The Effects of Lead (II) Nitrate on EPS Production by *Colwellia psychrerythraea* 34H and the Metal-Binding Capacity of the EPS under Freezing Conditions

Anaïs Gentilhomme

School of Oceanography

University of Washington

anais@agentilhomme.com

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Abstract

We examined how [Pb^{2+}] affects the growth of the bacterium, *Colwellia psychrerythraea* as well as the bacterium's ability to produce extracellular polysaccharide substances (EPS). In addition, we explored the potential binding capacity of EPS and [Pb^{2+}] through freezing trials that included both ice and liquid phases. Earlier studies have explored the effects of EPS on other bacteria or algae. In these experiments we focused on bacterial growth and the impacts on EPS production using the phenol-sulfuric acid method of Dubois et al. (1956) to measure EPS, an ICPMS machine to measure [Pb^{2+}], and a series of freezing experiments to examine selective retention of EPS or lead in ice. The growth data suggests that there may be a threshold or "tipping point" between 0.1 mg/L and 1 mg/L of [Pb^{2+}] up to which there is more EPS produced than when there is no lead present. However, at a high lead amount (1 mg/L), the culture was completely unable to grow or produce EPS. At low levels of lead (0.1 mg/L) there was possible binding activity between EPS and the heavy metal. This effect was not evident when 1 mg/L of lead was introduced to the system, which we attribute to as an oversaturation of the EPS such that no binding could take place.

Introduction

Lead Pb^{2+} has a wide range of negative effects at toxic levels ranging from behavioral and neurological damage to environmental bioaccumulation at toxic levels. For example, mussels and barnacles have been observed to accumulate as much as 10 parts per million (ppm) of Pb^{2+}, which was found to be detrimental to their growth, trigger anemia, and reduce egg-hatching (Lenntech, 2016). Studies of marine bacteria have also shown that "seawater bacteria from lead-contaminated cultures [can] adapt to lead pollution" (Tan, 1980). In aqueous chemistry lead nitrate readily dissolves in water and dissociates into [Pb^{2+}] and [NO_3^-] to give a clear colorless solution (Lenntech, 2016) which is not readily observable in the environment.

One way that organisms protect themselves and adapt, especially microorganisms in harsh environments, is through the production of extracellular polysaccharide substances (EPS) (Ewert et al., 2013). These defensive mechanisms provide protection not only in harsh environmental factors, but also provide protection in the presence of man-made chemicals that contain traces of heavy metals such as Pb^{2+} (Ewert et al., 2013).

Previous studies have analyzed how EPS forms, as well as to environmental factors such as light and temperature affect its production. One culture-based study demonstrated that the EPS-producing freshwater cyanobacterium, *Microcystis aeruginosa*, has a high tolerance to [Pb^{2+}]; it was also stimulated to produce more [EPS] at the highest metal concentrations tested (Bi et al., 2013). Although a low temperature of 4°C was used in that study, freezing conditions and the impacts of ice formation were not considered (Bi et al. 2013). The purpose of this experiment was to test the effects that different concentrations of lead may have on [EPS] production and its ability to bind to the cryoprotectant compound. My first hypothesis examines

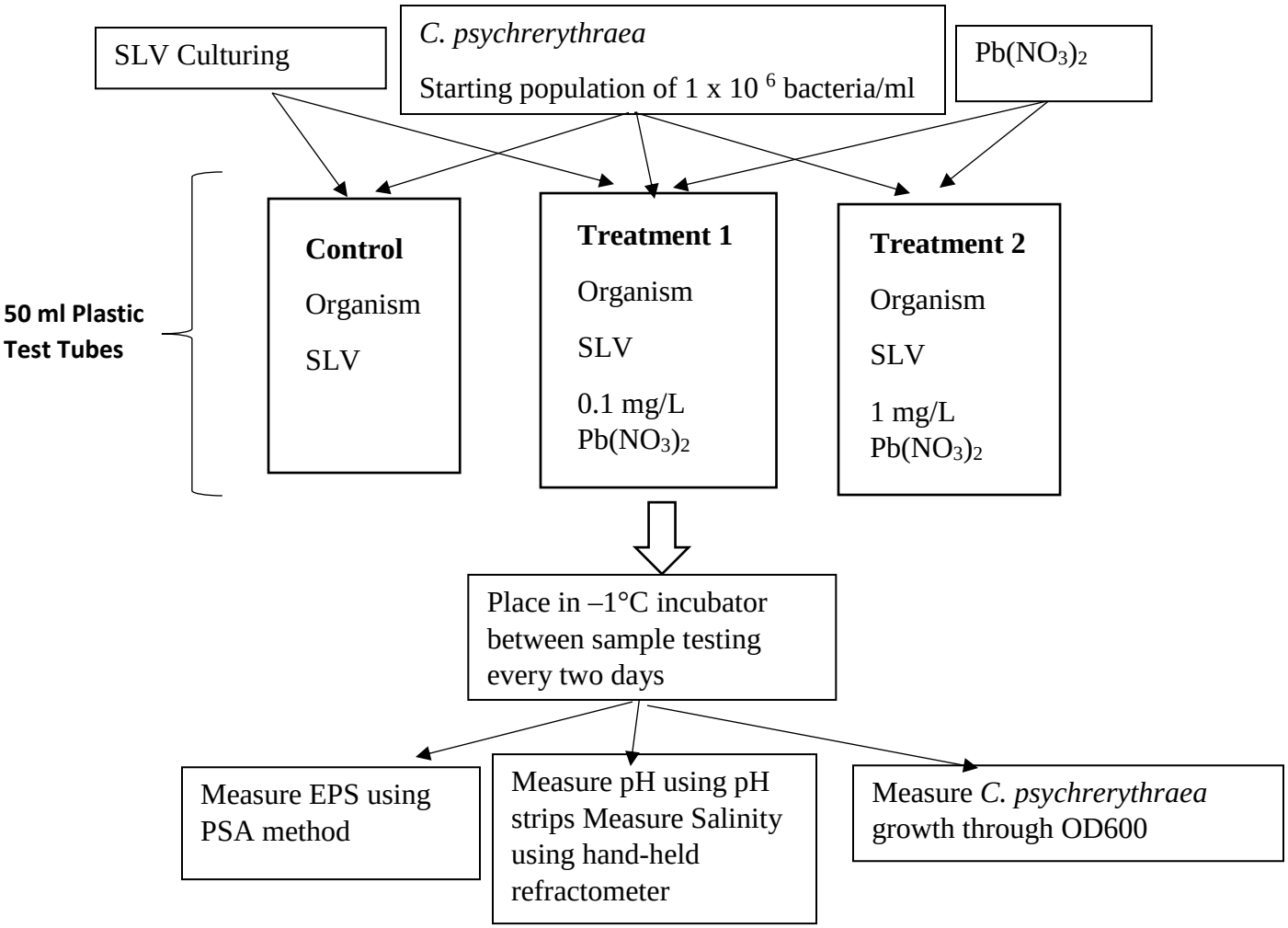
how lead will affect the growth of the model cold-adapted bacterium producing the EPS, *Colwellia psychrerythraea*, as well as the amount of EPS produced. I wanted to test the hypothesis that the addition of lead would simulate an increase in the production of [EPS] in freezing conditions. My second hypothesis aimed to predict to where the lead and EPS would migrate during the freezing process: into the ice or the remaining liquid water, with the hypothesis that lead would be greater in the liquid than in the ice while [EPS] would be greater in the ice than in liquid. Determining whether [Pb^{2+}] concentrates in the seawater or the sea ice, as well as its impact on [EPS] production, not only has environmental implications but may also influence the lives of those organisms, from microbes to humans, that live at high latitudes and depend on (uncontaminated) sea ice for their subsistence.

Marine bacteria like C. *psychrerythraea* rely upon EPS as a natural cryoprotectant, but it is not known whether they also depend on EPS for protection from heavy metals. In order to see how [Pb^{2+}] may impact C. *psychrerythraea* [EPS] production and its ability to bind to the [EPS] produced, our experimental design included freezing artificial seawater from the surface of the experimental container to the bottom, to simulate ice growth in nature. In addition, where heavy metals such as [Pb^{2+}], concentrate, whether in the ice or the waters beneath it, has not been examined previously. Based on previous literature from fresh water (Sun et al. 2008), the hypothesis that the addition of [Pb^{2+}] will simulate an increase in the production of [EPS] is testable under these lab created conditions.

Methods

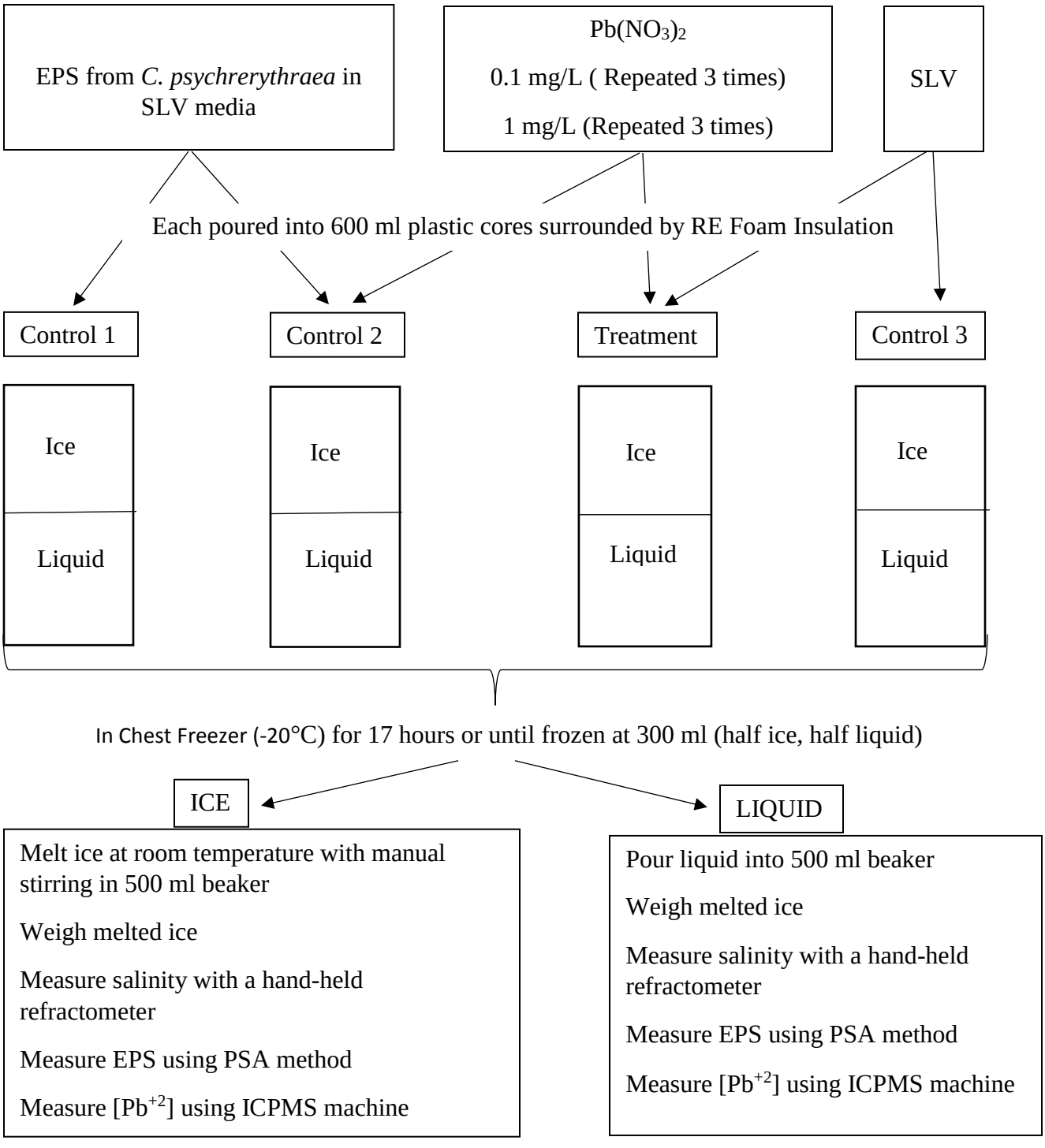
This research was conducted from January to March 2017, with the help of Jody Deming and her lab manager Shelly Carpenter, in the Deming Lab at the University of Washington.

Experimental Design: Effect of EPS and *C. psychrerythraea* growth due to [Pb^{+2}]



This experiment lasted 3 weeks, with sample being taken every two days to allow the organism time to produce sufficient [EPS] to maximize the chances of observing a clear impact of the lead exposure

Experimental Design: Allocation of Lead and EPS within Ice and Liquid



Media Testing

To determine which media would allow *C. psychrerythraea* to grow efficiently and produce EPS, three different media were tested over a period of 10 days. The three media were ALV, made of ammonium nitrate, calcium - L lactate, and vitamins (Tang et al. unpublished data), SLV made of sarcosine, calcium- L lactate, and vitamins (Collins et al. 2013), and LYE made of lactic acid, yeast extract, and artificial seawater (Marx et al. 2009). For each medium, 250 ml was placed into three 500 ml bottles and inoculated with a culture of *C. psychrerythraea* to a starting population of 1×10^{6} bacteria/ ml. The optical density was measured at 600 nm which measured the growth of the culture, and at 490 nm which measures the amount of EPS in the solution, every two days. The results shown in figure 1, indicate that SLV is the best medium for both factors.

Culturing Conditions

Effect of EPS and C. psychrerythraea growth due to [Pb^{+2}]

C. psychrerythraea was first cultured according to the procedures described by Marx et al. (2009). A stock culture of the bacterium was inoculated into SLV medium consisting of sarcosine, calcium L-lactate and vitamin solution (Collins et al. 2013) prepared in an artificial seawater (ASW) buffer (Marx et al., 2009) and grown at –1°C in a temperature-controlled incubator with a stirring rod for continuous mixing.

Allocation of Lead and EPS within Ice and Liquid

Concentrated EPS was prepared by growing 3600 mL of *C. psychrerythraea* in SLV until the culture reached an Optical Density (600nm) measurement of at least 0.5 according to the PSA method set by Dubois et al. (1956).A volume of 250 ml of the mixture was centrifuged

using a 5RC Corbal GSA rotor at 4,400 rpm for 15 minutes at 4°C to remove cells from the supernatant. The solution was then filtered using a 0.2 µ filter to eliminate any leftover cells and to retain only the EPS.

EPS Measurement

The starting EPS in the cultures was measured as the equivalent of the total concentration of glucose using the phenol sulfuric acid (PSA) method as described by Dubois et al. (1956). These results, measured as an Optical Density (490 nm) can be converted to µg/ml of EPS through a standard glucose curve. This curve was constructed by adding known amounts of glucose (µg/ml) to different tubes containing deionized water and then measuring optical density each tube using a spectrometer. This comparison allowed us to match the amount of known glucose that was added to a corresponding measured optical density (490nm) value. All subsequent optical density measurements can then be converted to glucose equivalents using this curve.

Pb Measurement

[Pb^{2+}] was analyzed by Dr. Dongsen Xue at the University of Washington School of Environmental and Forest Sciences with the use of an Inductively Couple Plasma Mass Spectrometry (ICPMS) machine. The method to test the [EPS] is known as EPA 200.7 which is standard analytical method certified by the State of Washington.

Calculations - Scaling to Salinity

In order to make the data comparable between the trials of the high and low levels of added lead, the lead and the EPS measured in the ice and liquid of all cores had to be scaled to their respective salinity. This was done by calculating the effective segregation coefficient (K_{eff})

which describes "the proportion by which solutes are retained in sea ice" (Ewert et al. 2011). Where the solute in the melted ice is represented by $[X]_{ice}$ and the concentration in the source solution is represented by $[X]_{source}$.

$$K_{eff\ (salinity)} = \frac{Salinity_{ice}}{Salinity_{source}} \qquad K_{eff\ (EPS)} = \frac{[EPS]_{\ ice}}{[EPS]_{\ source}} \qquad K_{eff\ (Lead)} = \frac{[Lead]_{\ ice}}{[Lead]_{\ source}}$$

Subsequently, to assess how each component interacts with the other, the "segregation coefficients of different solutes can be normalized to generate an enrichment index" or I_x (Ewert et al. 2011)

$$I_{EPS} = \frac{K_{eff(EPS)}}{K_{eff(salinity)}} \qquad I_{EPS} = \frac{K_{eff(Lead)}}{K_{eff(salinity)}}$$

Results

Preliminary results from the experiment show that the addition of a high amount of lead to the culture severely stunts bacterial growth (Figure 2, A). In addition, the production of [EPS] over time shows that, with an increase in added lead to each treatment, EPS production was also severely stunted with values close to 0 µg/ml (Figure 2, B). The ratio of EPS to cells, demonstrates that from about day 15 to day 20, cells could have been producing a higher amount of EPS with a low concentration of lead than with no lead (Figure 2, C). These results indicate that at a level of lead lower than 0.1 mg/L cells might produce more EPS over a longer period than without any lead at all (Figure 3). However, to be certain that this difference is real, more trials are needed so that confidence intervals cold be created.

When the Enrichment Index, the factor by which a given solute is concentrated in ice, is equal to one, the [EPS] and [Pb^{2+}] are expelled from the ice at the same proportion. When the index is above one, then the [EPS] or [Pb^{2+}] are selectively enriched in the ice and therefore selectively depleted in the liquid. EPS is more likely to be retained in the ice if a low concentration of lead was added to the solution (Figure 3, A). We found that the EPS enrichment index for just SLV and for SLV with $PbNO_3$ were both close to 0 which was expected as no EPS had been added (Figure 3, A). These two control cores, confirmed that the experimental process involved little to no contamination of EPS (Figure 3, A). The lead enrichment index which confirms the result that lead and EPS were more likely to be retained in the ice and therefore depleted in the liquid if both are present versus only one (Figure 3, B). These findings are presented by the averages for each control and treatment (Table 1).

Figure 4 demonstrates similar graphs to figure 3, except in this case there was a higher added concentration of lead nitrate (1.0 mg/L). The enrichment index of EPS is frequently below one, meaning that EPS and lead are selectively depleted from the ice during trial one and two (Figure 4, A). Moreover, the lack of consistency in the data demonstrates that at a higher level of lead, EPS and lead seem to have no connection or binding ability. As in trial two [EPS] was slightly more enriched in the ice than when lead was added, while in trial one and three the opposite pattern was observed (Figure 4, A). The lead enrichment index for lead at a higher concentration; when EPS is involved the lead is more enriched in the ice than when there is no EPS (Figure 4, B).

Discussion

Both parts of the experiment demonstrated that there are interactions between lead and EPS. The interactions could be either chemical or biological. Figure 2, C first demonstrated this relationship when an amount of lead nitrate between 0.1 and 1.0 mg/L induced the production of more EPS per cell than when there was no lead present. This result is similar to an experiment done by Bi et al. (2013) which depicted that when lead was added, the EPS content of the " group decreased significantly for the first [6 days] and then increased to the level of the control group at eight days". Their result, from fresh water bacteria shows the same final trend as our experiment. Our data indicate that there may be a threshold or "tipping point" of amount of $[Pb^{+2}]$ that can be added to a culture of this marine bacterium that would make it produce more EPS per cell than when there is no lead. Additional study is required to quantify this point in order to demonstrate how much lead this organism could tolerate without it impeding or stopping its growth and becoming toxic. Finding this precise level would be helpful when examining this specific heavy metal, a key environmental issue for the Arctic ecosystem.

As we showed in our experiments, at a high amount, lead stunted the growth of the culture so severely that no development was observed. This result was very different from that obtained when the same concentration of lead was added to the cyanobacterium, M. *aeruginosa,* in which case growth actually increased (Bi et al. 2013). These opposing results indicate that different types of bacteria have different reactions to this heavy metal. The cyanobacterium must have an internal process that detoxifies, or else completely isolates itself from the lead's destructive properties which, *C. psychrerythraea* lacks. This is significant as we can infer from our results that an increase amount of lead in the environment could negatively impact the

bacteria currently functioning in the Artic, yet trigger mass growth of bacteria such as *M. aeruginosa* that can be a threat to freshwater systems (Bi et al. (2013).

Considering the negative impact that such concentrations of lead can have on both the organism and an ecosystem, it was important to determine whether most of the lead would migrate to ice or migrate to liquid in the Arctic. It was already known that under simulated natural conditions EPS was preferentially retained in the ice rather than the liquid (Ewert et al. 2011). Indeed, the data shown in figures 3 and 4 along with Table 1 were consistent with these findings, as the EPS enrichment indices for the majority of the trials were above 1 whenever there was only EPS present in the starting solution. The lead enrichment indices showed that lead was not enriched in the ice unless EPS was present, and that the lead enrichment index was always higher than when just SLV and lead were present. This pattern, however, is only the case for the low level of lead that was tested; at the high lead level the presence of EPS did not typically lead to higher lead enrichment indices.

Other studies, have explored the binding capacity of heavy metals to EPS. One study showed that loosely bound EPS or flocculating EPS demonstrate strong binding properties for heavy metals to alcohol, carboxyl and amino which are components in the structure of EPS (Sun et al. 2009). These authors, however, only experimented with EPS loosely bound aerobic granules while the bacterial strain used in this study, *C. psychrerythraea*, may have more tightly bound EPS of a different composition (Sun et al. 2009). Another study by Dobrowolski et al. (2017) which researched the absorption of several metal ions, one of them being Pb^{2+} by EPS from the bacterial strain, *Rhodococcus opacus* supports that explanation. They found that the binding properties were due to an absorption mechanism of the metal ions onto EPS (Dobrowolski et al. 2017). This mechanism is likely due to an electrostatic attraction “a surface

complex formation and chemical interaction between the functional groups" such as amino or hydroxyl groups of the bacterial extracellular polymers. There could be a different binding mechanism for the EPS of *C. psychrerythraea*, though other consistencies between these findings the types of EPS hint to this concentration effect instead.

One view, which we considered at the start of this study, was that as the ice matrix grows the lead would be rejected from it as salinity is rejected from. However these findings demonstrate that a relationship exists between EPS and lead: that ice tends to retain lead (Krembs et al., 2011). Our finding may have important climate change implications; as less ice forms in the Polar Regions, less lead may be removed from the liquid ocean and retained in the ice. As long as lead is held in the ice, organisms in the ocean below may be at least temporarily protected from it. However, when ice no longer forms due to global warming, the flora and fauna of the ocean would lose this protection and may be unable to adapt to the changing ocean conditions, especially if those conditions include an increase anthropogenic input of lead contaminants into the ocean, which would negatively affect organisms in terms of their general behavior and their ability to grow an reproduce (Lenntech, 2016). The finding that lead and the EPS produced by aerobic cold-adapted bacteria are able to bind together at low levels is relevant in considering possible mitigation methods. Through the binding of EPS lead or other heavy metals to EPS, we can begin to explore the potential beginning of a technology for the recovery and removal of the metal ions from industrial waste streams (Sun et al. 2009). This technique, known as biosorption would remove contaminants from the environment through "a number of passive accumulation processes, such as ion exchange, complexation, microprecipitation, absorption and desorption" (Waite et al. 2016). However, due to the shear amounts of processes

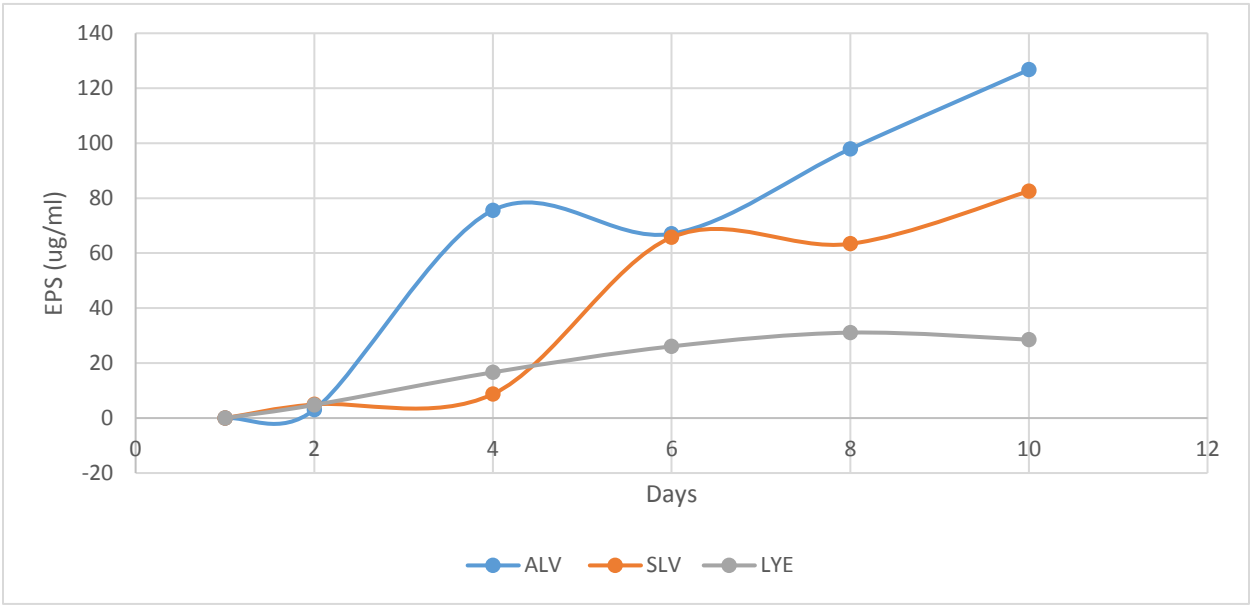
and external factors involved in this method more time and experimentation is needed to conclude it as a concrete metal removal technique.

However at higher concentrations of lead which we explored, this correlation between the retention of the heavy metal is no longer present (Figure 4). The variability of the EPS enrichment index when both lead and EPS are present demonstrate this loss, as it ranges from being strongly retained in the ice to be strongly depleted from it (Figure 4, top). As EPS saturated with lead was being expelled from the growing ice to compensate for this oversaturation of lead, it would also mean that the organisms that thrive in the ice due to this the protective presence of EPS (Marx et al., 2009) would also be short changed. This would jeopardize the ability of photosynthetic organisms to survive, where they have a better position to capture light for photosynthesis and be safe from predators (Krembs et al. 2011).

Thus, this study has demonstrated the delicate nature of the balance between an organism, the EPS it needs for cryoprotective and other reasons (Marx et al, 2009), and the amount of lead input into the environment. With too much lead the EPS retention properties are overwhelmed, leaving other species of bacteria or algae potentially endangered by the excess unbound lead. We may thus conclude that careful examination of fluid and ice concentrations of lead must be measured as different levels of lead result in contrasting impacts.

Figures

A



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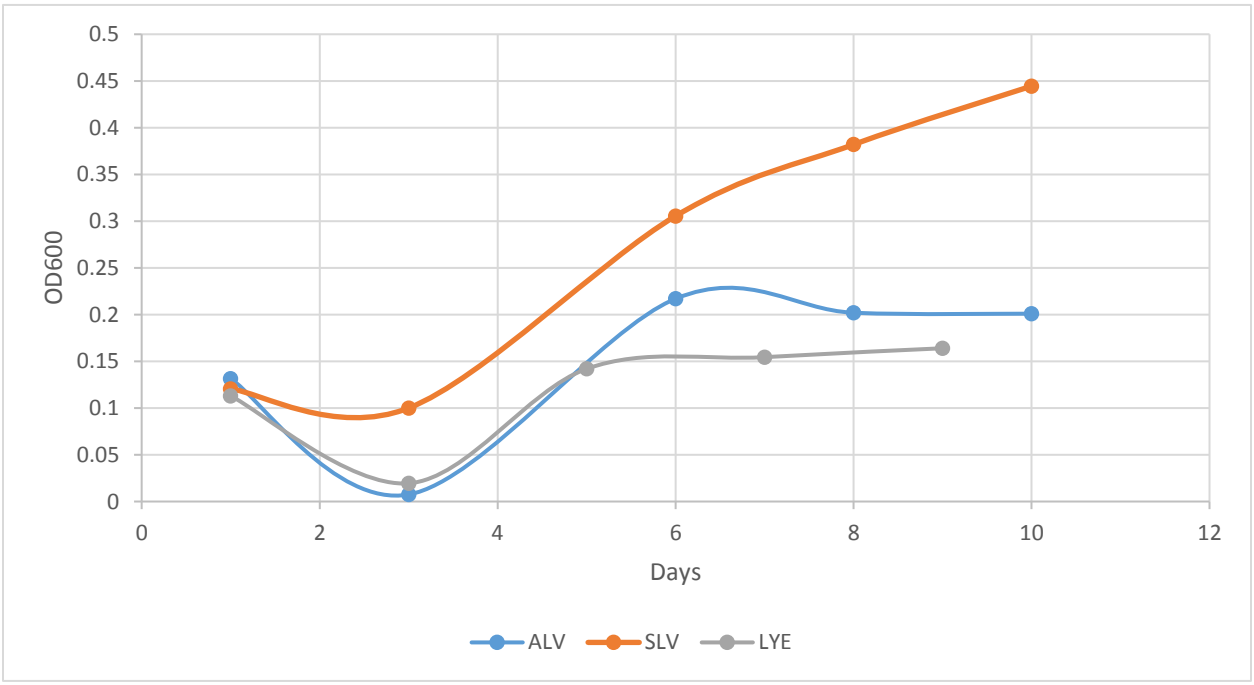

Figure 1. EPS production over time by three different media (top) and the growth of the culture over time measured as the optical density at 600 nm.

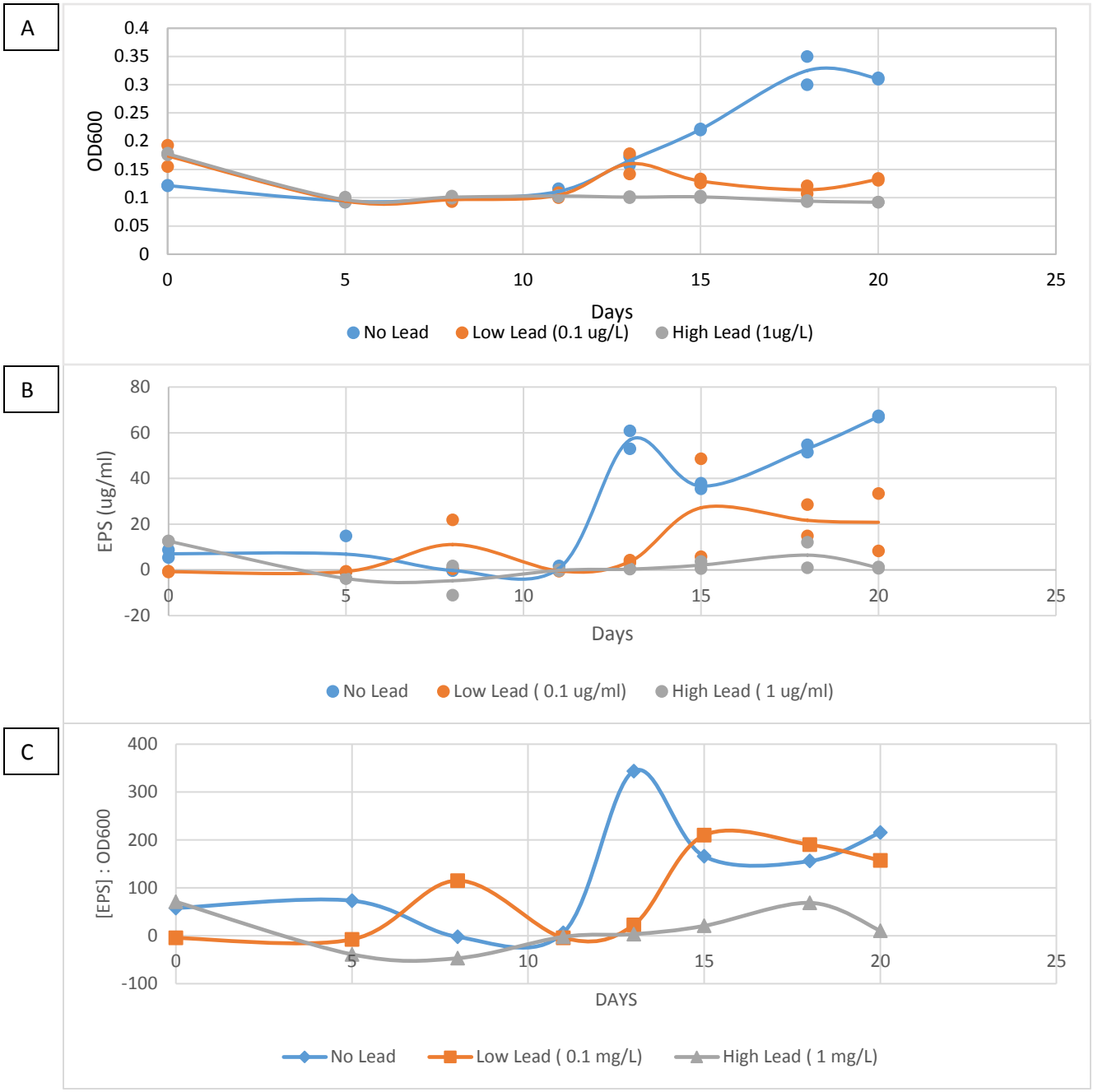

Figure 2. Depiction of bacterial growth (measured by optical density, where an OD600 of 0.1 is equivalent to 1 x 10^6 cells/ml), in the upper graph, EPS production in the middle graph, and the ratio of [EPS] to OD600 over 20 days at -1 °C in the lower graph, with no lead added, a low level of lead added (0.1 mg/L $PbNO_3$), and a 10-fold higher level of lead added (1.0 mg/L $PbNO_3$). When there appears to be only a single point in the upper two graphs both points are nearly identical.

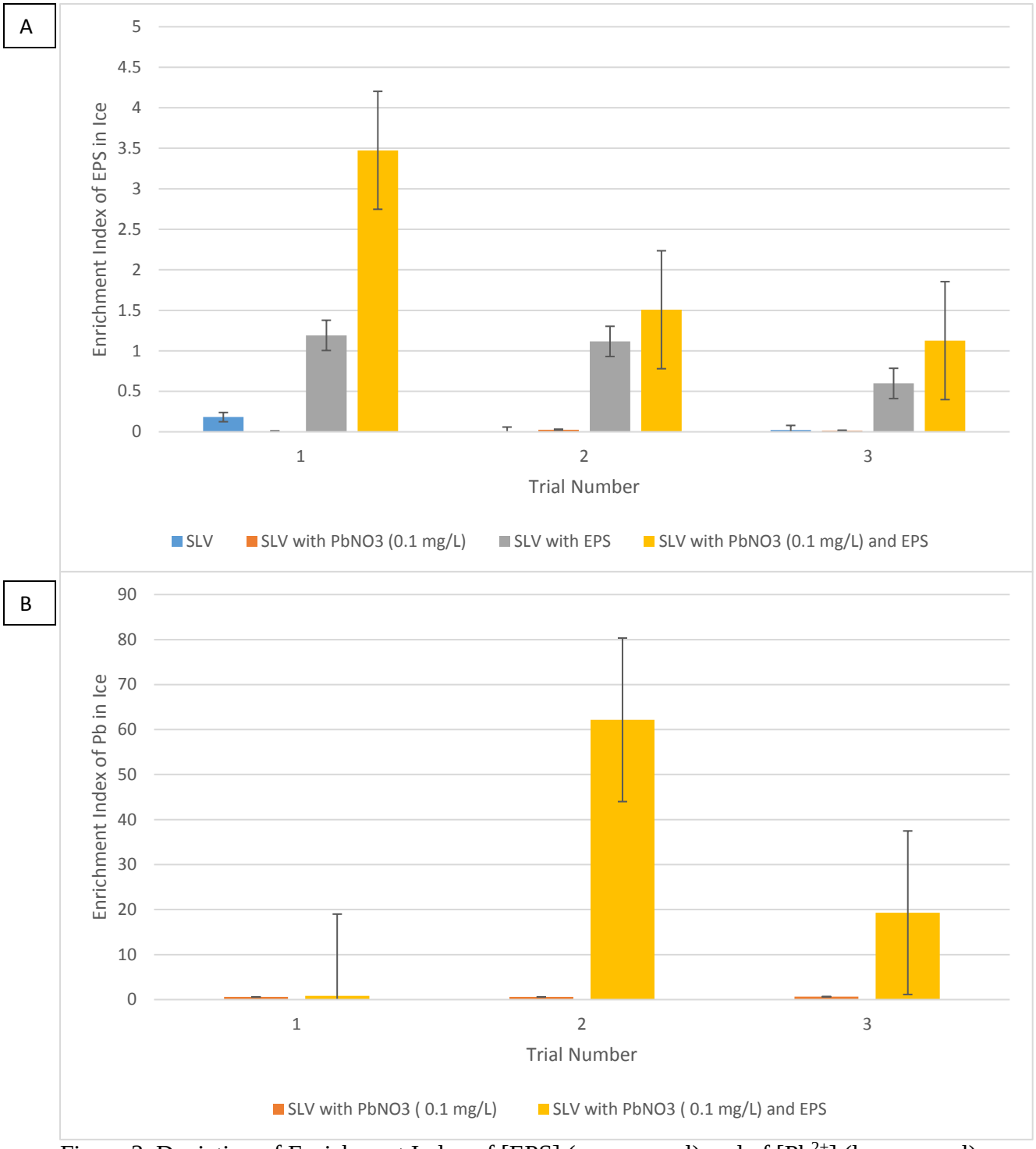

Figure 3. Depiction of Enrichment Index of [EPS] (upper panel) and of [Pb^{2+}] (lower panel) scaled to Salinity for trials with a low level of lead added (0.1 mg/L $PbNO_3$).

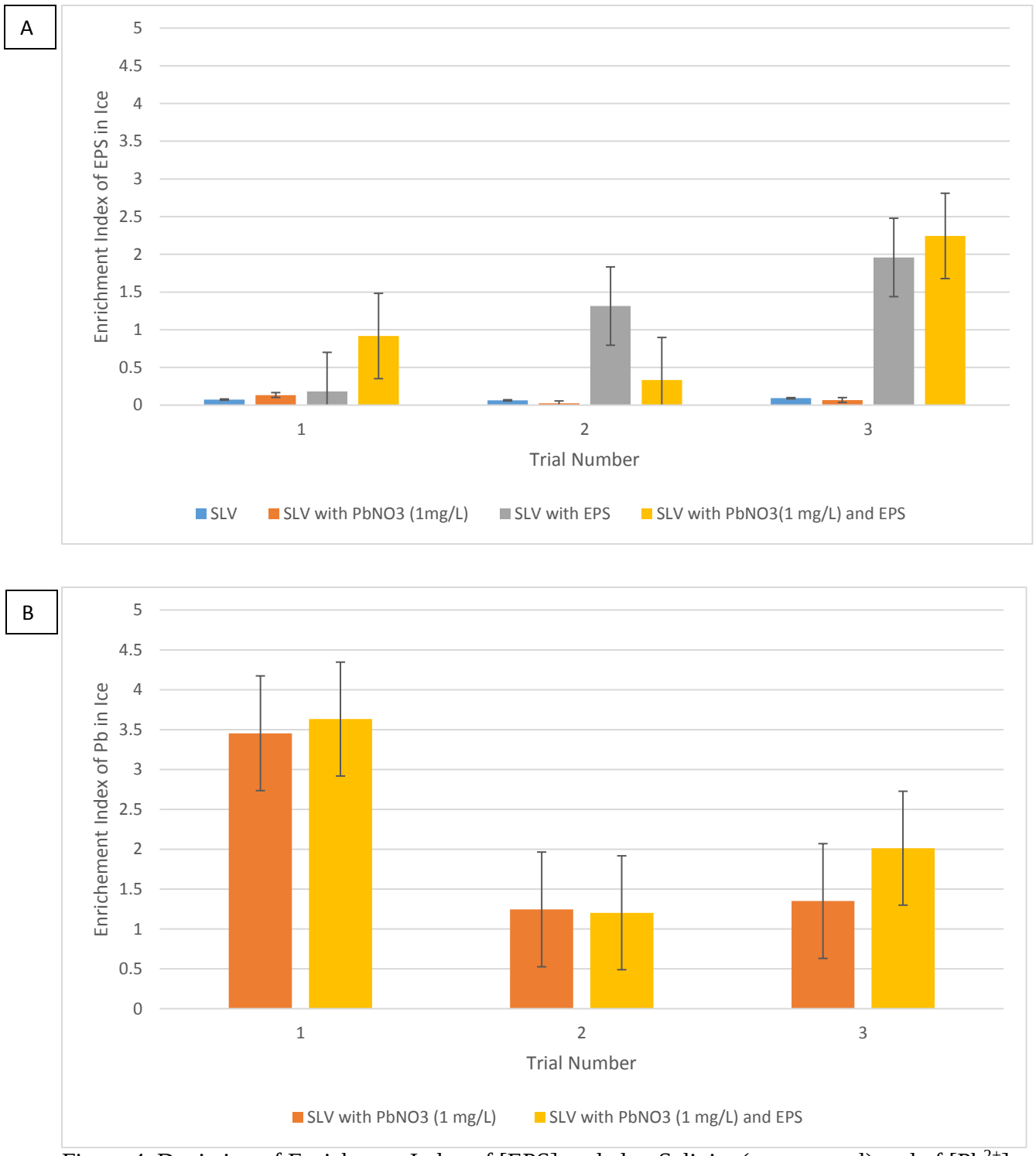

Figure 4. Depiction of Enrichment Index of [EPS] scaled to Salinity (upper panel) and of [Pb^{2+}] (lower panel) scaled to Salinity for Trial with a high level of lead added (1.0 mg/L of $PbNO_3$).

Tables

	EPS enrichment index (low lead)			
	SLV	SLV with $Pb(NO_3)_2$	SLV with EPS	SLV with $Pb(NO_3)_2$ and EPS
Trial 1	0.18	0.01	1.19	3.48
Trial 2	0.002	0.03	1.12	1.51
Trial 3	0.02	0.01	0.62	1.13
Mean	0.07 ± 0.10	0.02 ± 0.01	0.98 ± 0.31	2.04 ± 1.26
	Lead Enrichment Index (Low Lead)			
Trial 1	0	0.58	0	0.83
Trial 2	N/A	0.59	N/A	62.2
Trial 3	N/A	0.64	N/A	19.3
Mean	0	0.61 ± 0.3	0	27.4 ± 31.5

	EPS enrichment index (high lead)			
	SLV	SLV with $Pb(NO_3)_2$	SLV with EPS	SLV with $Pb(NO_3)_2$ and EPS
Trial 1	0.07	0.13	0.18	0.92
Trial 2	0.06	0.02	1.31	0.33
Trial 3	0.09	0.07	1.96	2.24
Mean	0.08 ± 0.01	0.08 ± 0.06	1.15 ± 0.90	1.16 ± 1.00
	Lead Enrichment Index (High Lead)			
Trial 1	0	3.46	0	3.63
Trial 2	N/A	1.24	N/A	1.20
Trial 3	N/A	1.35	N/A	2.01
Mean ± 1.2	0	2.02 ± 1.25	0	2.28 ± 1.24

Table 1. EPS and Lead Enrichment Index at all trials for every control and treatment at low lead (top) and high lead (bottom)

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