Enhancement of Bioleaching Using AHL Mediated Quorum Sensing

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Thesis submitted to the

University of Ottawa

In partial fulfillment of the requirements for the M.Sc. degree in Earth Sciences

uOttawa

L'Université canadienne
Canada's university

Abstract

Biomining is a maturing technology that uses the activity of sulfur and iron oxidising microorganisms to liberate valuable metals from ores but suffers from slow reaction kinetics. Increasing the reaction kinetics of a biomining process could produce significant bottom line improvements for mining companies worldwide and encourage the use of biomining as a green mining technology. Quorum sensing molecules have been shown to successfully modulate the behaviours of biomining bacteria in manners that may be able to improve bioreactor retention times. This study tests the potential for two different quorum sensing treatments to improve the nickel leaching ability of a biomining bacterial consortium. A novel method of delivering quorum sensing treatments to bacterial cultures is described while doubt is cast on established methods. Laboratory scale bioreactors were constructed and the leaching of nickel into solution was followed via ICP-AES to quantify improvements in bioleaching ability. Similar bioreactors were used to exhibit the inhibitory effect that a commonly used organic solvent can have on the leaching ability of bioleaching consortia. Ultimately a qualitative improvement in the bioleaching of nickel is produced using a mixture of tetradecanoyl-acylhomoserine lactone (C14-AHL) and its two derivatives, but the use of C14-AHL alone did not improve bioleaching kinetics. Use of small volumes of the solvent DMSO produced large inhibitory effects on the leaching of nickel by bioleaching consortia.

Résumé

La bioprospection minière est une technologie écologique bien connue qui utilise l'activité des micro-organismes qui oxydent le soufre et le fer afin de libérer des métaux précieux des minerais mais qui souffre aussi de cinétique de réaction lente. Augmenter la cinétique de réaction du processus de bioprospection minière pourrait produire des améliorations financières importantes pour des sociétés minières à travers le monde et encourager l'utilisation de bioprospection minière comme une technologie minière verte. Il s'est avéré que les molécules de détection du quorum modulent le comportement des bactéries de bioprospection minière avec succès sous des conditions qui pourraient améliorer le délai de réaction du bioréacteur. Cette étude analyse la capacité d'améliorer l'aptitude de la lixiviation de nickel avec un consortium bactérien de bioprospection minière sous deux conditions différentes. On décrit une nouvelle méthode de distribution des traitements de détection du quorum aux cultures bactériennes en émettant des doutes quant aux méthodes établies. Des bioréacteurs à l'échelle du laboratoire ont été construits et la lixiviation de nickel en solution a été suivie via ICP-AES afin de quantifier les améliorations de la capacité de biolixiviation. Des bioréacteurs similaires ont été utilisés afin d'exposer les effets inhibiteurs qu'un solvant biologique canonique typique pourrait affecter la capacité de lixiviation de consortiums de biolixiviation. En fin de compte, une petite mais importante amélioration de biolixiviation de nickel est produite en utilisant un mélange de tétradécanoyl-acylhomosérine lactone (C14-AHL) et ses deux dérivés, mais l'utilisation de C14-AHL seul n'a pas amélioré les cinétiques de biolixiviation. L'utilisation de petites quantités de solvant DMSO a produit des effets inhibiteurs importants sur la lixiviation du nickel par des consortiums de biolixiviation.

Acknowledgements

This thesis is produced in partial fulfillment of the requirements for the master's degree in Earth Sciences completed by myself, Alexander Dewar. I would like to thank my incredibly patient supervisors; Dr. Rory Cameron and Professor Danielle Fortin, as well as the staff at CanmetMINING and my colleagues amongst the Fortin laboratory group, all of whom supported me immensely throughout this project.

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1.1 What is biomining?

Biomining refers to the extraction of metals from ores using microorganisms in a controlled setting. Biomining can be applied to ores containing base metals such as copper, nickel, or zinc, or it can be used to help extract precious metals such as gold, silver or platinum. Commercial biomining almost exclusively utilizes iron-sulfide ores and provides a method of processing low quality ores en-masse (2, 3). Biomining processes benefit from low operating costs but suffer from very slow reaction kinetics and long reactor retention times. The two primary types of commercial biomining operations occur in either enormous heterogenous heaps of low-quality ore stacked dozens of meters high and many kilometers long, or in homogenous stirred-tank reactors which are generally reserved for higher grade ore concentrates. Biomining environments rapidly develop acidity due to the oxidation of sulfides which limits both the spontaneous oxidation of iron as well as the diversity of organisms that can live in the environment. This environment fosters microorganisms that can catalyze the aqueous leaching process through the regeneration of ferric iron. Ferric iron is capable of oxidizing mineral bound ferrous iron through either the thiosulfate mechanism or the polysulfide mechanism; the former mechanism can be catalyzed only by ferric iron and the latter by ferric iron as well as protons. For more details regarding the mechanisms of iron-sulfide leaching, see the following references (4, 5).

Biomining microorganisms can be active individually as free-floating planktonic cells, or sessile in microbial mats known as biofilms. Biofilms are known to protect microorganisms from environmental stressors such as desiccation, starvation, and oxidative stress and are considered to be prerequisite for mineral leaching to occur (5, 6). This is because microbial biofilms provide an enlarged reaction space in which the chemical oxidation of iron and sulfur may occur (4). Differences exist in the biofilms generated by different species of biomining microorganisms and biofilms present from the pre-colonization of minerals by one species can enhance or inhibit the subsequent colonization of that mineral by new species in a phenomenon known generally as bacterial succession (7). The production of biofilms by microorganisms is controlled in part by intercellular chemical messaging systems that are together known as quorum sensing (8, 9).

1.2 What is quorum sensing?

The term quorum sensing (QS) describes one or more systems of communication used by microbes to regulate gene expression in response to population density, largely these genes control phenotypic responses known as “group behaviours”(10). Group behaviours such as bioluminescence, virulence, or biofilm production are costly and unrewarding when initiated by singular cells but are powerful and beneficial when performed by large groups of organisms in concert. QS is controlled by the secretion of “autoinducer” (AI) molecules by individual organisms, which vary in shape and chemistry. They are constitutively produced by many microbes that possess QS abilities, and either freely diffuse across cellular membranes or are actively transported. AIs are detected by receptor proteins that are stabilized by the presence of their cognate AI molecule, together forming a stable complex molecule. The stabilized receptor proteins then bind DNA promotors that modify the transcription of the corresponding QS-regulated genes (9). As the population of QS microbes increase in an environment, the concentration of autoinducer molecules will increase accordingly, until eventually a threshold concentration is reached and synchronous changes in gene expression are initiated across the entire

population of microbes simultaneously. It is interesting to note that there appears to be many more microorganisms with the molecular machinery that allows them to "listen" and respond to QS autoinducer signals than there are microorganisms that actually produce QS AIs (11, 12). Some relevant biomining microorganisms such as *Leptosprillum spp* are thought to have this ability to respond to QS signals, despite not being able to produce said signals themselves. (13).

There are three primary systems of bacterial quorum sensing: AI-1 which utilizes acylhomoserine lactone (AHL) autoinducers, AI-2 which utilizes 4,5-dihydroxy-2,3-pentanedione (DPD) and its derivatives as autoinducers, and a third system uses autoinducer peptides (14–16). AI-1, and autoinducer peptide systems are typically found in gram-negative and gram-positive bacteria, respectively. The AI-2 QS system can be found in both gram-negative and gram-positive bacteria and are thought to serve as a means of interspecies communication. Other niche quorum sensing systems are known to exist, but none have been found that are currently thought to be relevant to a biomining environment (17). To date, no QS systems have been found to be functional in a biomining or biomining relevant archaeon, and no evidence has been found of the AI-2 type QS system in *Acidithiobacillus ferrooxidans,* the most well studied biomining microorganism (7, 16, 18).

The QS system most thoroughly described is the AI-1 system which uses acylhomoserine lactones (AHLs) as autoinducers to convey intercellular information. There are many different AHLs although they all share a similar basic chemical structure, seen in Figure 1.

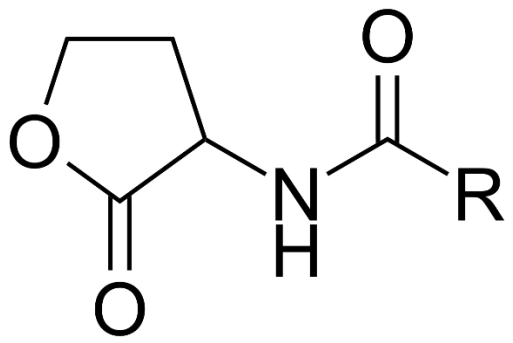

Figure 1: The basic chemical structure of an acylhomoserine lactone (AHL). The R group can be a chain of carbons of any length between 4 and 20, typically having an even number of carbon atoms.

The differences between AHLs arise from their variable R groups, which consist of hydrocarbon chains that range in length between 4 and 18 carbons (19). In addition to their variable length, the carbon chain of any given AHL can be modified at the third carbon with one of two chemical substitutions, either keto- or hydroxyl- groups. Typically AHLs have an R group with an even number of carbon atoms, rather than an odd number.

The AI-1/AHL QS system is commonly found in gram-negative bacteria, a group that includes many prominent genera of biomining bacteria such as: *Leptospirillum, Acidithiobacillus,* and *Thermobacillus,* although it has been established that AHL QS does not occur in thermophilic bacteria as AHLs are not heat stable (12). Long chain AHLs are known to be less soluble in aqueous solutions than their short chain counterparts (e.g., 14 vs 4 carbons), and the long chain varieties are also thought to be

more heat stable (20). The AHL component of the AI-1 QS system has been shown to function in a variety of gram-negative mesophilic biomining bacteria, and the potential to utilize the corresponding AHL autoinducers to improve the leaching kinetics of biomining operations will be the focus of this review.

1.3 How QS Manipulations Might Improve the Bottom Line

The potential for bacterial quorum sensing to be manipulated in order to improve the kinetics of biomining systems has been suggested by numerous experts in the field of biohydrometallurgy (13, 16, 21). Preliminary work has focused on the use of exogenously added AHL signals intended to manipulate the AI-1 QS system to encourage bacterial attachment to the mineral surface, as well as to promote exopolymeric substance (EPS) and biofilm production. Previous studies have shown that QS manipulation via exogenously added AHLs can initiate earlier bacterial attachment to mineral surfaces, as well as increase the amount of EPS produced by bioleaching bacteria (6, 22). Thus far most research regarding the role of QS in biomining has been performed using pure cultures of a single bacterial species, or in some cases binary cultures of two bacterial species. Few studies have investigated the effects of exogenously added AHLs on diverse communities of microorganisms that more realistically represent the communities responsible for biomining in industrial settings (22). However, recently reported results regarding the increased mineral-surface colonization and improved iron-sulfide leaching ability of biomining bacteria exposed to exogenously added AHLs suggest that this could be a promising avenue of research that could ultimately allow microbiologists to improve the bottom line for mining process plants by improving the mineral leaching kinetics of bacterial consortia (6).

1.4 Previous Work with Quorum Sensing Systems in Biomining Environments

There are limited studies that have addressed specifically the topic of QS in biomining bacteria. These studies have tried to shed light on the ways in which common biomining bacteria respond to various QS signals, and to some extent investigate the ability to improve iron-sulfide mineral bioleaching using AHL signals. Chiefly these studies use AHL QS signals, both natural and synthetic, to explore the potential for the manipulation of cell adhesion, exopolysaccharide production, and biofilm development in biomining bacteria. A few of the most relevant studies will be summarized here, and their implications highlighted (6, 13, 23, 24).

The AHL QS system was first described in the marine bioluminescent bacterium *Vibrio fischeri,* which has since become the paradigm for AHL mediated QS (25). In *V. fischeri,* there are two proteins, LuxI & LuxR, which control the expression of genes required for bioluminescence. LuxI is the autoinducer synthase which produces the AHL autoinducer 3-oxohexanoyl-acyl-homoserine lactone (3-oxo-C6-AHL), whereas LuxR is the DNA binding transcriptional regulator**.** The AHL molecule passively diffuses in and out of cells and increases in concentration within the environment concurrent with rising cellular density. When the AHL signal reaches a threshold concentration, its binding to LuxR allows the LuxR-AHL complex to bind DNA and induce the expression of bioluminescence genes. Active transport of autoinducers out of the cell prevents short-circuiting of the AHL signalling system. The transcriptional response only engages once the influx of AHLs into the cell overwhelms the constitutive AHL export (25). This paradigm of AHL quorum sensing found in *V. fischeri* sets the stage for the investigation of quorum sensing in other bacteria, such as those involved in biomining operations. Few, if any, biomining microbes exhibit bioluminescence; instead these acidophiles utilize QS to precisely regulate processes

such as the attachment to mineral surfaces, the excretion of EPS, and the formation of biofilms (24). Proteobacteria that utilize AHL QS sensing almost universally contain homologs of LuxI which produce AHLs, and homologs of LuxR to detect and respond to AHLs (26).

Acidithiobacillus ferrooxidans is a chemolithoautotrophic acidophile that is often found in acid mine drainage sites and biomining environments. Formerly known as *Thiobacillus ferrooxidans*, the gammaproteobacteria *At. ferrooxidans* has been shown to possess a type AI-1 QS system (13, 22). Like the AHL based QS system present in *V. fischeri,* there are four main molecular components present in the AHL system of *At. ferrooxidans.* There is an AHL synthase protein, AfeI, analogous to the LuxI protein from *V. fischeri*, as well as a transcriptional regulator, AfeR, analogous to the LuxR transcriptional regulator protein, there is also a palindromic DNA sequence to which the AferR-AHL complex binds, and finally there is the AHL signalling molecule component (13, 27). The AfeI protein is capable of synthesizing nine different AHLs of various acyl chain lengths, each of which are between eight and sixteen carbons (28). The AHLs synthesized by *At. ferrooxidans* are sometimes modified by the addition of substitutions at the third carbon in the carbon chain, these substitutions can include oxo- and hydroxyl- moieties.

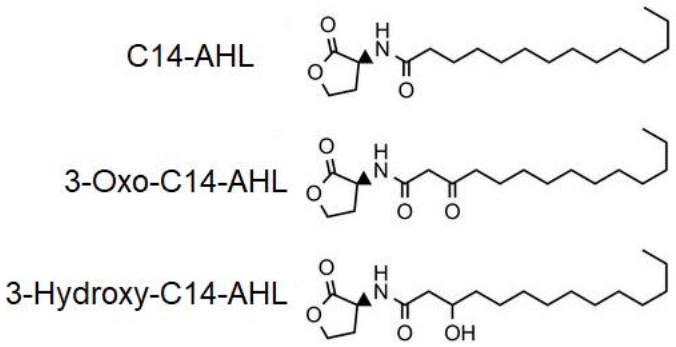

Figure 2: The chemical structures of the unsubstituted C14-AHL, the 3-oxo-C14-AHL, and 3-hydroxy-C14-AHL molecules.

At. ferrooxidans is not the only biomining bacterium that produces or responds to AHL QS signals. By testing for its ability to activate the AHL bioreporter strain *Agrobacterium tumefaciens, At. thiooxidans* has also been found to produce AHL signaling molecules, primarily synthesizing short-chain AHLs of 6 or 8 carbons (13). Bioinformatic analysis of partial and complete genomes of a *Leptospirillum* species revealed an AI-1 type QS locus comprised of two genes thought to encode an AHL synthase and a transcriptional regulator. When screened via the *A. tumefaciens* bioreporter *Leptospirillum ferrooxidans* was found not to produce AHL signals, although the authors suggested that *L. ferrooxidans* may still be able to perceive and respond to AHL signals (13). This result was later supported by findings from (Bellenberg, et al., 2014b) which showed that a pure culture of *L. ferrooxidans* had improved pyrite leaching ability in the presence of exogenously added AHLs of carbon chain length 14 (C14-AHL). A separate study showed that although *Leptospirillum ferriphilum* did not produce AHL signals, its attachment to pyrite was increased in the presence of the quorum sensing signal C14-AHL

The effect of various long-chain AHLs on the colonization and leaching of pyrite by biomining bacteria was studied by Bellenberg et al. (6). The researchers tracked the leaching of iron into solution

to determine how extensively pyrite samples were bioleached. The bacteria studied included *At. ferrooxidans* ATCC 23270, as well as strains of *Acidithiobacillus ferrivorans, Acidiferrobacter* sp, *Acidiferrobacter ferrooxydans*, and *L. ferrooxidans*. Bacteria were inoculated into shake-flasks as pure and binary cultures and pyrite chips were provided as the sole energy source for the bacteria. To assess the role of AI-1 quorum sensing on bioleaching in these pure and binary cultures, several AHLs were tested at 5 µM concentrations including the C8-, C12-, C14-, C16- and C18-AHLs. The authors found that different species of bacteria did not always respond to each AHL in the same manner. For instance, the pyrite leaching by pure cultures of *At. ferrivorans*, and *Acidoferrobacter* sp. was strongly inhibited by a mixture of C14-AHLs, while the same C14-AHL mixture improved the leaching of pyrite by pure cultures of *At. ferrooxidans* and *L. ferrooxidans* (6). Pyrite bioleaching was slightly improved for *At. ferrooxidans* by treatment with C8-, C14-, and C16/C18-AHLs, but no treatment improved the pyrite leaching of this bacterium by more than 6%. Pyrite leaching by *L. ferrooxidans* was improved using treatments of C8-, C12-, and C14-AHLs by 14%, 17% and 12%, respectively. The sulfur oxidizing species *At. thiooxidans* was shown to respond to C8- and C10-AHLs by increasing its attachment to sulfur coupons. These results helped to establish the effectiveness of long chain AHLs as a potential option for improving the leaching behaviours of pure cultures of bacteria.

Regarding the binary cultures of two bacteria grown in co-culture by Bellenberg et al. (6): pyrite leaching was not significantly enhanced by the presence of a C14-AHL mixture in a binary culture of *A. ferrooxidans* and *L. ferrooxidans*. The combined culture of *L. ferrooxidans* and *Acidiferrobacter* spp. initially expressed highly reduced pyrite leaching ability, far inferior to the leaching ability of either constituent pure culture, indicating an antagonistic interaction between the two species. Interestingly, this inhibition was largely relieved by treatment with C8-, C14- and C16/C18-AHLs, which restored the pyrite leaching ability of this binary culture to 16%, 39%, and 35% of the control values initially achieved by pure cultures of *L. ferrooxidans*, up from the paltry 5% initially reached by this binary culture. Many of the combinations of bacteria and AHLs that were tested resulted in no significant change of pyrite leaching, and the remaining combinations produced decreases in pyrite leaching. These results will not be discussed at length in this literature review as the aim of this review is to highlight potential opportunities to improve bacterially mediated mineral leaching.

The work of Bellenberg et al (6) tested AHL mixtures of various chain lengths, using the unsubstituted, 3-hydroxy-substituted, and 3-oxo-substituted forms against a range of single and binary bacterial cultures. Some AHL mixtures expedited mineral leaching behaviors, while other AHLs inhibited mineral leaching behaviours. Results varied depending upon which species of bacteria were tested. Table 1 attempts to summarize the findings of Bellenberg et al (6). It is worthy of note that the mixture of C14-AHL and its 3-oxo and 3-hydroxy derivatives (henceforth known as C14-MIX) succeeded in improving the leaching kinetics of *Leptospirillum ferrooxidans* cultures when *L. ferrooxidans* was grown alone, or in a binary culture with *Acidithiobacillus ferrooxidans.* The treatment responsible for the most improvements in pyrite leaching ability was the C14-MIX treatment, which seemed to work particularly well with the *L. ferrooxidans* strain, and to a lesser extent, the *A. ferrooxidans* strain.

Table 1: A summary of the findings of Bellenberg et al (2014b), regarding the effects of various AHL treatments on the pyrite leaching behaviour of several pure and binary bacterial cultures. Treatment with the C14-MIX AHL cocktail was most often associated with improvements in pyrite bioleaching.

	AHL Treatment			
Bacteria in Culture	C8-MIX	C12-MIX	C14-MIX	C16-MIX + C18-MIX
Acidithiobacillus ferrooxidans	Slight Increase	Slight Decrease	Slight Increase	Slight
Acidithiobacillus ferrivorans	Unclear/No difference	Major Decrease	Major Decrease	Unclear/No difference
Acidiferrobacter sp.	Unclear/No difference	Major Decrease	Major Decrease	N/A
Leptospirillum ferrooxidans	Increase	Increase	Increase	Decrease
Acidithiobacillus ferrooxidans and *Leptospirillum ferrooxidans*	Unclear/No difference	Unclear/No difference	Slight Increase	Unclear/No difference
Acidithiobacillus ferrooxidans and *Acidithiobacillus ferrivorans*	Unclear/No difference	Unclear/No difference	Unclear/No difference	Unclear/No difference
Acidithiobacillus ferrooxidans and *Acidiferrobacter ferrooxidans*	Unclear/No difference	Unclear/No difference	Unclear/No difference	Unclear/No difference
Leptospirillum ferrooxidans *and* *Acidiferrobacter* sp.	Increase	Unclear/No difference	Major Increase	Major Increase
Leptospirillum ferrooxidans *and* *Acidithiobacillus ferrivorans*	Unclear/No difference	Slight Decrease	Unclear/No difference	Unclear/No difference

To improve the efficiency of biomining processes, González et al (2013) investigated the use of exogenously added AHL QS signals to modulate the attachment of *At. ferrooxidans* to pyrite and reduced sulfur substrates. A number of AHL molecules were tested for their ability to promote the attachment of *At. ferrooxidans, At. thiooxidans,* or *L. ferriphilum* to either pyrite or sulfur surfaces. In addition to attachment, the leaching of pyrite grains and sulfur coupons was studied while in the presence of various biomining organisms and cultures. A pure culture of *A. ferrooxidans* and one biomining community containing *At. thiooxidans*, *L. ferriphilum*, and, *At. ferrooxidans* were subjected to incubations and attachment experiments, with or without the addition of AHLs.

Single AHL molecules were tested with *At. ferrooxidans* grown on elemental sulfur coupons, with and without the addition of unsubstituted C14-AHL at a concentration of 5 µM. When the sulfur coupons were stained and visualized via fluorescence microscopy, the results clearly indicated that increased levels of attachment to sulfur coupons were reached in the presence of the C14-AHL additive. Similar results were found for the single AHLs C12-AHL, and 3-hydroxy-C12-AHL, and 3-hydroxy-C14-AHL. The AHLs 3-oxo-C12-AHL and 3-oxo-C14-AHL were also tested but were not found to improve adhesion of *At. ferrooxidans* to sulfur coupons. Overall, the authors of this study found that the attachment of *At. ferrooxidans* to sulfur prills could indeed be improved by the addition of certain AHLs (22).

To test whether the adhesion of *At. ferrooxidans* to pyrite coupons could be modulated by the addition of exogenous AHL molecules, a mixture of 3-oxo-C14-AHL, 3-hydroxy-C14-AHL, and C14-AHL was tested and visualized under atomic force microscopy and electrostatic force microscopy, revealing that the mineral adhesion of *At. ferrooxidans* was increased in the presence of this mixture as compared to untreated controls. No similar increase of attachment was found when *At. ferrooxidans* was grown in the presence of an analogous mixture of C12-AHLs. A summary of the AHLs tested on the pure strain of *At. ferrooxidans* can be viewed in Table 2.

Table 2: The effect of 5 µM doses of various AHLs on the attachment of a pure culture of A. ferrooxidans ATCC 23270 to either pyrite or sulfur substrates by González et al. (2013). The AHLs used in the present study are in bold.

At. ferrooxidans ATCC 23270	Pyrite	Sulfur
C12-AHL	-	Improved attachment
3-oxo-C12-AHL	-	No change in attachment
3-Hydroxy-C12-AHL	-	Improved attachment
C14-AHL	-	**Improved attachment**
3-oxo-C14-AHL	-	**No change in attachment**
3-Hydroxy-C14-AHL	-	**Improved attachment**
Mix of 3 C12-AHLs	No change in attachment	-
Mix of 3 C14-AHLs (C14-MIX)	**Improved attachment**	-

Gonzalez et al. also studied an *At. ferrooxidans* strain growing in an industrially relevant biomining community that was extracted from a bioreactor present at the Biotechnology Center of the Universidad Católica del Norte in Antofogasta, Chile. The community was grown on pyrite coupons and exposed to the C14-AHL at 5 µM. *At. ferrooxidans* was highlighted using a fluorescent probe specific to its 16s rRNA gene and visualized using fluorescence microscopy. Consistent with the results found using pure cultures of *At. ferrooxidans,* the presence of C14-AHL increased adhesion of the *At. ferrooxidans* cells to pyrite. Two other members of the biomining community were assessed for behavioural changes in the presence of C14-AHL using qPCR. *At. thiooxidans* was not found to exhibit altered behaviour in the presence of the C14-AHL molecule, but *L. ferriphilum* was found to have a ten-fold reduction in planktonic cells suggesting that *L. ferriphilum* had increased its adherence to the pyrite substrate by ten-fold in response to C14-AHL. This experiment is particularly relevant as it studied an *At. ferrooxidans* species present in a true biomining community, as opposed to a pure or binary culture. The finding that *L. ferriphilum* enhanced its attachment to pyrite is the first report of multiple different species of biomining bacteria simultaneously responding to an exogenously added AHL while present in an industrially relevant biomining community.

In short, González et al. (2013) showed the modulation of *At. ferrooxidans* attachment to pyrite or sulfur using various AHLs at concentrations of 5 µM, primarily these effects were mediated by the hydroxylated or unsubstituted forms of the C12 and C14 AHLs, rather than the oxo-AHLs. Pure cultures of *At. ferrooxidans* ATCC 23270 were used as well as a strain growing in a commercially relevant biomining culture. In pure culture experiments, several long-chain AHLs and AHL mixtures were able to modulate the attachment of *A. ferrooxidans* to sulfur or pyrite coupons, these results were summarized in Table 2. The strain of *At. ferrooxidans* present in the industrial biomining community responded to doses of 5µM of unsubstituted C14-AHL, increasing its attachment to pyrite. *L. ferriphilum* also appeared to increase its attachment to pyrite when exposed to the single C14-AHL molecule, increasing its attachment to pyrite ten-fold in the presence of the C14-AHL treatment. Thus, the work of González et al. (2013) clearly indicates that the attachment of biomining bacteria to pyrite or sulfur substrates can be increased using the addition of exogenous synthetic quorum sensing AHL molecules. Specifically, the bacteria *At. ferrooxidans* and *L. ferriphilum* were encouraged to attach to pyrite and sulfur substrates using various individual C12 and C14 AHLs, as well as a mixture C14-AHLs. This finding sets the stage for future research regarding the enhancement of biomining processes using QS autoinducers and suggests the biomining bacteria *At. ferrooxidans* and *L. ferriphilum* as primary candidates for manipulation using long-chain AHLs.

The molecular response of *At. ferrooxidans* to QS AHLs was elucidated by Mamani et al (2016) who used bioinformatics and transcriptomics in conjunction with an AHL analog to identify the genes involved in QS response (24). Using DNA microarray experiments a total of 141 genes (approximately 4.5% of the *At. ferrooxidans* genome) from planktonic cells were found to respond to the AHL analog tetrazole 9c. Of the 141 genes found to respond to QS, 42.5% (60 genes) were also found to be related to biofilm formation. In sessile *At. ferrooxidans* cells, however, only four genes with known functions were found to respond to the AHL analog. Notably, the most upregulated gene was *afeI*, known to encode an AHL synthase. Other genes were found to respond to the AHL analog even though they do

not possess the canonical AfeR binding site, indicating that there may be undiscovered transcriptional regulators that are involved in *At. ferrooxidans* QS response.

Mamani et al. (2016) also found that the AHL analog was more effective at stimulating biofilm formation at 3 days than the natural 3-hydroxy-C14-AHL. It was shown that tetrazole 9c could initiate biofilm formation 2 days earlier than untreated cells (3 days as opposed to 5), and lead to the production of many different AHLs, some of which have been shown to be detected by secondary colonizers like *At. thiooxidans* (Bellenberg et al., 2014b; Mamani et al., 2016). This study exemplifies the potential for behaviour modification of *At. ferrooxidans* using AHL QS manipulation and provides insight as to which biomolecular pathways are involved in the QS response for this bacterium.

Building upon the research produced by other experts in the field; Chabert et al. (24) (applied the mixture of 3-oxo-C14-AHL, 3-hydroxy-C14-AHL, and unsubstituted C14-AHL to improve the design of microbial fuel cells colonized with *At. ferrooxidans* ATCC 23270 grown on carbon felt electrodes. Microbial fuel cells convert chemical energy into electricity and are catalyzed by the extracellular transfer of electrons by a bacterium, in this case *At. ferrooxidans*. The bacterium was cultured using either reduced sulfur or ferrous iron as an energy source. The C14-MIX treatment was the same as that previously described and used by Bellenberg et al, (6) and Gonzalez et al (22). The authors compared biofilms grown with and without additional C14-MIX, showing that electrode biofilm coverage was improved by the addition of a C14-MIX treatment used at 5µM. An increase in current intensity confirmed that electron transfer can be improved by the addition of exogenous synthetic C14-AHLs. The authors also suggest that QS may initiate changes in the metabolism of *A. ferrooxidans,* which may also have contributed to increased current intensities. This study adds to the body of knowledge suggesting the effectiveness of modulating the colony and biofilm formation of *At. ferrooxidans* using 5µM doses of C14-AHL mixtures.

1.5 State of Research & the Solvent Dilemma

The manipulation of QS in biomining microbes has primarily utilized the C14-AHL molecule and its keto and hydroxyl derivatives, 3-oxo-C14-AHL and 3-hydroxy-C14-AHL. Mixtures of these three molecules have been applied to *At. ferrooxidans* at 5 µM concentrations in several studies, consistently showing the ability of this AHL mixture to improve *At. ferrooxidans* attachment to, and biofilm colonization of, substrates including pyrite and elemental sulfur. This finding is supported by data from Mamani et al (2016) who provide evidence that the AI-1 QS system can regulate 141 genes in *At. ferrooxidans,* 60 of which are related to biofilm formation (24). Other notable biomining species have been shown to respond to the C14-AHL molecule and a mixture of C14-AHLs. The single C14-AHL molecule improved the attachment of *L. ferriphilum* to pyrite, while a C14-AHL mixture was able to improve the pyrite leaching ability of *L. ferrooxidans* in pure and binary cultures (6, 22, 23).

By improving attachment to minerals, as well as enhancing the production of EPS and biofilm formation, exogenously added AHLs provide a promising means of improving the kinetics of bacterially mediated iron-sulfide mineral leaching. In some cases AHLs have been successfully used to improve the bioleaching of pyrite, but few studies have replicated this phenomenon in diverse cultures of biomining bacteria that more accurately represent industrial biomining communities (6). Studies have thus far almost exclusively focused on pure and binary cultures of biomining bacteria that are grown on substrates limited to pyrite or elemental sulfur and have not utilized other iron-sulfide minerals. In

addition to applying AHLs to diverse communities of biomining bacteria, future studies need to verify the applicability of this biotechnology to new mineral substrates that are more representative of the complex ores and mineral concentrates that are used in mineral processing facilities.

A complicating factor for this field of research is the limited solubility of AHLs in water and aqueous solutions (20)**.** AHLs are sparingly soluble in water, as such, working solutions of AHLs are typically generated in organic solvents such as dimethyl sulfoxide (DMSO), ethyl acetate, or dimethyl formamide (22, 29). Other solvents can include alcohols or aqueous buffers, although alcohol solvents are not recommended as they are likely to open the lactone ring, destroying the AHL molecule (30). Aqueous buffers are also unsuitable as they do not dissolve hydroxylated AHLs (31). The most common solvent used in bioleaching studies is DMSO(6, 22–24). DMSO has universal solvent properties and is commonly used to dissolve hydrophobic crystals for microbiological assays (29). DMSO is often used in bioleaching studies to generate standard solutions of dissolved AHLs, which are then added to live cultures of bacteria (22, 24).

The use of DMSO as a solvent for C14-AHLs results in significant concentrations of DMSO being added to the bioleaching culture. For instance, Gonzalez et al (2013) report using a 50µM solution of C14-AHLs dissolved in DMSO (22). This 50µM standard solution is diluted ten-fold to reach the desired final concentration of 5µM C14-AHLs in their bioleaching experiments. This implies that 10% of the final solution volume for the bioleaching cultures consists of DMSO. This method is referenced repeatedly in the literature on this topic, even though it is unclear what effect such concentrations of DMSO have on the microorganisms used in bioleaching studies of this nature (6, 21, 23, 24). DMSO has previously been shown to inhibit microbial growth for long term incubations (>24 hours) of other bacteria at similar concentrations (29, 32). Data presented in the present study suggests that very low doses of DMSO inhibit bioleaching consortia, which could complicate the pursuit of an enhanced bioleaching system (Figure 3) and calls into question some of the established methods used for this type of research. A number of experiments performed for the purposes of this study were unsuccessful due to microbial inhibition due to contact with the organic solvent DMSO or other organic solvents. To avoid the inhibition of the microorganisms responsible for bioleaching activities a novel method of delivering C14-AHLs to bioleaching cultures was developed and used for this project.

Successful cultures of bioleaching acidophiles can be monitored by measuring the oxidation/reduction potential (ORP) of the bulk solution. Oxidation/reduction potential can be considered a measurement of a solutions ability to oxidize chemical species. Referenced against the Standard Hydrogen Electrode (SHE), which has been arbitrarily assigned an electrode potential of zero volts, ORP is measured using volts or millivolts. It is not feasible to use the SHE as a laboratory tool however, and ORP values are measured using more robust electrodes. The internal reference electrode of the ORP probe becomes the baseline of measurement, rather than the SHE.

Ferric iron is a powerful oxidizing agent, but typically has limited solubility at circumneutral pH. However, at extremely acidic pHs (below pH of ~2.5) the solubility of ferric iron is increased dramatically and the redox couple of ferric iron and ferrous iron can become a key determinate in the ORP of iron containing solutions. In the bioleaching solutions generated for this study, ORP is chiefly determined by the ratio of ferric iron to ferrous iron (33). Successfully inoculated bioleaching cultures will rapidly climb in ORP over the course of the first few weeks as the acidophilic chemoautotrophs regenerate ferric iron

and facilitate the reaction of ferric iron with mineral-bound ferrous iron. Unsuccessful or otherwise inhibited bioleaching cultures will quickly plateau in ORP value.

Thus, ORP can be a convenient tool for measuring the success of bioleaching cultures. Successful cultures will rapidly produce ferric iron, which is a powerful oxidant and will subsequently raise the ORP of the bioleaching system. Bioleaching cultures that are inhibited will plateau in ORP in the first week or two of experimentation, and this plateau can be indication that the experiment aught to be terminated, as the unsuccessful cultures may produce unusable data.

The project presented here attempts to validate the potential for quorum sensing molecules to improve the recovery of valuable metals from an iron-sulfide ore in a bioleaching environment. An iron-sulfide ore was bioleached by a commercially relevant culture of bioleaching bacteria, with and without the addition of AHL autoinducers. C14-AHL molecules were selected based on previous studies that have established their effectiveness at improving the colonization of iron-sulfide minerals by canonical bioleaching bacteria (6, 22, 23). The AHLs are delivered to the bioleaching consortia using a novel method of ore pre-treatment to avoid microbial inhibition by contact with a toxic inorganic solvent. The nickel bearing iron-sulfide ore and bacterial community structure to be used have previously been characterized by CanmetMINING, Natural Resources Canada (1, 34). The bacterial community structure as determined in Cameron et al. (2010) consists largely of multiple strains of *Acidithiobacillus ferrooxidans,* an *Acidiphilium* species, and an unknown mine water bacterium that is closely related to *Acidithiobacillus* (35).

2- Objectives and hypotheses

The primary objective of this study was use QS signals to improve the rate at which a biomining reactor could extract base metals from an iron-sulfide ore. As previously described, other research groups have had success in modulating the attachment and leaching behaviours of bioleaching microbes, chiefly *Acidithiobacillus ferrooxidans,* using exogenously added C14-AHLs. This leads to the hypothesis that C14-AHLs can be added to bioleaching reactors to improve the metal leaching efficiency of the reactor. If C14-AHLs can modulate the bacterial behaviours that govern bioleaching efficiency, then the addition of C14-AHLs to a bioleaching reactor should improve metal leaching at specific timepoints as compared to controls.

Methods of delivery for C14-AHLs described in the literature were found to be insufficient for the task at hand. The solvent DMSO is widely used to dissolve AHL molecules but established methods of delivery indicate that large quantities of DMSO are delivered to the final bioleaching reactor during experimentation, a situation deemed unacceptable for this project as even small volumes of DMSO are capable of inhibiting bioleaching reactor efficiency. Included in this report is indicating that low quantities of DMSO can inhibit the activity of bioleaching cultures. To achieve the primary objective of enhancing bioleaching efficiency, it was necessary to develop a novel method of delivery for AHLs into a bioleaching culture that would prevent unintentional inhibition of the bioleaching culture due to contact with organic solvents. To this end a novel process of C14-AHL delivery was devised utilizing the solvent ethyl acetate to dissolve and deliver the AHL molecules with subsequent removal of the ethyl acetate solvent “vehicle” via evaporation in a convection oven.

Two different AHL treatments were applied to bioleaching reactors throughout the course of the project. The single molecule N-tetradecanoyl-L-homoserine lactone (C14-AHL) was tested at a concentration of 5µM, and a mixture of the three different C14-AHLs was also tested. The mixture is known as C14-MIX, and consisted of equal parts C14-AHL, 3-oxo-C14-AHL, and 3-hydroxy-C14-AHL. The C14-MIX was delivered at a total concentration of 5µM. It was hypothesized that the addition of these C14 treatments to bioleaching reactors would result in increased leaching of the nickel bearing iron-sulfide ore as compared to controls.

3- Methods

3.1 Nickel Bearing Iron-Sulfide Ore

The ore used for this project was a nickel bearing iron-sulfide ore that contained ~0.8% nickel by mass, with 83% of the nickel reporting to **pentlandite**, **$(Fe, Ni)_9S_8$**, and 16% reporting to **pyrrhotite**, **$Fe_{(1-x)}S$**. Pyrite accounted for less than one percent of the ore's mass, and less than one percent of the nickel present in the ore was present in pyrite. The ore was previously characterized in great detail via electron probe x-ray microanalysis (EPMA) and further details regarding "Ore 1" are available in (1). Tables 3-5 show the results of Electron Probe X-Ray Microanalysis and Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) for the ore. The ore was crushed using a jaw-crusher to a particle size such that 80% of the material would pass through a sieve with pore sizes of 147µM, also known as a 100 Tyler mesh.

Table 3: The mineralogical composition of the nickel bearing iron-sulfide ore used throughout this project, adapted from (1)***.***

Mineral or mineral group	**Mass in %**
Amphibole/pyroxene	19.6
Carbonates (calcite, dolomite, ankerite)	0.1
Chalcopyrite	0.7
Chlorite	1.7
Oxides (magnetite, hematite, ilmenite, chromite)	9.0
Feldspars	23.2
Pentlandite	3.0
Pyrrhotite	33
Pyrite	0.1
Quartz	7.0
Talc	0.5
Others (apatite, danalite, epidote, mica, titanite)	2.1

Table 4: Chemical composition of the primary nickel-bearing phases and distribution of nickel adapted from (1)***.***

Mineral	**Mass in % ± Standard Deviation**
Pentlandite	36.3 ± 0.7
Pyrrhotite	0.7 ± 0.1
Pyrite	~0
Proportion of nickel reporting to pentlandite (%)	83
Proportion of nickel reporting to pyrrhotite (%)	16
Proportion of nickel reporting to pyrite (%)	~0

Table 5: Chemical composition of the nickel sulphide ores, adapted from (1)***.***

Element	**Mass in % ± Standard Deviation**
Nickel	0.79 ± 0.02
Magnesium	2.74 ± 0.02
Copper	0.224 ± 0.003
Cobalt	0.0274 ± 0.0001
Iron	14.0 ± 0.2

3.2 Bioleaching Maintenance Culture Use and Preparation

The culture used in this study was initially derived from water and soil samples collected from mining related locations in Sudbury, Ontario during a visit in October 2006 (34). These samples were enriched using a media designed to culture iron-oxidizing microbes, and a media designed to culture sulfur-oxidizing microbes. After the enrichment in 2006 the culture was introduced to a low-grade nickel bearing iron-sulfide ore, at that time the culture contained *Acidthiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospirillum ferriphilum,* and at least one *Acidiphilium* species, among others (36). The bioleaching consortium has been serially subcultured since 2006 and was conditioned to the ore used in this study starting in May 2018.

The most recent phylogenetic analysis was performed in 2010 and indicated that 30°C cultures were largely dominated by a strain of *Acidithiobacillus ferrooxidans (DQ529309)*, and an uncultured mine water bacterium (DQ469216) closely related to a strain of *Acidithiobacillus thiooxidans* (99% bootstrap value) (34, 35). The 30°C cultures also hosted a species very closely related to a strain of

Leptospirillum ferriphilum (100% bootstrap value) as well as what appears to be a species of *Acidiphilium (100% bootstrap value).*

Maintenance cultures were retained and serially subculturing for the duration of experimentation which began in May of 2018 and ended in March of 2020. Subcultures were created every two weeks, with pH adjustment performed daily to within +/- 0.05 of pH 2. pH. pH adjustments were made by adding dropwise either a sulfuric acid solution or a sodium bicarbonate solution. Five grams of ore was included as an energy source and growth media was conditioned for 24 hours prior to inoculation to mitigate pH fluctuations that might inhibit bacterial growth. 1mL of slurry from a 14-day old maintenance cultures was inoculated into conditioned media via 1mL micropipette. Previous enumerations of viable bacterial cells using the Most Probable Number (MPN) technique estimate that a 14 day culture of this nature would be in logarithmic growth phase and contain between $1x10^8$ iron oxidizing cells/mL and roughly $1x10^9$ sulfur oxidizing cells/mL (34). ORP measurements were made using a WTW Multiline P4 handheld meter and referenced against the Silver-Silver Chloride reference electrode.

3.3 Experimental Design & Assembly of Shake-Flask Bioreactors

The primary experimental design used shake-flask bioreactors to assess the ability of various treatment additives to improve nickel extraction via bioleaching at selected time points. Triplicate reactors were used for treatment and control groups. Shake-flask assembly was performed as follows: Reactors were built in 250 mL Erlenmeyer flasks. Five grams of dry ore were added to the bottom of each dry flask, and AHL solutions were applied to the dry ore by micropipette. Flasks were then dried in an oven set to 40°C for 16 hours to evaporate the solvent vehicle. After evaporation of the ethyl acetate, 98 mL of a media modified from Tuovenin & Kelly (1973) was added to each flask (37). The media, known as mTK media, contained 0.5g/L $(NH_4)_2SO_4$, 0.5g/L KH_2PO_4, and 0.5g/L $MgSO_4*7H_2O$. The growth medium was acidified using 1mL of 10% sulfuric acid in order to meet the immediate acid demand of the 5 grams of ore inside the flask. Flasks were then conditioned at the desired experimental temperature for 24 hours to minimize pH fluctuations that might inhibit bacterial growth. After 24 hours the flasks were adjusted to within +/- 0.05 of pH 2. After pH adjustments were made in the morning, two or three hours were allowed for the shake flasks to adjust to the change, and then the first ORP measurements & 5mL samples were taken for liquid analysis by (ICP-AES). Flasks were then replenished with 5mL of growth media that had been warmed to room temperature, flasks were replenished by weight to within 0.1g. After the first liquid sample had been extracted and the media replenished, the live cultures of bacteria were added from a maintenance culture that was in logarithmic growth phase using a 1mL micropipette.

3.4 Application and Generation of Acylhomoserine Lactone Standard Solution

Standard solutions of AHLs were distributed over 5g of dry ore in a 250mL Erlenmeyer flask using a micropipette to apply a dose of 5 µM of AHL. After the AHL solution was applied, the solvent was driven off via evaporation in a convection oven set to 40°C for 16 hours overnight. Control flasks were treated in the same manner but dosed with an equivalent volume of ethyl acetate solvent instead of AHL solution. After evaporating for 16 hours growth media was added to the ore. See calculation 1 in the appendix for an example of this treatment.

The 3-Hydroxy-C14-AHL used in this study is not sufficiently water soluble to create a water-based standard solution. The solvents dimethyl-sulfoxide (DMSO) and diethyl ether were found to be unsuitable due to their tendencies to inhibit bacterial growth or not completely dissolve the AHL, respectively. The solvent ethyl acetate was found to be effective at dissolving 3-hydroxy-C14-AHL and could be removed via evaporation to limit its negative impact on the bacterial cultures.

The solubility of 3-hydroxy-C14-AHL in ethyl acetate was determined empirically in-lab, and it was found that 1.8 mg of 3-hydroxy-C14-AHL dissolved in a minimum volume of 5mL of ethyl acetate, suggesting that 3-hydroxy-C14-AHL has solubility of roughly 0.36 g/L. See calculation 2 in the appendix for an example of this method.

Small masses of AHL crystals (~2 micrograms) were weighed into a microcentrifuge tube using an analytical balance, and subsequently dissolved using 5mL of ethyl acetate. In this manner standard solutions of the three AHLs were generated in ethyl acetate. Standard solutions of the three different AHLs were combined to create the C14-MIX standard solution. Standard solutions were then applied to the dry flasks as described above. See calculation 3 in the appendix for an example of this method.

3.5 Sampling of Bioleaching Shake Flasks

Liquid samples of 5mL were drawn using a 5mL micropipette, filtered using a 0.22µm syringe filter, and deposited into 10mL glass scintillation vials. Samples were preserved by acidification with 4 drops of concentrated nitric acid delivered by dropping vertically with a glass Pasteur pipette. Samples were refrigerated until an entire experiment could be analyzed simultaneously by ICP-MS performed by the NRCan Analytical Services Group. Liquid levels within the reactor vessels were replenished using 5mL of mTK media which had been warmed to room temperature as to not thermally shock the bacterial culture.

3.6 DMSO Toxicity Experiment

The ability of DMSO to inhibit the bioleaching culture used for this project was assessed using bioleaching flasks that were incubated with varying concentrations of DMSO in an experiment termed the “DMSO toxicity experiment”. The concentrations of DMSO tested were 1.0%, 0.5%, 0.1% and 0.05% and were all much lower than those used by QS researchers Gonzalez et al (2013) (22). Bacterial cultures containing DMSO were compared to two different controls which both contained zero percent DMSO. One control was inoculated with the bacterial culture, while the other was not; these flasks were known as “abiotic” and “control”, respectively.

The DMSO toxicity experiment was performed between April 10th and May 2nd, 2019 at the Center for Advanced Research in Environmental Genomics on the University of Ottawa campus. In contrast to other experiments reported here, the DMSO toxicity test was performed using duplicate flasks, rather than triplicate. In addition, the flasks were incubated at 37°C rather than 30°C. These modifications were made due to logistical constraints resulting from the conducting of this experiment at University of Ottawa facilities, rather than the laboratories at CanmetMINING. Furthermore, samples from this experiment were analyzed by the Geochemistry Laboratories in the Advanced Research Complex on the University of Ottawa campus rather than the Analytical Services Group at CanmetMINING.

Bioleaching flasks were constructed using 250mL Erlenmeyer flasks. Five grams of ore were used to provide energy for the bioleaching microorganisms, 1mL, 0.5mL, 0.1mL or 0.05mL of DMSO was added to make up the required concentration and mTK media was added to each flask to make up a total volume of 98mL (37). The growth medium was acidified using 1mL of 10% sulfuric acid to meet the immediate acid demand of the of ore inside the flask. Flasks were then conditioned at the desired experimental temperature for 24 hours to minimize pH fluctuations that might inhibit bacterial growth. After 24 hours the flasks were adjusted to within +/- 0.05 of pH 2. After pH adjustments were made in the morning, two or three hours were allowed for the shake flasks to adjust to the change and then the first ORP measurements & 5mL samples were taken for subsequent liquid analysis by ICP-AES. Flasks were then replenished with 5mL of growth media that had been warmed to room temperature, flasks were replenished by weight to within 0.1g. After the first liquid sample had been extracted and the media replenished, the 1mL of live bacteria were added from a maintenance culture that was in logarithmic growth phase using a 1mL micropipette. Samples were collected from culture flasks six times over the course of 23 days.

3.7 Statistical Analysis

Statistical analysis of ICP-AES and ORP data was performed using the one-way analysis of variance (ANOVA) function available in Minitab 14. An 80% confidence interval was deemed appropriate as the biological nature of the experiments incurred a lot of inherent variance and relatively few replicates were created. The study seeks to explore potential manipulations in mineral leaching where even small improvements in metal acquisition could potentially scale up to enormous improvements for the bottom line. An 80% confidence interval is appropriate for preliminary investigations where the expected deviations might be minimal, and due to the relatively low number of replicates, relevant results might otherwise be dismissed using a higher confidence interval. Data points shown in graphs represent average values of all replicates performed, and metal extraction curves have been modified by appropriate dilution factors to account for small deviations from the intended 100mL volume.

Given the 80% confidence interval used for this project, a p value of less than 0.200 for an ANOVA test means that the null hypothesis, that the mean values are not different, is rejected and the alternate hypothesis, that the mean values are different, is accepted. A p value of less than 0.200 is indicative of a statistically significant effect that is not caused by the inherent variance of the bioleaching systems.

4- Results & Discussion

4.1 Project Summary

Bioleaching systems harness the activity of chemolithoautotrophic microorganisms to liberate metal from rock, further refining is used to turn the metal into a marketable product. The addition of quorum sensing autoinducers to bioleaching systems has previously been shown to improve mineral colonization by bioleaching cultures and, in some cases, improve the leaching efficiency of the bioleaching culture (6, 13, 22, 23). Previous research on this topic has suffered from a limited diversity of substrates on which bioleaching cultures were grown, typically utilizing either chips of pure pyrite or coupons of pure S^0. Additionally, few studies have addressed the viability of quorum sensing manipulations in cultures of diverse microorganisms, instead focusing on single and binary bacterial

strains. Commercial bioleaching environments typically use a complex ore comprised of many minerals and a diverse culture of bioleaching microorganisms representing far more than two species of bacteria (38, 39). Thus, while the field of research until now has thoroughly established the ability to use quorum sensing to manipulate few bacteria on simple substrates, it is still unclear if the same can be done for diverse cultures of microorganisms with ores of a more complex nature. Therefore, this project's primary objective was to determine if quorum sensing manipulation could be used to improve the efficiency of an industrially relevant bioleaching system that utilized both a diverse bioleaching culture, and an ore of realistic composition.

A well characterized ore and an established bioleaching culture were used in conjunction with QS AHLs acquired from Sigma-Adritch to perform a series of bioleaching experiments designed to improve bioleaching metal recovery via the exogenous addition of quorum sensing signals (34, 36). Bioleaching shake-flask reactors were constructed on a small scale in 250mL Erlenmeyer flasks. Shake flasks were treated with the quorum sensing signal C14-AHL and its derivatives using a novel method designed to alleviate microbial inhibition from organic solvent toxicity. Shake flasks were maintained at constant pH for three to four weeks and liquid samples were extracted for analysis by ICP-AES at six or seven points throughout the experiment. Two experimental conditions were tested: the treatment of cultures with 5µM of unsubstituted C14-AHL, and the treatment of cultures with a mixture of 3-oxo-C14-AHL, 3-hydroxy-C14-AHL and unsubstituted C14-AHL (C14-MIX) at a total concentration of 5µM.

Additionally, a simple experiment was carried out to display the potential for organic solvents to inhibit the activity of the bioleaching culture. The solvent DMSO was widely used in the literature for the delivery of AHLs to bioleaching cultures, but the referenced methods suggested an unacceptably high amount of the organic solvent was being delivered to the final bioleaching solution (22). Since the inhibition of the bioleaching consortium could have masked any metal leaching benefit provided by the QS AHL treatment it was determined that an alternative method for the delivery of AHLs needed to be devised. Included in this report is the evidence that small volumes of DMSO can have a profound impact on the metal leaching efficiency of bioleaching cultures. An alternative method for the delivery of AHLs to bioleaching cultures is proposed that does not necessitate the addition of inhibitory organic solvents directly to the microbial culture.

4.2 DMSO Toxicity Experiment

The established methods for delivering C14-AHLs to microbial cultures result in up to 10% of the working volume of solution being comprised of DMSO (22). It was suspected that this would result in inhibitions of bioleaching activity by the bioleaching cosortia, as microbial inhibition by DMSO has been well documented in the past (29, 32, 40). A leaching experiment using treatments with various concentrations of DMSO was performed in order to confirm the suspicion that DMSO would inhibit the bioleaching activities of the microbial cultures used for this project, this was known as the DMSO toxicity experiment.

The DMSO toxicity experiment was performed to determine the extent to which small volumes of DMSO could inhibit the bioleaching ability of the culture used for this project. Six different experimental conditions were constructed by dosing microbial cultures with varying concentrations of DMSO and were respectively known as: 1.0 % DMSO, 0.5% DMSO, 0.1% DMSO, 0.05% DMSO, abiotic, and control. Figure 3 displays the concentration of nickel in solution over the course of the experiment.

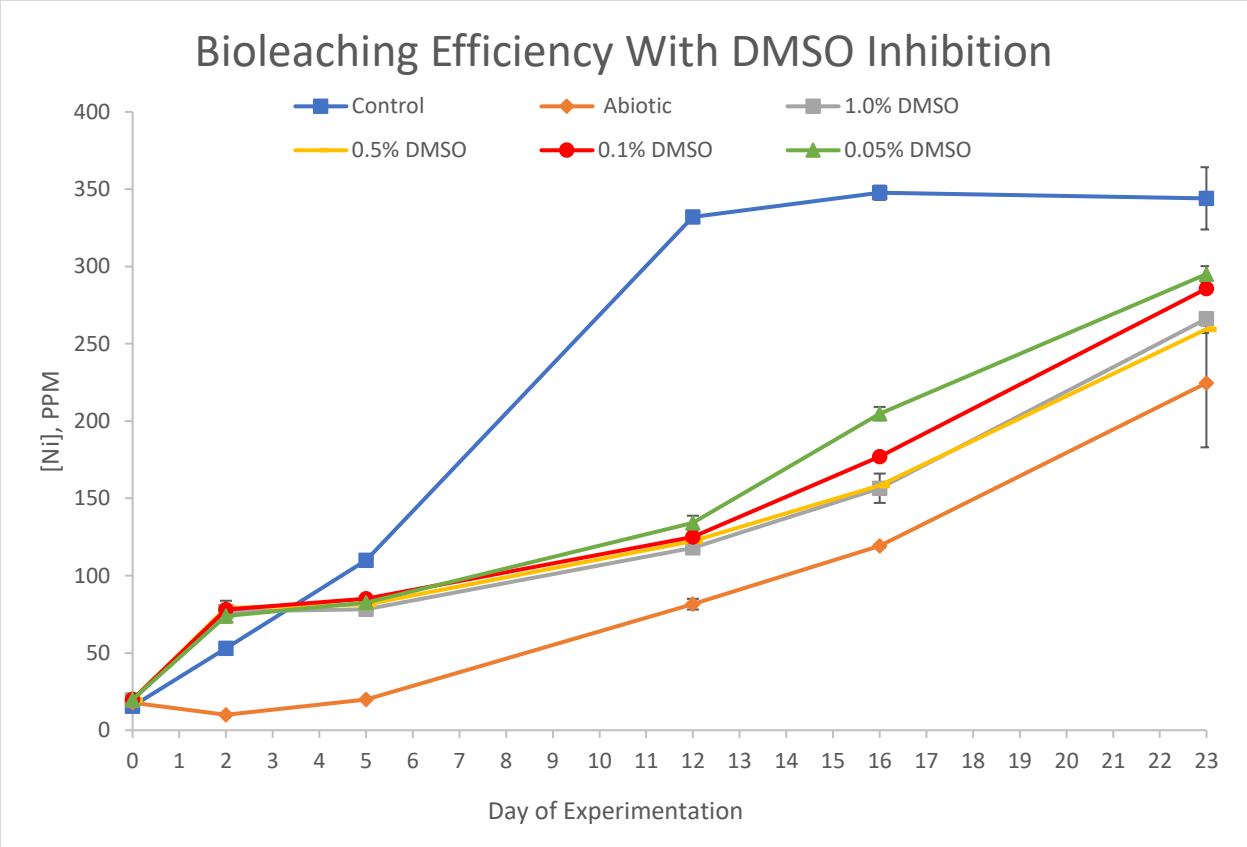

Figure 3: Nickel leaching data from DMSO toxicity experiment. Six different leaching conditions were constructed. Five flasks were inoculated with bioleaching microorganisms, and one was not (orange diamonds). Four bioleaching flasks were inhibited with various concentrations of DMSO. Blue squares represent a bioleaching culture with no added DMSO, orange diamonds represent an uninoculated leaching flask with no added DMSO, grey squares represent a bioleaching flask with 1.0% DMSO, the yellow line represents a bioleaching flask with 0.5% DMSO, red circles represent a bioleaching flask with 0.1% DMSO, and green triangles represent a bioleaching flask with 0.05% DMSO. Data points represent the average of two replicates. Error bars indicate one standard deviation.

Early on during the experiment, on day 2, the flasks treated with DMSO leached significantly more nickel than the control flask or the abiotic flask, while the abiotic flask leached much less nickel than any other flask. For the remainder of the experiment the control flask was the most effective at leaching nickel, while the abiotic flask was the least effective. The control flask reached nickel in solution value of 347 ppm by day 12, which corresponds to about 88% of the nickel available in the reactor (see calculation 4 in the appendix for details). At that time the control flask values for nickel in solution plateaued significantly, as it appeared to leach almost all the available nickel. During the experiment each DMSO containing flask behaved similarly to each other, but a significant difference between the 0.05% (green triangles) DMSO flask and the 1.0% DMSO (grey stars) flask did arise on days 12 and 16.

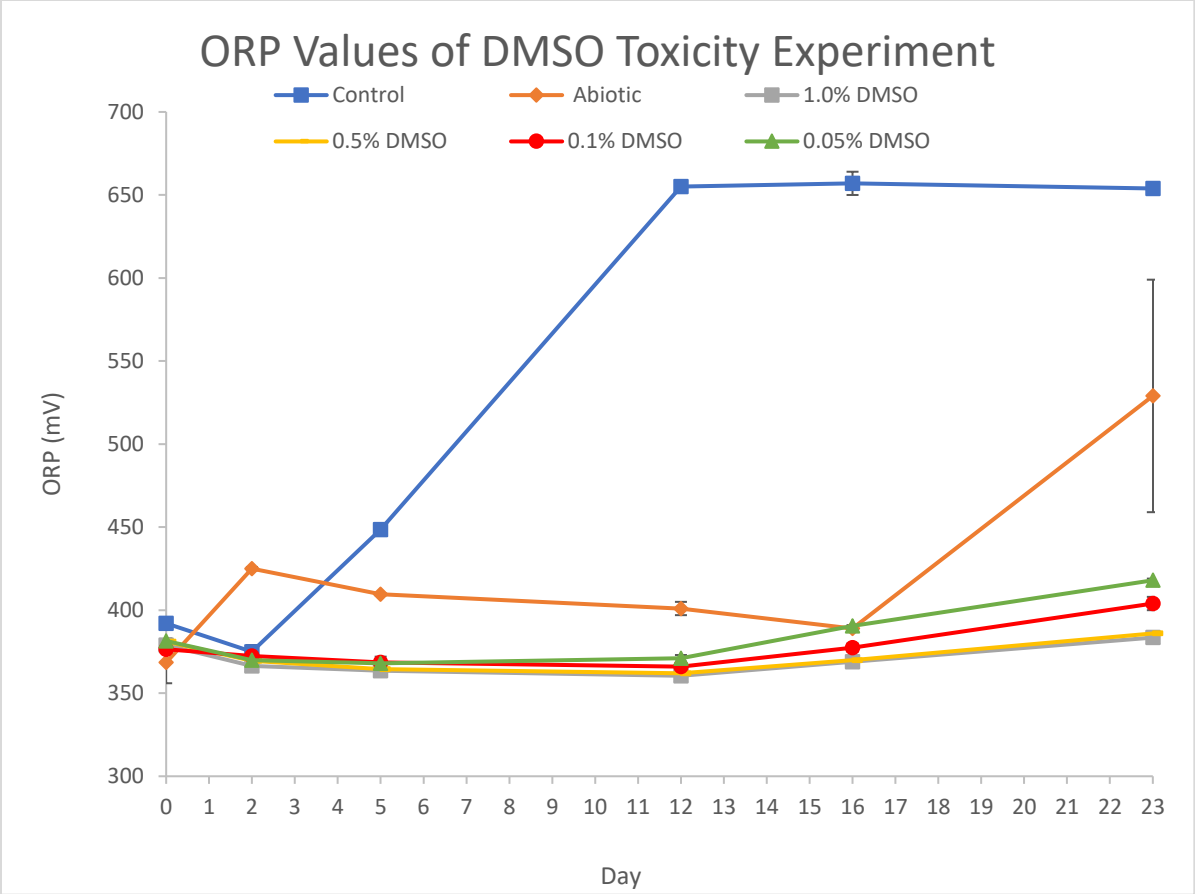

Figure 4: The average ORP values obtained during the DMSO Toxicity Experiment. Error bars represent one standard deviation.

For the majority of the experiment the control group flasks had the highest ORP (Figure 4, appendix 8.2.1.2). The abiotic flask had a higher ORP than the DMSO containing flasks for all days except day 16. For all times measured after day 2 the DMSO containing flasks were significantly lower in ORP than the control flask. On days 12 and 16 the flasks with the lowest concentration of DMSO (0.05%) had a significantly higher ORP than the 1% and 0.5% DMSO flasks.

For the majority of the DMSO toxicity experiment bioleaching cultures treated with any concentration of DMSO were less effective at leaching nickel than the untreated control flasks. The exception to this is early in the experiment on day 2, where each DMSO containing flask leached more nickel than the control flask (Figure 3). It is unclear why this improvement in nickel leaching occurred, however the phenomenon did not persist for long and disappeared by the next measurement. By the end of the experiment the DMSO inhibited flasks were approaching the control flask values for nickel in solution but this is largely due to the fact that the control flask had plateaued in its nickel leaching, having leached almost all of the available nickel by day 12. The DMSO containing flasks never reached the value of nickel in solution that the control flask had reached as soon as day 12. In short, volumes of DMSO as low as 0.05% were potent inhibitors of microbial bioleaching.

Early in the experiment (Figure 3, orange line) the abiotic flask had significantly depressed bioleaching behaviour, but by the end of the experiment (day 23) there was no significant difference between the abiotic flask and the DMSO treated flasks. The data garnered from the abiotic treatment group shows the difference in leaching behaviour between bioleaching flasks that are inoculated with a bioleaching culture, and those that are not. Inoculated flasks leach much more nickel than those flasks that are not inoculated with bioleaching microbes, especially in the first two weeks of the process.

The DMSO toxicity experiment shows quite clearly the potential for organic solvents to inhibit the activity of bioleaching microorganisms, while also showing the important role of the microbial inoculum in the initiation of leaching. Bioleaching cultures treated with doses of DMSO as low as 0.05% were less than 50% as effective as untreated controls. Interestingly, a slight dose-response phenomenon was exposed, as flasks containing 0.05% DMSO leached significantly more nickel than flasks containing 1% DMSO when measured during the experiment, indicating that the inhibition in nickel leaching ability of the biological constoria was proportional to the amount of DMSO included in the culture. Future studies could explore more closely the dose-response curve of bioleaching consortia exposed to DMSO or other organic solvents.

Other researchers have addressed the issue of organic solvent toxicity by including equal amounts of organic solvent in their treatment and control groups(6, 13, 22, 23). This practice serves to ensure that all treatment groups are equally inhibited, but fails to provide the bioleaching cultures with an ideal growth environment, and could potentially mask improvements in bioleaching that would otherwise be conveyed by the QS treatments. Previous studies have shown that DMSO can be a potent microbial inhibitor when used for long-term assays of 22 hours or more (29). Microbial inhibion by DMSO likely has its origins in the increased membrane fluidity caused by the incorporation of DMSO into the cellular membrane, or by the generation of reactive oxygen species (40). Future work with QS in bioleaching environments would be served well by avoiding the use of DMSO as a solvent for delivery of chemical treatments.

4.3 Ethyl Acetate Ore Pre-Treatment

The small volumes of DMSO used in this experiment ranged from 0.05% to 1% and any volume of DMSO tested massively inhibited the ability of bioleaching cultures to extract nickel (Figure 3). The volumes of DMSO used in this experiment were many times lower than those reportedly used by Gonzalez et al (2013) in their studies of QS in bioleaching, a study which is repeatedly referenced in related literature, calling into question some of the established methods for the use of QS AHLs in bioleaching research (22–24). For this reason the method of ore pre-treatment with ethyl acetate was developed and used to deliver the C14-AHL treatments. Ore pre-treatment with ethyl acetate was found not to significantly inhibit the bioleaching behaviours of the microbial cultures when used in either the C14-AHL experiment or the C14-MIX experiment (Figures 6 ad 8, respectively). This led to the conclusion that the delivery of long chain AHLs to microbial bioleaching cultures should be performed using an ore-pretreatment method rather than dosing with an organic solvent vehicle when possible, in order to prevent the inhibition of the bioleaching cultures by contact with organic solvents. Ethyl acetate appeared to be a suitable solvent as it solvates long chain unsubstituted AHLs as well as AHLs with keto and hydroxyl moeities, and ethyl acetate is a volatile solvent that can be removed from experimental conditions using a well ventilated convection oven.

The development of the ore pre-treatment method required a volatile solvent suitable for solvating AHLs and also subsequent removal of the solvent by evaporation. Prior to ethyl acetate, one such solvent used was diethyl ether which was found to be capable of solvating the C14-AHL molecule, as well as the 3-Oxo-C14-AHL molecule, but could not suitably solvate the 3-hydroxy-C14-AHL, which ultimately led to the choice of ethyl acetate as the solvent of choice for ore pre-treatment, rather than diethyl ether.

One experiment performed using the ore pre-treatment method with diethyl ether failed to produce usable results and was terminated early based upon discouraging ORP measurements(Figure 5). Originally the experiment was intended to measure the effect of C14-AHL on the bioleaching ability of the microbial community but unfortunately the diethyl ether vehicle was not fully evaporated before adding mTK medium into the shake flask bioreactor. This mistake lead to significant levels of the solvent coming into contact with the microbial culture, which consequently inhibited the growth and activity of the microbial culture, and ultimately the experiment was terminated prematurely.

Diethyl ether was used to deliver C14-AHLs to three replicate bioleaching flasks. Three other flasks were treated with just diethyl ether and no AHLs, while a final three flasks were not treated, and used as a control. ORP data from this experiment is displayed in Figure 5. Notably the C14-AHL treated group does not rise in ORP over time, a clear indication that the microbial culture is not performing as it should and not producing high concentrations of ferric iron. Normally ferric iron is responsible for raising the ORP of the solution. A low ORP value that is not increasing indicates that ferric iron is not being regenerated by the chemoautotrophic bioleaching culture.

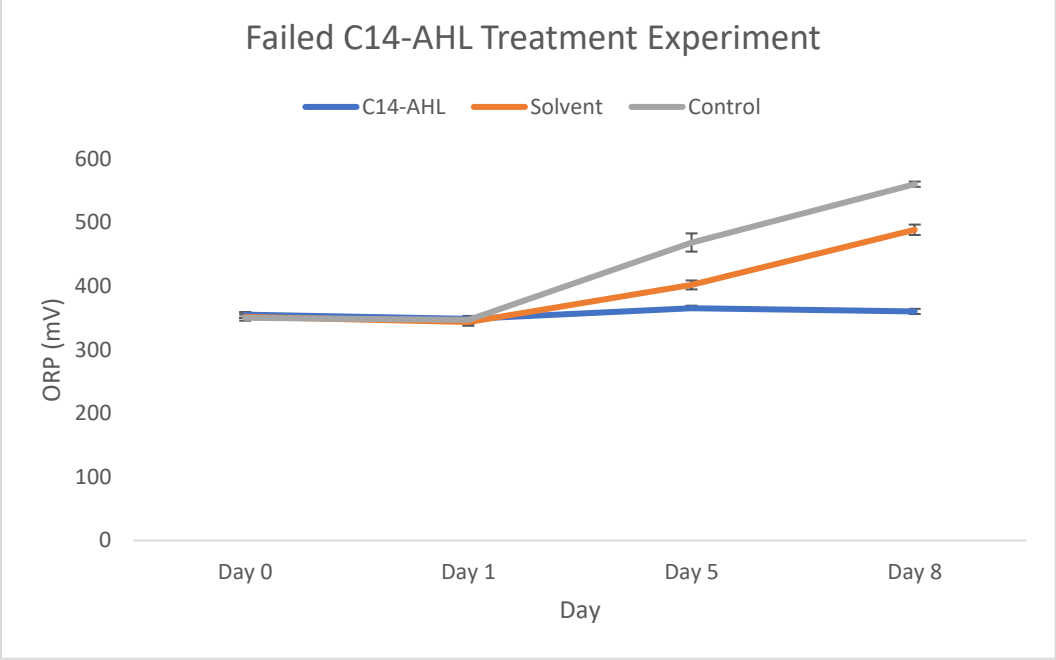

Figure 5: The ORP data from a failed bioleaching experiment where C14-AHL was delivered to the ore via pre-treatment with the solvent diethyl ether.

As indicated by Figures 4, 7 and 9, ORP values of succesful bioleaching cultures climb rapidly beyond 400mV, usually within the first week. The flat blue line at ~350mV that represents the C14-AHL

treatment group clearly indicates that the experiment would not provide useful data. This was due to the diethyl ether solvent not being fully evaporated, which subsequently led to the decision to evaporate the delivery solvent overnight, for 16 hours, rather than the 2 hours provided for this experiment. This and other failed experiments extended the developmental period for the ore pre-treatment method, an unfortunate hinderence for the completion of other aspects of this project.

There is a concern regarding the fate of AHL molecules subjected to prolonged baking in a convection oven set to 40 degrees celsius for 16 hours. The AHL molecules could degrade on exposure to atmospheric air and high temperature. For this reason a positive control was sought out to verify the succesful delivery of the AHL molecules. The bacterium *Rhizobium radiobacter* strain NTL4(pZLR4) was purchased from the American Type Culture Collection (ATCC) to act as a bioreporter for AHL signals. The bacterium produces a blue pigment on contact with AHLs and could be used to confirm the continued viability of the AHL treament after the overnight evaporation. Unfortunately due to constraints imposed during the COVID-19 pandemic the culturing and use of the *Rhizobium rabiobacter* strain was not possible.

4.4 C14-AHL Treatment

The potential for C14-AHL to improve bioleaching efficiency was tested by delivering 5µM of C14-AHL to bioleaching cultures and measuring the nickel leached into solution. The three treatment groups for this experiment are known as: C14-AHL, Solvent Control, and Untreated Control. C14-AHL refers to the flasks treated with 5µM of C14-AHL, Solvent Control refers to flasks treated with ethyl acetate of the same volume as was used to deliver the C14-AHL molecule to the C14-AHL flasks, and Untreated Control was not treated with C14-AHL or ethyl acetate. The results of this experiment are displayed in (Figure 6).

The data points presented in Figure 6 are the average values of two experiments that were performed from September 19th to October 11th, and October 31st to November 21st, 2019.

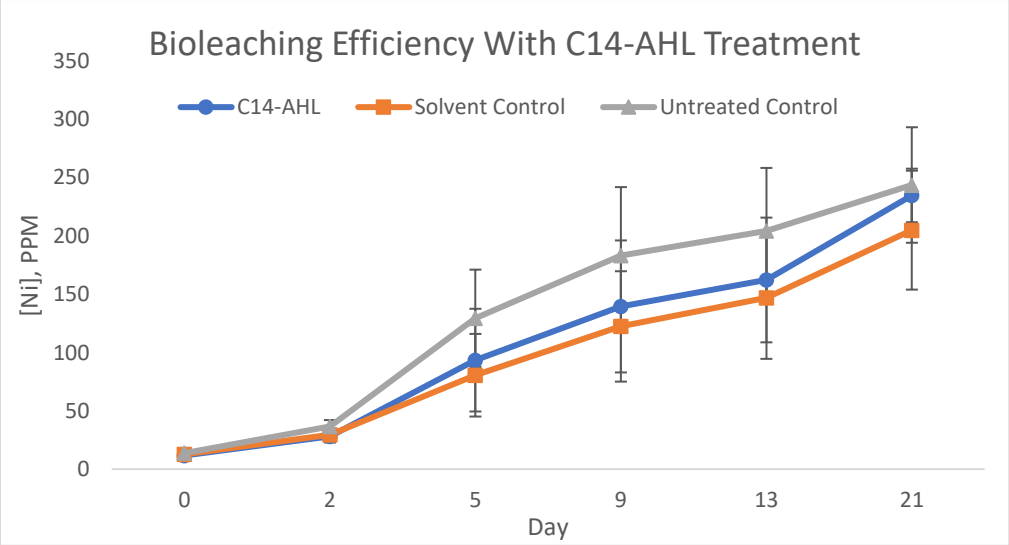

Figure 6: Nickel in solution values after treatment of bioleaching flasks with 5µM C14-AHL. Three different bioleaching flasks were constructed. Blue squares represent flasks treated with 5µM C14-AHL

delivered via ore pre-treatment using ethyl acetate. Orange triangles represent a culture that was pre-treated with ethyl acetate, but no QS molecules. Grey circles represent a bioleaching culture that was not pre-treated in any manner. The data points represent average values from two experiments, each performed with triplicate flasks. Error bars represent one standard deviation.

The treatment of bioleaching flasks with C14-AHL alone failed to produce a statistically significant improvement in bioleaching effectiveness at any of the six time points measured (Figure 6). There was no statistically significant difference between treatment groups at any time point measured.

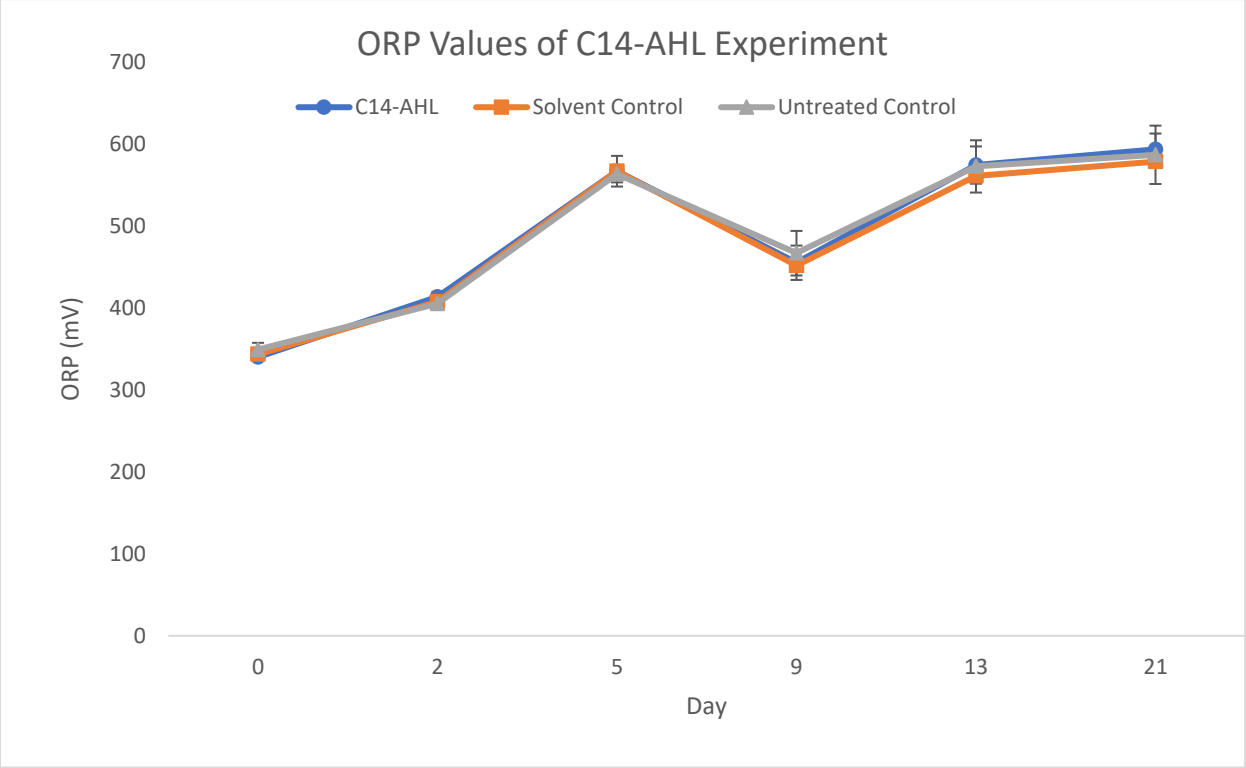

Figure 7: The average ORP values obtained during the C14-AHL experiment. No significant differences were detected between treatment groups. Error bars represent one standard deviation.

The ORP values recorded during the C14-AHL experiment did not significantly differ between groups when tested using one-way ANOVA. No significant effects on nickel leaching or ORP were detected when using the C14-AHL treatment at 5µM.

Analysis of bioleaching experiments using one-way ANOVA indicated that the treatment of bioleaching flasks with 5µM of unsubstituted C14-AHL did not have any significant effect, positive or negative, on bioleaching efficiency (Figure 6). These results were consistent across each experiment performed during this project (Figures 5 and 7). It is well established that C14-AHL can improve the attachment of bioleaching microorganisms to mineral surfaces, but it has been previously shown that these alterations in attachment behaviour do not always coicide with improved mineral leaching. Bellenberg et al (2013) describe just such an interaction involving C14-AHLs and the bacteria *At. ferrooxidans* and *Acidiferrobacter spp*, the group measured an improvement in the biofilm coverage of a pyrite substrate, but with no increase in pyrite dissolution (41). It is also possible that the C14-AHL

treatment did not result in increased attachment of the bioleaching bacteria to the substrate, it is even possible that the treatment inhibited microbial colonization of the substrate. It is an unfortunate limitation of the present study that no data is available regarding the attachment behaviours of the microbes used. In light of this limitation, all that can be confidently stated about the C14-AHL treatment is that it was not effective at improving the bioleaching kinetics of the system.

4.5 C14-MIX Treatment

The mixture of 3-oxo-C14-AHL, 3-hydroxy-C14-AHL and unsubstituted C14-AHL was tested for its ability to improve bioleaching efficiency at a total concentration of 5µM. The mixture of C14-AHLs was delivered as previously described using ethyl acetate. This experiment also reexamined the potential for C14-AHL to improve bioleaching efficiency by once again dosing bioleaching cultures with 5µM of C14-AHL. The four treatment groups for this experiment were known as: C14-AHL, C14-MIX, Solvent Control and Untreated Control.

C14-AHL refers to the flasks treated with 5µM of C14-AHL, C14-MIX refers to flasks treated with 3-oxo-C14-AHL, 3-hydroxy-C14-AHL and unsubstituted C14-AHL in equal portions, for a total concentration of 5µM. Solvent Control refers to flasks treated with ethyl acetate of the same volume as was used to deliver the C14-AHL molecules, and Untreated Control was not treated with C14-AHLs or ethyl acetate. Figure 8 displays the average nickel concentration of each test condition for the course of this experiment.

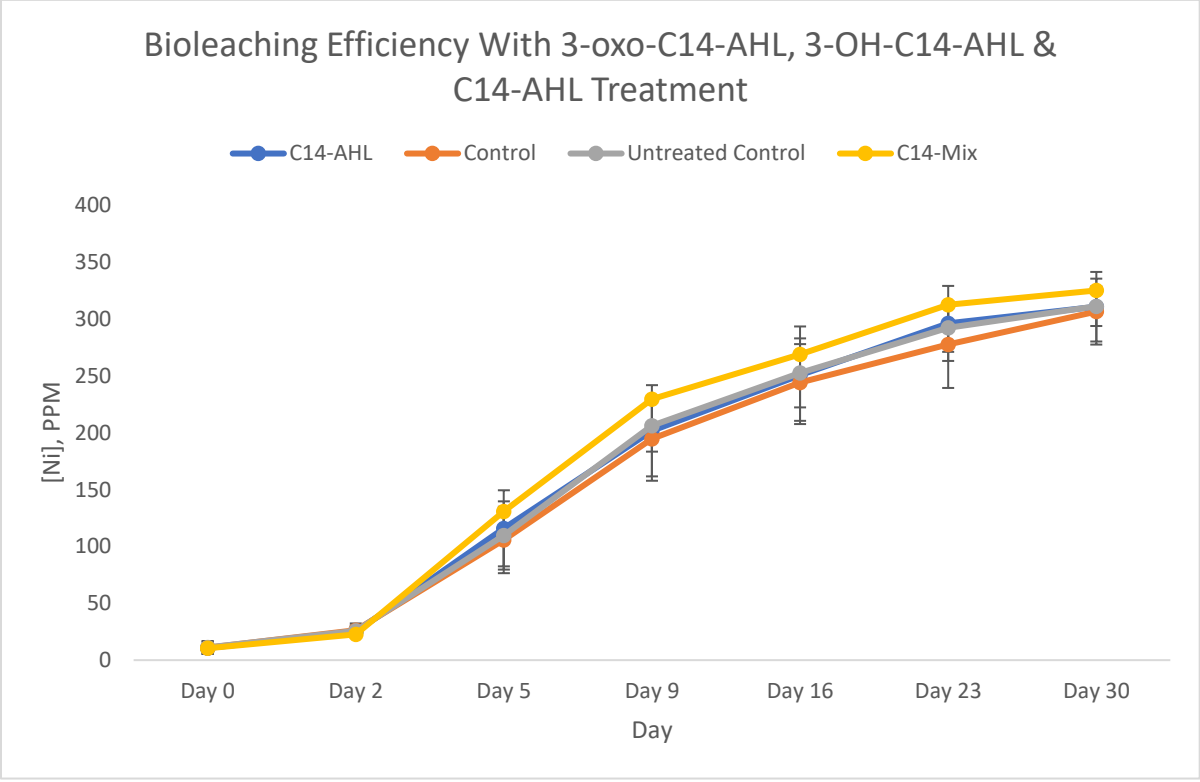

Figure 8: Nickel in solution values after treatment of bioleaching flasks with 5µM C14-AHL or 5µM of a mix containing C14-AHL, 3-oxo-C14-AHL and 3-hydroxy-C14-AHL. Four different bioleaching conditions were tested. Blue circles represent treatment with 5µM C14-AHL delivered via ore pre-treatment using

ethyl acetate. Orange crosses represent a culture that was pre-treated with ethyl acetate, but no QS molecules. Grey triangles represent a control bioleaching culture that was not treated in any manner. Yellow diamonds represent a culture treated with a mixture of C14-AHLs. The data points represent average values from three experiments, each performed with triplicate flasks.

At no measured time point during the experiment did the flasks treated with the single C14-AHL molecule record a significantly different value for nickel leaching as compared to the solvent control flasks or the untreated control flasks. This result is consistent with that of the previously described C14-AHL experiment (Section 4.2).

Significant results for C14-MIX were detected on testing days 9 and 30, with respective p values of 0.138 and 0.180 (Figure 8, appendix 8.2.3.1d & 8.2.3.1g). On these days the C14-MIX treatment reached a level of nickel in solution significantly greater than that of the solvent control flask, although it failed to reach a level of nickel in solution significantly greater than the untreated control flasks. There were no other significant effects to report.

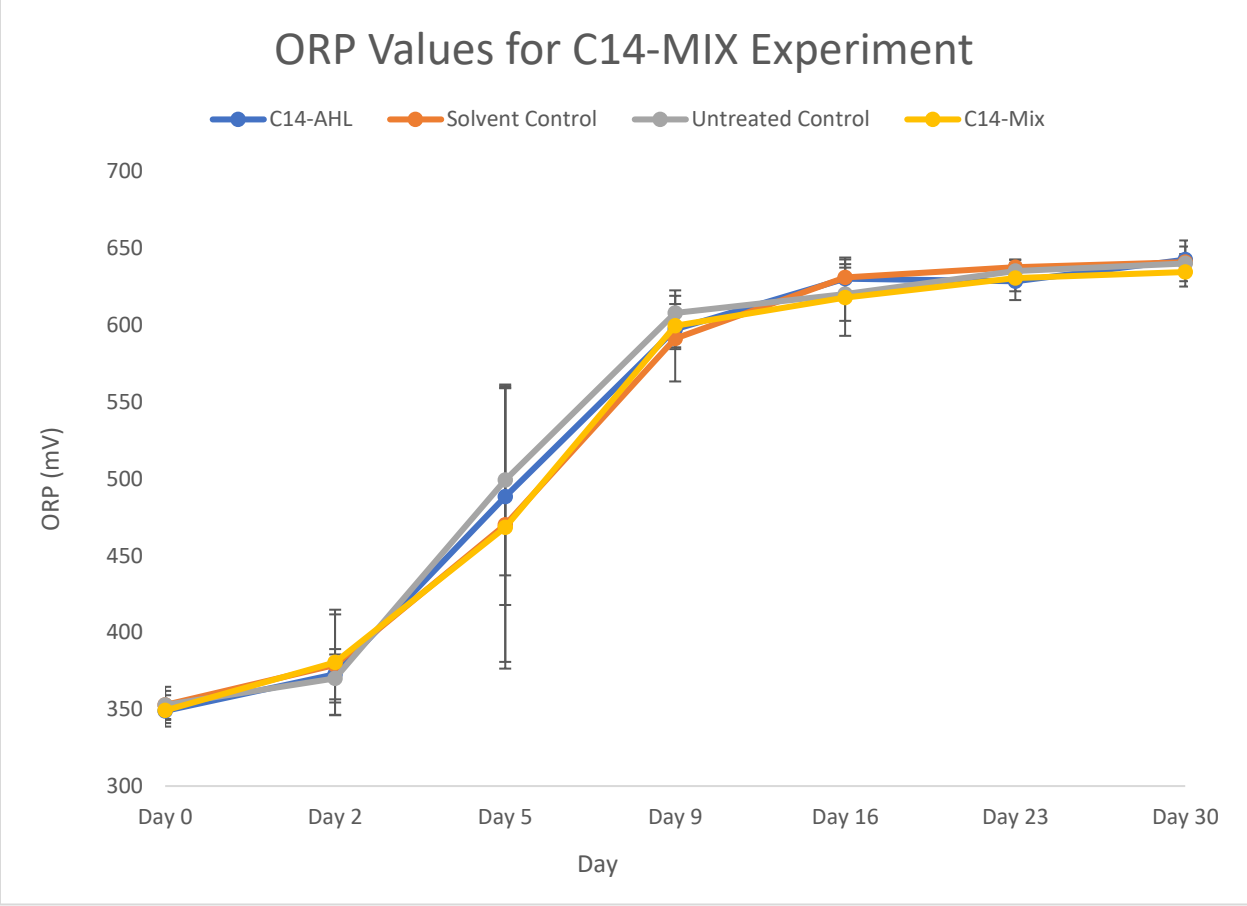

Figure 9: The average ORP values obtained during the C14-MIX experiment. ANOVA tests did not reveal any significant differences between the experimental conditions.

The ORP values recorded during the C14-MIX experiment did not significantly differ between groups when tested using one-way ANOVA.

During the C14-MIX experiment, the C14-AHL treatment once again failed to produce measurable impacts on nickel leaching. Treatment with 5µM of C14-MIX produced significant improvements in nickel leaching by the biological consortium on days 9 and 23 when measured with an 80% confidence interval. ORP values did not significantly change during treatment with C14-MIX.

Bioleaching experiments performed with a mixture of C14-AHL and its derivatives (3-oxo-C14-AHL and 3-hydroxy-C14-AHL) indicated that a 5µM dose of C14-MIX significantly increased the amount of nickel leached at two different time points when measured with an 80% confidence interval(Figures 7, appendix 8.2.3.1d & 8.2.3.1g). The improvement did not reach significance when measured using a confidence interval of 90%. The results of the C14-MIX experiment lend some support to the hypothesis that a mixture of C14-AHLs can be added to bioleaching reactors to improve the metal leaching efficiency of the bioleaching reactor. This result indicates that C14-MIX is a more potent treatment for the manipulation of microbial bioleaching behaviours than treatment with the single C14-AHL molecule, as the C14-AHL treatment did not produce any measurable effect on nickel leaching.

To be brief: the results of the C14-AHL and C14-MIX treatments indicate that C14-MIX used at 5µM is a more promising treatment than the C14-AHL molecule alone. Given the 80% confidence interval used for this study, it would be prudent to gather more data in order to improve confidence in the effect of the C14-MIX on bioleaching behaviours.

Recent bioleaching studies using AHLs have primarily used the same mixture of C14-AHL and its two derivatives rather than the single C14-AHL molecule for the bioleaching enhancement of *At. ferrooxidans* (6, 23). More recently genetic modifications of *At. ferrooxidans* have been made allowing researchers to construct strains which overexpress the *qs-I* operon, responsible for encoding both the AfeI AHL synthetase protein, and the AfeR transcriptional regulator protein. This QS overexpression strain was capable of increased biofilm formation and bioleaching on a sulfur substrate (42). The manipulation of *At. ferrooxidans* and other biomining bacteria through the addition of QS AHLs or the generation of mutant strains continues to appear promising, but still has barriers to pass before it is economically viable.

There are major limitations to the present study: the lack of data regarding the mineral surface behaviours of the bioleaching microorganisms (i.e., attachment, colonization, biofilm formation), the age of the 16s rRNA sequence data for the bioleaching culture, and the lack of positive control for the ore-pretreatment process. Information regarding the mineral attachment behaviour of the bioleaching microbes would serve to corroborate perceived improvements in the bioleaching activity of the microbes by providing context regarding the behaviour of the microbes, while a more recent genomic snapshot of the specific bacteria present in the bioleaching cultures would help to pinpoint which bacteria were or were not responsive to the QS treatments. It is unfortunately unclear whether or not the ore pre-treatment procedure was succesful in delivering intact dosages of AHL molecules.

Specific efforts were made to address these shortcomings of the project; by performing Most Probable Number (MPN) attachment tests designed to determine the effect of the AHL treatments on bacterial attachment to mineral surfaces, by harvesting 16s rRNA sequence data for planktonic, sessile, and whole bacterial cultures. Finally, attempts were made to produce a positive control for the presence of AHL chemicals. This is a prudent step for the generation of a new protocol to deliver the AHL chemicals, as a 16 hour incubation could potentially deactivate the AHL molecules. The bacterium

Rhizodium radiobacter produces a blue pigment in the presence of AHLs, and can thus be used a positive control to determine if the AHL signals were sucessfully delivered. Unfortunately, constraints imposed by the COVID-19 pandemic prevented the completion of the MPN attachment tests, the acquistion of 16s rRNA sequence data and the implementation of the positive control for the ore pre-treatment method.

5- Conclusion

This project sought to improve biomining reactor efficiency using quorum sensing molecules known as homoserine lactones. Two different experimental conditions were tested, treatment with 5μM doses of unsubstituted C14-AHL, and treatment with a 5μM mixture of the homoserine lactones 3-oxo-C14-AHL, 3-hydroxy-C14-AHL and C14-AHL. In addition to AHL QS treatments, a simple toxicity experiment was performed to confirm the potential for the solvent DMSO to inhibit the leaching behaviour of a biomining consortium. To apply AHL molecules to the bacterial cultures without exposing the bacteria to toxic organic solvents a novel method of AHL delivery was developed.

Results of bioleaching experiments designed to test the C14-AHL and C14-MIX treatments showed that treatment with C14-AHL had little to no effect on the leaching of nickel from the ore, while the C14-MIX treatment produced significant improvements in nickel leaching. Therefore, the treatment of mixed C14-AHL, 3-oxo-C14-AHL, 3-hydroxy-C14-AHL appears to be the more promising candidate for future endeavours in this field of study, rather than treatment with C14-AHL alone.

Established methods for delivering AHLs to bacterial cultures rely on the use of DMSO as a solvent vehicle. These methods were implemented and the effect of the DMSO on the bioleaching ability of the biomining reactors was quantified. Massive inhibition of the bioleaching culture's ability to leach nickel was found even using low doses of DMSO. These results lead to the generation of a novel method of delivering QS AHLs, and highlighted limitations of previously established methods.

An ethyl acetate solvent was used to dissolve AHLs to create a standard solution, which was used to apply AHLs directly to the dry ore, after which the solvent was evaporated, leaving AHLs applied directly to the surface of the ore. This process eliminated any potential for negative interactions between the biomining microorganisms and the organic solvents that are required to work with AHLs. Future researchers using exogenously provided AHL molecules to manipulate bioleaching bacteria should consider using similar pre-treatment applications for their AHLs although it would be prudent to first verify the ability of the ore pre-treatment method to deliver intact AHLs using a positive control.

Overall this study serves to pave the way for future research using AHL mediated QS to enhance bioleaching processes. The well established use of DMSO to deliver AHL treatments was criticized and an alternative method was developed, while promising results were found using a treatment of 5μM of the homoserine lactones 3-oxo-C14-AHL, 3-hydroxy-C14-AHL and C14-AHL. Future studies would be served well by verifying the success of the ore pre-treatment method as well as providing 16s rRNA sequence data for their microbial cultures, as well as applying microbial techniques like MPN to investigate the mineral surface behaviour of the microbial communities, prior to scaling-up experiment, performing column experiments to emulate bioheap leaching environments, or exploring new AHL treatments.

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7.0 Appendix

7.1 Sample Calculations:

7.1.1 Calculation 1: Delivery of C14-AHL example

Delivery of 5 µM of C14-AHL

Concentration of C14-AHL standard solution: **1.1×10^{-3} mol/L**.

Desired final dosage of C14-AHL: **5 µM**

Final volume of working solution (Reaction vessel) = **100 mL**

Volume of standard solution delivered to dry ore: **Unknown (V_1)**

1.1×10^{-3} mol/L C14-AHL standard solution = **1100 µM**

1100 µM = C_1

5 µM = C_2

100 mL = V_2

$C_1V_1 = C_2V_2$

1100 µM * V_1 = 5 µM * 100 mL

1100 µM * V_1 = 500 µM*mL

V_1 = 500 µM*mL / 1100 µM

V_1 = 0.454545 mL

V_1 = 454 µL

In this example, 454 microliters of C14-AHL standard solution would be delivered to the dry flask, before having the ethyl acetate solvent evaporated in a convection oven, and then being diluted in 100 mL of media. Control flasks would be treated with 454 microliters of pure ethyl acetate.

7.1.2 Calculation 2: Determination of 3-Hydroxy-C14-AHL Solubility & Standard Solution Preparation

The preparation of a standard AHL solution, in this case 3-hydroxy-C14-AHL (3-OH-C14-AHL), in ethyl acetate.

Standard solutions were prepared in 5 mL of ethyl acetate as follows.

Molar mass of 3-OH-C14-AHL: **327.45 g/mol**

Weight of 3-OH-C14-AHL Measured: **1.8 mg**

1.8 mg 3-OH-C14-AHL / 5 mL Ethyl Acetate

=0.36 mg/mL

=0.36 g/L / 327.45 g/mol

=0.0011 mol/L

=1100 µmol/L

1.8 milligrams of 3-hydroxy-C14-AHL crystal would dissolve in 5mL of ethyl acetate producing a standard solution of **$1.1x10^{-3}$ mol/L.**

7.1.3 Calculation 3: Delivery of C14-MIX example

The delivery of the C14-MIX required the use of three different standard solutions consisting of of the AHLs: C14-AHL, 3-oxo-C14-AHL, and 3-hydroxy-C14-AHL, altogether delivered to a final concentration of 5 µM. Below is an example of how the C14-MIX cocktail was constructed from the constituent standard solutions. In contrast to the delivery of the 5 µM C14-AHL treatment, each of the three components of the C14-MIX cocktail are delivered to ~1.67 µM, totaling to a final concentration of 5 µM.

Concentration of C14-AHL Standard Solution: **1700 µM**

1700 µM = C_1, 5 µM = C_2, 100 mL = V_2

$C_1V_1 = C_2V_2$

1700 µM * V_1 = 1.6667 µM * 100 mL

1700 µM * V_1 = 166.67 µM*mL

V_1 = 166.67 µM*mL / 1700 µM

V_1 = 0.0980 mL

V_1 = 98 µL

The C14-AHL component of the C14-MIX cocktail would have required 98 microliters of the 1700 µM C14-AHL standard solution.

Concentration of 3-oxo-C14-AHL Standard Solution: **1000 µM**

1000 µM = C_1, 5 µM = C_2, 100 mL = V_2

V_1 = 166.67 µM*mL / 1000 µM

V_1 = 0.16667 mL

V_1 = 167 µL

The 3-oxo-C14-AHL component of the C14-MIX cocktail would have required 167 microliters of the 1000 µM 3-oxo-C14-AHL standard solution.

Concentration of 3-OH-C14-AHL Standard Solution: **1100 µM**

1100 µM = C_1, 5 µM = C_2, 100 mL = V_2

V_1 = 166.67 µM*mL / 1100 µM

V_1 = 0.15152 mL

V_1 = 152 µL

The 3-OH-C14-AHL component of the C14-MIX cocktail would have required 152 microliters of the 1100 µM 3-OH-C14-AHL standard solution.

Altogether the C14-MIX cocktail would, in this example, be comprised of 98µL of the C14-AHL standard solution, 167µL of the 3-oxo-C14-AHL standard solution, and 152µL of the 3-OH-C14-AHL standard solution. These small volumes of solution would be mixed before being delivered to the dry flask, before having the ethyl acetate solvent evaporated in a convection oven, and then being diluted in 100 mL of media. Control flasks would be treated with (98 + 167 + 152) 417 microliters of pure ethyl acetate.

7.1.4 Calculation 4:

All values of nickel in solution are reported in parts per million as determined by ICP-AES. At times the proportion of nickel that has been leached from the ore is referenced, this number is derived from the known chemical composition of the ore (Tables 3-5), the mass of ore added to each bioleaching flask (5 grams) and the volume of solution in each flask (100mL).

Percentage of nickel in ore (0.79%) * weight of ore (mg) / volume of shake flask bioreactor (in L)

= 0.0079 * 5000mg / 0.1L

=395 mg/L

=395 ppm

Each bioleaching flask constructed during each experiment throughout this project was functionally identical, aside from the various treatments, meaning that each flask would have the same theoretical yield of 395 ppm nickel if 100% of the nickel contained within the iron-sulfide ore was leached into solution.

7.2. ANOVA and Tukey's Post-Hoc Analysis of Experimental Data

7.2.1.1 One-Way Anova and Tukey's Post-Hoc Analysis for DMSO Experiment Nickel Data.

Treatment 1: Abiotic

Treatment 2: Control

Treatment 3: 1% DMSO

Treatment 4: 0.5% DMSO

Treatment 5: 0.1% DMSO

Treatment 6: 0.05% DMSO

7.2.1.1a One-way ANOVA: Day0 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   5  31.14  6.23  5.35  0.032
Error       6   6.98  1.16
Total      11  38.12

S = 1.078   R-Sq = 81.69%   R-Sq(adj) = 66.43%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
1      2  17.804  2.570              (----*-----)
2      2  15.541  0.325  (-----*----)
3      2  19.595  0.029                      (-----*----)
4      2  19.721  0.496                       (-----*----)
5      2  19.877  0.084                         (----*-----)
6      2  19.953  0.122                         (-----*----)
                          --------+---------+---------+---------+-
                               16.0      18.0      20.0      22.0

Pooled StDev = 1.078


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%
```

```
Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
2          -5.199  -2.263  0.673       (------*-------)
3          -1.145   1.791  4.727                (------*-------)
4          -1.019   1.917  4.853                (-------*------)
5          -0.862   2.074  5.009                 (------*-------)
6          -0.787   2.149  5.085                 (------*-------)
                                  --------+---------+---------+---------+-
                                       -4.0       0.0       4.0       8.0


Treatment = 2 subtracted from:

Treatment  Lower  Center  Upper  --------+---------+---------+---------+-
3          1.118   4.054  6.990                     (------*------)
4          1.244   4.180  7.116                     (------*-------)
5          1.401   4.337  7.273                      (------*------)
6          1.476   4.412  7.348                      (------*------)
                                 --------+---------+---------+---------+-
                                      -4.0       0.0       4.0       8.0


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
4          -2.810   0.126  3.062            (------*-------)
5          -2.653   0.282  3.218            (-------*------)
6          -2.578   0.358  3.294             (------*------)
                                  --------+---------+---------+---------+-
                                       -4.0       0.0       4.0       8.0


Treatment = 4 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
5          -2.780   0.156  3.092            (------*-------)
6          -2.704   0.232  3.167            (-------*------)
                                  --------+---------+---------+---------+-
                                       -4.0       0.0       4.0       8.0


Treatment = 5 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
6          -2.861   0.075  3.011            (------*-------)
                                  --------+---------+---------+---------+-
                                       -4.0       0.0       4.0       8.0
```

7.2.1.1b One-way ANOVA: Day2 versus Treatment

```
Source     DF      SS      MS      F      P
Treatment   5  7387.0  1477.4  82.73  0.000
Error       6   107.2    17.9
Total      11  7494.1

S = 4.226   R-Sq = 98.57%   R-Sq(adj) = 97.38%

                      Individual 80% CIs For Mean Based on
                      Pooled StDev
```

```
Level  N    Mean  StDev  -------+---------+---------+---------+--
1      2   9.994  0.967  (-*-)
2      2  53.003  0.664                    (--*-)
3      2  76.722  9.993                              (-*--)
4      2  79.113  0.515                               (--*-)
5      2  78.086  1.946                               (-*-)
6      2  73.827  1.369                             (-*-)
                       -------+---------+---------+---------+--
                              20        40        60        80

Pooled StDev = 4.226


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  ---+---------+---------+---------+------
2          31.504  43.009  54.514                        (--*---)
3          55.223  66.727  78.232                                (---*---)
4          57.614  69.118  80.623                                 (---*---)
5          56.587  68.092  79.597                                 (---*---)
6          52.328  63.832  75.337                               (---*---)
                              ---+---------+---------+---------+------
                               -30         0        30        60


Treatment = 2 subtracted from:

Treatment   Lower  Center   Upper  ---+---------+---------+---------+------
3          12.214  23.718  35.223              (---*---)
4          14.605  26.110  37.614               (---*---)
5          13.578  25.083  36.588               (---*---)
6           9.319  20.823  32.328             (---*---)
                              ---+---------+---------+---------+------
                               -30         0        30        60


Treatment = 3 subtracted from:

Treatment    Lower  Center   Upper  ---+---------+---------+---------+------
4           -9.114   2.391  13.896           (---*---)
5          -10.140   1.365  12.869           (--*---)
6          -14.400  -2.895   8.610         (---*---)
                              ---+---------+---------+---------+------
                               -30         0        30        60


Treatment = 4 subtracted from:

Treatment    Lower  Center   Upper  ---+---------+---------+---------+------
5          -12.531  -1.026  10.478          (---*--)
6          -16.791  -5.286   6.219        (---*---)
                              ---+---------+---------+---------+------
                               -30         0        30        60


Treatment = 5 subtracted from:
```

```
Treatment    Lower  Center  Upper  ---+---------+---------+---------+------
6          -15.764  -4.260  7.245          (---*--)
                                  ---+---------+---------+---------+------
                                   -30         0        30        60
```

7.2.1.1c One-way ANOVA: Day5 versus Treatment

```
Source     DF      SS      MS       F      P
Treatment   5  8927.1  1785.4  164.27  0.000
Error       6    65.2    10.9
Total      11  8992.3

S = 3.297   R-Sq = 99.27%   R-Sq(adj) = 98.67%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ---+---------+---------+---------+------
1      2   19.81   0.90  (*)
2      2  109.85   4.51                                      (*)
3      2   78.28   4.44                        (*-)
4      2   81.34   0.88                         (-*)
5      2   85.18   4.09                           (*)
6      2   82.54   2.62                          (*)
                         ---+---------+---------+---------+------
                           25        50        75       100

Pooled StDev = 3.30


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment  Lower  Center  Upper  --+---------+---------+---------+-------
2          81.06   90.04  99.01                                     (--*-)
3          49.50   58.47  67.45                               (--*-)
4          52.55   61.53  70.50                                (--*-)
5          56.40   65.37  74.35                                 (--*-)
6          53.75   62.73  71.70                                (--*-)
                               --+---------+---------+---------+-------
                               -35         0        35        70


Treatment = 2 subtracted from:

Treatment   Lower  Center   Upper  --+---------+---------+---------+-------
3          -40.54  -31.57  -22.59  (--*--)
4          -37.49  -28.51  -19.53   (--*-)
5          -33.64  -24.67  -15.69    (--*--)
6          -36.29  -27.31  -18.34    (-*--)
                               --+---------+---------+---------+-------
                               -35         0        35        70


Treatment = 3 subtracted from:
```

```
Treatment  Lower  Center  Upper  --+---------+---------+---------+-------
4          -5.92    3.06  12.03           (--*-)
5          -2.07    6.90  15.88            (--*--)
6          -4.72    4.25  13.23            (-*--)
                              --+---------+---------+---------+-------
                              -35         0        35        70


Treatment = 4 subtracted from:

Treatment  Lower  Center  Upper  --+---------+---------+---------+-------
5          -5.13    3.84  12.82            (-*--)
6          -7.78    1.20  10.17           (-*--)
                              --+---------+---------+---------+-------
                              -35         0        35        70


Treatment = 5 subtracted from:

Treatment   Lower  Center  Upper  --+---------+---------+---------+-------
6          -11.62   -2.65   6.33           (-*--)
                               --+---------+---------+---------+-------
                               -35         0        35        70
```

7.2.1.1d One-way ANOVA: Day12 versus Treatment

```
Source     DF       SS       MS       F      P
Treatment   5  80946.9  16189.4  545.19  0.000
Error       6    178.2     29.7
Total      11  81125.1

S = 5.449   R-Sq = 99.78%   R-Sq(adj) = 99.60%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+
1      2   81.55   4.89  (*
2      2  332.08   3.47                                      *)
3      2  117.93   3.87       (*)
4      2  122.37   2.70        *)
5      2  125.12   8.79        (*)
6      2  134.16   6.53         (*)
                        ---------+---------+---------+---------+
                               140       210       280       350

Pooled StDev = 5.45


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  -----+---------+---------+---------+----
2          235.70  250.54  265.37                                    (*)
3           21.54   36.38   51.21                  (*)
```

```
4           25.99   40.82   55.66                   (*)
5           28.73   43.57   58.40                   (*)
6           37.78   52.61   67.45                    (*
                                  -----+---------+---------+---------+----
                                    -150         0       150       300


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper  -----+---------+---------+---------+----
3          -228.99  -214.16  -199.32  (*)
4          -224.55  -209.72  -194.88  (*)
5          -221.80  -206.97  -192.13  (*)
6          -212.76  -197.92  -183.09   (*)
                                      -----+---------+---------+---------+----
                                        -150         0       150       300


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  -----+---------+---------+---------+----
4          -10.39    4.44  19.28               (*)
5           -7.64    7.19  22.03               (*)
6            1.40   16.24  31.07                (*)
                                  -----+---------+---------+---------+----
                                    -150         0       150       300


Treatment = 4 subtracted from:

Treatment   Lower  Center  Upper  -----+---------+---------+---------+----
5          -12.09    2.75  17.58               (*)
6           -3.04   11.79  26.63                (*)
                                  -----+---------+---------+---------+----
                                    -150         0       150       300


Treatment = 5 subtracted from:

Treatment  Lower  Center  Upper  -----+---------+---------+---------+----
6          -5.79    9.04  23.88               (*)
                                 -----+---------+---------+---------+----
                                   -150         0       150       300
```

7.2.1.1e One-way ANOVA: Day16 versus Treatment

```
Source     DF       SS       MS       F      P
Treatment   5  64612.1  12922.4  152.61  0.000
Error       6    508.1     84.7
Total      11  65120.2

S = 9.202   R-Sq = 99.22%   R-Sq(adj) = 98.57%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      2  119.16   1.65  (*)
2      2  347.72   6.51                                   (-*)
3      2  156.56  13.43       (*-)
4      2  158.33   2.01       (-*)
```

```
5      2  177.11  15.52          (*-)
6      2  204.84   6.13              (*-)
                        ----+---------+---------+---------+-----
                          140       210       280       350

Pooled StDev = 9.20


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  --------+---------+---------+---------+-
2          203.50  228.56  253.61                                    (-*-)
3           12.35   37.40   62.46                      (-*-)
4           14.12   39.17   64.22                      (-*-)
5           32.89   57.95   83.00                        (-*-)
6           60.62   85.68  110.73                          (-*-)
                                 --------+---------+---------+---------+-
                                      -120         0       120       240


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper  --------+---------+---------+---------+-
3          -216.20  -191.15  -166.10  (-*-)
4          -214.44  -189.39  -164.33  (-*-)
5          -195.66  -170.61  -145.56    (-*-)
6          -167.93  -142.88  -117.83      (-*-)
                                 --------+---------+---------+---------+-
                                      -120         0       120       240


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
4          -23.29    1.77  26.82                  (-*-)
5           -4.51   20.54  45.59                    (-*-)
6           23.22   48.27  73.32                      (-*-)
                                 --------+---------+---------+---------+-
                                      -120         0       120       240


Treatment = 4 subtracted from:

Treatment  Lower  Center  Upper  --------+---------+---------+---------+-
5          -6.28   18.78  43.83                  (--*-)
6          21.45   46.51  71.56                     (-*-)
                                 --------+---------+---------+---------+-
                                      -120         0       120       240


Treatment = 5 subtracted from:

Treatment  Lower  Center  Upper  --------+---------+---------+---------+-
6           2.68   27.73  52.78                   (-*-)
                                 --------+---------+---------+---------+-
                                      -120         0       120       240
```

7.2.1.1f One-way ANOVA: Day23 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   5  16080  3216  4.34  0.051
Error       6   4446   741
Total      11  20525

S = 27.22   R-Sq = 78.34%   R-Sq(adj) = 60.29%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
1      2  224.67  58.92   (-----*----)
2      2  344.10  28.48                          (-----*----)
3      2  266.19   3.18             (----*-----)
4      2  259.46   3.43           (-----*----)
5      2  285.73   9.37                (----*-----)
6      2  295.10   7.25                 (-----*-----)
                        -+---------+---------+---------+--------
                        200       250       300       350

Pooled StDev = 27.22


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  ------+---------+---------+---------+---
2           45.32  119.43  193.53                       (------*------)
3          -32.59   41.52  115.62               (------*-------)
4          -39.31   34.79  108.89              (------*-------)
5          -13.05   61.06  135.16                 (------*-------)
6           -3.67   70.43  144.54                  (------*------)
                           ------+---------+---------+---------+---
                              -100         0       100       200


Treatment = 2 subtracted from:

Treatment    Lower  Center   Upper  ------+---------+---------+---------+---
3          -152.01  -77.91   -3.81   (------*-------)
4          -158.74  -84.64  -10.53  (-------*------)
5          -132.48  -58.37   15.73     (------*-------)
6          -123.10  -48.99   25.11      (------*-------)
                            ------+---------+---------+---------+---
                               -100         0       100       200


Treatment = 3 subtracted from:

Treatment   Lower  Center   Upper  ------+---------+---------+---------+---
4          -80.83   -6.73   67.38          (------*-------)
5          -54.57   19.54   93.64             (------*------)
6          -45.19   28.92  103.02             (-------*------)
```

```
                                  ------+---------+---------+---------+---
                                     -100         0       100       200


Treatment = 4 subtracted from:

Treatment   Lower  Center   Upper  ------+---------+---------+---------+---
5          -47.84   26.27  100.37             (-------*------)
6          -38.46   35.64  109.75              (-------*------)
                                  ------+---------+---------+---------+---
                                     -100         0       100       200


Treatment = 5 subtracted from:

Treatment   Lower  Center  Upper  ------+---------+---------+---------+---
6          -64.73    9.38  83.48            (------*------)
                                 ------+---------+---------+---------+---
                                    -100         0       100       200
```

7.2.1.2 One-Way Anova and Tukey's Post-Hoc Analysis for DMSO ORP Data.

Treatment 1: Abiotic

Treatment 2: Control

Treatment 3: 1% DMSO

Treatment 4: 0.5% DMSO

Treatment 5: 0.1% DMSO

Treatment 6: 0.05% DMSO

7.2.1.2a One-way ANOVA: Day0 versus Treatment

```
Source     DF     SS     MS     F      P
Treatment   5  584.8  117.0  2.16  0.188
Error       6  325.5   54.3
Total      11  910.3

S = 7.365   R-Sq = 64.24%   R-Sq(adj) = 34.44%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+
1      2  368.50  17.68  (-------*------)
2      2  392.00   0.00                        (------*------)
3      2  379.00   1.41             (------*------)
4      2  381.00   1.41               (------*------)
5      2  376.50   2.12          (-------*------)
6      2  381.50   2.12               (-------*------)
                        ---------+---------+---------+---------+
                               370       380       390       400

Pooled StDev = 7.37


Tukey 80% Simultaneous Confidence Intervals
```

```
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment    Lower  Center   Upper  --------+---------+---------+---------+-
2            3.449  23.500  43.551                    (---------*---------)
3           -9.551  10.500  30.551              (---------*---------)
4           -7.551  12.500  32.551               (---------*---------)
5          -12.051   8.000  28.051             (---------*---------)
6           -7.051  13.000  33.051               (----------*---------)
                                  --------+---------+---------+---------+-
                                        -20         0        20        40


Treatment = 2 subtracted from:

Treatment    Lower   Center  Upper  --------+---------+---------+---------+-
3          -33.051  -13.000  7.051   (----------*---------)
4          -31.051  -11.000  9.051    (----------*---------)
5          -35.551  -15.500  4.551  (---------*---------)
6          -30.551  -10.500  9.551     (---------*---------)
                                  --------+---------+---------+---------+-
                                        -20         0        20        40


Treatment = 3 subtracted from:

Treatment    Lower  Center   Upper  --------+---------+---------+---------+-
4          -18.051   2.000  22.051          (---------*---------)
5          -22.551  -2.500  17.551        (---------*---------)
6          -17.551   2.500  22.551          (---------*---------)
                                  --------+---------+---------+---------+-
                                        -20         0        20        40


Treatment = 4 subtracted from:

Treatment    Lower  Center   Upper  --------+---------+---------+---------+-
5          -24.551  -4.500  15.551       (---------*---------)
6          -19.551   0.500  20.551         (---------*---------)
                                  --------+---------+---------+---------+-
                                        -20         0        20        40


Treatment = 5 subtracted from:

Treatment    Lower  Center   Upper  --------+---------+---------+---------+-
6          -15.051   5.000  25.051           (----------*---------)
                                  --------+---------+---------+---------+-
                                        -20         0        20        40
```

7.2.1.2b One-way ANOVA: Day2 versus Treatment

```
Source     DF      SS     MS      F      P
Treatment   5  4996.8  999.4  94.43  0.000
Error       6    63.5   10.6
Total      11  5060.3
```

```
S = 3.253   R-Sq = 98.75%   R-Sq(adj) = 97.70%

                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
1      2  425.00   1.41                              (-*)
2      2  375.00   5.66      (-*)
3      2  366.50   3.54   (*-)
4      2  369.50   2.12    (-*)
5      2  372.50   3.54      (*-)
6      2  370.00   0.00    (-*-)
                          --------+---------+---------+---------+-
                                380       400       420       440

Pooled StDev = 3.25


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment    Lower   Center    Upper  -------+---------+---------+---------+--
2          -58.856  -50.000  -41.144      (---*---)
3          -67.356  -58.500  -49.644   (---*--)
4          -64.356  -55.500  -46.644    (---*--)
5          -61.356  -52.500  -43.644     (---*---)
6          -63.856  -55.000  -46.144    (---*---)
                             -------+---------+---------+---------+--
                                  -50       -25         0        25


Treatment = 2 subtracted from:

Treatment    Lower  Center  Upper  -------+---------+---------+---------+--
3          -17.356  -8.500  0.356                    (---*--)
4          -14.356  -5.500  3.356                     (---*--)
5          -11.356  -2.500  6.356                      (---*---)
6          -13.856  -5.000  3.856                     (---*---)
                            -------+---------+---------+---------+--
                                 -50       -25         0        25


Treatment = 3 subtracted from:

Treatment   Lower  Center   Upper  -------+---------+---------+---------+--
4          -5.856   3.000  11.856                          (--*---)
5          -2.856   6.000  14.856                           (--*---)
6          -5.356   3.500  12.356                          (--*---)
                            -------+---------+---------+---------+--
                                 -50       -25         0        25


Treatment = 4 subtracted from:

Treatment   Lower  Center   Upper  -------+---------+---------+---------+--
5          -5.856   3.000  11.856                          (--*---)
6          -8.356   0.500   9.356                        (--*---)
```

```
                                  -------+---------+---------+---------+--
                                       -50       -25         0        25


Treatment = 5 subtracted from:

Treatment    Lower  Center  Upper  -------+---------+---------+---------+--
6          -11.356  -2.500  6.356                      (---*---)
                                  -------+---------+---------+---------+--
                                       -50       -25         0        25
```

7.2.1.2c One-way ANOVA: Day5 versus Treatment

```
Source     DF        SS       MS       F      P
Treatment   5  12100.42  2420.08  326.30  0.000
Error       6     44.50     7.42
Total      11  12144.92

S = 2.723   R-Sq = 99.63%   R-Sq(adj) = 99.33%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
1      2  409.50   0.71                    (*)
2      2  448.50   3.54                                  (*-)
3      2  363.50   2.12  (*-)
4      2  364.50   0.71   (*)
5      2  368.50   4.95    (*-)
6      2  368.00   1.41    (*)
                        ------+---------+---------+---------+---
                            375       400       425       450

Pooled StDev = 2.72


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment    Lower   Center    Upper  --------+---------+---------+---------+-
2           31.586   39.000   46.414                           (-*)
3          -53.414  -46.000  -38.586          (-*)
4          -52.414  -45.000  -37.586           (*)
5          -48.414  -41.000  -33.586           (-*)
6          -48.914  -41.500  -34.086           (-*)
                                  --------+---------+---------+---------+-
                                        -50         0        50       100


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper  --------+---------+---------+---------+-
3          -92.414  -85.000  -77.586  (*)
4          -91.414  -84.000  -76.586  (*-)
5          -87.414  -80.000  -72.586   (*)
6          -87.914  -80.500  -73.086  (-*)
```

```
                                    --------+---------+---------+---------+-
                                          -50         0        50       100


Treatment = 3 subtracted from:

Treatment   Lower  Center   Upper  --------+---------+---------+---------+-
4          -6.414   1.000   8.414                  (*-)
5          -2.414   5.000  12.414                   (*)
6          -2.914   4.500  11.914                  (-*)
                                   --------+---------+---------+---------+-
                                         -50         0        50       100


Treatment = 4 subtracted from:

Treatment   Lower  Center   Upper  --------+---------+---------+---------+-
5          -3.414   4.000  11.414                  (-*)
6          -3.914   3.500  10.914                  (-*)
                                   --------+---------+---------+---------+-
                                         -50         0        50       100


Treatment = 5 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
6          -7.914  -0.500  6.914                (-*)
                                  --------+---------+---------+---------+-
                                        -50         0        50       100
```

7.2.1.2d One-way ANOVA: Day12 versus Treatment

```
Source     DF        SS       MS        F      P
Treatment   5  135607.8  27121.6  3828.92  0.000
Error       6      42.5      7.1
Total      11  135650.3

S = 2.661   R-Sq = 99.97%   R-Sq(adj) = 99.94%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
1      2  401.00   5.66       *
2      2  655.00   0.00                                      *
3      2  360.50   0.71  *
4      2  362.00   0.00  *)
5      2  366.00   1.41  (*
6      2  371.00   2.83   *)
                         -----+---------+---------+---------+----
                            400       480       560       640

Pooled StDev = 2.66


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%
```

```
Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  ---------+---------+---------+---------+
2          246.75  254.00  261.25                                    (*
3          -47.75  -40.50  -33.25                  *)
4          -46.25  -39.00  -31.75                  (*
5          -42.25  -35.00  -27.75                  (*
6          -37.25  -30.00  -22.75                   *)
                                  ---------+---------+---------+---------+
                                        -160         0       160       320


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper  ---------+---------+---------+---------+
3          -301.75  -294.50  -287.25  (*
4          -300.25  -293.00  -285.75  (*
5          -296.25  -289.00  -281.75  (*
6          -291.25  -284.00  -276.75   *)
                                     ---------+---------+---------+---------+
                                           -160         0       160       320


Treatment = 3 subtracted from:

Treatment  Lower  Center  Upper  ---------+---------+---------+---------+
4          -5.75    1.50   8.75                   *)
5          -1.75    5.50  12.75                   *)
6           3.25   10.50  17.75                    (*
                               ---------+---------+---------+---------+
                                     -160         0       160       320


Treatment = 4 subtracted from:

Treatment  Lower  Center  Upper  ---------+---------+---------+---------+
5          -3.25    4.00  11.25                   *)
6           1.75    9.00  16.25                    (*
                               ---------+---------+---------+---------+
                                     -160         0       160       320


Treatment = 5 subtracted from:

Treatment  Lower  Center  Upper  ---------+---------+---------+---------+
6          -2.25    5.00  12.25                   *)
                               ---------+---------+---------+---------+
                                     -160         0       160       320
```

7.2.1.2e One-way ANOVA: Day16 versus Treatment

```
Source     DF        SS       MS        F      P
Treatment   5  129452.0  25890.4  1350.80  0.000
Error       6     115.0     19.2
Total      11  129567.0

S = 4.378   R-Sq = 99.91%   R-Sq(adj) = 99.84%

                        Individual 80% CIs For Mean Based on
                        Pooled StDev
```

```
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      2  389.00   0.00    (*
2      2  657.00   9.90                                      *)
3      2  369.00   1.41  *)
4      2  370.00   1.41  *)
5      2  377.50   3.54   *)
6      2  390.50   0.71    (*
                         ----+---------+---------+---------+-----
                           400       480       560       640

Pooled StDev = 4.38


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper     +---------+---------+---------+---------
2          256.08  268.00  279.92                                          (*)
3          -31.92  -20.00   -8.08                      (*
4          -30.92  -19.00   -7.08                      (*)
5          -23.42  -11.50    0.42                      (*)
6          -10.42    1.50   13.42                       (*)
                                  +---------+---------+---------+---------
                                -300      -150         0       150


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper
3          -299.92  -288.00  -276.08
4          -298.92  -287.00  -275.08
5          -291.42  -279.50  -267.58
6          -278.42  -266.50  -254.58

Treatment     +---------+---------+---------+---------
3             (*)
4             (*)
5              *)
6              (*)
              +---------+---------+---------+---------
           -300      -150         0       150


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper     +---------+---------+---------+---------
4          -10.92    1.00  12.92                       (*)
5           -3.42    8.50  20.42                        (*
6            9.58   21.50  33.42                         *)
                                  +---------+---------+---------+---------
                               -300      -150         0       150


Treatment = 4 subtracted from:

Treatment  Lower  Center  Upper     +---------+---------+---------+---------
5          -4.42    7.50  19.42                       (*
```

```
6           8.58   20.50  32.42                          *)
                                  +---------+---------+---------+---------
                                -300      -150         0       150


Treatment = 5 subtracted from:

Treatment  Lower  Center  Upper     +---------+---------+---------+---------
6           1.08   13.00  24.92                        (*)
                                  +---------+---------+---------+---------
                                -300      -150         0       150
```

7.2.1.2f One-way ANOVA: Day23 versus Treatment

```
Source     DF      SS     MS      F      P
Treatment   5  117180  23436  14.29  0.003
Error       6    9843   1640
Total      11  127023

S = 40.50   R-Sq = 92.25%   R-Sq(adj) = 85.79%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
1      2  529.00  98.99                (---*---)
2      2  654.00   1.41                          (---*----)
3      2  383.50   2.12  (---*---)
4      2  386.00   1.41  (----*---)
5      2  404.00   5.66    (---*----)
6      2  418.00   1.41      (---*---)
                         ------+---------+---------+---------+---
                             400       500       600       700

Pooled StDev = 40.50


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 96.55%


Treatment = 1 subtracted from:

Treatment    Lower   Center   Upper  ---------+---------+---------+---------+
2            14.74   125.00  235.26                     (----*-----)
3          -255.76  -145.50  -35.24        (-----*----)
4          -253.26  -143.00  -32.74        (-----*----)
5          -235.26  -125.00  -14.74         (-----*----)
6          -221.26  -111.00   -0.74          (----*-----)
                                  ---------+---------+---------+---------+
                                        -200         0       200       400


Treatment = 2 subtracted from:

Treatment    Lower   Center    Upper  ---------+---------+---------+---------+
3          -380.76  -270.50  -160.24  (----*-----)
4          -378.26  -268.00  -157.74  (-----*----)
5          -360.26  -250.00  -139.74   (-----*----)
```

```
6          -346.26  -236.00  -125.74    (----*-----)
                                    ---------+---------+---------+---------+
                                           -200         0       200       400


Treatment = 3 subtracted from:

Treatment    Lower  Center   Upper  ---------+---------+---------+---------+
4          -107.76    2.50  112.76               (----*-----)
5           -89.76   20.50  130.76                (----*-----)
6           -75.76   34.50  144.76                (-----*----)
                                   ---------+---------+---------+---------+
                                         -200         0       200       400


Treatment = 4 subtracted from:

Treatment   Lower  Center   Upper  ---------+---------+---------+---------+
5          -92.26   18.00  128.26              (-----*----)
6          -78.26   32.00  142.26               (-----*----)
                                   ---------+---------+---------+---------+
                                         -200         0       200       400


Treatment = 5 subtracted from:

Treatment   Lower  Center   Upper  ---------+---------+---------+---------+
6          -96.26   14.00  124.26              (-----*----)
                                   ---------+---------+---------+---------+
                                         -200         0       200       400
```

7.2.2.1 One-Way Anova and Tukey's Post-Hoc Analysis for C14-AHL Nickel Data.

Treatment 1: C14-AHL Group
Treatment 2: Solvent Control Group
Treatment 3: Untreated Control Group

7.2.2.1a One-way ANOVA: Day 0 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2  11.93  5.97  5.04  0.021
Error      15  17.78  1.19
Total      17  29.71

S = 1.089   R-Sq = 40.17%   R-Sq(adj) = 32.19%


                        Individual 80% CIs For Mean Based on Pooled
                        StDev
Level  N    Mean  StDev    -+---------+---------+---------+--------
1      6  11.749  0.827    (-------*------)
2      6  12.727  1.010                (------*-------)
3      6  13.743  1.361                             (-------*------)
                          -+---------+---------+---------+--------
                        11.20     12.00     12.80     13.60

Pooled StDev = 1.089
```

```
Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ----+---------+---------+---------+-----
2          -0.160   0.978  2.116               (-------*------)
3           0.857   1.994  3.132                       (------*-------)
                                  ----+---------+---------+---------+-----
                                   -1.5       0.0       1.5       3.0


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ----+---------+---------+---------+-----
3          -0.121   1.017  2.154               (-------*------)
                                  ----+---------+---------+---------+-----
                                   -1.5       0.0       1.5       3.0
```

7.2.2.1b One-way ANOVA: Day 2 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2   57.2  28.6  0.94  0.413
Error      15  457.2  30.5
Total      17  514.4

S = 5.521   R-Sq = 11.12%   R-Sq(adj) = 0.00%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
1      6  28.205  5.394  (---------*---------)
2      6  29.427  5.693      (---------*---------)
3      6  32.447  5.472                (---------*---------)
                         ------+---------+---------+---------+---
                            27.0      30.0      33.0      36.0

Pooled StDev = 5.521


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%


Treatment = 1 subtracted from:

Treatment   Lower  Center   Upper  --------+---------+---------+---------+-
2          -4.548   1.222   6.991           (----------*-----------)
3          -1.528   4.242  10.011                 (----------*-----------)
                                  --------+---------+---------+---------+-
                                       -5.0       0.0       5.0      10.0


Treatment = 2 subtracted from:
```

```
Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
3          -2.750   3.020  8.790               (----------*----------)
                                  --------+---------+---------+---------+-
                                       -5.0       0.0       5.0      10.0
```

7.2.2.1c One-way ANOVA: Day 5 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2    730   365  0.21  0.812
Error      15  25907  1727
Total      17  26638

S = 41.56   R-Sq = 2.74%   R-Sq(adj) = 0.00%


                        Individual 80% CIs For Mean Based on
                        Pooled StDev
Level  N   Mean  StDev  -+---------+---------+---------+--------
1      6  93.45  44.03           (--------------*--------------)
2      6  80.52  38.80  (--------------*--------------)
3      6  94.55  41.68            (--------------*--------------)
                        -+---------+---------+---------+--------
                        60        75        90       105

Pooled StDev = 41.56


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
2          -56.36  -12.93  30.51  (--------------*-------------)
3          -42.33    1.11  44.54       (-------------*--------------)
                                  ---------+---------+---------+---------+
                                         -30         0        30        60


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
3          -29.40   14.03  57.47            (--------------*-------------)
                                  ---------+---------+---------+---------+
                                         -30         0        30        60
```

7.2.2.1d One-way ANOVA: Day 9 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2   1072   536  0.17  0.844
Error      15  46695  3113
Total      17  47767

S = 55.79   R-Sq = 2.24%   R-Sq(adj) = 0.00%
```

```
                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      6  139.49  56.57          (---------------*--------------)
2      6  122.36  51.82  (--------------*--------------)
3      6  137.84  58.77          (--------------*--------------)
                          ----+---------+---------+---------+-----
                            100       120       140       160

Pooled StDev = 55.79


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
2         -75.44  -17.13  41.18  (--------------*-------------)
3         -59.96   -1.65  56.66       (--------------*-------------)
                                  ---------+---------+---------+---------+
                                         -40         0        40        80


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
3         -42.83   15.48  73.79           (--------------*-------------)
                                  ---------+---------+---------+---------+
                                         -40         0        40        80
```

7.2.2.1e One-way ANOVA: Day 13 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2   1103   552  0.18  0.835
Error      15  45345  3023
Total      17  46448

S = 54.98   R-Sq = 2.37%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
1      6  162.18  53.46          (--------------*--------------)
2      6  146.89  57.32  (--------------*--------------)
3      6  164.55  54.08           (--------------*--------------)
                          --+---------+---------+---------+-------
                          120       140       160       180

Pooled StDev = 54.98


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%
```

```
Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
2          -72.75  -15.29  42.17   (-------------*--------------)
3          -55.08    2.38  59.84       (--------------*-------------)
                                  ---------+---------+---------+---------+
                                         -40         0        40        80


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
3          -39.79   17.67  75.13           (-------------*--------------)
                                  ---------+---------+---------+---------+
                                         -40         0        40        80
```

7.2.2.1f One-way ANOVA: Day 21 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2   2740  1370  0.67  0.524
Error      15  30486  2032
Total      17  33225

S = 45.08   R-Sq = 8.25%   R-Sq(adj) = 0.00%


                         Individual 80% CIs For Mean Based on Pooled
                         StDev
Level  N    Mean  StDev    +---------+---------+---------+---------
1      6  234.64  22.95                  (-----------*-----------)
2      6  204.89  55.85    (-----------*-----------)
3      6  215.19  49.51         (-----------*-----------)
                           +---------+---------+---------+---------
                         180       200       220       240

Pooled StDev = 45.08


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  --+---------+---------+---------+-------
2          -76.87  -29.75  17.36  (------------*-------------)
3          -66.57  -19.45  27.67     (------------*-------------)
                                  --+---------+---------+---------+-------
                                  -70       -35         0        35


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  --+---------+---------+---------+-------
3          -36.81   10.31  57.42             (-------------*------------)
                                  --+---------+---------+---------+-------
                                  -70       -35         0        35
```

7.2.2.2 One-Way Anova and Tukey's Post-Hoc Analysis for C14-AHL ORP Data.

Treatment 1: C14-AHL Group
Treatment 2: Solvent Control Group
Treatment 3: Untreated Control Group

7.2.2.2a One-way ANOVA: Day 0 versus Treatment

```
Source     DF    SS   MS     F      P
Treatment   2   253  126  1.11  0.354
Error      15  1704  114
Total      17  1957

S = 10.66   R-Sq = 12.92%   R-Sq(adj) = 1.31%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev   ---+---------+---------+---------+------
1      6  340.00   9.49   (---------*--------)
2      6  340.83  10.68    (---------*---------)
3      6  348.33  11.69                (---------*--------)
                          ---+---------+---------+---------+------
                         336.0     342.0     348.0     354.0

Pooled StDev = 10.66
```

7.2.2.2b One-way ANOVA: Day 2 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2    253   126  0.04  0.964
Error      15  51138  3409
Total      17  51390

S = 58.39   R-Sq = 0.49%   R-Sq(adj) = 0.00%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      6  413.33  62.58       (---------------*---------------)
2      6  408.33  58.02    (---------------*---------------)
3      6  404.17  54.26  (---------------*---------------)
                         ----+---------+---------+---------+-----
                           380       400       420       440

Pooled StDev = 58.39


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 90.97%
```

7.2.2.2c One-way ANOVA: Day 5 versus Treatment

```
Source     DF     SS    MS     F      P
```

```
Treatment   2    169    85  0.02  0.976
Error      15  51875  3458
Total      17  52044

S = 58.81   R-Sq = 0.33%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ---+---------+---------+---------+------
1      6  510.83  62.88    (---------------*---------------)
2      6  506.67  60.39  (---------------*---------------)
3      6  514.17  52.67      (---------------*---------------)
                          ---+---------+---------+---------+------
                           480       500       520       540

Pooled StDev = 58.81
```

7.2.2.2d One-way ANOVA: Day 9 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2    411   206  0.07  0.936
Error      15  46513  3101
Total      17  46924

S = 55.69   R-Sq = 0.88%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ---+---------+---------+---------+------
1      6  555.83  57.31        (--------------*--------------)
2      6  544.17  61.35  (--------------*--------------)
3      6  549.17  47.48    (--------------*--------------)
                          ---+---------+---------+---------+------
                           520       540       560       580

Pooled StDev = 55.69
```

7.2.2.2e One-way ANOVA: Day 13 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   2    308   154  0.06  0.943
Error      15  39192  2613
Total      17  39500

S = 51.12   R-Sq = 0.78%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
1      6  574.17  46.41       (-------------*-------------)
2      6  565.00  58.99   (-------------*------------)
3      6  565.83  46.95   (-------------*-------------)
                           -+---------+---------+---------+--------
                          540       560       580       600
```

```
Pooled StDev = 51.12
```

7.2.2.2f One-way ANOVA: Day 21 versus Treatment

```
Source     DF     SS   MS     F      P
Treatment   2   1169  585  0.60  0.563
Error      15  14688  979
Total      17  15857

S = 31.29   R-Sq = 7.37%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
1      6  608.33  18.62             (-----------*----------)
2      6  590.83  38.26  (-----------*----------)
3      6  591.67  33.57   (----------*-----------)
                          --------+---------+---------+---------+-
                                585       600       615       630

Pooled StDev = 31.29
```

7.2.3.1 One-Way Anova and Tukey's Post-Hoc Analysis for C14-MIX Nickel Data.

Treatment 1: C14-AHL Group
Treatment 2: Solvent Control Group
Treatment 3: Untreated Control Group
Treatment 4: C14-MIX Group

7.2.3.1a One-way ANOVA: Day 0 versus Treatment

```
Source     DF      SS    MS     F      P
Treatment   3     2.9   1.0  0.03  0.993
Error      32  1001.0  31.3
Total      35  1003.8

S = 5.593   R-Sq = 0.28%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
1      9  10.643  5.454   (---------------*---------------)
2      9  10.984  5.862     (---------------*---------------)
3      9  11.189  6.002      (----------------*---------------)
4      9  10.469  4.998  (---------------*---------------)
                          ------+---------+---------+---------+---
                              9.0      10.5      12.0      13.5

Pooled StDev = 5.593


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%
```

```
Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
2          -4.990   0.342  5.674     (--------------*--------------)
3          -4.786   0.546  5.878     (---------------*--------------)
4          -5.506  -0.174  5.158   (---------------*--------------)
                                  -------+---------+---------+---------+--
                                      -3.5       0.0       3.5       7.0


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
3          -5.127   0.204  5.536    (---------------*--------------)
4          -5.847  -0.516  4.816  (---------------*--------------)
                                  -------+---------+---------+---------+--
                                      -3.5       0.0       3.5       7.0


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
4          -6.052  -0.720  4.612  (--------------*--------------)
                                  -------+---------+---------+---------+--
                                      -3.5       0.0       3.5       7.0
```

7.2.3.1b One-way ANOVA: Day 2 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   3   77.1  25.7  1.00  0.404
Error      32  819.3  25.6
Total      35  896.4

S = 5.060   R-Sq = 8.60%   R-Sq(adj) = 0.04%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
1      9  24.987  4.728           (--------*--------)
2      9  26.466  6.193                 (--------*--------)
3      9  25.790  5.023              (--------*--------)
4      9  22.591  4.058  (-------*--------)
                          --------+---------+---------+---------+-
                               22.5      25.0      27.5      30.0

Pooled StDev = 5.060


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
2          -3.345   1.479  6.303            (---------*---------)
```

```
3          -4.021   0.803  5.627           (---------*--------)
4          -7.220  -2.396  2.427     (--------*---------)
                                  -------+---------+---------+---------+--
                                      -5.0       0.0       5.0      10.0


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
3          -5.500  -0.676  4.148        (---------*--------)
4          -8.699  -3.875  0.949  (--------*---------)
                                  -------+---------+---------+---------+--
                                      -5.0       0.0       5.0      10.0


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  -------+---------+---------+---------+--
4          -8.023  -3.199  1.625   (---------*--------)
                                  -------+---------+---------+---------+--
                                      -5.0       0.0       5.0      10.0
```

7.2.3.1c One-way ANOVA: Day 5 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   3   3280  1093  0.97  0.421
Error      32  36246  1133
Total      35  39526

S = 33.66   R-Sq = 8.30%   R-Sq(adj) = 0.00%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+
1      9  115.95  35.48         (--------*---------)
2      9  105.71  30.98  (--------*---------)
3      9  109.66  31.82    (---------*---------)
4      9  130.80  36.05                  (---------*---------)
                         ---------+---------+---------+---------+
                                 105       120       135       150

Pooled StDev = 33.66


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
2          -42.33  -10.24  21.84       (----------*---------)
3          -38.37   -6.29  25.80        (----------*----------)
4          -17.23   14.86  46.94               (----------*----------)
                                  ---------+---------+---------+---------+
                                         -30         0        30        60
```

```
Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
3          -28.13    3.96  36.04            (---------*----------)
4           -6.99   25.10  57.18                   (---------*----------)
                                  ---------+---------+---------+---------+
                                         -30         0        30        60


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
4          -10.94   21.14  53.23                (----------*----------)
                                  ---------+---------+---------+---------+
                                         -30         0        30        60
```

7.2.3.1d One-way ANOVA: Day 9 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   3   6177  2059  1.97  0.138
Error      32  33436  1045
Total      35  39613

S = 32.32   R-Sq = 15.59%   R-Sq(adj) = 7.68%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
1      9  201.75  42.56      (--------*--------)
2      9  194.78  39.25  (--------*--------)
3      9  206.20  24.18         (--------*--------)
4      9  229.66  15.58                       (--------*-------)
                         -------+---------+---------+---------+--
                              192       208       224       240

Pooled StDev = 32.32


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
2          -37.78   -6.97  23.85          (--------*--------)
3          -26.36    4.46  35.27             (--------*--------)
4           -2.90   27.92  58.73                    (--------*--------)
                                  ---------+---------+---------+---------+
                                         -35         0        35        70


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  ---------+---------+---------+---------+
3          -19.39   11.43  42.24               (--------*--------)
4            4.07   34.88  65.70                     (--------*--------)
                                  ---------+---------+---------+---------+
```

```
                                    -35         0        35        70

Treatment = 3 subtracted from:

Treatment  Lower  Center  Upper  ---------+---------+---------+---------+
4          -7.36   23.46  54.27                  (--------*--------)
                                 ---------+---------+---------+---------+
                                        -35         0        35        70
```

7.2.3.1e One-way ANOVA: Day 16 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   3   4042  1347  1.03  0.394
Error      32  42006  1313
Total      35  46048

S = 36.23   R-Sq = 8.78%   R-Sq(adj) = 0.23%


                         Individual 80% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+
1      9  245.33  49.03    (----------*---------)
2      9  241.66  34.74  (---------*----------)
3      9  248.43  31.67      (----------*---------)
4      9  268.97  25.24                   (---------*----------)
                         ---------+---------+---------+---------+
                                240       255       270       285

Pooled StDev = 36.23


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
2         -38.21   -3.67  30.87         (---------*---------)
3         -31.43    3.11  37.65           (---------*---------)
4         -10.89   23.65  58.19                 (---------*---------)
                                --------+---------+---------+---------+-
                                      -35         0        35        70


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
3         -27.77    6.77  41.31           (---------*---------)
4          -7.23   27.32  61.86                 (---------*---------)
                                --------+---------+---------+---------+-
                                      -35         0        35        70


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
```

```
4          -14.00   20.54  55.08               (---------*---------)
                                    --------+---------+---------+---------+-
                                          -35         0        35        70
```

7.2.3.1f One-way ANOVA: Day 23 versus Treatment

```
Source     DF     SS    MS     F      P
Treatment   3   3224  1075  1.20  0.324
Error      32  28547   892
Total      35  31771

S = 29.87   R-Sq = 10.15%   R-Sq(adj) = 1.72%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
1      9  302.33  40.58           (--------*-------)
2      9  287.96  33.11  (--------*--------)
3      9  298.31  24.70         (--------*--------)
4      9  314.39  14.67                   (--------*-------)
                          -------+---------+---------+---------+--
                               285       300       315       330

Pooled StDev = 29.87


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
2          -42.85  -14.37  14.10      (--------*---------)
3          -32.49   -4.02  24.46         (---------*--------)
4          -16.41   12.06  40.54               (--------*---------)
                                    --------+---------+---------+---------+-
                                          -30         0        30        60


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
3          -18.12   10.36  38.83              (--------*---------)
4           -2.04   26.44  54.91                   (---------*--------)
                                    --------+---------+---------+---------+-
                                          -30         0        30        60


Treatment = 3 subtracted from:

Treatment   Lower  Center  Upper  --------+---------+---------+---------+-
4          -12.39   16.08  44.55                (--------*---------)
                                    --------+---------+---------+---------+-
                                          -30         0        30        60
```

7.2.3.1g One-way ANOVA: Day 30 versus Treatment

```
Source     DF     SS   MS     F      P
Treatment   3   2081  694  1.74  0.180
Error      32  12794  400
Total      35  14875

S = 20.00   R-Sq = 13.99%   R-Sq(adj) = 5.93%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      9  309.34  29.35       (-------*--------)
2      9  304.59  20.07  (--------*-------)
3      9  309.71  17.41       (--------*-------)
4      9  324.81   5.66                      (--------*--------)
                          ----+---------+---------+---------+-----
                            300       310       320       330

Pooled StDev = 20.00


Tukey 80% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Treatment

Individual confidence level = 94.84%


Treatment = 1 subtracted from:

Treatment   Lower  Center  Upper    +---------+---------+---------+---------
2          -23.81   -4.75  14.31             (---------*--------)
3          -18.70    0.36  19.43                (--------*---------)
4           -3.60   15.47  34.53                       (---------*--------)
                             +---------+---------+---------+---------
                           -40       -20         0        20


Treatment = 2 subtracted from:

Treatment   Lower  Center  Upper    +---------+---------+---------+---------
3          -13.95    5.12  24.18                (---------*--------)
4            1.16   20.22  39.28                         (--------*---------)
                             +---------+---------+---------+---------
                           -40       -20         0        20


Treatment = 3 subtracted from:

Treatment  Lower  Center  Upper    +---------+---------+---------+---------
4          -3.96   15.10  34.16                     (---------*--------)
                             +---------+---------+---------+---------
                           -40       -20         0        20
```

7.2.3.2 One-Way Anova for C14-MIX ORP Data.

Treatment 1: C14-AHL Group
Treatment 2: Solvent Control Group
Treatment 3: Untreated Control Group
Treatment 4: C14-MIX Group

7.2.3.2a One-way ANOVA: Day 0 versus Treatment

```
Source     DF    SS   MS     F      P
Treatment   3   125   42  0.41  0.750
Error      32  3294  103
Total      35  3419

S = 10.15   R-Sq = 3.66%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
1      9  348.89  10.83  (------------*-----------)
2      9  352.78  12.53             (------------*------------)
3      9  352.78   9.72             (------------*------------)
4      9  349.22   6.55   (------------*-----------)
                          ------+---------+---------+---------+---
                            346.5     350.0     353.5     357.0

Pooled StDev = 10.15
```

7.2.3.2b One-way ANOVA: Day 2 versus Treatment

```
Source     DF     SS   MS     F      P
Treatment   3    672  224  0.29  0.833
Error      32  24817  776
Total      35  25489

S = 27.85   R-Sq = 2.64%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
1      9  372.78  17.34     (-----------*-----------)
2      9  378.89  34.80           (-----------*-----------)
3      9  370.00  16.58  (-----------*-----------)
4      9  380.56  36.27            (------------*-----------)
                          --+---------+---------+---------+-------
                          360       370       380       390

Pooled StDev = 27.85
```

7.2.3.2c One-way ANOVA: Day 5 versus Treatment

```
Source     DF      SS    MS     F      P
Treatment   3    2483   828  0.14  0.938
Error      32  194252  6070
Total      35  196735

S = 77.91   R-Sq = 1.26%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
```

```
1      9  511.67  70.49        (-------------*------------)
2      9  495.67  86.46  (------------*-------------)
3      9  515.00  58.84         (-------------*-------------)
4      9  498.33  91.52   (------------*-------------)
                          -----+---------+---------+---------+----
                             475       500       525       550

Pooled StDev = 77.91
```

7.2.3.2d One-way ANOVA: Day 9 versus Treatment

```
Source     DF     SS   MS     F      P
Treatment   3   1285  428  1.12  0.356
Error      32  12234  382
Total      35  13519

S = 19.55   R-Sq = 9.50%   R-Sq(adj) = 1.02%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
1      9  597.22  13.72        (-------*--------)
2      9  591.11  29.56  (-------*--------)
3      9  607.78  15.63                  (--------*-------)
4      9  599.56  14.93          (--------*-------)
                          -------+---------+---------+---------+--
                               590       600       610       620

Pooled StDev = 19.55
```

7.2.3.2e One-way ANOVA: Day 16 versus Treatment

```
Source     DF     SS   MS     F      P
Treatment   3    225   75  0.18  0.908
Error      32  13256  414
Total      35  13481

S = 20.35   R-Sq = 1.67%   R-Sq(adj) = 0.00%


                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev   ---+---------+---------+---------+------
1      9  617.78  20.48      (------------*-----------)
2      9  622.78  16.79             (------------*-----------)
3      9  616.11  16.16   (------------*------------)
4      9  617.78  26.35      (------------*-----------)
                           ---+---------+---------+---------+------
                          609.0     616.0     623.0     630.0

Pooled StDev = 20.35
```

7.2.3.2f One-way ANOVA: Day 23 versus Treatment

```
Source     DF      SS    MS     F      P
Treatment   3   202.8  67.6  0.98  0.415
Error      32  2211.1  69.1
Total      35  2413.9
```

```
S = 8.312   R-Sq = 8.40%   R-Sq(adj) = 0.00%

                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
1      9  628.89  10.54  (----------*---------)
2      9  635.00   5.59                    (---------*----------)
3      9  633.33   7.07               (----------*---------)
4      9  630.56   9.17       (----------*---------)
                          ----+---------+---------+---------+-----
                          626.5     630.0     633.5     637.0

Pooled StDev = 8.31
```

7.2.3.2g One-way ANOVA: Day 30 versus Treatment

```
Source     DF      SS    MS     F      P
Treatment   3   113.9  38.0  0.44  0.726
Error      32  2761.1  86.3
Total      35  2875.0

S = 9.289   R-Sq = 3.96%   R-Sq(adj) = 0.00%

                          Individual 80% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+
1      9  638.89   9.28               (-----------*-----------)
2      9  638.33   6.12             (-----------*----------)
3      9  638.33  13.46             (-----------*----------)
4      9  634.44   6.35  (-----------*----------)
                          ---------+---------+---------+---------+
                               633.5     637.0     640.5     644.0

Pooled StDev = 9.29
```

7.3 Raw Data

Raw data is reported in units of either PPM for values of nickel in solution, as provided by ICP-AES, or in mV for values of ORP as reported by a Hach portable ORP meter.

7.3.1 DMSO Toxicity Experiment Nickel Data

Control			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.15333	0.153105	15.54063
	0.14823		

	0.15665		
	0.15421		
	0.15917	0.157708	
	0.15156		
	0.16062		
	0.15948		
12-Apr	0.5334	0.53473	53.00325
	0.53238		
	0.53763		
	0.53551		
	0.52565	0.525335	
	0.52331		
	0.52858		
	0.5238		
15-Apr	1.1313	1.13035	109.8475
	1.1291		
	1.1331		
	1.1279		
	1.0685	1.0666	
	1.0652		
	1.067		
	1.0657		
22-Apr	3.3547	3.345375	332.0838
	3.3367		
	3.358		
	3.3321		
	3.3096	3.2963	
	3.2833		
	3.3036		
	3.2887		
26-Apr	3.4415	3.4311	347.715
	3.4237		
	3.4371		
	3.4221		
	3.534	3.5232	
	3.5107		
	3.5308		
	3.5173		
2-May	3.654	3.642375	344.0975
	3.627		
	3.6545		
	3.634		

	3.2559	3.239575	
	3.2282		
	3.2575		
	3.2167		

Abiotic			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.16127	0.159868	17.80388
	0.15367		
	0.16418		
	0.16035		
	0.19554	0.19621	
	0.19272		
	0.19879		
	0.19779		
12-Apr	0.093416	0.093107	9.99435
	0.091103		
	0.094197		
	0.093712		
	0.10646	0.10678	
	0.10579		
	0.10734		
	0.10753		
15-Apr	0.19129	0.191765	19.80938
	0.19109		
	0.1934		
	0.19128		
	0.20418	0.204423	
	0.20335		
	0.20517		
	0.20499		
22-Apr	0.85401	0.850025	81.54788
	0.84625		
	0.85213		
	0.84771		
	0.78154	0.780933	
	0.77679		
	0.78524		
	0.78016		
26-Apr	1.206	1.203275	119.16
	1.2024		

	1.2076		
	1.1971		
	1.1793	1.179925	
	1.1811		
	1.1867		
	1.1726		
2-May	1.8331	1.830025	224.6688
	1.8289		
	1.8382		
	1.8199		
	2.6688	2.66335	
	2.6546		
	2.6816		
	2.6484		

1.0% DMSO			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.19562	0.196155	19.59488
	0.19519		
	0.19862		
	0.19519		
	0.19576	0.195743	
	0.19441		
	0.19795		
	0.19485		
12-Apr	0.83809	0.837875	76.72163
	0.83796		
	0.83864		
	0.83681		
	0.69653	0.696558	
	0.69385		
	0.69987		
	0.69598		
15-Apr	0.7517	0.751395	78.28013
	0.74877		
	0.75527		
	0.74984		
	0.81534	0.814208	
	0.8123		
	0.81721		

	0.81198		
22-Apr	1.1531	1.151875	117.925
	1.1516		
	1.1573		
	1.1455		
	1.2094	1.206625	
	1.2009		
	1.2134		
	1.2028		
26-Apr	1.4771	1.4707	156.5638
	1.4677		
	1.4775		
	1.4605		
	1.663	1.660575	
	1.6608		
	1.6693		
	1.6492		
2-May	2.6453	2.639375	266.1875
	2.6304		
	2.6555		
	2.6263		
	2.6946	2.684375	
	2.6688		
	2.7016		
	2.6725		

0.5% DMSO			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.20093	0.200718	19.721
	0.19903		
	0.20257		
	0.20034		
	0.19431	0.193703	
	0.18891		
	0.19604		
	0.19555		
12-Apr	0.7954	0.794773	79.11275
	0.79239		
	0.79642		
	0.79488		

	0.78867	0.787483	
	0.78502		
	0.7891		
	0.78714		
15-Apr	0.81055	0.807178	81.33738
	0.80592		
	0.80577		
	0.80647		
	0.82239	0.81957	
	0.81533		
	0.82373		
	0.81683		
22-Apr	1.2443	1.24275	122.3688
	1.2409		
	1.2487		
	1.2371		
	1.2055	1.204625	
	1.2054		
	1.2099		
	1.1977		
26-Apr	1.5722	1.5691	158.3288
	1.5684		
	1.5753		
	1.5605		
	1.6011	1.597475	
	1.5931		
	1.6057		
	1.59		
2-May	2.5794	2.570325	259.4588
	2.5585		
	2.5841		
	2.5593		
	2.6263	2.61885	
	2.6074		
	2.6368		
	2.6049		

0.1% DMSO			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.19895	0.19937	19.87738

	0.19818		
	0.20072		
	0.19963		
	0.19816	0.198178	
	0.19715		
	0.19995		
	0.19745		
12-Apr	0.79267	0.79462	78.08625
	0.79578		
	0.7968		
	0.79323		
	0.76719	0.767105	
	0.7645		
	0.7702		
	0.76653		
15-Apr	0.88035	0.880728	85.18113
	0.87793		
	0.88624		
	0.87839		
	0.82386	0.822895	
	0.81787		
	0.82578		
	0.82407		
22-Apr	1.3154	1.31335	125.1175
	1.3114		
	1.3188		
	1.3078		
	1.1879	1.189	
	1.1879		
	1.1954		
	1.1848		
26-Apr	1.8827	1.880825	177.105
	1.8788		
	1.8913		
	1.8705		
	1.6652	1.661275	
	1.6537		
	1.6713		
	1.6549		
2-May	2.9323	2.92355	285.7263
	2.9103		
	2.9423		

	2.9093		
	2.8012	2.790975	
	2.7787		
	2.8102		
	2.7738		

0.05% DMSO			
	Raw Data (in PPM, 100* Diluted)	**Average of 4 measurements**	**Reported (After 100* Dilution Factor, 2 replicates)**
10-Apr	0.19938	0.200385	19.9525
	0.19999		
	0.20197		
	0.2002		
	0.19796	0.198665	
	0.1985		
	0.19988		
	0.19832		
12-Apr	0.75004	0.747945	73.82663
	0.74828		
	0.75031		
	0.74315		
	0.73018	0.728588	
	0.72626		
	0.73019		
	0.72772		
15-Apr	0.80666	0.80681	82.53513
	0.80788		
	0.80886		
	0.80384		
	0.8459	0.843893	
	0.84047		
	0.84669		
	0.84251		
22-Apr	1.3887	1.3878	134.16
	1.3901		
	1.3947		
	1.3777		
	1.3007	1.2954	
	1.2915		
	1.3011		
	1.2883		

26-Apr	2.0943	2.091725	204.835
	2.0833		
	2.1018		
	2.0875		
	2.0099	2.004975	
	2.0016		
	2.0136		
	1.9948		
2-May	2.9125	2.89975	295.1025
	2.8872		
	2.9158		
	2.8835		
	3.0163	3.0023	
	2.9873		
	3.0187		
	2.9869		

7.3.2 DMSO Toxicity Experiment ORP Raw Data

Day	Abiotic		Control		1.0% DMSO		0.5% DMSO		0.1% DMSO		0.05% DMSO	
0	356	381	392	392	378	380	382	380	378	375	383	380
2	424	426	379	371	364	369	368	371	370	375	370	370
5	409	410	451	446	365	362	365	364	372	365	367	369
12	397	405	655	655	361	360	362	362	367	365	369	373
16	389	389	664	650	368	370	369	371	380	375	391	390
23	459	599	653	655	382	385	385	387	408	400	419	417

7.3.3 C14-AHL Treatment Raw Ni Data

	Experiment - Sept 19			Experiment - Oct 31		
	C14 1	C14 2	C14 3	C14 1	C14 2	C14 3

0	12.74596	11.47345	12.51979	12.06381	10.74818	10.9414
2	33.42217	28.90894	35.17728	26.93333	22.46586	22.32535
5	145.3252	96.74007	147.018	68.16	51.82109	51.61838
9	198.7062	157.3642	204.2445	120.5	84.1086	72.00799
13	209.2118	181.7069	224.4295	156.9307	109.2245	91.54691
21	255.1513	218.1564	269.8963	228.3151	225.755	210.5894

	Experiment - Sept 19			**Experiment - Oct 31**		
	Solvent 1	Solvent 2	Solvent 3	Solvent 1	Solvent 2	Solvent 3
0	14.09	11.2056	12.16392	13.50529	12.72358	12.67121
2	31.78954	22.24551	23.86704	31.38972	37.80037	29.47042
5	50.56247	43.76812	68.88933	93.17087	150.9488	75.79107
9	128.3433	64.28714	70.96098	143.3639	205.6275	121.5672
13	177.9424	78.64136	88.02353	176.6928	226.165	133.8604
21	251.5723	129.3706	156.8373	216.2545	277.2	198.0989

	Experiment - Sept 19			**Experiment - Oct 31**		
	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3
0	13.48059	11.39632	14.59082	15.24716	13.29354	14.45082
2	30.53787	22.85573	31.45978	37.35456	36.60209	35.87224
5	68.09405	41.99104	69.17951	126.6631	109.0583	152.3402
9	119.0286	43.07079	115.9	181.835	209.3846	157.8089
13	147.7921	73.23353	153.9	189.3538	232.3098	190.7397
21	221.3474	120.597	218.4631	234.1644	267.1233	229.4709

7.3.4 C14-AHL Treatment Raw ORP Data

Experiment 19th-Sep									
Day	**C14 1**	**C14 2**	**C14 3**	**DE 1**	**DE 2**	**DE 3**	**Control 1**	**Control 2**	**Control 3**
0	335	330	330	345	330	325	340	330	350
2	350	355	365	365	345	360	350	350	365

5	480	450	435	445	455	455	480	455	465
9	535	500	485	535	465	480	535	475	530
13	565	530	510	570	480	505	555	485	550
23	595	590	590	600	545	540	590	530	585

Experiment 31st-Oct									
Day	**C14 1**	**C14 2**	**C14 3**	**DE 1**	**DE 2**	**DE 3**	**Control 1**	**Control 2**	**Control 3**
0	350	350	345	350	350	360	350	355	370
2	470	460	480	450	475	450	460	470	435
5	565	570	565	560	580	560	565	565	560
9	615	585	615	585	615	570	615	615	610
13	615	610	615	610	605	595	615	615	615
23	630	625	620	620	620	610	625	620	630

7.3.5 C14-MIX Treatment Raw Ni Data

	17-Jul			**12-Mar**			**15-May**		
	C14-AHL 1	**C14-AHL 2**	**C14-AHL 3**	**C14-AHL 1**	**C14-AHL 2**	**C14-AHL 3**	**C14-AHL 1**	**C14-AHL 2**	**C14-AHL 3**
Day 0	17.455	18.5	17.71	7.23	7.42	7.4	7.24	6.55	6.28
Day 2	26.3703	30.4989	28.5388	27.27968	27.94162	27.67065	18.72064	19.2011	18.66342
Day 6	66.014	77.856	93.531	106.653	160.9319	160.9437	110.835	154.0751	112.6995
Day 9	150.495	223.912	234.541	180.1558	245.1551	259.1463	156.0181	213.7349	152.5545
Day 16	179.545	246.573	254.829	240.5	325.4875	310.2478	222.7527	261.1437	214.0038
Day 23	231.506	287.345	296.617	289.8049	350.1667	338.8672	271.3914	307.6188	292.1539
Day 30	263.493	309.183	318.068	314.818	354.3	365.2378	287.6706	297.5643	287.3563

	17-Jul			**12-Mar**			**15-May**		
	Solvent 1	Solvent 2	Solvent 3	Solvent 1	Solvent 2	Solvent 3	Solvent 1	Solvent 2	Solvent 3
Day 0	18.55	17.89	19.79	7.83	7.94	6.7	6.32	7.12	6.72
Day 2	31.0407	30.6169	34.8698	30.85074	29.03555	24.73505	19.25864	20.02592	17.76076
Day 6	96.853	92.374	79.466	129.7282	72.9806	66.27636	122.6238	139.4349	151.6097
Day 9	221.175	196.187	192.258	240.0878	191.754	99.34565	195.649	201.8435	214.6956
Day 16	249.108	240.713	232.317	289.2	258.3292	161.5635	236.6955	258.4578	271.5855
Day 23	292.404	292.549	289.305	327.745	293.8639	182.5005	284.3147	282.487	252.7037
Day 30	311.912	311.961	306.939	350.0228	313.872	233.9648	297.857	320.0112	312.5124

	17-Jul			**12-Mar**			**15-May**		
	Untreated 1	Untreated 2	Untreated 3	Untreated 1	Untreated 2	Untreated 3	Untreated 1	Untreated 2	Untreated 3
Day 0	19.03	20.47	17.74	6.3	6.32	6.96	8.3	8.39	7.19
Day 2	29.9888	34.2252	29.558	26.86164	22.25777	27.22602	19.05233	21.9582	20.9821
Day 6	75.095	85.242	95.07	108.4154	76.69974	118.4732	173.2294	138.8429	115.8781

Day 9	181.945	192.258	245.7	215.3225	210.9	200.3571	233.5034	207.7003	168.1356
Day 16	210.881	203.231	260.957	281.2412	250.175	281.3249	293.7162	263.8098	228.5963
Day 23	274.941	251.597	295.523	285.1283	290.6036	321.5363	320.8486	306.4	283.0239
Day 30	296.982	271.567	312.462	308.2822	314.7849	329.425	326.7452	330.1036	311.2762

	17-Jul			**12-Mar**			**15-May**		
	Mix 1	Mix 2	Mix 3	Mix 1	Mix 2	Mix 3	Mix 1	Mix 2	Mix 3
Day 0	17	17.03	16.89	6.46	5.76	6.67	9.44	6.65	8.32
Day 2	26.9692	27.1893	27.2035	23.44349	19.56537	23.77493	20.07807	16.62499	18.46891
Day 6	88.338	85.74	87.873	168.6533	166.8513	138.3316	172.1891	124.9694	144.2918
Day 9	228.806	217.509	228	245.0892	258.2765	235.0498	225.6254	203.898	224.6952
Day 16	257.494	246.126	254.714	306.32	313.29	278.83	263.9059	245.5611	254.5341
Day 23	301.441	299.9	297.273	338.12	337.53	305.98	309.0968	312.2119	311.8224
Day 30	321.949	325.97	320.996	332.36	335.58	329.86	319.7324	321.3331	318.5274

7.3.6 C14-MIX Treatment Raw ORP Data

	Experiment 17-Jul											
Day	**C14-AHL 1**	**C14-AHL 2**	**C14-AHL 3**	**Solvent 1**	**Solvent 2**	**Solvent 3**	**Control 1**	**Control 2**	**Control 3**	**Mix 1**	**Mix 2**	**Mix 3**
Day 0	360	370	355	360	360	380	355	375	355	355	360	355

Day 2	360	365	340	345	345	360	365	355	340	345	345	345
Day 5	450	420	390	385	370	390	420	465	435	370	375	385
Day 9	585	595	595	590	575	575	580	615	605	585	590	600
Day 16	620	625	620	615	615	625	585	625	625	610	615	625
Day 23	635	640	645	645	640	640	630	635	650	645	640	635
Day 30	650	645	655	640	645	650	650	655	650	625	640	645

7.3.7 Failed Experiment ORP Raw Data

	Day 0	**Day 1**	**Day 5**	**Day 8**
C14 1	**360**	**345**	**360**	**365**
C14 2	355	350	365	360
C14 3	350	350	370	355
Solvent 1	340	336	360	450
Solvent 2	355	350	375	440
Solvent 3	350	340	360	460
Control 1	350	340	470	565
Control 2	350	345	450	555
Control 3	350	355	485	560