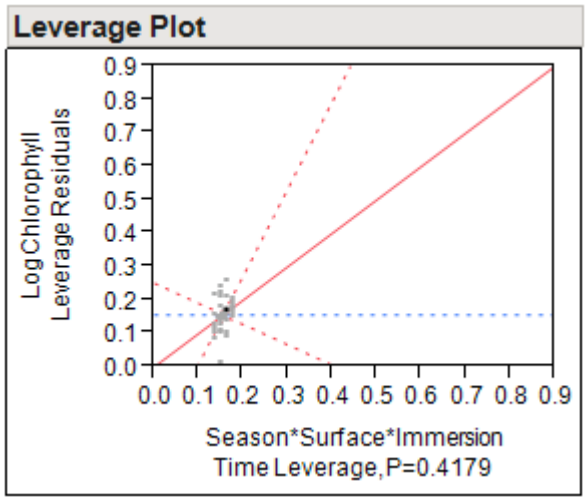
LSMeans Differences Tukey HSD

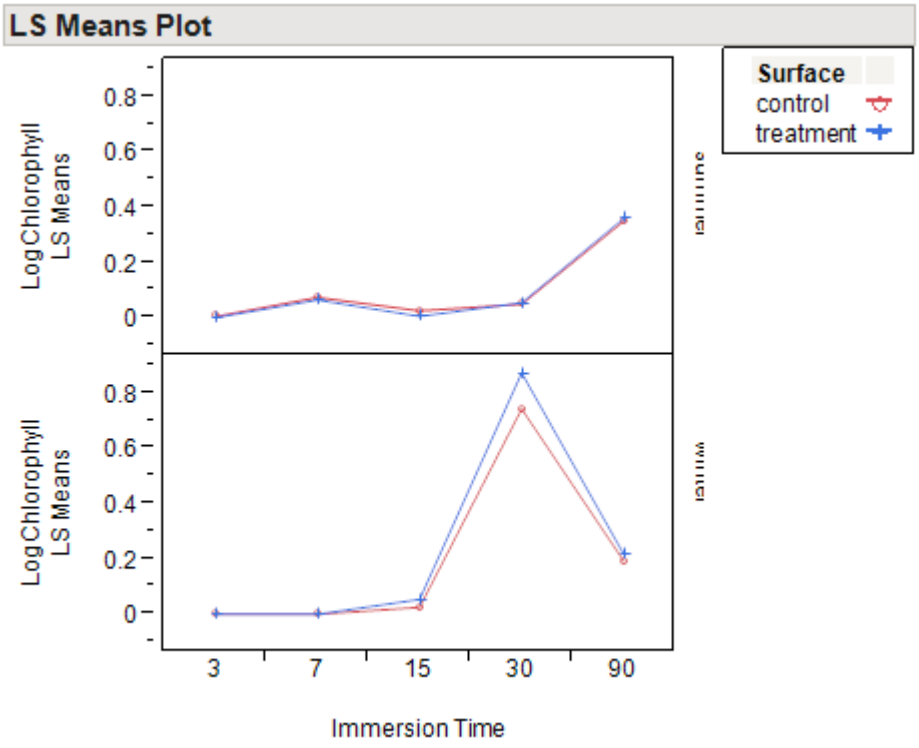
α= 0.050 Q= 3.34782

Level			Least Sq Mean
treatment,30	A		0.46443302
control,30	A		0.39687040
treatment,90		B	0.28915693
control,90		B	0.27163433
control,7			C 0.03750208
treatment,7			C 0.03306589
treatment,15			C 0.03037238
control,15			C 0.02589824
control,3			C 0.00257392
treatment,3			C 0.00065713

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

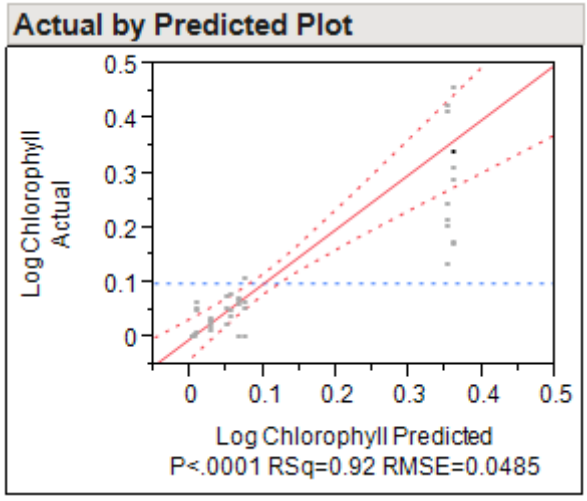
α= 0.050 Q= 3.78834

Level							Least Sq Mean
winter,treatment,30	A						0.87385490
winter,control,30	A						0.74358312
summer,treatment,90		B					0.36059453
summer,control,90		B	C				0.35069584
winter,treatment,90			C	D			0.21771934
winter,control,90				D	E		0.19257281
summer,control,7					E	F	0.07458980
summer,treatment,7					E	F	0.06564888
summer,treatment,30						F	0.05501114
winter,treatment,15						F	0.05436483
summer,control,30						F	0.05015769
summer,control,15						F	0.02617460
winter,control,15						F	0.02562188
summer,treatment,15						F	0.00637994
summer,control,3						F	0.00514691
summer,treatment,3						F	0.00129724
winter,treatment,7						F	0.00048289
winter,control,7						F	0.00041437
winter,treatment,3						F	0.00001702
winter,control,3						F	0.00000093

Levels not connected by same letter are significantly different.

RESPONSE LOG CHLOROPHYLL – SUMMER ONLY

Whole Model



Summary of Fit

RSquare	0.915595
RSquare Adj	0.877613
Root Mean Square Error	0.048491
Mean of Response	0.09957
Observations (or Sum Wgts)	30

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	0.51013273	0.056681	24.1059
Error	20	0.04702691	0.002351	Prob > F
C. Total	29	0.55715964		<.0001*

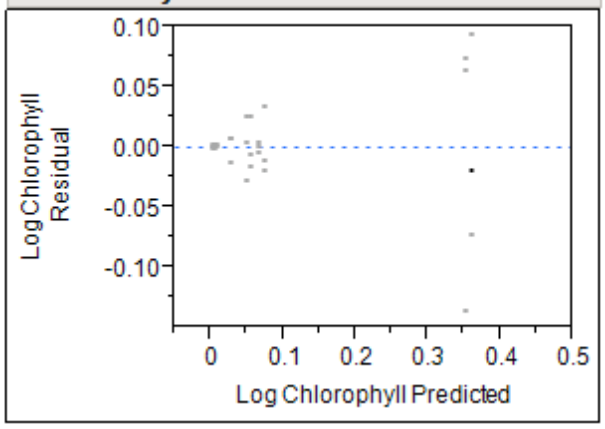
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0032221	0.019796	0.16	0.8723
Surface[control]	0.0019248	0.019796	0.10	0.9235
Immersion Time[7-3]	0.0668973	0.027996	2.39	0.0268*
Immersion Time[15-7]	-0.053842	0.027996	-1.92	0.0688
Immersion Time[30-15]	0.0363071	0.027996	1.30	0.2094
Immersion Time[90-30]	0.3030608	0.027996	10.83	<.0001*
Surface[control]*Immersion Time[7-3]	0.0025456	0.027996	0.09	0.9285
Surface[control]*Immersion Time[15-7]	0.0054269	0.027996	0.19	0.8483
Surface[control]*Immersion Time[30-15]	-0.012324	0.027996	-0.44	0.6645
Surface[control]*Immersion Time[90-30]	-0.002523	0.027996	-0.09	0.9291

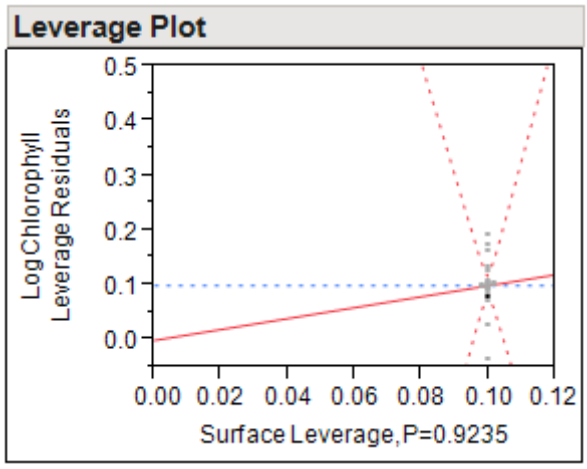
Effect Tests

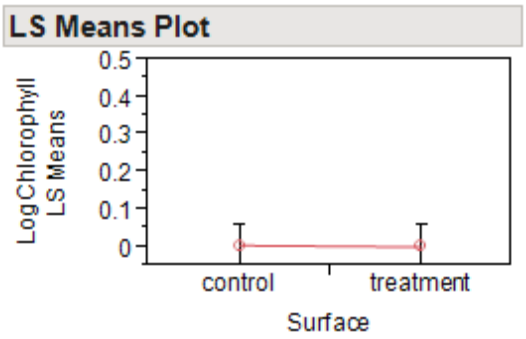
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Surface	1	1	0.00002223	0.0095	0.9235
Immersion Time	4	4	0.50922054	54.1414	<.0001*
Surface*Immersion Time	4	4	0.00081679	0.0868	0.9855

Residual by Predicted Plot



Surface Type





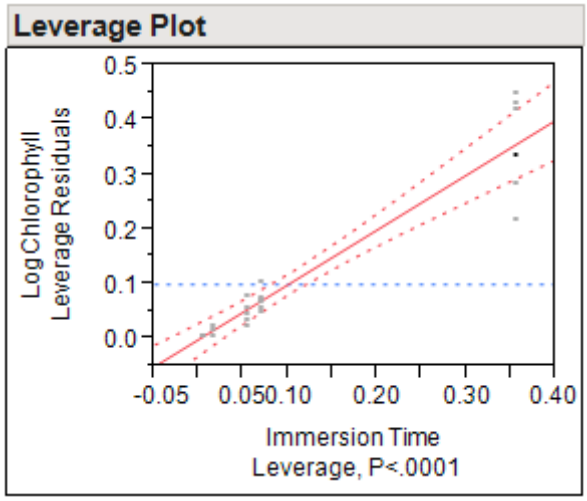
LSMeans Differences Student's t

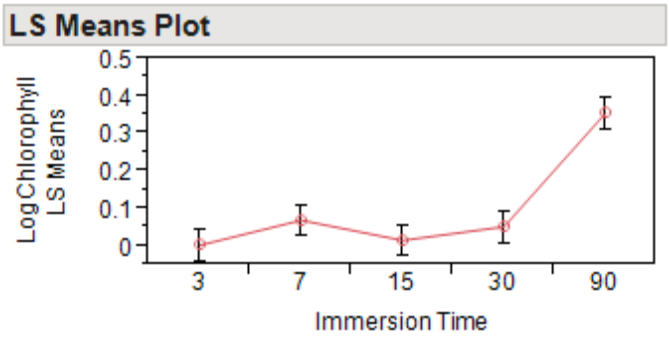
α= 0.050 t= 2.08596

Level		Least Sq Mean
control	A	0.00514691
treatment	A	0.00129724

Levels not connected by same letter are significantly different.

Immersion Time





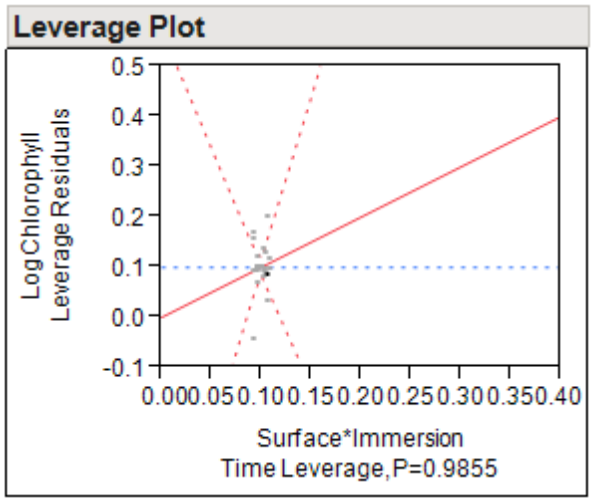
LSMeans Differences Tukey HSD

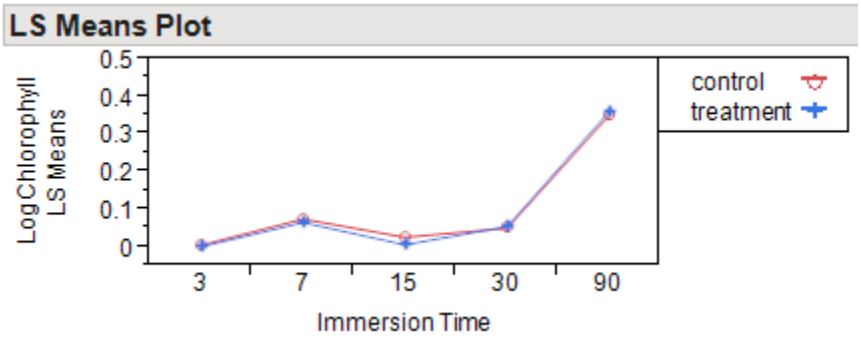
α= 0.050 Q= 2.99238

Level		Least Sq Mean
90	A	0.35564519
7	B	0.07011934
30	B	0.05258442
15	B	0.01627727
3	B	0.00322207

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





LSMeans Differences Tukey HSD

α= 0.050 Q= 3.54111

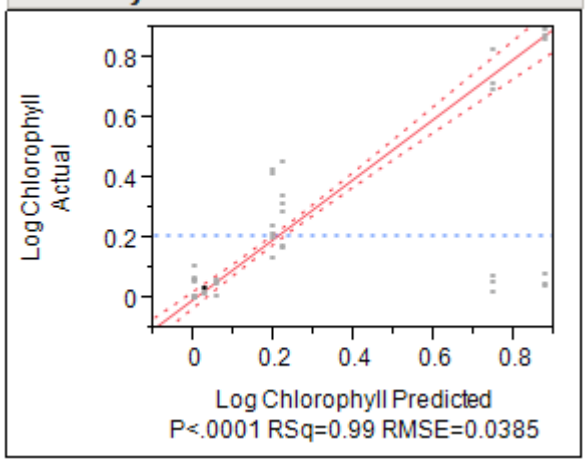
Level		Least Sq Mean
treatment,90	A	0.36059453
control,90	A	0.35069584
control,7	B	0.07458980
treatment,7	B	0.06564888
treatment,30	B	0.05501114
control,30	B	0.05015769
control,15	B	0.02617460
treatment,15	B	0.00637994
control,3	B	0.00514691
treatment,3	B	0.00129724

Levels not connected by same letter are significantly different.

RESPONSE LOG CHLOROPHYLL – WINTER ONLY

Whole Model

Actual by Predicted Plot



Summary of Fit

RSquare	0.989798
RSquare Adj	0.985207
Root Mean Square Error	0.038525
Mean of Response	0.210863
Observations (or Sum Wgts)	30

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	2.8800112	0.320001	215.6054
Error	20	0.0296840	0.001484	Prob > F
C. Total	29	2.9096952		<.0001*

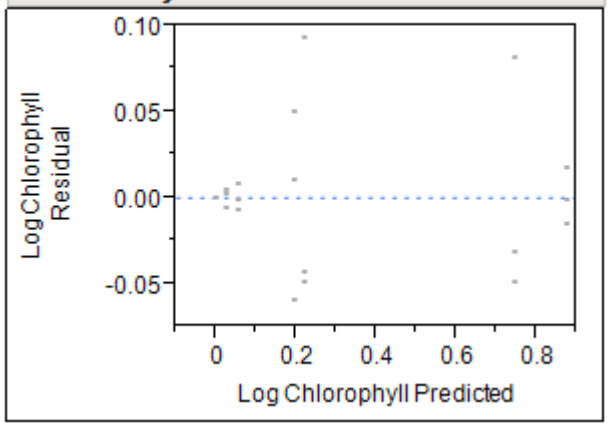
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	8.9776e-6	0.015728	0.00	0.9996
Surface[control]	-8.046e-6	0.015728	-0.00	0.9996
Immersion Time[7-3]	0.0004396	0.022243	0.02	0.9844
Immersion Time[15-7]	0.0395447	0.022243	1.78	0.0906
Immersion Time[30-15]	0.7687257	0.022243	34.56	<.0001*
Immersion Time[90-30]	-0.603573	0.022243	-27.14	<.0001*
Surface[control]*Immersion Time[7-3]	-2.621e-5	0.022243	-0.00	0.9991
Surface[control]*Immersion Time[15-7]	-0.014337	0.022243	-0.64	0.5265
Surface[control]*Immersion Time[30-15]	-0.050764	0.022243	-2.28	0.0336*
Surface[control]*Immersion Time[90-30]	0.0525626	0.022243	2.36	0.0284*

Effect Tests

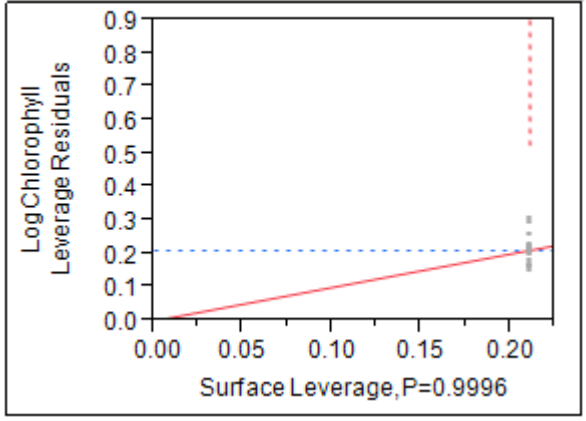
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Surface	1	1	3.8846e-10	0.0000	0.9996
Immersion Time	4	4	2.8523674	480.4558	<.0001*
Surface*Immersion Time	4	4	0.0174599	2.9410	0.0461*

Residual by Predicted Plot

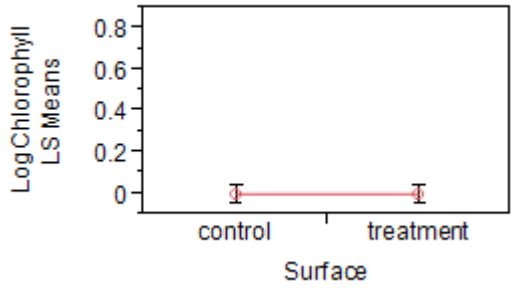


Surface Type

Leverage Plot



LS Means Plot



LSMeans Differences Student's t

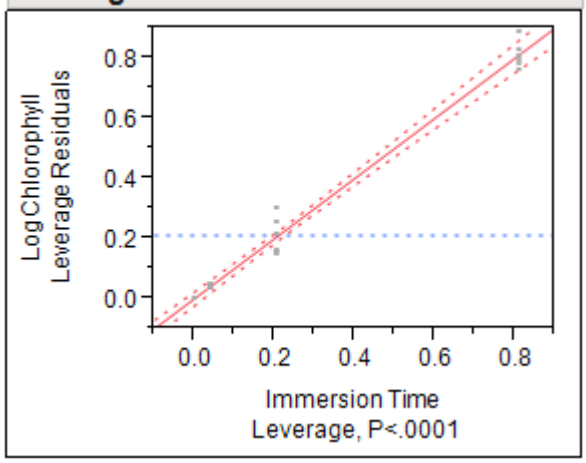
α= 0.050 t= 2.08596

Level		Least Sq Mean
treatment	A	0.00001702
control	A	0.00000093

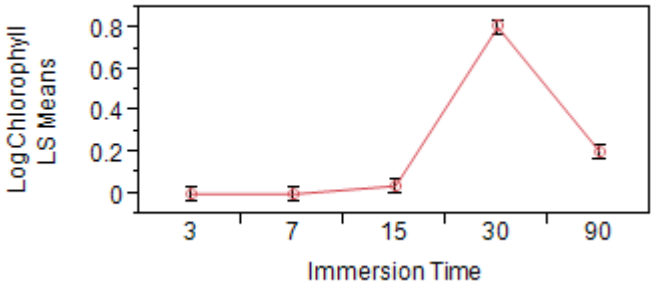
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



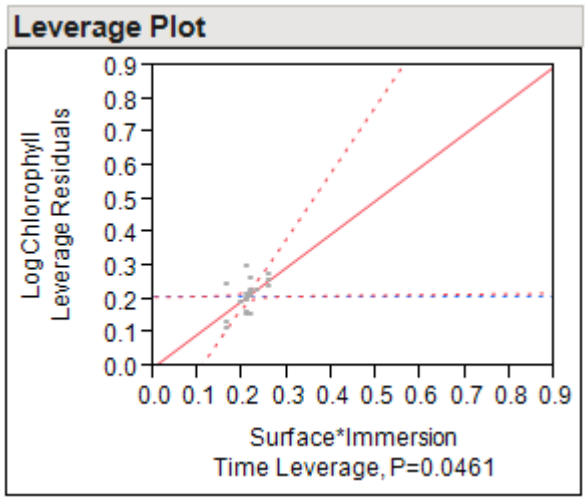
LSMeans Differences Tukey HSD

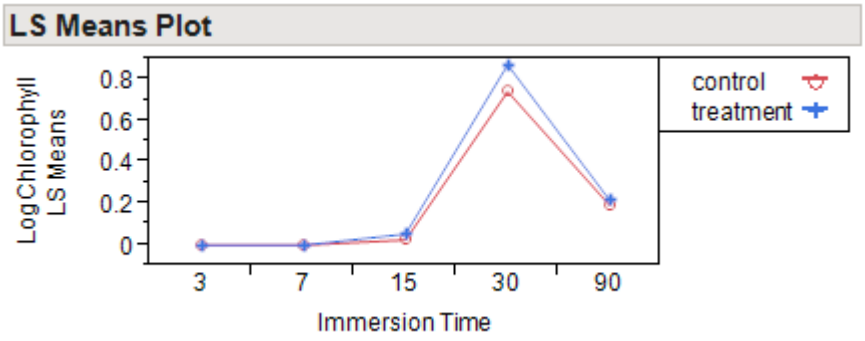
α= 0.050 Q= 2.99238

Level			Least Sq Mean
30	A		0.80871901
90		B	0.20514607
15		C	0.03999335
7		C	0.00044863
3		C	0.00000898

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





LSMeans Differences Tukey HSD

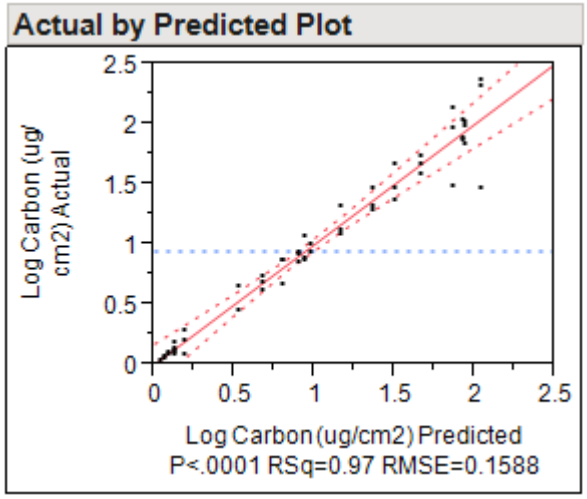
α= 0.050 Q= 3.54111

Level			Least Sq Mean
treatment,30	A		0.87385490
control,30		B	0.74358312
treatment,90		C	0.21771934
control,90		C	0.19257281
treatment,15		D	0.05436482
control,15		D	0.02562188
treatment,7		D	0.00048289
control,7		D	0.00041437
treatment,3		D	0.00001702
control,3		D	0.00000093

Levels not connected by same letter are significantly different.

RESPONSE LOG CARBON

Whole Model



Summary of Fit	
RSquare	0.96697
RSquare Adj	0.950879
Root Mean Square Error	0.15883
Mean of Response	0.943878
Observations (or Sum Wgts)	59

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	28.802958	1.51595	60.0924
Error	39	0.983850	0.02523	Prob > F
C. Total	58	29.786808		<.0001*

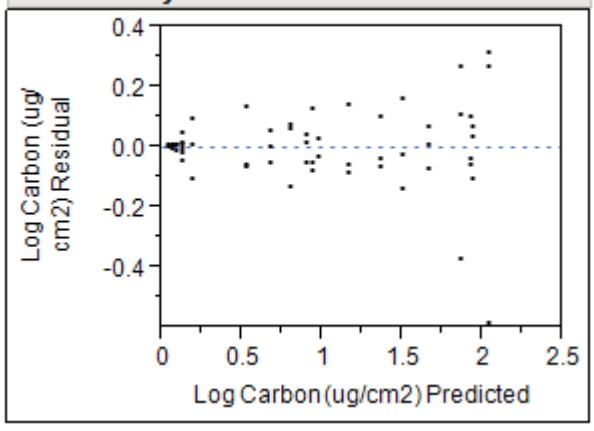
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.1022167	0.04585	2.23	0.0316*
Season[summer]	0.0548667	0.04585	1.20	0.2387
Surface[control]	0.0191333	0.04585	0.42	0.6787
Immersion Time[7-3]	0.4163833	0.066838	6.23	<.0001*
Immersion Time[15-7]	0.3968167	0.066838	5.94	<.0001*
Immersion Time[30-15]	0.43325	0.064842	6.68	<.0001*
Immersion Time[90-30]	0.488	0.064842	7.53	<.0001*
Season[summer]*Surface[control]	0.00765	0.04585	0.17	0.8684
Season[summer]*Immersion Time[7-3]	0.3595333	0.066838	5.38	<.0001*
Season[summer]*Immersion Time[15-7]	-0.731317	0.066838	-10.94	<.0001*
Season[summer]*Immersion Time[30-15]	0.2299167	0.064842	3.55	0.0010*
Season[summer]*Immersion Time[90-30]	-0.029667	0.064842	-0.46	0.6498
Surface[control]*Immersion Time[7-3]	-0.045533	0.066838	-0.68	0.4997
Surface[control]*Immersion Time[15-7]	0.28165	0.066838	4.21	0.0001*
Surface[control]*Immersion Time[30-15]	-0.454917	0.064842	-7.02	<.0001*
Surface[control]*Immersion Time[90-30]	0.133	0.064842	2.05	0.0470*
Season[summer]*Surface[control]*Immersion Time[7-3]	-0.01825	0.066838	-0.27	0.7863
Season[summer]*Surface[control]*Immersion Time[15-7]	-0.16515	0.066838	-2.47	0.0180*
Season[summer]*Surface[control]*Immersion Time[30-15]	0.4704167	0.064842	7.25	<.0001*
Season[summer]*Surface[control]*Immersion Time[90-30]	-0.451333	0.064842	-6.96	<.0001*

Effect Tests

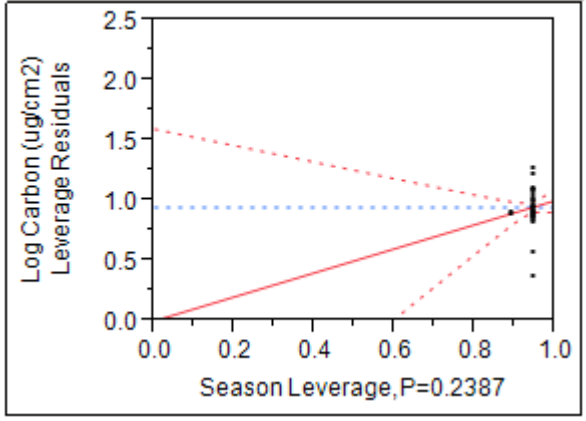
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	0.036124	1.4320	0.2387
Surface	1	1	0.004393	0.1741	0.6787
Immersion Time	4	4	21.964743	217.6716	<.0001*
Season*Surface	1	1	0.000702	0.0278	0.8684
Season*Immersion Time	4	4	3.304011	32.7429	<.0001*
Surface*Immersion Time	4	4	1.324809	13.1289	<.0001*
Season*Surface*Immersion Time	4	4	1.705198	16.8986	<.0001*

Residual by Predicted Plot

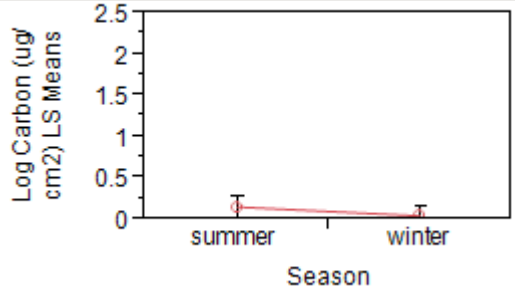


Season

Leverage Plot



LS Means Plot



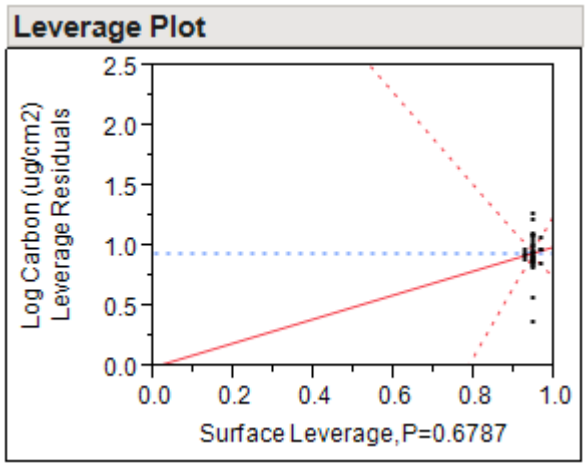
LSMeans Differences Student's t

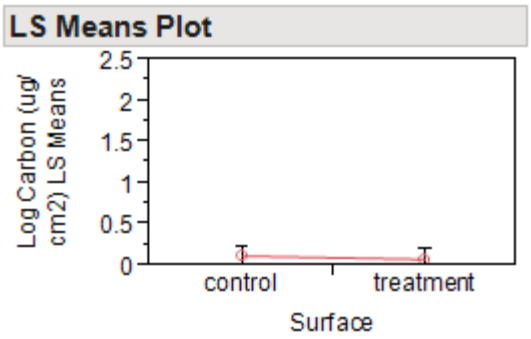
α= 0.050 t= 2.02269

Level		Least Sq Mean
summer	A	0.15708333
winter	A	0.04735000

Levels not connected by same letter are significantly different.

Surface Type





LSMeans Differences Student's t

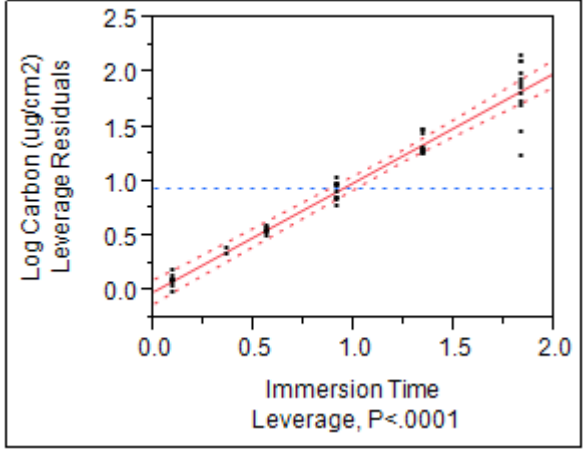
α= 0.050 t= 2.02269

Level		Least Sq Mean
control	A	0.12135000
treatment	A	0.08308333

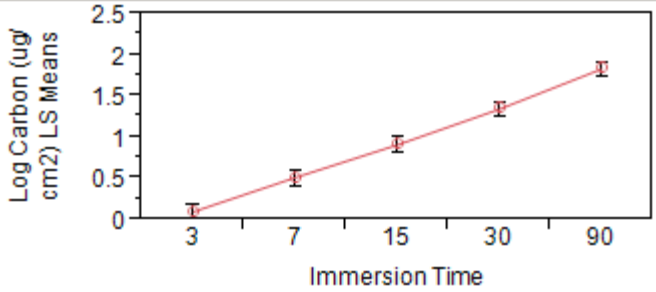
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



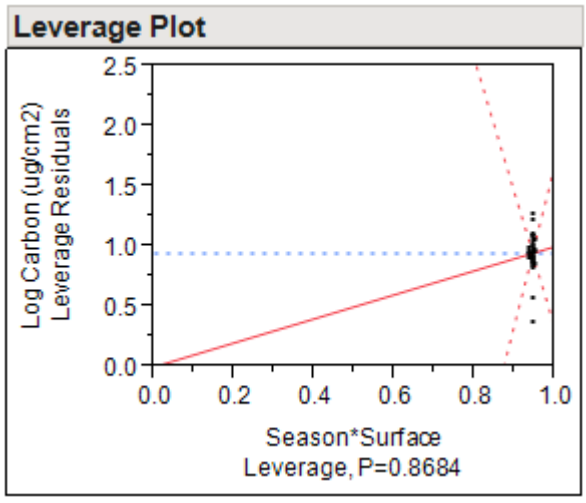
LSMeans Differences Tukey HSD

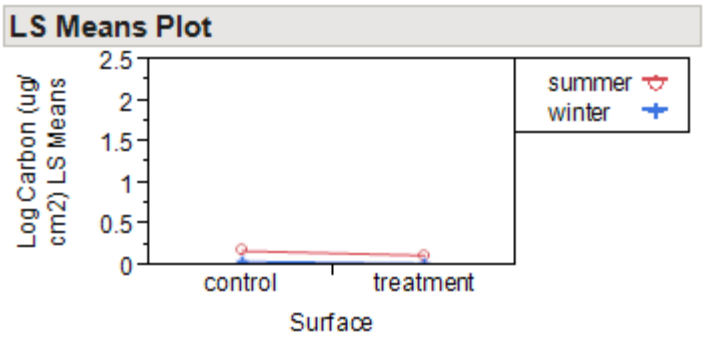
α= 0.050 Q= 2.85949

Level					Least Sq Mean
90	A				1.8366667
30		B			1.3486667
15			C		0.9154167
7				D	0.5186000
3				E	0.1022167

Levels not connected by same letter are significantly different.

Season*Surface Type





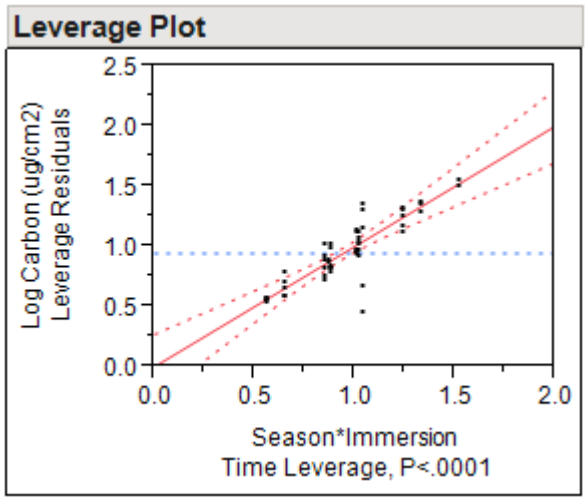
LSMeans Differences Tukey HSD

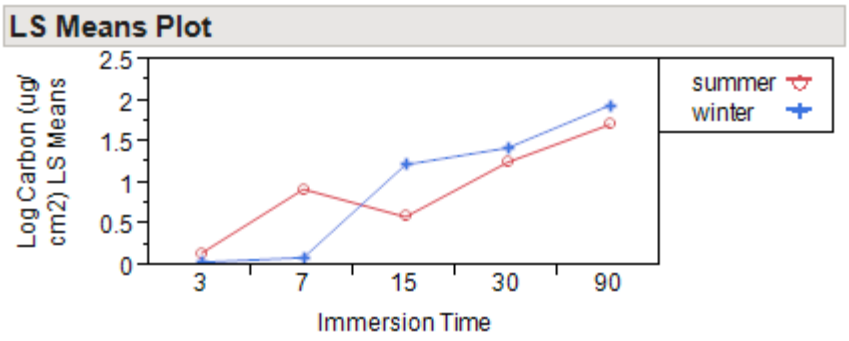
α= 0.050 Q= 2.68337

Level		Least Sq Mean
summer,control	A	0.18386667
summer,treatment	A	0.13030000
winter,control	A	0.05883333
winter,treatment	A	0.03586667

Levels not connected by same letter are significantly different.

Season*Immersion Time





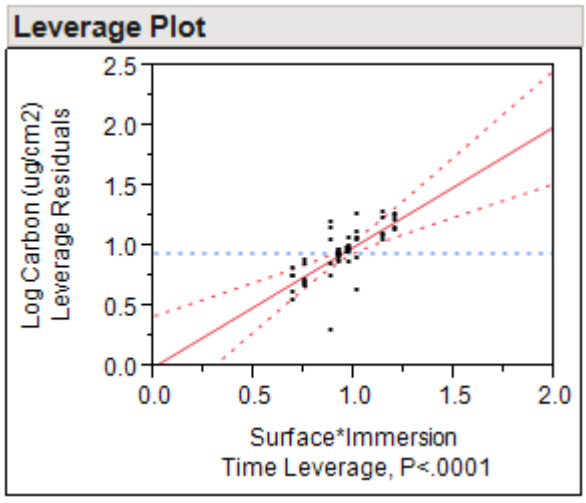
LSMeans Differences Tukey HSD

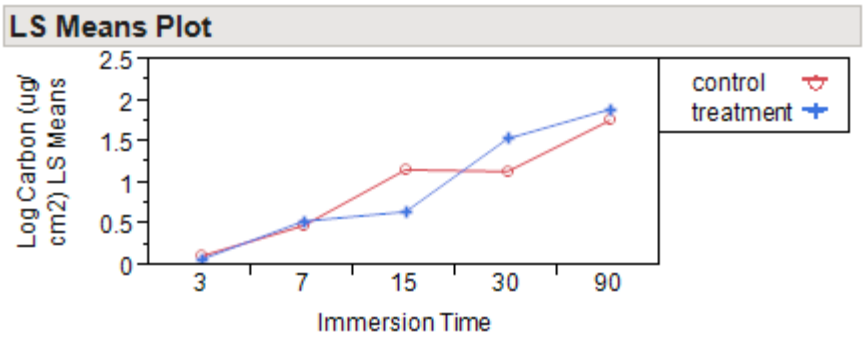
α= 0.050 Q= 3.35265

Level						Least Sq Mean
winter,90	A					1.9533333
summer,90	A	B				1.7200000
winter,30		B	C			1.4356667
summer,30			C			1.2616667
winter,15			C	D		1.2323333
summer,7				D		0.9330000
summer,15					E	0.5985000
summer,3					F	0.1570833
winter,7					F	0.1042000
winter,3					F	0.0473500

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





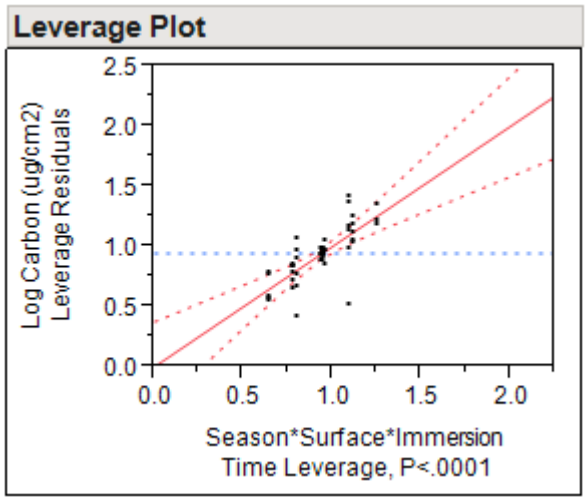
LSMeans Differences Tukey HSD

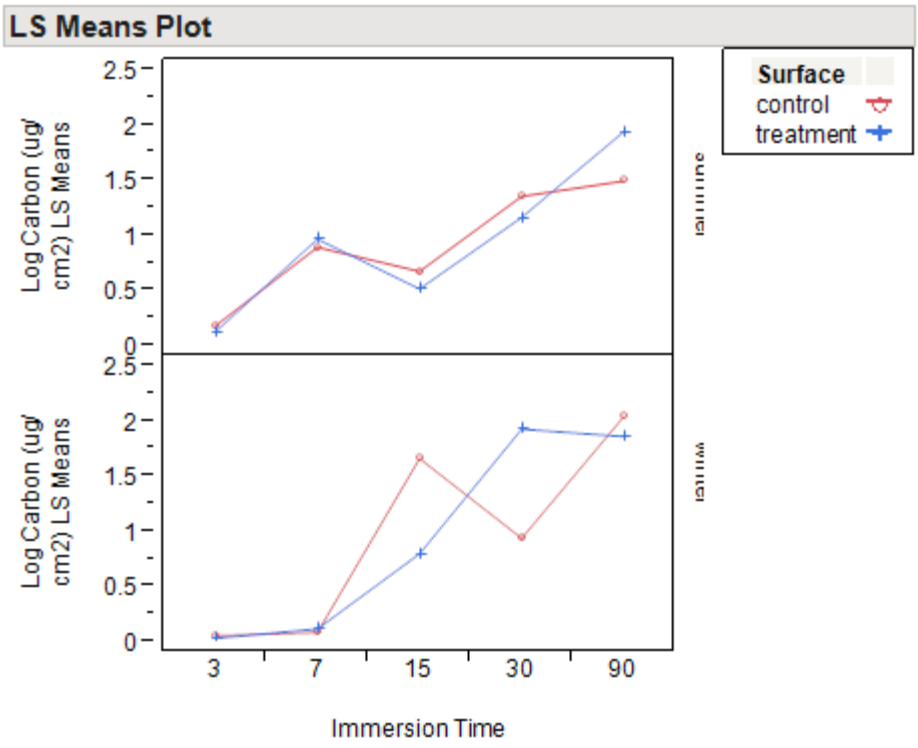
α= 0.050 Q= 3.35265

Level					Least Sq Mean
treatment,90	A				1.9033333
control,90	A	B			1.7700000
treatment,30		B			1.5483333
control,15			C		1.1706667
control,30			C		1.1490000
treatment,15				D	0.6601667
treatment,7				D	0.5450000
control,7				D	0.4922000
control,3					E 0.1213500
treatment,3					E 0.0830833

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

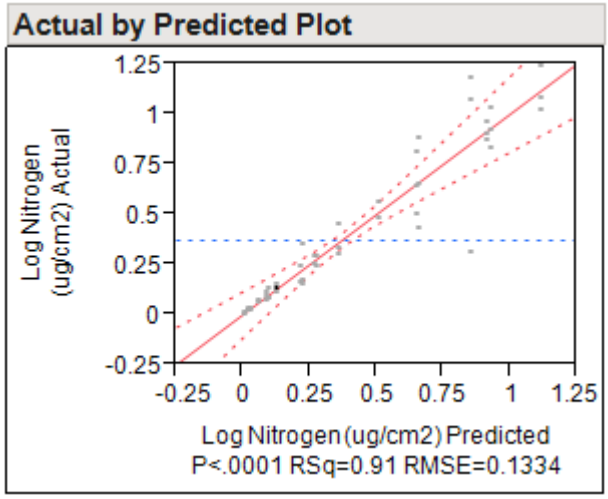
α= 0.050 Q= 3.7947

Level								Least Sq Mean
winter,control,90	A							2.0433333
summer,treatment,90	A	B						1.9433333
winter,treatment,30	A	B						1.9300000
winter,treatment,90	A	B						1.8633333
winter,control,15	A	B	C					1.6633333
summer,control,90		B	C	D				1.4966667
summer,control,30			C	D	E			1.3566667
summer,treatment,30				D	E	F		1.1666667
summer,treatment,7				D	E	F	G	0.9700000
winter,control,30					E	F	G	0.9413333
summer,control,7					E	F	G	0.8960000
winter,treatment,15						F	G	0.8013333
summer,control,15						F	G	0.6780000
summer,treatment,15							G	H 0.5190000
summer,control,3								H 0.1838667
summer,treatment,3								H 0.1303000
winter,treatment,7								H 0.1200000
winter,control,7								H 0.0884000
winter,control,3								H 0.0588333
winter,treatment,3								H 0.0358667

Levels not connected by same letter are significantly different.

RESPONSE LOG NITROGEN

Whole Model



Summary of Fit	
RSquare	0.91329
RSquare Adj	0.871046
Root Mean Square Error	0.13337
Mean of Response	0.372037
Observations (or Sum Wgts)	59

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	7.3067227	0.384564	21.6197
Error	39	0.6937192	0.017788	Prob > F
C. Total	58	8.0004420		<.0001*

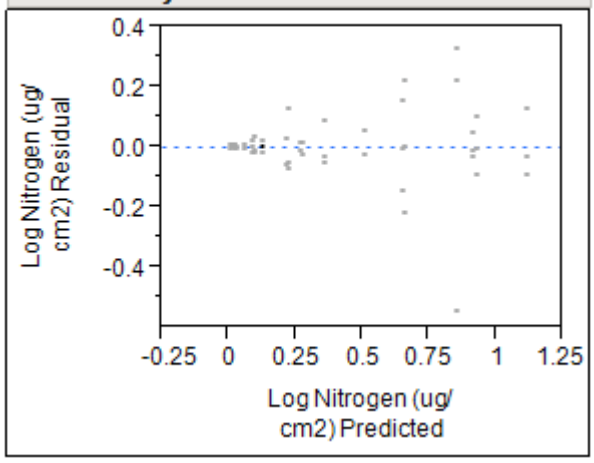
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0452425	0.038501	1.18	0.2471
Season[summer]	0.0287075	0.038501	0.75	0.4604
Surface[control]	0.0122742	0.038501	0.32	0.7516
Immersion Time[7-3]	0.1008825	0.056124	1.80	0.0800
Immersion Time[15-7]	0.1914	0.056124	3.41	0.0015*
Immersion Time[30-15]	0.2130583	0.054448	3.91	0.0004*
Immersion Time[90-30]	0.2216667	0.054448	4.07	0.0002*
Season[summer]*Surface[control]	0.0022092	0.038501	0.06	0.9545
Season[summer]*Immersion Time[7-3]	0.0975842	0.056124	1.74	0.0900
Season[summer]*Immersion Time[15-7]	-0.350767	0.056124	-6.25	<.0001*
Season[summer]*Immersion Time[30-15]	0.107225	0.054448	1.97	0.0561
Season[summer]*Immersion Time[90-30]	0.1351667	0.054448	2.48	0.0175*
Surface[control]*Immersion Time[7-3]	-0.011533	0.056124	-0.21	0.8383
Surface[control]*Immersion Time[15-7]	0.1812333	0.056124	3.23	0.0025*
Surface[control]*Immersion Time[30-15]	-0.367558	0.054448	-6.75	<.0001*
Surface[control]*Immersion Time[90-30]	0.1655	0.054448	3.04	0.0042*
Season[summer]*Surface[control]*Immersion Time[7-3]	-0.001034	0.056124	-0.02	0.9854
Season[summer]*Surface[control]*Immersion Time[15-7]	-0.167533	0.056124	-2.99	0.0049*
Season[summer]*Surface[control]*Immersion Time[30-15]	0.426275	0.054448	7.83	<.0001*
Season[summer]*Surface[control]*Immersion Time[90-30]	-0.378667	0.054448	-6.95	<.0001*

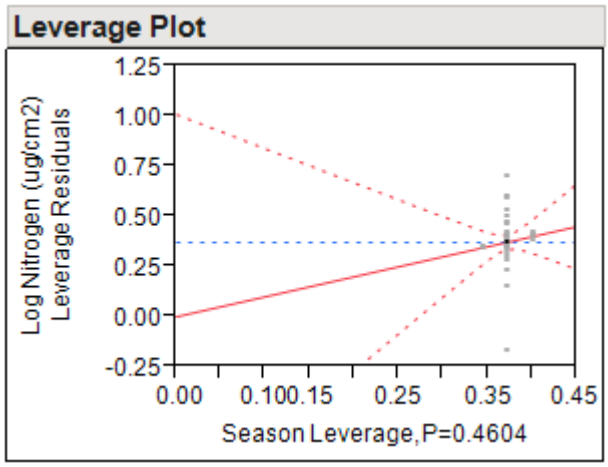
Effect Tests

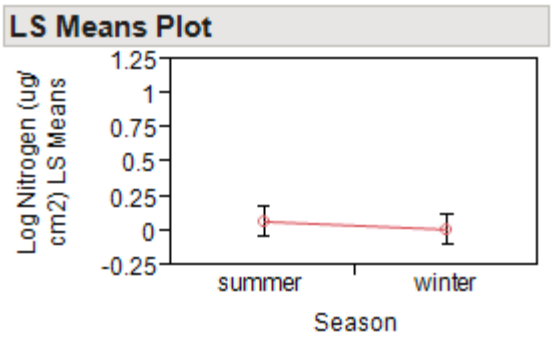
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	0.0098894	0.5560	0.4604
Surface	1	1	0.0018079	0.1016	0.7516
Immersion Time	4	4	4.1441262	58.2444	<.0001*
Season*Surface	1	1	0.0000586	0.0033	0.9545
Season*Immersion Time	4	4	0.8714515	12.2480	<.0001*
Surface*Immersion Time	4	4	0.8170426	11.4833	<.0001*
Season*Surface*Immersion Time	4	4	1.3108919	18.4242	<.0001*

Residual by Predicted Plot



Season





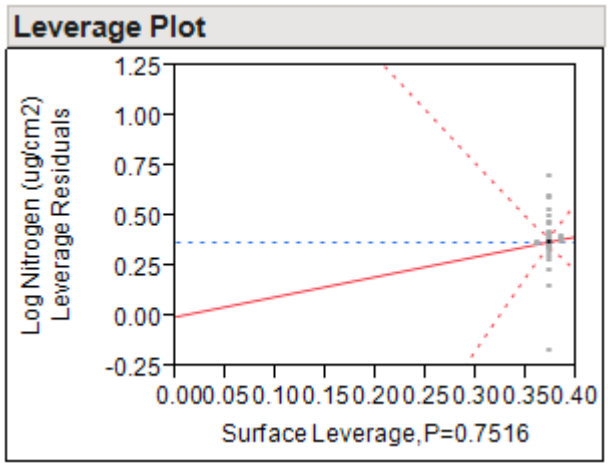
LSMeans Differences Student's t

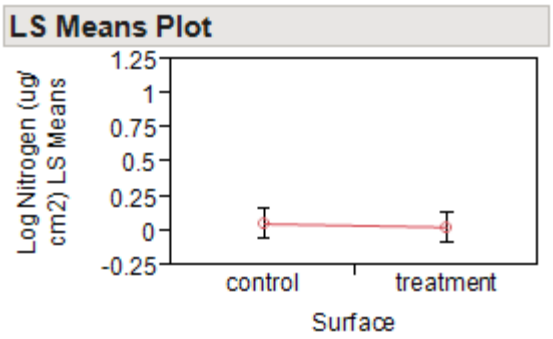
α= 0.050 t= 2.02269

Level		Least Sq Mean
summer	A	0.07395000
winter	A	0.01653500

Levels not connected by same letter are significantly different.

Surface Type





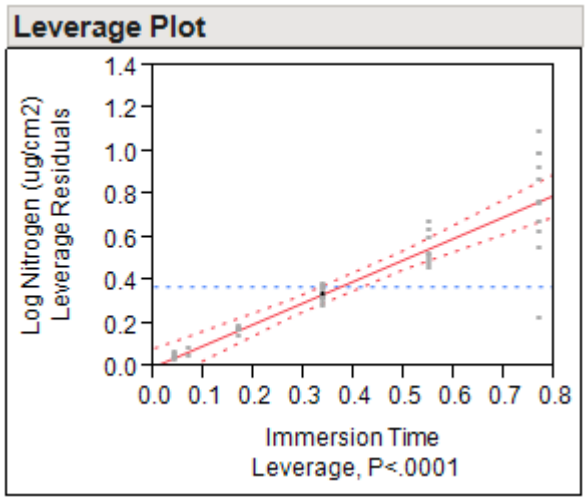
LSMeans Differences Student's t

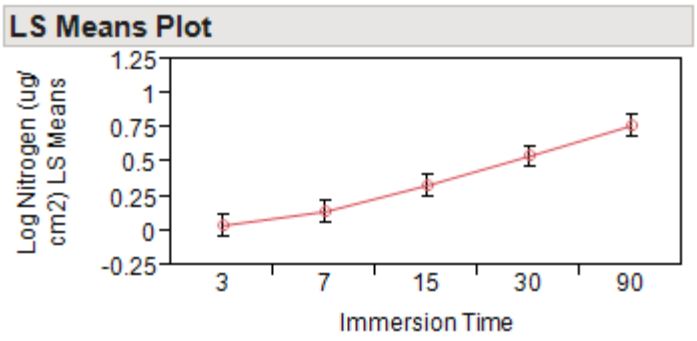
α= 0.050 t= 2.02269

Level		Least Sq Mean
control	A	0.05751667
treatment	A	0.03296833

Levels not connected by same letter are significantly different.

Immersion Time





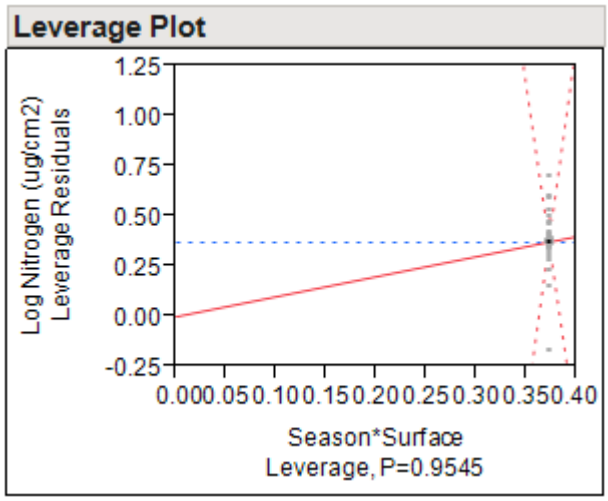
LSMeans Differences Tukey HSD

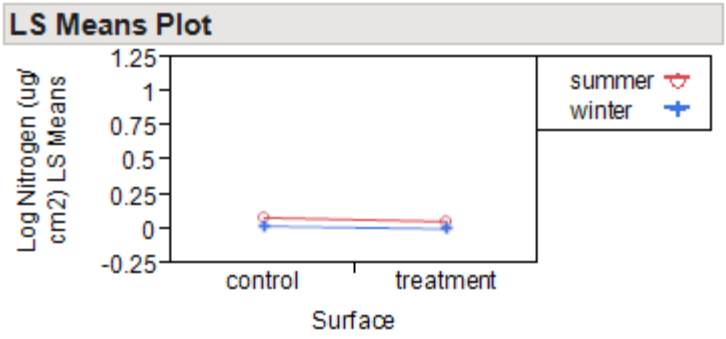
α= 0.050 Q= 2.85949

Level				Least Sq Mean
90	A			0.77225000
30		B		0.55058333
15			C	0.33752500
7			D	0.14612500
3			D	0.04524250

Levels not connected by same letter are significantly different.

Season*Surface Type





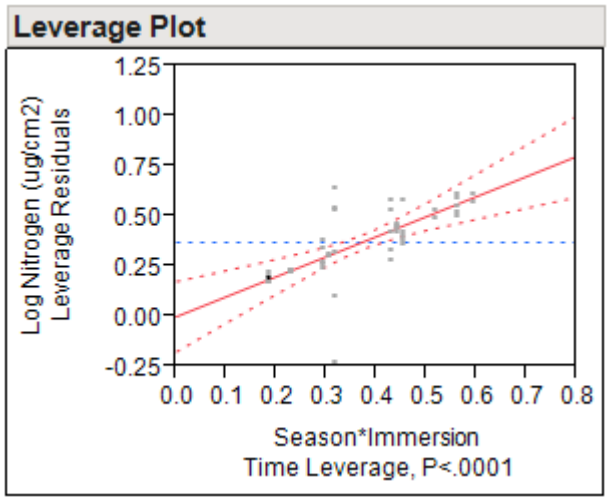
LSMeans Differences Tukey HSD

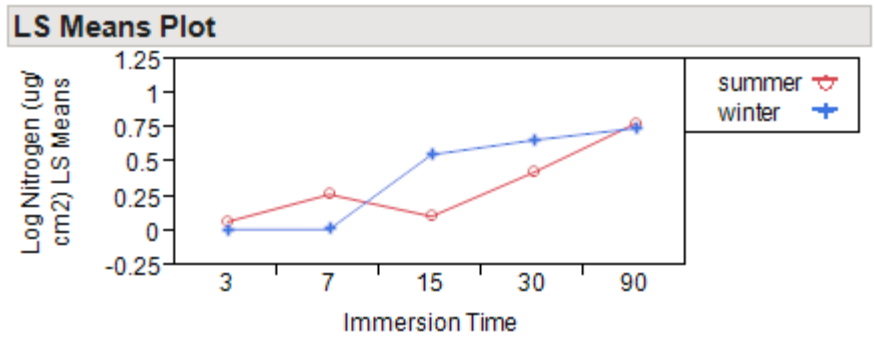
α= 0.050 Q= 2.68337

Level		Least Sq Mean
summer,control	A	0.08843333
summer,treatment	A	0.05946667
winter,control	A	0.02660000
winter,treatment	A	0.00647000

Levels not connected by same letter are significantly different.

Season*Immersion Time





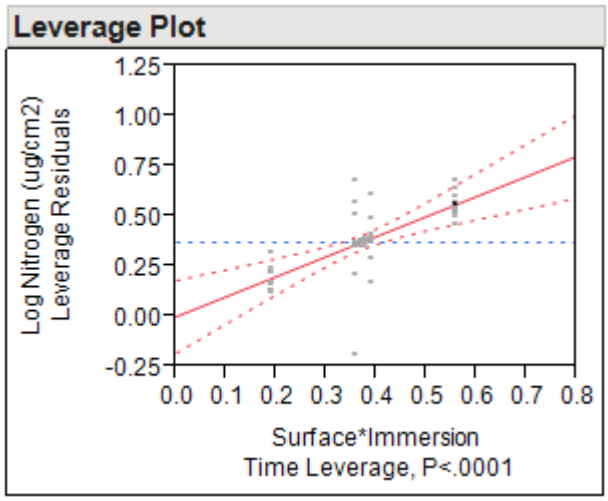
LSMeans Differences Tukey HSD

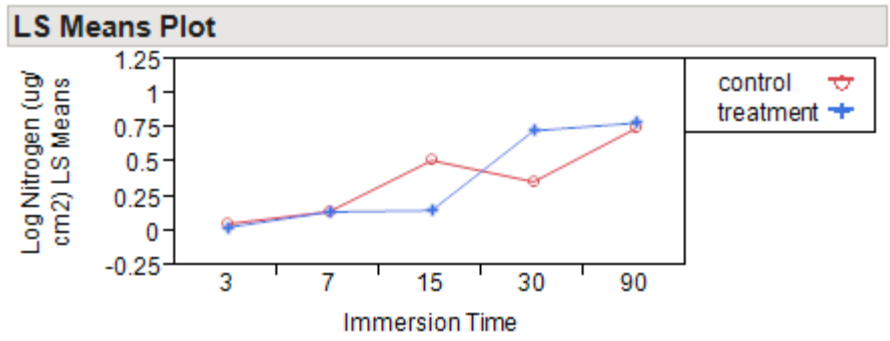
α= 0.050 Q= 3.35265

Level				Least Sq Mean
summer,90	A			0.79016667
winter,90	A			0.75433333
winter,30	A	B		0.66783333
winter,15	A	B		0.56200000
summer,30		B	C	0.43333333
summer,7			C D	0.27241667
summer,15			D	0.11305000
summer,3			D	0.07395000
winter,7			D	0.01983333
winter,3			D	0.01653500

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





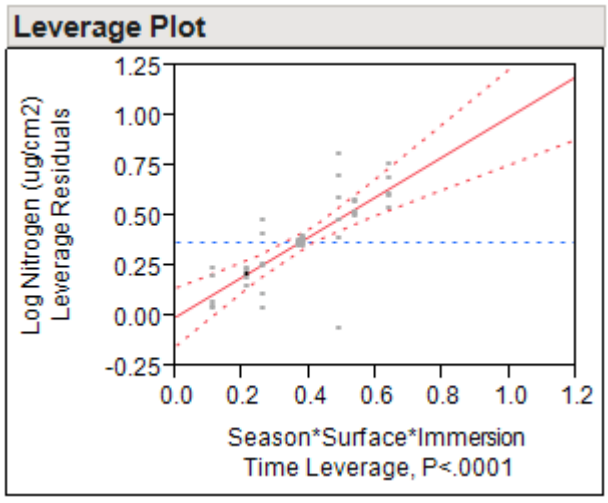
LSMeans Differences Tukey HSD

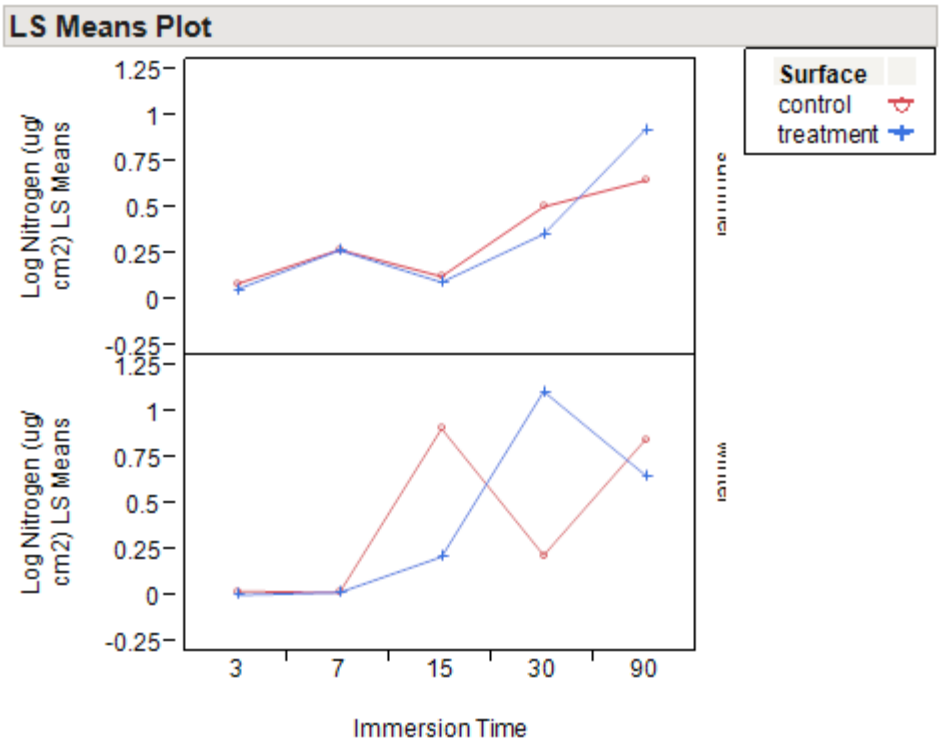
α= 0.050 Q= 3.35265

Level					Least Sq Mean
treatment,90	A				0.79233333
control,90	A	B			0.75216667
treatment,30	A	B			0.73616667
control,15		B	C		0.51950000
control,30			C	D	0.36500000
treatment,15				D	E 0.15555000
control,7				D	E 0.14686667
treatment,7				D	E 0.14538333
control,3					E 0.05751667
treatment,3					E 0.03296833

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

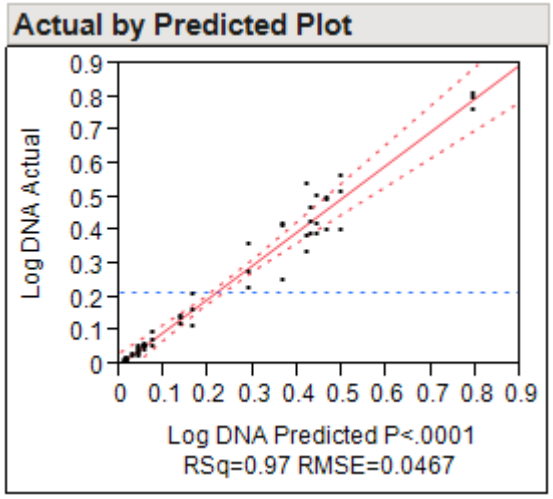
α= 0.050 Q= 3.7947

Level							Least Sq Mean
winter,treatment,30	A						1.1133333
summer,treatment,90	A	B					0.9290000
winter,control,15	A	B	C				0.9103333
winter,control,90	A	B	C				0.8530000
winter,treatment,90		B	C	D			0.6556667
summer,control,90		B	C	D			0.6513333
summer,control,30			C	D	E		0.5076667
summer,treatment,30				D	E	F	0.3590000
summer,control,7				D	E	F	0.2743333
summer,treatment,7				D	E	F	0.2705000
winter,control,30					E	F	0.2223333
winter,treatment,15					E	F	0.2136667
summer,control,15					E	F	0.1286667
summer,treatment,15					E	F	0.0974333
summer,control,3						F	0.0884333
summer,treatment,3						F	0.0594667
winter,control,3						F	0.0266000
winter,treatment,7						F	0.0202667
winter,control,7						F	0.0194000
winter,treatment,3						F	0.0064700

Levels not connected by same letter are significantly different.

RESPONSE LOG TOTAL DNA

Whole Model



Summary of Fit	
RSquare	0.971191
RSquare Adj	0.957507
Root Mean Square Error	0.046709
Mean of Response	0.216027
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	2.9419025	0.154837	70.9712
Error	40	0.0872675	0.002182	Prob > F
C. Total	59	3.0291700		<.0001*

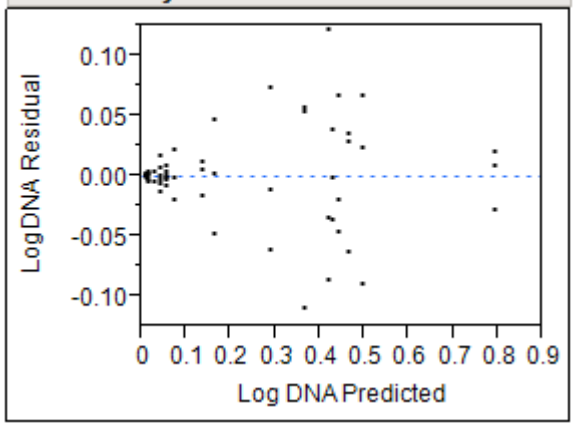
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0225088	0.013484	1.67	0.1029
Season[summer]	0.0114927	0.013484	0.85	0.3991
Surface[control]	0.0024864	0.013484	0.18	0.8546
Immersion Time[7-3]	0.0585357	0.019069	3.07	0.0038*
Immersion Time[15-7]	-0.026902	0.019069	-1.41	0.1660
Immersion Time[30-15]	0.3319655	0.019069	17.41	<.0001*
Immersion Time[90-30]	0.1502229	0.019069	7.88	<.0001*
Season[summer]*Surface[control]	0.0026977	0.013484	0.20	0.8424
Season[summer]*Immersion Time[7-3]	0.0551537	0.019069	2.89	0.0062*
Season[summer]*Immersion Time[15-7]	-0.05819	0.019069	-3.05	0.0040*
Season[summer]*Immersion Time[30-15]	0.0186819	0.019069	0.98	0.3331
Season[summer]*Immersion Time[90-30]	-0.106782	0.019069	-5.60	<.0001*
Surface[control]*Immersion Time[7-3]	0.0046223	0.019069	0.24	0.8097
Surface[control]*Immersion Time[15-7]	-0.015665	0.019069	-0.82	0.4162
Surface[control]*Immersion Time[30-15]	0.0682391	0.019069	3.58	0.0009*
Surface[control]*Immersion Time[90-30]	0.0097141	0.019069	0.51	0.6133
Season[summer]*Surface[control]*Immersion Time[7-3]	0.0046571	0.019069	0.24	0.8083
Season[summer]*Surface[control]*Immersion Time[15-7]	-0.009384	0.019069	-0.49	0.6253
Season[summer]*Surface[control]*Immersion Time[30-15]	-0.007596	0.019069	-0.40	0.6925
Season[summer]*Surface[control]*Immersion Time[90-30]	-0.097	0.019069	-5.09	<.0001*

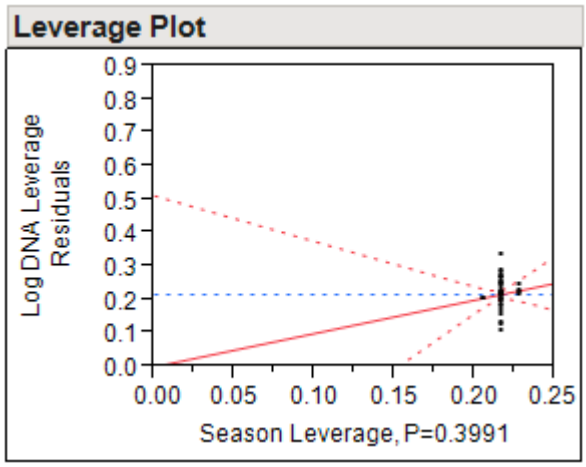
Effect Tests

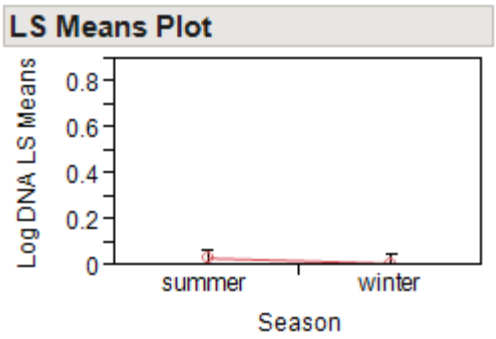
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	0.0015850	0.7265	0.3991
Surface	1	1	0.0000742	0.0340	0.8546
Immersion Time	4	4	2.5607836	293.4407	<.0001*
Season*Surface	1	1	0.0000873	0.0400	0.8424
Season*Immersion Time	4	4	0.1379103	15.8032	<.0001*
Surface*Immersion Time	4	4	0.0614609	7.0428	0.0002*
Season*Surface*Immersion Time	4	4	0.1102130	12.6293	<.0001*

Residual by Predicted Plot



Season





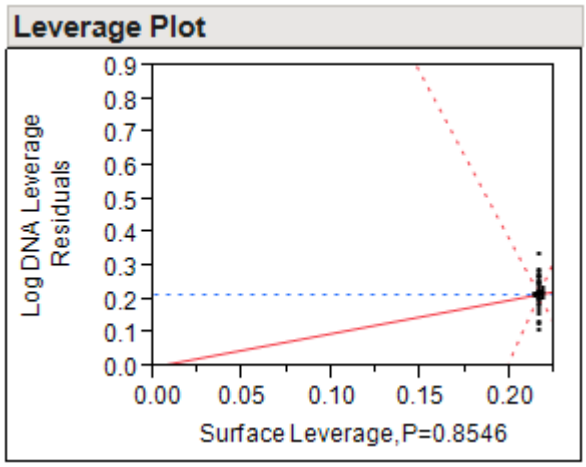
LSMeans Differences Student's t

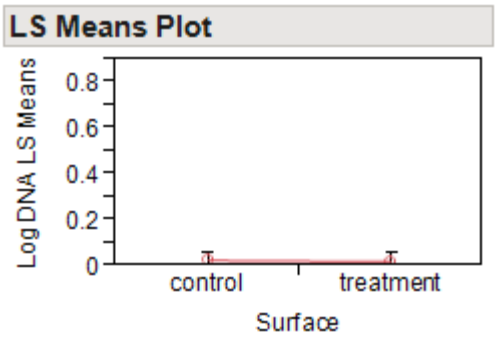
α= 0.050 t= 2.02108

Level		Least Sq Mean
summer	A	0.03400148
winter	A	0.01101609

Levels not connected by same letter are significantly different.

Surface Type





LSMeans Differences Student's t

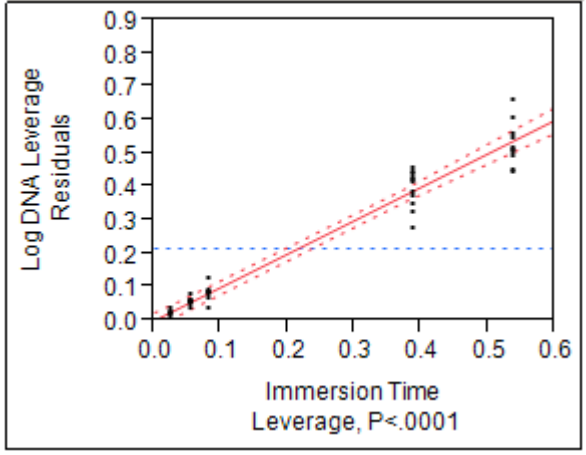
α= 0.050 t= 2.02108

Level		Least Sq Mean
control	A	0.02499519
treatment	A	0.02002237

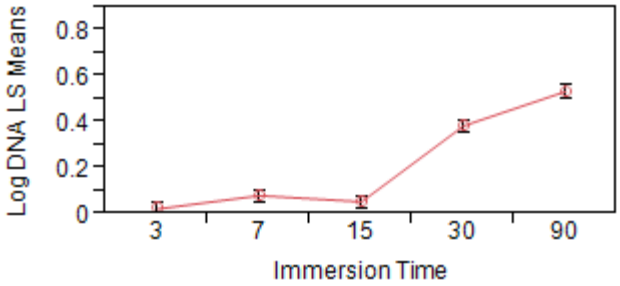
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



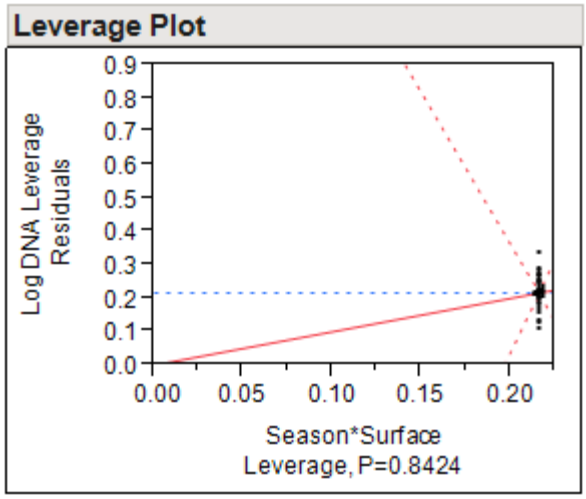
LSMeans Differences Tukey HSD

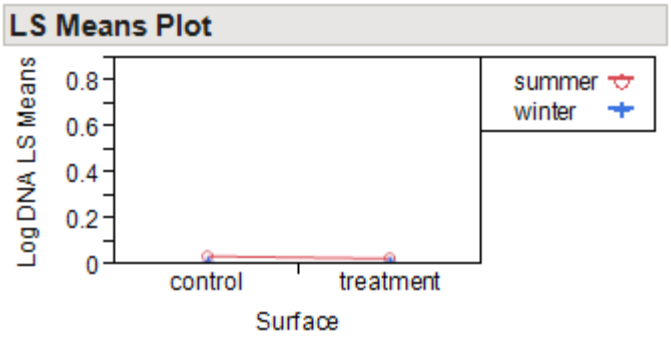
α= 0.050 Q= 2.85609

Level				Least Sq Mean
90	A			0.53633140
30		B		0.38610849
7			C	0.08104451
15			C	D 0.05414298
3				D 0.02250878

Levels not connected by same letter are significantly different.

Season*Surface Type





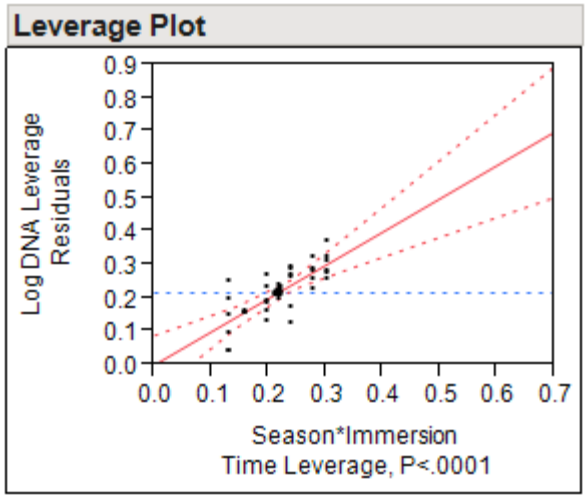
LSMeans Differences Tukey HSD

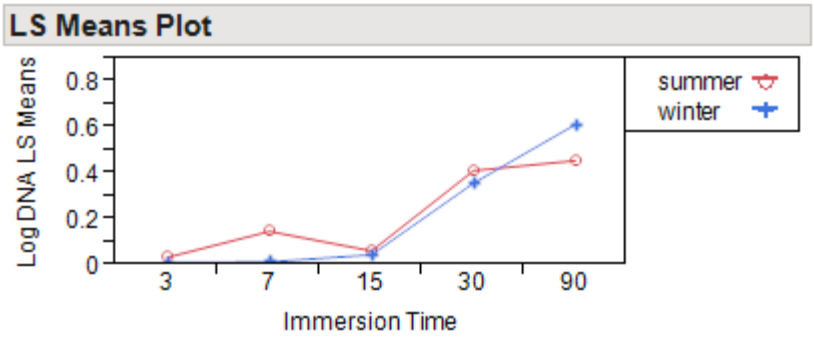
α= 0.050 Q= 2.68042

Level		Least Sq Mean
summer,control	A	0.03918560
summer,treatment	A	0.02881735
winter,treatment	A	0.01122740
winter,control	A	0.01080478

Levels not connected by same letter are significantly different.

Season*Immersion Time





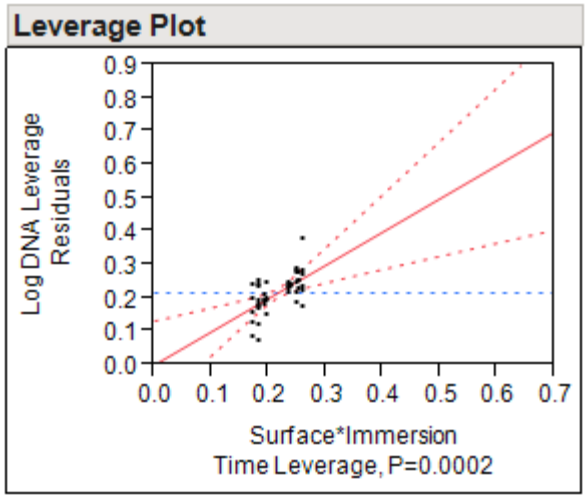
LSMeans Differences Tukey HSD

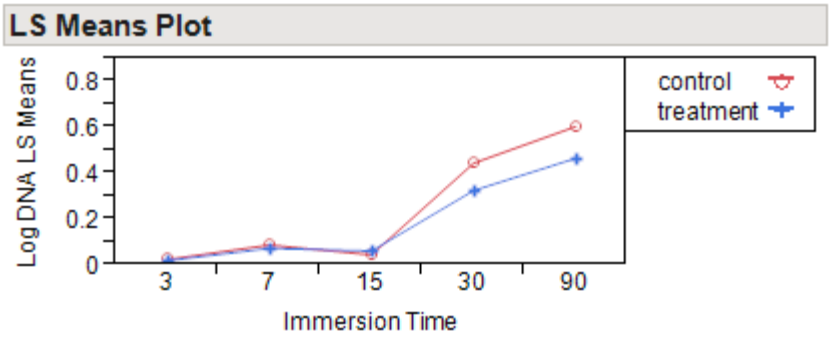
α= 0.050 Q= 3.34782

Level					Least Sq Mean
winter,90	A				0.61597521
summer,90		B			0.45668759
summer,30		B	C		0.41324648
winter,30			C		0.35897050
summer,7				D	0.14769094
summer,15				D	E 0.06259902
winter,15					E 0.04568694
summer,3					E 0.03400148
winter,7					E 0.01439807
winter,3					E 0.01101609

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





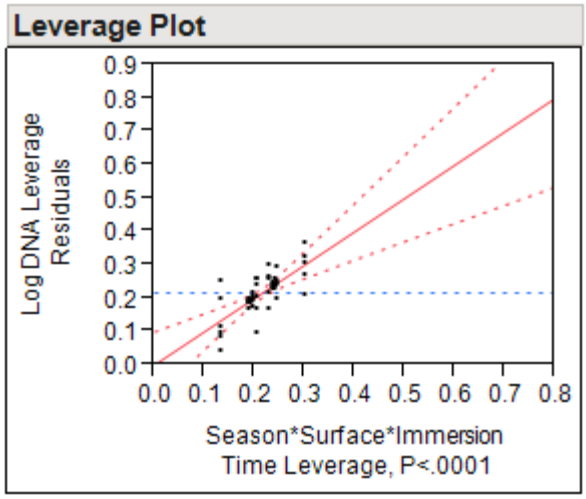
LSMeans Differences Tukey HSD

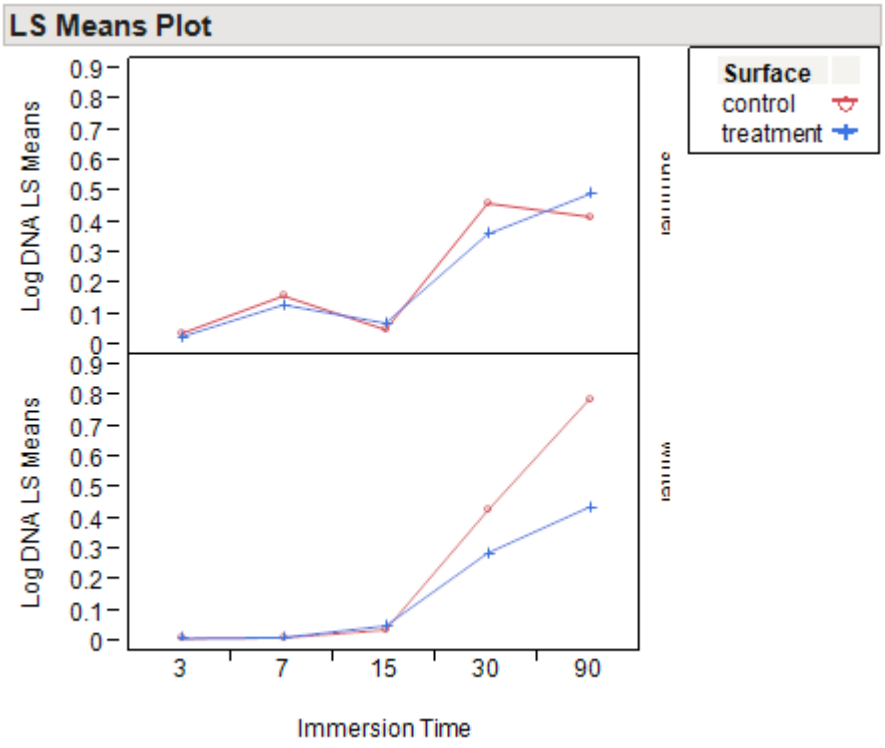
α= 0.050 Q= 3.34782

Level				Least Sq Mean
control,90	A			0.60572856
treatment,90		B		0.46693424
control,30		B		0.44579158
treatment,30			C	0.32642540
control,7			D	0.08815326
treatment,7			D	0.07393576
treatment,15			D	0.06269897
control,15			D	0.04558700
control,3			D	0.02499519
treatment,3			D	0.02002237

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

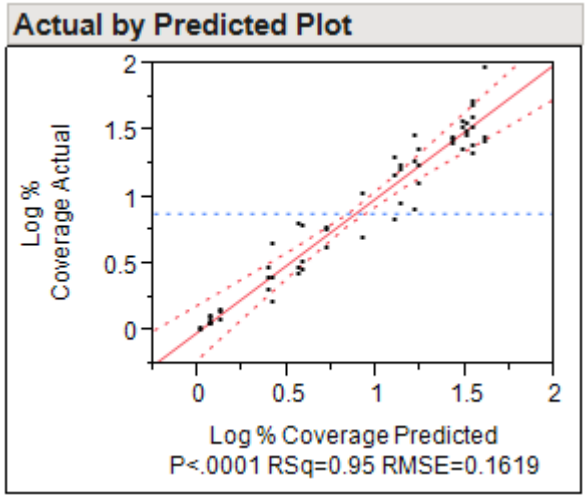
α= 0.050 Q= 3.78834

Level							Least Sq Mean
winter,control,90	A						0.79199736
summer,treatment,90		B					0.49391542
summer,control,30		B					0.46330460
winter,treatment,90		B					0.43995306
winter,control,30		B	C				0.42827856
summer,control,90		B	C				0.41945975
summer,treatment,30		B	C				0.36318836
winter,treatment,30			C	D			0.28966245
summer,control,7				D	E		0.16215450
summer,treatment,7					E	F	0.13322738
summer,treatment,15					E	F	0.07318412
winter,treatment,15					E	F	0.05221382
summer,control,15					E	F	0.05201393
summer,control,3					E	F	0.03918560
winter,control,15					E	F	0.03916006
summer,treatment,3					E	F	0.02881735
winter,treatment,7						F	0.01464413
winter,control,7						F	0.01415202
winter,treatment,3						F	0.01122740
winter,control,3						F	0.01080478

Levels not connected by same letter are significantly different.

RESPONSE LOG % COVERAGE

Whole Model



Summary of Fit

RSquare	0.945674
RSquare Adj	0.919869
Root Mean Square Error	0.161928
Mean of Response	0.881088
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	18.257494	0.960921	36.6472
Error	40	1.048833	0.026221	Prob > F
C. Total	59	19.306327		<.0001*

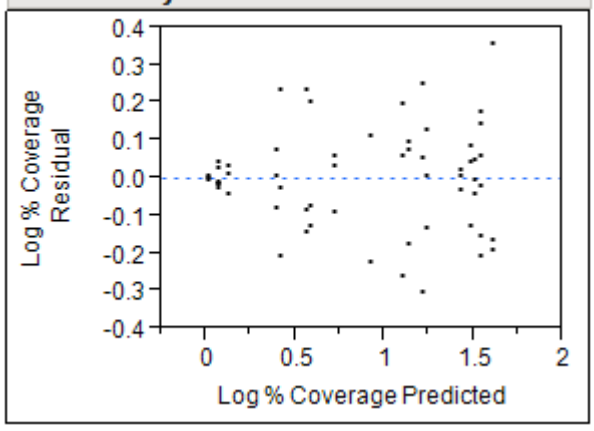
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.2223839	0.046745	4.76	<.0001*
Season[summer]	0.1823791	0.046745	3.90	0.0004*
Surface[control]	-0.020769	0.046745	-0.44	0.6592
Time[7-3]	0.3829829	0.066107	5.79	<.0001*
Time[15-7]	0.0896188	0.066107	1.36	0.1828
Time[30-15]	0.7230624	0.066107	10.94	<.0001*
Time[90-30]	0.046607	0.066107	0.71	0.4849
Season[summer]*Surface[control]	0.0073988	0.046745	0.16	0.8750
Season[summer]*Time[7-3]	0.3259004	0.066107	4.93	<.0001*
Season[summer]*Time[15-7]	-0.385902	0.066107	-5.84	<.0001*
Season[summer]*Time[30-15]	-0.184328	0.066107	-2.79	0.0081*
Season[summer]*Time[90-30]	0.1139449	0.066107	1.72	0.0925
Surface[control]*Time[7-3]	0.0163467	0.066107	0.25	0.8060
Surface[control]*Time[15-7]	0.0502023	0.066107	0.76	0.4521
Surface[control]*Time[30-15]	-0.012571	0.066107	-0.19	0.8501
Surface[control]*Time[90-30]	-0.123622	0.066107	-1.87	0.0688
Season[summer]*Surface[control]*Time[7-3]	0.0150065	0.066107	0.23	0.8216
Season[summer]*Surface[control]*Time[15-7]	0.032062	0.066107	0.49	0.6303
Season[summer]*Surface[control]*Time[30-15]	0.0352593	0.066107	0.53	0.5967
Season[summer]*Surface[control]*Time[90-30]	0.0175476	0.066107	0.27	0.7920

Effect Tests

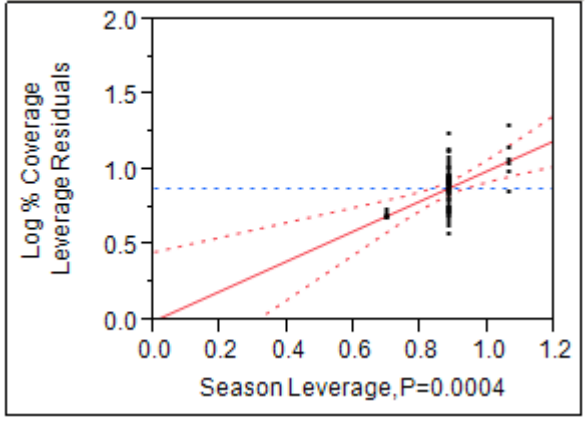
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	0.399145	15.2225	0.0004*
Surface	1	1	0.005176	0.1974	0.6592
Time	4	4	14.081087	134.2548	<.0001*
Season*Surface	1	1	0.000657	0.0251	0.8750
Season*Time	4	4	2.209687	21.0680	<.0001*
Surface*Time	4	4	0.138671	1.3221	0.2783
Season*Surface*Time	4	4	0.087111	0.8306	0.5137

Residual by Predicted Plot

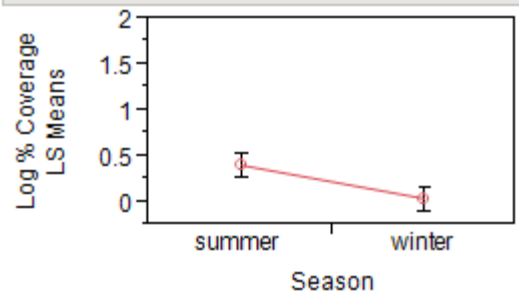


Season

Leverage Plot



LS Means Plot



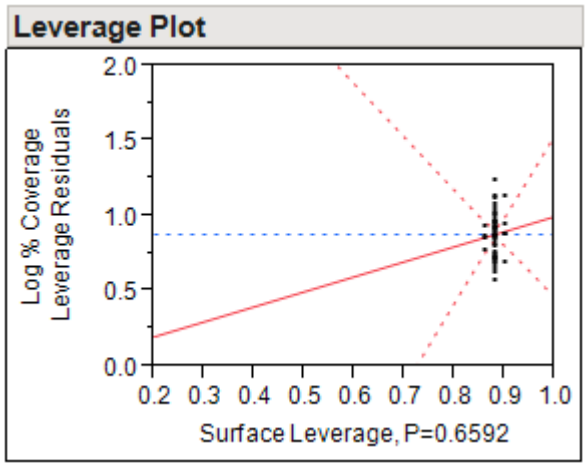
LSMeans Differences Student's t

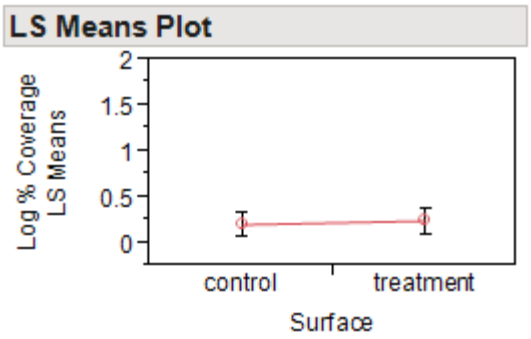
α= 0.050 t= 2.02108

Level		Least Sq Mean
summer	A	0.40476293
winter	B	0.04000479

Levels not connected by same letter are significantly different.

Surface Type





LSMeans Differences Student's t

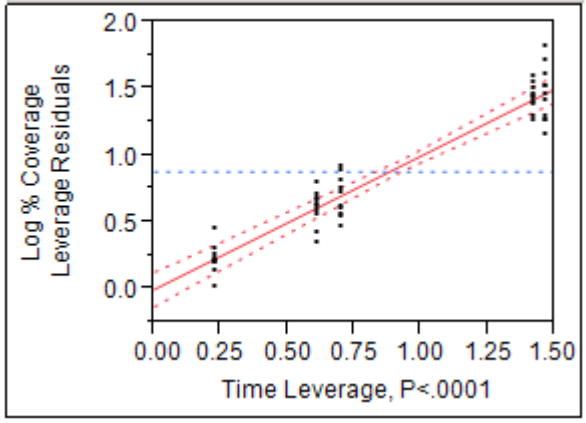
α= 0.050 t= 2.02108

Level		Least Sq Mean
treatment	A	0.24315331
control	A	0.20161441

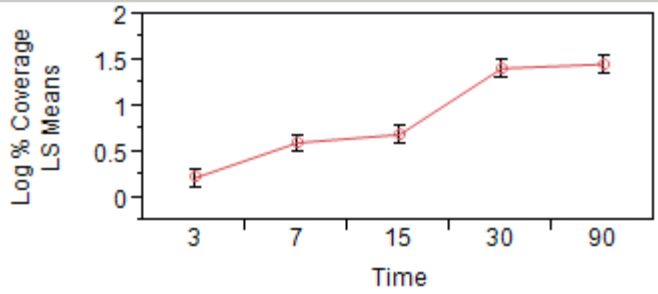
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



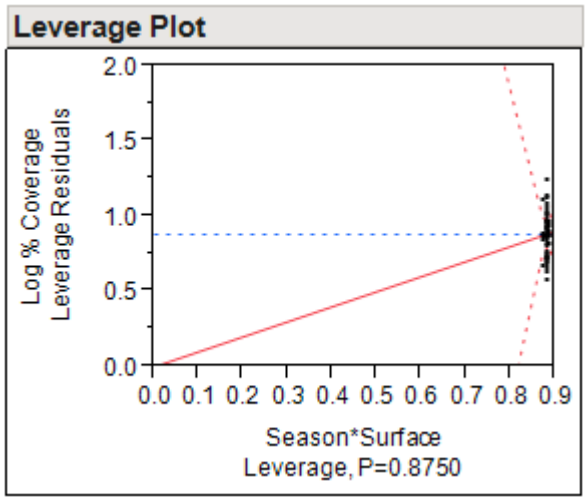
LSMeans Differences Tukey HSD

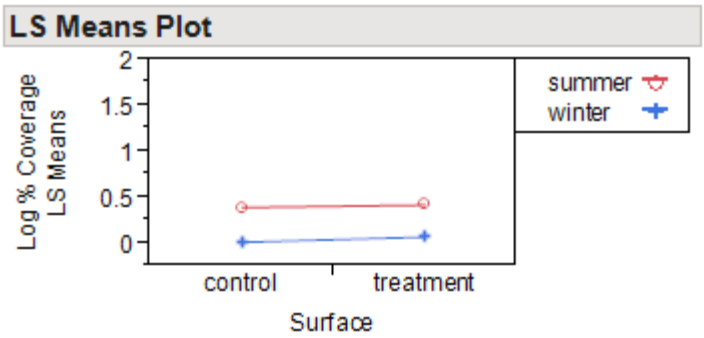
α= 0.050 Q= 2.85609

Level				Least Sq Mean
90	A			1.4646550
30	A			1.4180480
15		B		0.6949856
7		B		0.6053668
3			C	0.2223839

Levels not connected by same letter are significantly different.

Season*Surface Type





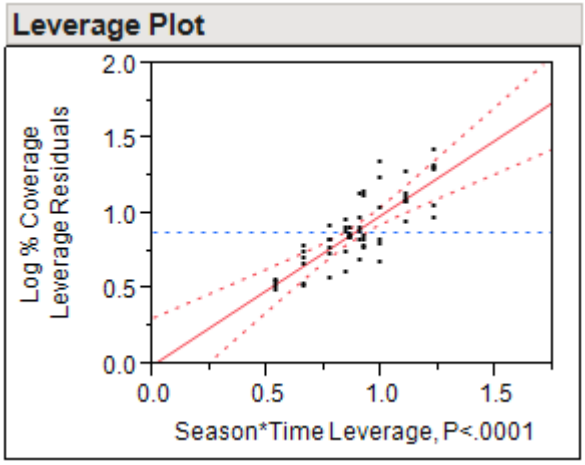
LSMeans Differences Tukey HSD

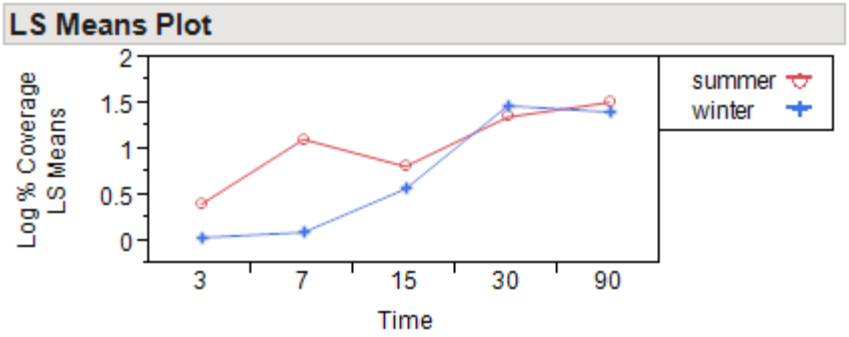
α= 0.050 Q= 2.68042

Level			Least Sq Mean
summer,treatment	A		0.41813357
summer,control	A		0.39139228
winter,treatment	A	B	0.06817305
winter,control		B	0.01183654

Levels not connected by same letter are significantly different.

Season*Immersion Time





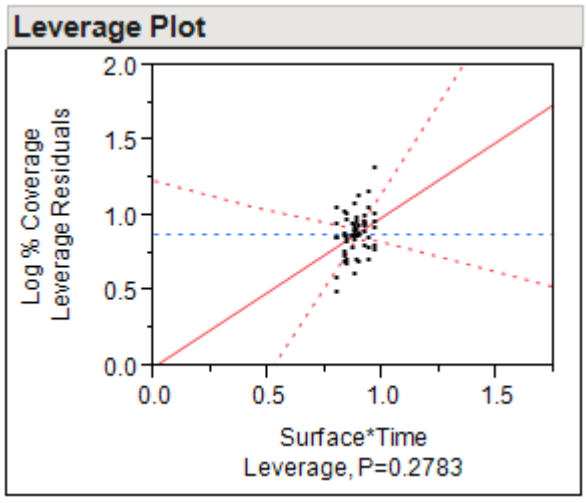
LSMeans Differences Tukey HSD

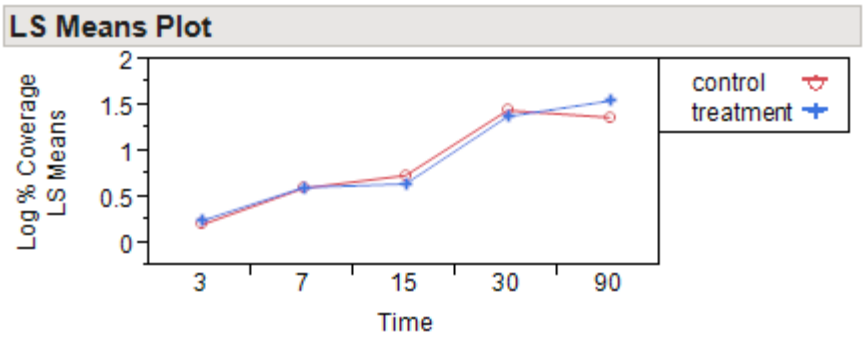
α= 0.050 Q= 3.34782

Level							Least Sq Mean
summer,90	A						1.5166489
winter,30	A						1.4799990
winter,90	A	B					1.4126611
summer,30	A	B					1.3560970
summer,7		B	C				1.1136463
summer,15			C	D			0.8173631
winter,15				D	E		0.5726081
summer,3					E	F	0.4047629
winter,7						F	G 0.0970873
winter,3							G 0.0400048

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





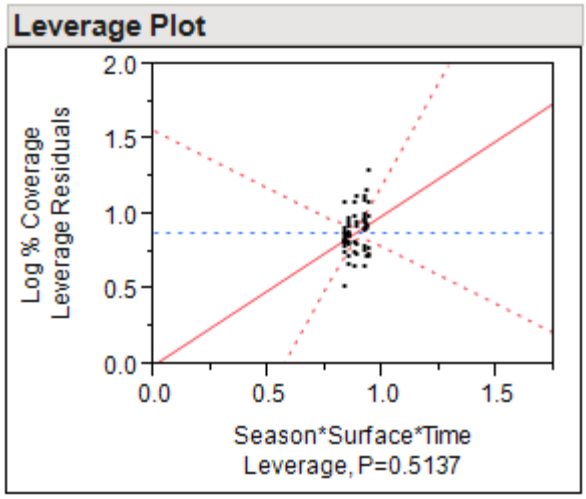
LSMeans Differences Tukey HSD

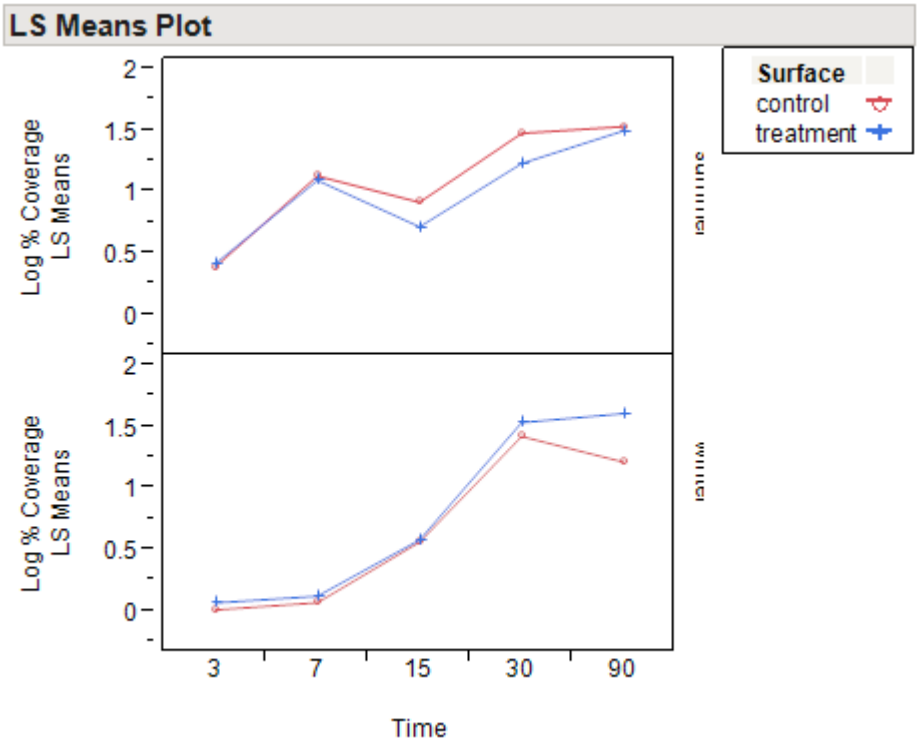
α= 0.050 Q= 3.34782

Level			Least Sq Mean
treatment,90	A		1.5550686
control,30	A		1.4512561
treatment,30	A		1.3848399
control,90	A		1.3742414
control,15		B	0.7407652
treatment,15		B	0.6492061
treatment,7		B	0.6097896
control,7		B	0.6009440
treatment,3		C	0.2431533
control,3		C	0.2016144

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

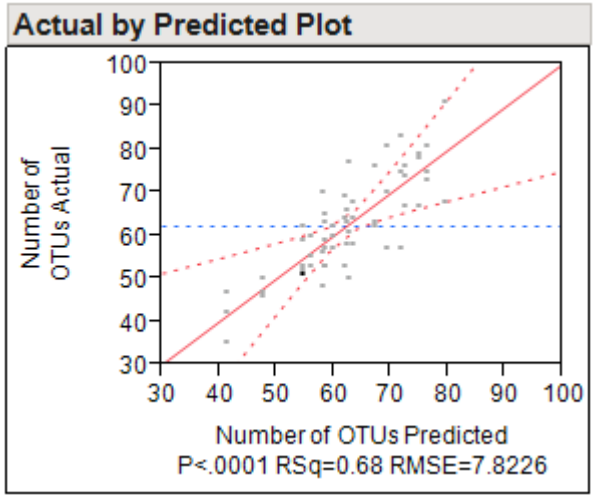
α= 0.050 Q= 3.78834

Level									Least Sq Mean
winter,treatment,90	A								1.6103490
winter,treatment,30	A	B							1.5365175
summer,control,90	A	B							1.5335095
summer,treatment,90	A	B							1.4997883
summer,control,30	A	B							1.4790318
winter,control,30	A	B							1.4234805
summer,treatment,30	A	B	C						1.2331623
winter,control,90	A	B	C	D					1.2149732
summer,control,7	A	B	C	D					1.1316288
summer,treatment,7		B	C	D					1.0956638
summer,control,15			C	D	E				0.9176100
summer,treatment,15				D	E	F			0.7171163
winter,treatment,15					E	F	G		0.5812959
winter,control,15					E	F	G	H	0.5639204
summer,treatment,3					E	F	G	H	I	0.4181336
summer,control,3						F	G	H	I	0.3913923
winter,treatment,7							G	H	I	0.1239154
winter,control,7								H	I	0.0702592
winter,treatment,3								H	I	0.0681730
winter,control,3									I	0.0118365

Levels not connected by same letter are significantly different.

RESPONSE NUMBER OF OTUs

Whole Model



Summary of Fit	
RSquare	0.678003
RSquare Adj	0.521133
Root Mean Square Error	7.822551
Mean of Response	62.20339
Observations (or Sum Wgts)	59

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	19	5025.0593	264.477	4.3221
Error	39	2386.5000	61.192	Prob > F
C. Total	58	7411.5593		<.0001*

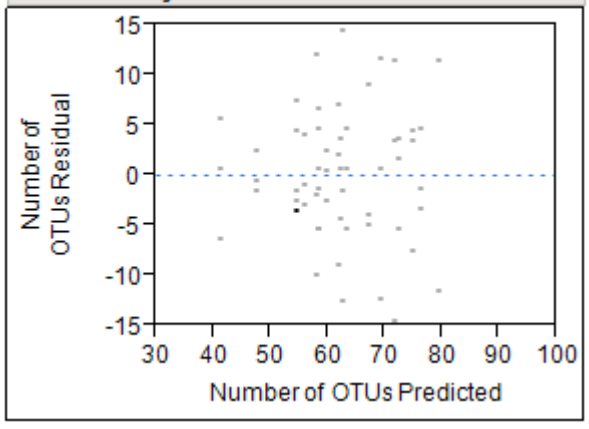
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	55.166667	2.258176	24.43	<.0001*
Season[summer]	-4	2.258176	-1.77	0.0843
Surface Type[control]	0.1666667	2.258176	0.07	0.9415
Immersion Time[7-3]	-1.666667	3.193543	-0.52	0.6047
Immersion Time[15-7]	8.75	3.193543	2.74	0.0092*
Immersion Time[30-15]	10.375	3.291829	3.15	0.0031*
Immersion Time[90-30]	-3.708333	3.291829	-1.13	0.2668
Season[summer]*Surface Type[control]	3.3333333	2.258176	1.48	0.1479
Season[summer]*Immersion Time[7-3]	-1.5	3.193543	-0.47	0.6412
Season[summer]*Immersion Time[15-7]	6.9166667	3.193543	2.17	0.0365*
Season[summer]*Immersion Time[30-15]	1.875	3.291829	0.57	0.5722
Season[summer]*Immersion Time[90-30]	-3.875	3.291829	-1.18	0.2463
Surface Type[control]*Immersion Time[7-3]	2.8333333	3.193543	0.89	0.3804
Surface Type[control]*Immersion Time[15-7]	-4.583333	3.193543	-1.44	0.1592
Surface Type[control]*Immersion Time[30-15]	0.9583333	3.291829	0.29	0.7725
Surface Type[control]*Immersion Time[90-30]	-5.958333	3.291829	-1.81	0.0780
Season[summer]*Surface Type[control]*Immersion Time[7-3]	0.3333333	3.193543	0.10	0.9174
Season[summer]*Surface Type[control]*Immersion Time[15-7]	-7.75	3.193543	-2.43	0.0200*
Season[summer]*Surface Type[control]*Immersion Time[30-15]	1.125	3.291829	0.34	0.7344
Season[summer]*Surface Type[control]*Immersion Time[90-30]	3.2083333	3.291829	0.97	0.3357

Effect Tests

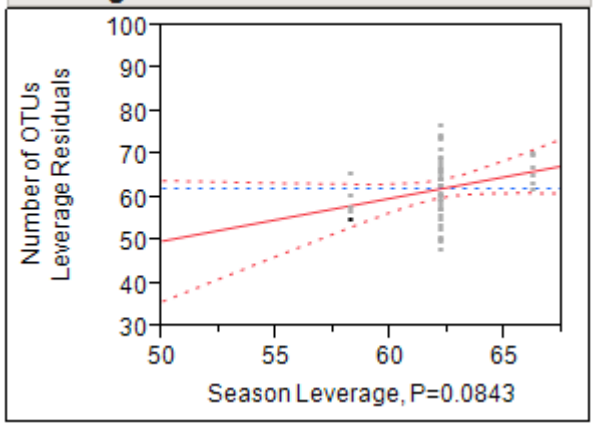
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Season	1	1	192.0000	3.1376	0.0843
Surface Type	1	1	0.3333	0.0054	0.9415
Immersion Time	4	4	3202.3258	13.0830	<.0001*
Season*Surface Type	1	1	133.3333	2.1789	0.1479
Season*Immersion Time	4	4	617.8485	2.5242	0.0562
Surface Type*Immersion Time	4	4	586.9091	2.3978	0.0666
Season*Surface Type*Immersion Time	4	4	588.1439	2.4029	0.0662

Residual by Predicted Plot

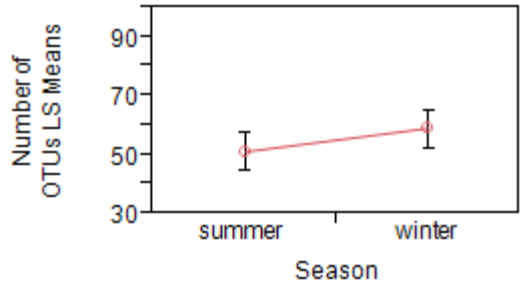


Season

Leverage Plot



LS Means Plot



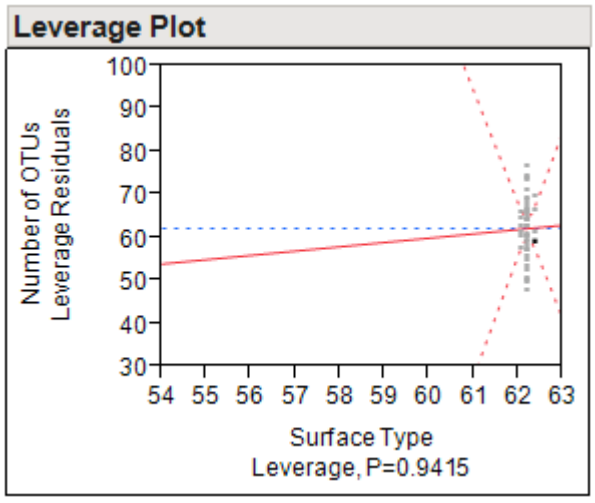
LSMeans Differences Student's t

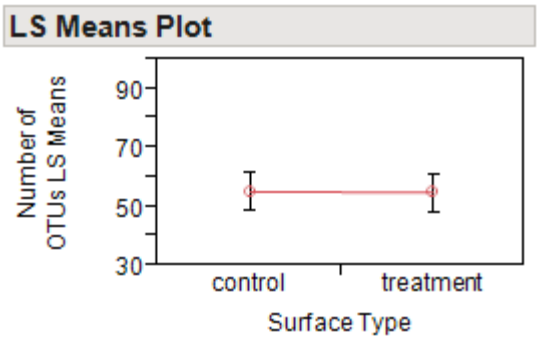
α= 0.050 t= 2.02269

Level		Least Sq Mean
winter	A	59.166667
summer	A	51.166667

Levels not connected by same letter are significantly different.

Surface Type





LSMeans Differences Student's t

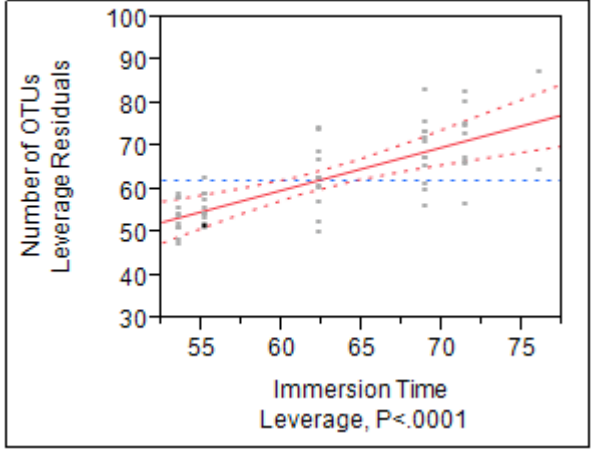
α= 0.050 t= 2.02269

Level		Least Sq Mean
control	A	55.333333
treatment	A	55.000000

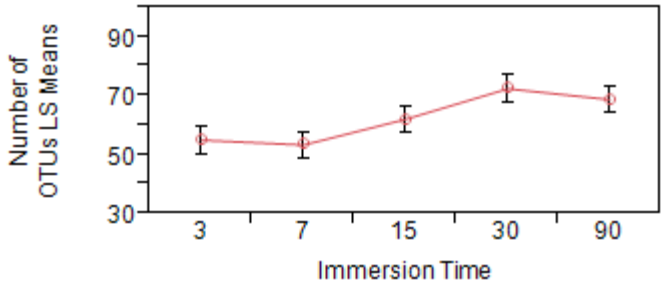
Levels not connected by same letter are significantly different.

Immersion Time

Leverage Plot



LS Means Plot



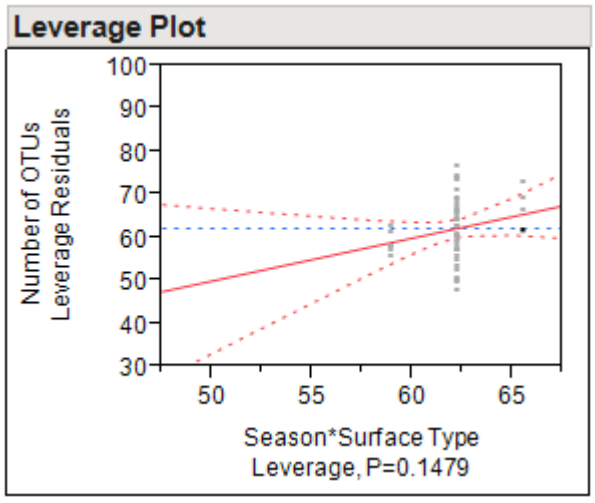
LSMeans Differences Tukey HSD

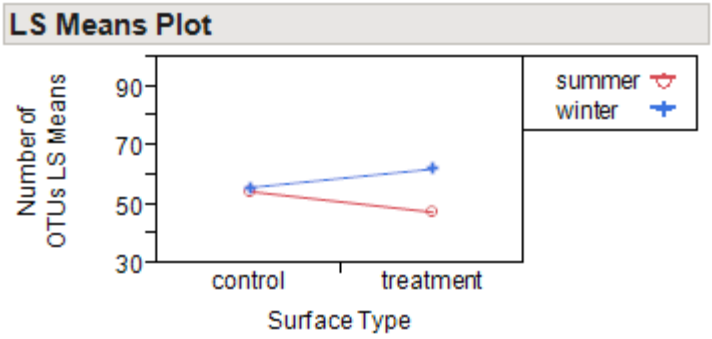
α= 0.050 Q= 2.85949

Level				Least Sq Mean
30	A			72.625000
90	A	B		68.916667
15		B	C	62.250000
3			C	55.166667
7			C	53.500000

Levels not connected by same letter are significantly different.

Season*Surface Type





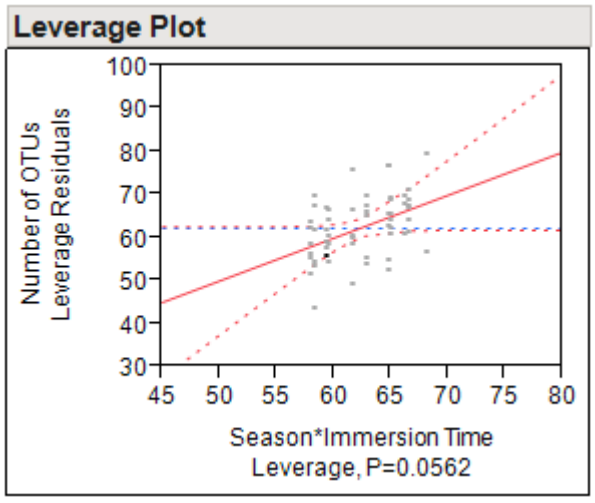
LSMeans Differences Tukey HSD

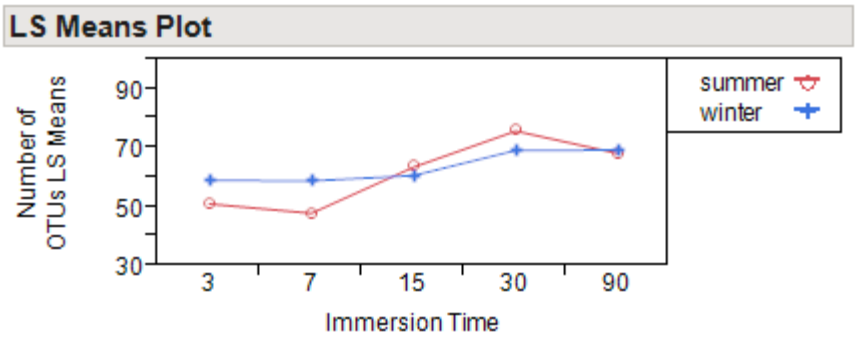
α= 0.050 Q= 2.68337

Level		Least Sq Mean
winter,treatment	A	62.333333
winter,control	A	56.000000
summer,control	A	54.666667
summer,treatment	A	47.666667

Levels not connected by same letter are significantly different.

Season*Immersion Time





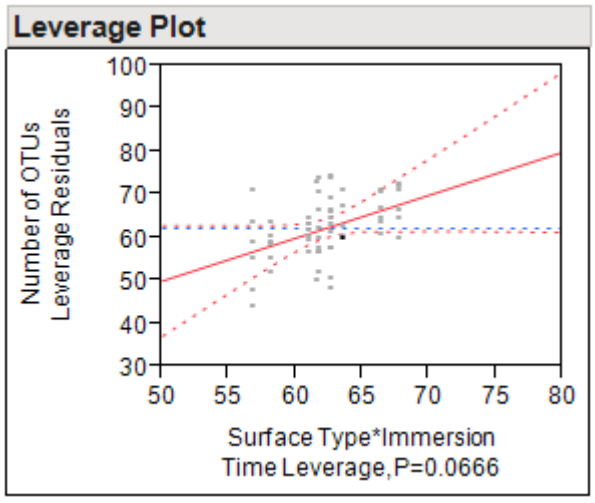
LSMeans Differences Tukey HSD

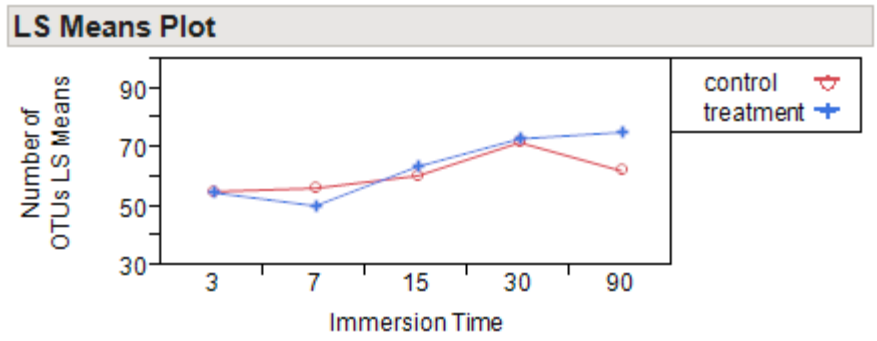
α= 0.050 Q= 3.35265

Level					Least Sq Mean
summer,30	A				75.916667
winter,90	A	B			69.500000
winter,30	A	B			69.333333
summer,90	A	B			68.333333
summer,15	A	B	C		63.666667
winter,15	A	B	C	D	60.833333
winter,3		B	C	D	59.166667
winter,7		B	C	D	59.000000
summer,3			C	D	51.166667
summer,7				D	48.000000

Levels not connected by same letter are significantly different.

Surface Type*Immersion Time





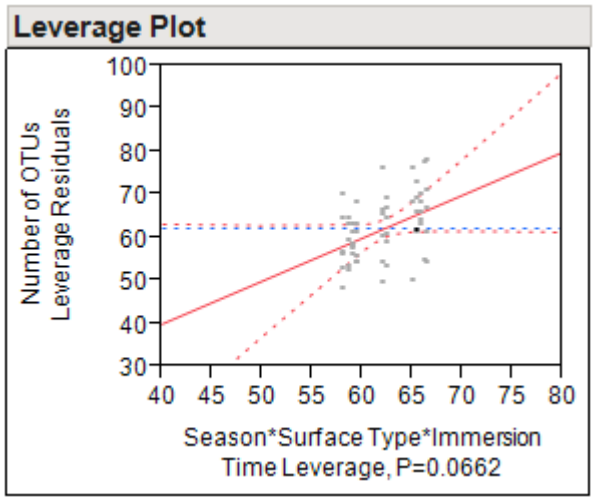
LSMeans Differences Tukey HSD

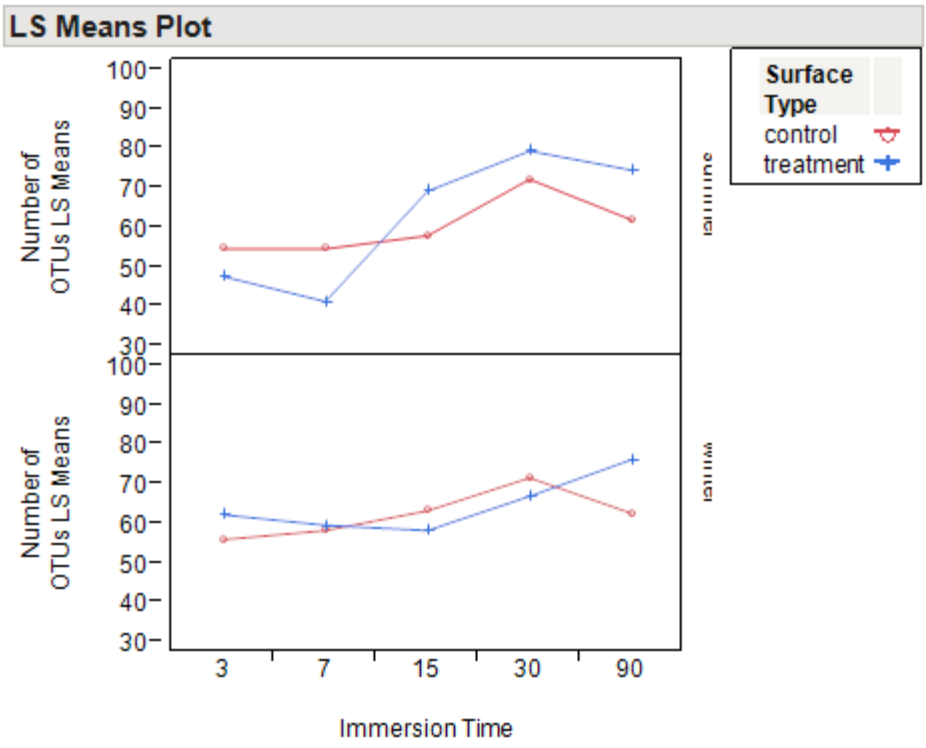
α= 0.050 Q= 3.35265

Level			Least Sq Mean
treatment,90	A		75.500000
treatment,30	A		73.250000
control,30	A		72.000000
treatment,15	A	B	63.833333
control,90	A	B	62.333333
control,15	A	B	60.666667
control,7		B	56.500000
control,3		B	55.333333
treatment,3		B	55.000000
treatment,7		B	50.500000

Levels not connected by same letter are significantly different.

Season*Surface Type*Immersion Time





LSMeans Differences Tukey HSD

α= 0.050 Q= 3.7947

Level				Least Sq Mean
summer,treatment,30	A			79.500000
winter,treatment,90	A			76.333333
summer,treatment,90	A			74.666667
summer,control,30	A			72.333333
winter,control,30	A	B		71.666667
summer,treatment,15	A	B		69.333333
winter,treatment,30	A	B		67.000000
winter,control,15	A	B	C	63.333333
winter,control,90	A	B	C	62.666667
winter,treatment,3	A	B	C	62.333333
summer,control,90	A	B	C	62.000000
winter,treatment,7	A	B	C	59.666667
winter,control,7	A	B	C	58.333333
winter,treatment,15	A	B	C	58.333333
summer,control,15	A	B	C	58.000000
winter,control,3	A	B	C	56.000000
summer,control,7	A	B	C	54.666667
summer,control,3	A	B	C	54.666667
summer,treatment,3		B	C	47.666667
summer,treatment,7			C	41.333333

Levels not connected by same letter are significantly different.