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Influence of Zinc on Bacteria and Enzyme Activities in Surface Waters

Influence of Zinc on Bacterial Populations and their Proteolytic Enzyme Activities in Freshwater Environments: A Cross-Site Comparison

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Abstract:

Temporal responses of indigenous bacterial populations and proteolytic enzyme (i.e. aminopeptidase) activities in the bacterioplankton assemblages from three separate freshwater environments were examined after exposure to varying zinc concentrations under controlled microcosm conditions. Zinc concentrations (ranging from 0 to 10μM) were added to water samples collected from the Kalamazoo River (KR), Rice Creek (RC) and Huron River (HR) and examined for bacterial abundance and aminopeptidase activities at various time intervals over a 48 h incubation period in the dark. The results showed that while the zinc concentrations did not significantly influence total bacterial counts directly, however aminopeptidase activities varied significantly over time to increasing zinc treatments. Also, ANOVA and linear regression analyses revealed significant positive relationships between bacterial numbers and their hydrolytic enzyme activities suggesting that both probably co-vary to increasing zinc concentrations in aquatic systems. The results from this study serve as additional evidence of the ecological role of Zn as an extracellular peptidase cofactor on the dynamics of bacterial assemblages in aquatic environments.

Key Words: metals, aminopeptidase, bacteria, freshwater

Introduction:

The dynamic changes in the abundance and distribution of bacterial populations, both temporally and spatially, in aquatic environments have been well documented (e.g., Leff et al. 1999, Olapade et al. 2005; 2006). Most important, changes in bacterial numbers and activities in aquatic systems have been attributed to combinations of factors including, in response to anthropogenic disturbances, availability of nutrients and organic matter as well as water chemistry characteristics (Webster 1997, Olapade et al. 2005, Tiquia 2010). In particular, many heterotrophic bacterial populations are known to numerically dominate in various environments due to the possession of wide arrays of extracellular enzymes that are used in effectively hydrolyzing and assimilating various complex organic compounds found in such systems (Hoppe 1983, Findlay et al. 1997, Azam 1998, Rao et al 1998, Keith and Arnosti 2001, Nagata 2008, Zimmerman 2013). Additionally, the proteolytic activities and community diversity of heterotrophic bacterial assemblages have been shown to be limited by the presence of metal pollutants in various contaminated environments (e.g., Goulder et al. 1980, Roane and Pepper 2000, Kelly et al. 2003).

Many metals, including zinc (Zn) are found ubiquitously in various environments, and are required essentially by bacteria as trace elements in order to function during their enzyme catalysis, molecule transportation and development of protein structure (Hughes and Poole 1989). However, some of these metals can also be found predominantly as cationic species where they accumulate and become toxic to microbial assemblages in aquatic systems (e.g., Roane and Pepper 2000). In particular, zinc concentrations have been recorded on average to be present at background levels of about 0.30 µM in freshwater system (e.g. Leppard 1981, Sigg 1985) and around 0.20 µM in seawater environments (Bidwell and Spotte 1985, Shaffer et al. 2004). The availability of zinc and other metals have been suggested to be highly dependent on various environmental components including pH, redox potentials and organic contents in such milieus (e.g., Roane and Pepper 2000). Therefore,

the main objective of this study was to determine the influence of various zinc concentrations on the bacterial abundance and their proteolytic enzymatic activities from three separate freshwater environments with differing watershed characteristics.

The three freshwater environments from which their bacterioplankton assemblages were examined in this study (i.e. Kalamazoo River {KR}, Rice Creek {RC} and Huron River {HR}) in response to Zn concentrations differ in many of their environmental and water chemistry properties (Figure 1). The sites were specifically selected to reflect differences in prior exposure and hence potential adaptations to anthropogenic disturbances as well as reveal any existing spatial variations among the watersheds. Comparatively, the RC site sampled is located downstream to a foundry industry in Albion, MI and have been exposed over the years to various effluents produced from metal castings; in contrast, the upstream site along the HR passes mostly through residential areas around Ann Arbor, while the KR site is along a recreational park on a college campus. Water samples were also collected during the spring and summer months to show temporal differences among the three sites. Leucine-aminopeptidase was utilized as substrate to measure proteolytic activities in this study because aminopeptidase is considered a zinc-dependent enzyme (Choudhury and Srivastava 2001) and also due to previous documentations of its relatively high hydrolysis rate by bacterial assemblages in aquatic systems (e.g. Obayashi and Suzuki 2008; Tequia 2011).

Materials and Methods:

Study Sites and Sample Collection:

Triplicate water samples were aseptically collected in sterile falcon tubes from three separate freshwater environments and stored at 4^0C until returned to the laboratory. The first study site was at Albion (42.242550 N -84.735417 W) along the Kalamazoo River, an extensive watershed along the

southwestern portions of the Lower Peninsula of Michigan that connects with Lake Michigan (Figure 1). The second study site was within the south branch of Rice Creek (42.298096 N -84.851372 W), a tributary of the Kalamazoo River between Albion and Marshall. The third study site was along the Huron River close to Ann Arbor (42.281389, -83.748333). These sites were selected because of their locations as well as the differences in their respective hydrodynamic and watershed characteristics (USEPA 2015; USGS 2015; HRWC 2015).

During sampling from May through June, various water chemistry properties including dissolved oxygen (DO), pH, conductivity, temperature and oxidation-reduction potential (ORP) were measured (see Table 1) at each study site using the YSI model 556 MPS multi-probe system (YSI Incorporated, USA). Ion chromatography was employed to quantify the presence of various anions including chloride, sulfate and nitrate using conductivity detectors and also standardized according to the various manufacturer's specifications at the Dow Analytical Laboratory (Albion College, USA).

Microcosm Experiment:

Microcosms to determine the effects of zinc on bath the indigenous bacterial populations as well as their proteolytic (aminopeptidase) enzyme activities were set up under controlled laboratory conditions by adding various concentrations of $ZnCl_2$ (ranging from 0, 0.1, 0.5, 1.0 to 10.0μM) in triplicates to the collected water samples as previously described (Obayashi and Suzuki 2008; Bong et al. 2010). The substrate, L-Leucine-7-amido4-methylcoumarin hydrochloride used to determine aminopeptidase activities in the samples were also added to the experimental tubes from a 1mM stock (Adipogen, CA) to yield a final concentration of 100 μM (Obayashi and Suzuki 2005). The experimental tubes containing the water samples and enzyme substrate were then incubated at room temperature in the dark before subsampling at 0, 4, 24 and 48 hours.

Bacterial Enumeration:

Total bacterial numbers in the subsamples were determined by concentrating onto 0.2µm pore-size black polycarbonate filters (Poretics, Livermore, CA) before staining with 200µL of 15µg/µL of DAPI (4',6 diamidino-2-phenylindole) solution for between 3 to 5 minutes. Filters were rinsed with sterile water and then mounted onto glass slides with Type FF immersion oil (Porter and Feig 1980, Olapade and Weage 2008). Approximately 300 to 500 stained bacterial cells in 10 separate fields were then counted under an epifluorescence microscope.

Aminopeptidase Activity:

300µL of each triplicate samples were collected after each designated incubation time to determine aminopeptidase activities and aseptically transferred into 96 well plates. The fluorescence of the hydrolytic product i.e. 7-amino-4-methylocuomarin (AMC) were then measured with a SpectraMax M2/M2e microplate reader (Molecular Devices, CA) at an excitation/emission wavelength of 360/460nm (Obayashi and Suzuki 2008; Bong et al. 2010). The concentrations of AMC in the samples were measured by using a standard calibration of AMC after deducting the blank florescence of each sample. Then, the hydrolysis rates of substrate were determined as the difference in the AMC change in the treatments minus the concentration of the non-enzymatically-produced AMC as previously described by Obayashi and Suzuki (2008).

***Statistical Data Analysis*:**

All statistical analyses were performed with SPSS for windows (version 22, SPSS Inc., Chicago, IL). The student t-tests and ANOVA analyses were performed to analyze the differences in bacterial abundance and aminopeptidase activity between the various zinc concentrations and sampling times during the study periods. Post-hoc tests were also carried out for pair-wise comparison using Bonferroni correction test. Relationships between bacterial populations and enzyme activity were further examined using linear regression and Pearson Correlation analysis. All

data sets were log-transformed before performing statistical analyses to normalize and enhance the data set to be better interpretable. Statistical significance alpha was set to 0.05.

Results

Environmental Variables

All water chemistry characteristics measured were variable among the sites except for dissolved oxygen (DO) concentrations. Water temperature ranged from an average of 13.05 in KR (May) to 21.25^{0}C in HR (June). Also, KR had both the lowest and the highest total bacterial numbers during the sampling periods between the three sites. Concentrations of the anions that were measured differed significantly (p<0.05) among the three studies sites throughout the sampling period (Table 1).

Bacterial Abundance

The total bacterial counts as determined by DAPI staining varied on average between 1.76 x 10^6 (0.5µM; 0h) and 3.08 x 10^6 cells/ mL (0µM; 0h) in the KR microcosms (Fig. 2A). In RC, the numbers varied from the lowest of 1.28 x 10^6 (10µM; 4h) to 2.91 x 10^6 cells/ mL (0µM; 24h, Fig. 2B), while in the HR the numbers ranged from 1.52 x 10^6 (0µM; 0h) to 6.61 x 10^6 cells/ mL (0µM; 24h, Fig. 2C). Generally, the total bacterial counts recorded in the three samples were very similar between the sites and did not vary significantly during exposure to the different zinc concentrations (KR: F= 1.76, p=0.146; RC: F= 1.77, p=0.14; HR: F= 2.41, p=0.56, Table 2). However, the TBC numbers recorded in all three sites over time intervals during the study period differed significantly (KR: F= 3.58, p=0.018; RC: F= 14.24, p<0.0001; HR: F= 9.92, p<0.0001). The results of the interactions between concentration and time revealed that there were no significant differences. Also, the post-hoc analysis of pair wise comparison of bacterial numbers to the various zinc concentrations showed the absence of any statistical significance in all three sites examined (Table 3).

The results of post-hoc tests on pair-wise comparison of mean bacterial numbers over the study period revealed significant differences in most of time intervals examined. The bacterial numbers were more variable between the communities enumerated in RC than those in HR and less so in KR, especially at the 0 through the 4h of exposure to the zinc treatments (Table 4).

Enzyme Activity

Results of aminopeptidase activities showed a range of 2.26 (0.5µM; 24h) to 12.18 $\log_{10}$/nmol/L/h (1.0µM; 48h) in samples from the KR (Fig. 3A). The aminopeptidase hydrolytic rate recorded in the samples from RC varied from 2.02 (1.0µM; 0h) to 17.73 $\log_{10}$/nmol/L/h (0µM; 48h), while those in HR were from 0.41 (10µM; 24h) to 31.28 $\log_{10}$/nmol/L/h (0µM; 48h, Fig. 3C). Comparatively, lesser hydrolytic enzyme activities were recorded with increasing zinc concentrations especially in the RC and HR samples. The results of ANOVA showed significant variations in aminopeptidase activities to zinc concentrations (KR: F= 3.04, p=0.023; RC: F= 4.48, p<0.003; HR: F= 14.83, p<0.0001) and between sampling time intervals (KR: F= 33.10, p=0.018; RC: F= 44.80, p<0.0001; HR: F= 80.62, p<0.0001) as shown in Table 2. Post-hoc tests using Bonferroni correction revealed no significant differences to zinc concentrations in the KR samples, there were differences especially between the control and the highest concentration e.g. 10µM in both RC and HR (p<0.05, Table 3). However, significant variations were mostly recorded in hydrolytic activities after 4 hour of incubation higher incubation in all the three samples (Table 4).

Relationship between Bacterial Density and Aminopeptidase Enzyme Activity

The results of ANOVA and linear regression analyses between total bacterial numbers and aminopeptidase activities revealed positive relationships in the KR (R^2=0.28; p=0.016), in RC (R^2=0.46; p=0.009) as well as in HR (R^2=0.45; p=0.01). The slopes of the linear regression equations between the bacterial numbers and enzyme activities in the three sites were 1.3334, 2.6145 and 2.0647 for KR, RC and HR, respectively (Fig 4). These results suggest that both bacterial numbers

and their hydrolytic enzyme activities in the three sites probably co-vary to increasing zinc concentrations under the microcosm condition during the study period.

Discussion

Microbial assemblages in freshwater systems are strongly influenced by various environmental factors including complex nutrients and organic compounds. Hence heterotrophic bacterial populations have to produce several extracellular enzymes for the hydrolysis of complex, high-molecular-weight organic polymers to make these compounds bioavailable for assimilation in aquatic systems. This present study was designed to examine the effects of increasing zinc concentrations on indigenous bacterial assemblages within the bacterioplankton assemblages from three different freshwater environments as well as on the hydrolytic activities of aminopeptidase. This is especially of significant ecological importance due to the fact that some aminopeptidases are known to be zinc-dependent, while some others are not; therefore relative Zn manipulations could potentially provide a better insight regarding the importance of metal-dependent aminopeptidases to microbial assemblages and organic matter cycling in various aquatic systems (e.g. Fukuda et al 2000). In general, the results obtained from the microcosm studies revealed that while increasing zinc concentrations may not have significantly influenced total bacterial counts directly, however in contrast, aminopeptidase activities varied significantly over time to increasing Zn treatments, especially within the RC and HR, microcosms. This results somewhat corroborates earlier studies that have also observed tolerance of high zinc concentrations by indigenous bacterial populations in freshwater environments (e.g. Colwell et al. 1989, Admiraal et al. 1999). In their study that examined short-term toxicity of zinc to bacteria in a metal polluted stream, Admiraal et al. (1999) recorded higher Zn tolerance and resilience among the bacterial populations at the metal stressed sites as compared to populations enumerated in the upstream site. They also found that bacterial activities

showed pronounced sensitivity to high zinc concentration at the upstream site relative to the polluter and the downstream site along the stream. Colwell et al. (1989) attributed zinc tolerance and adaptation by the epilithic bacterial populations in their study to the accumulation of Zn in the structural milieu of the biofilms within the assemblages. Similarly, Fukuda et al. (2000) also found strong positive correlations between cell-specific aminopeptidase activity and concentrations of dissolved zinc in the upper layer across the Pacific.

The differences observed in the hydrolytic enzyme activities to Zn exposure, despite no significant variation in bacterial populations, between the three freshwater systems may probably be due to differences in the water chemistry characteristics among the studied sites, in particular organic carbon availability. Previous studies have documented contrasting responses of various extracellular enzyme activities to organic carbon influxes into freshwater environments (e.g., Ainsworth and Goulder 2000, Harbott and Grace 2005, Tiquia 2011, Brown and Goulder 1999). For instance, while some studies have recorded high activities of leucine aminopeptidase and other extracellular enzyme activities in freshwater sites that were exposed to high anthropogenic disturbances (Ainsworth and Goulder 2000Harbott and Grace 2005, Tiquia 2011), in total contrast to these studies, Brown and Goulder (1999) found the activity of aminopeptidase to be largely indifferent to fish farm effluent discharge in the River Hull, north-east of England.

The lack of bacterial community response to increasing Zn exposure as observed here suggest high levels of resilience by the indigenous bacterial assemblages in the three bacterioplankton assemblages examined. However, metal tolerance by bacterial populations is not at all uncommon, given previous documentations of such metal resistance in several aquatic environments (e.g., Suzuki et al.1992, Paulsson et al. 2000). Suzuki et al. (1992) observed a gradual decrease in viable bacterial numbers in samples of natural seawater over a week of incubation, whereas 93% and 26% of bacterial cells in tributyltin or cadmium-treated samples were still viable and tolerant after 2 weeks of

incubation, respectively. Therefore, the relatively strong positive correlations between total bacterial numbers and aminopeptidase activities in the presence of zinc over the study period, is probably indicative of a selection for those bacterial populations that are capable of hydrolytic activities using the Zn-dependent, aminopeptidase enzyme among the bacterioplankton assemblages examined. Furthermore, the slight differences observed in slopes of the linear relationship between the bacterial numbers and the hydrolytic activities in this study, could be reflective of differences in prior Zn exposure among the sites. Specifically, the RC site located downstream to a foundry industry had the strongest relationship between the bacterial numbers and hydrolytic activities in the presence of zinc compared to the other two sites examined in this study. Overall, this present study reveals that while the relatively high Zn concentrations used may not have had detectable effects on bacterial abundance, it however influenced total bacterial hydrolytic activities. These results further serve as additional evidence of the ecological role of Zn as an extracellular peptidase cofactor on the dynamics of bacterial assemblages as well as validate earlier suggestions regarding the need to examine both bacterial numbers and their activities as ecological indices of freshwater water health statuses to pollution pressures in aquatic environments (e.g. Harbott and Grace 2005).

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References

Admiraal, W., Blanck H., Jong BM., Guasch H., Ivorra N., Lehmann V., Nystrom BAH, Paulsson M., Sabater S. 1999. Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream. Wat. Res. 33:1989-1996.

Ainsworth, A.M. and Goulder, R. 2000. Downstream change in leucine aminopeptidase activity and leucine assimilation by epilithic microbiota along the River Swale, Northern England Sci Total Environ 251-251:191-204.

Azam, F. 1998. Microbial control of oceanic carbon flux: The plot thickens. Science 288:694-696.

Bidwell, J.P. and Spotte, S. 1985. Artificial seawaters: Formulas and methods. Jones and Bartlett. Boston MA.

Bong, C.W., Malfatti F., Azam F., Obayashi Y. and Suzuki S. 2010. The effect of zinc exposure on the bacterial abundance and proteolytic activity in seawater. Eds Hamamura N. et al.: Interdisciplinary Studies on Environmental Chemistry-Biological Responses to Contamination pp 57-63.

Brown, S.E., and Goulder, R.1999. Change in riverine epilithic extracellular enzyme activity in response to fish farm effluent. Letters Appl Microbiol 29:385-388.

Choudhury R, Srivastava S. 2001. Zinc resistance mechanisms in bacteria Curr Sci 81:768-775.

Colwell, F.S., Hornor, S.G., Cherry, D.S. 1989. Evidence of structural and functional adaptation in epilithon exposed to zinc. Hydrobiologia 171:79-90.

Ferris, F.G., Schultze S., Witten, T.C., Fyfe, W.S, and Beveridge T.J. 1989. Metal Interaction with biofilms in acidic and neutral pH environments. Applied and Environmental Microbiology 55:1249-1257.

Findlay, S, Hickey, CW, and Quin JM. 1997. Microbial enzymatic response to catchment-scale variations in supply of dissolved organic carbon. New Zealand J Maar Freshwater Res 31:701-706.

Fuduka, R., Sohrin, Y., Saotome, N., Fuduka, H., Nagata, T., and Koike, I. 2000. East-west gradient in ectoenzyme activities in the subarctic Pacific: Possible regulation by zinc. Linmol Oceangr. 45:930-939.

Goulder R, Blanchard AS, Sanderson PL, Wright B. 1980. Relationships between heterotrophic bacteria and pollution in an industrialized estuary Water Res 14:591-601.

Harbott EL, Grace MR. 2005. Extracellular enzyme response to bioavailability of dissolved organic C in streams of varying catchment urbanization. J N Am Benthol Soc 24:588-601.

Hoppe, H.G. 1983. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. Mar Ecol Prog Ser 11:299-308.

HRWC, 2015. Huron River Watershed Council. Water quality monitoring (http://www.hrwc.org/our-work/programs/water-quality-monitoring/), Michigan, USA Accessed July 8, 2015

Hughes, M.N., Poole RK 1989. Metals and microorganisms. Chapman & Hill, NY.

Keith, SC, Arnosti C. 2001. Extracellular enzyme activity in a river-bay-shelf transect: variations in polysaccharide hydrolysis ratio with substrate and size class. Aquat Microb Ecol 24:243-253.

Kelly, J, Haeggblom M, Tate RL. 2003. Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipid fatty acid profiles. Biol Fert Soils 38:65-71.

Leff, L.G., Brown, B.J., Lemke MJ. 1999. Spatial and temporal changes in bacterial assemblages of the Cuyahoga River. Ohio J Sci 99:44-48.

Leppard, G.G. 1981. Trace element speciation in surface waters. Plenum Press, NY.

Martinez, J. and Farooq, A. 1993. Aminopeptidase in marine chroococcoid cyanobacteria. Applied and Environmental Microbiology 59:3701-3707.

Nagata, T. 2008. Organic matter-bacteria interactions in seawater pp 207-241. In Microbial Ecology of the Oceans. 2nd ed. Kirchman DL ed. John Wiley & Sons, NY.

Obayashi, Y and Suzuki, S. 2005. Proteolytic enzymes in coastal surface seawater: significant activity of endopeptidases and exopeptidases. Limnology and Oceanography 50:722-726.

Obayashi, Y and Suzuki, S. 2008. Occurrence of exo- and endopeptidases in dissolved and particulate fractions of coastal seawater. Aquatic Microbial Ecology 50: 231-237.

Olapade, O.A., Gao X, Leff LG. 2005. Abundance of three bacterial populations in selected streams. Microb Ecol 49:461-467.

Olapade, O.A., Brothers A., Crissman M, Gao X, Leff LG. 2006. Comparison of planktonic microbial communities among nine North American streams. Arch Hydrobiol 165:221-239.

Olapade, OA. and Weage EA. 2010. Comparison of fecal indicator bacterial populations in surface waters of the Kalamazoo River, USA. Microbes Environ. 25 (1) 41-44.

Paulsson, M., Nystrom B., Blanck H. 2000. Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosm study, Aquatic Toxicol 47:243-257.

Pennanen, T, Frostegard A, Fritze H, and Baath E. 1996. Phospholipids fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. Applied and Environmental Microbiology 62:420-428.

Porter, KG and Feig YS. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943-948.

Rao, MB, Tanksale AM, Ghatge MS and Deshpande VV. 1998. Molecular biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews 62: 597-635.

Roane, T.M. and Pepper, IL. 2000. Microorganisms and metal pollutants pp 403-424. In Environmental Microbiology. Maier RM, Pepper IL, Gerba CP editors. Academic Press

Shaffer, MM, Hoffman, SR, Overdier, JT, Arfmstrong, DE. 2004. Physical and kinetic speciation of copper and zinc in three geochemically contrasting marine estuaries. Environ Sci Technol 38: 3810-3819.

Sigg, L. 1985. Metal transfer mechanisms in lakes; the role of settling particles. In Chemical processes in Lakes Stumm W. ed. John Wiley, NY.

Suzuki, S., Fuagawa, T., Takma K. 1992. Occurrence of tributyltin tolerant bacteria in tributyltin or cadmium containing seawater Appl Environ Microbiol 58: 3410-3412.

USGS, 2015. United States Geological Survey. Water data for the nation (http://waterdata.usgs.gov/nwis), Michigan, USA. Accessed July 8, 2015

Tiqua, SM. 2010. Metabolic diversity of the heterotrophic microorganisms and potential link to pollution of the Rouge River Environ Pollut 158:1435-1443.

Tiqua, SM. 2011. Extracellular hydrolytic enzyme activities of the heterotrophic microbial communities of the Rouge River: An approach to evaluate ecosystem response to urbanization. Microbial Ecology 62:679-689.

USEPA 2015.United States Environmental Protection Agency. Kalamazoo River area of concern. <http://epa.gov/greatlakes/aoc/kalriv/index.html >. Accessed July 8, 2015.

Webster JR, Meyer JL, Wallace JB, Benfield EF. 1997. Organic matter dynamics in Hugh White Creek, Coweeta Hydrologic laboratory, North Carolina, USA. J N Amer Benthol Soc 16:74-77.

Zimmerman, A.E., Martiny, A.C. and Allison, S.D. 2013. Microdiversity of extracellular enzyme genes among sequenced prokaryotic genomes. ISME J 7:1187-1199.

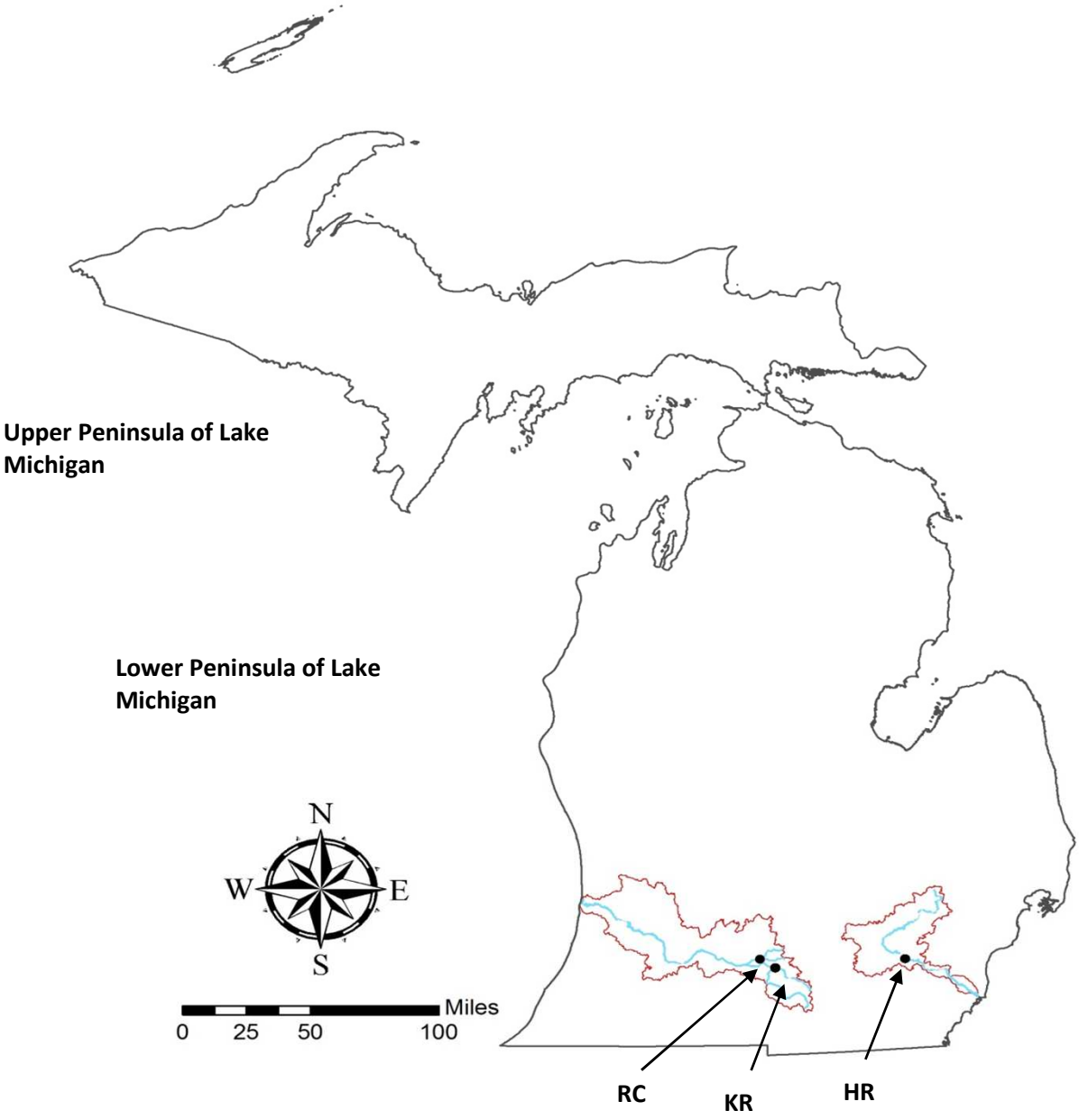
Figures

Figure 1. Map of the State of Michigan (USA) showing location of water sampling sites, along the Kalamazoo River (KR), Rice Creek (RC) and Huron River (HR), all located within the Lower Peninsula of the state

Figure 2. Changes in bacterial abundance in water samples from the Kalamazoo River (A), Rice Creek (B) and Huron River (C) observed while exposed to zinc concentrations over time.

Figure 3. Changes in aminopeptidase activities in water samples from the Kalamazoo River (A), Rice Creek (B) and Huron River (C) observed while exposed to zinc concentrations over time.

Figure 4. Linear relationship between bacterial abundance and aminopeptidase activities in water samples from the Kalamazoo River (●), Rice Creek (□) and Huron River (▲) observed under microcosm study.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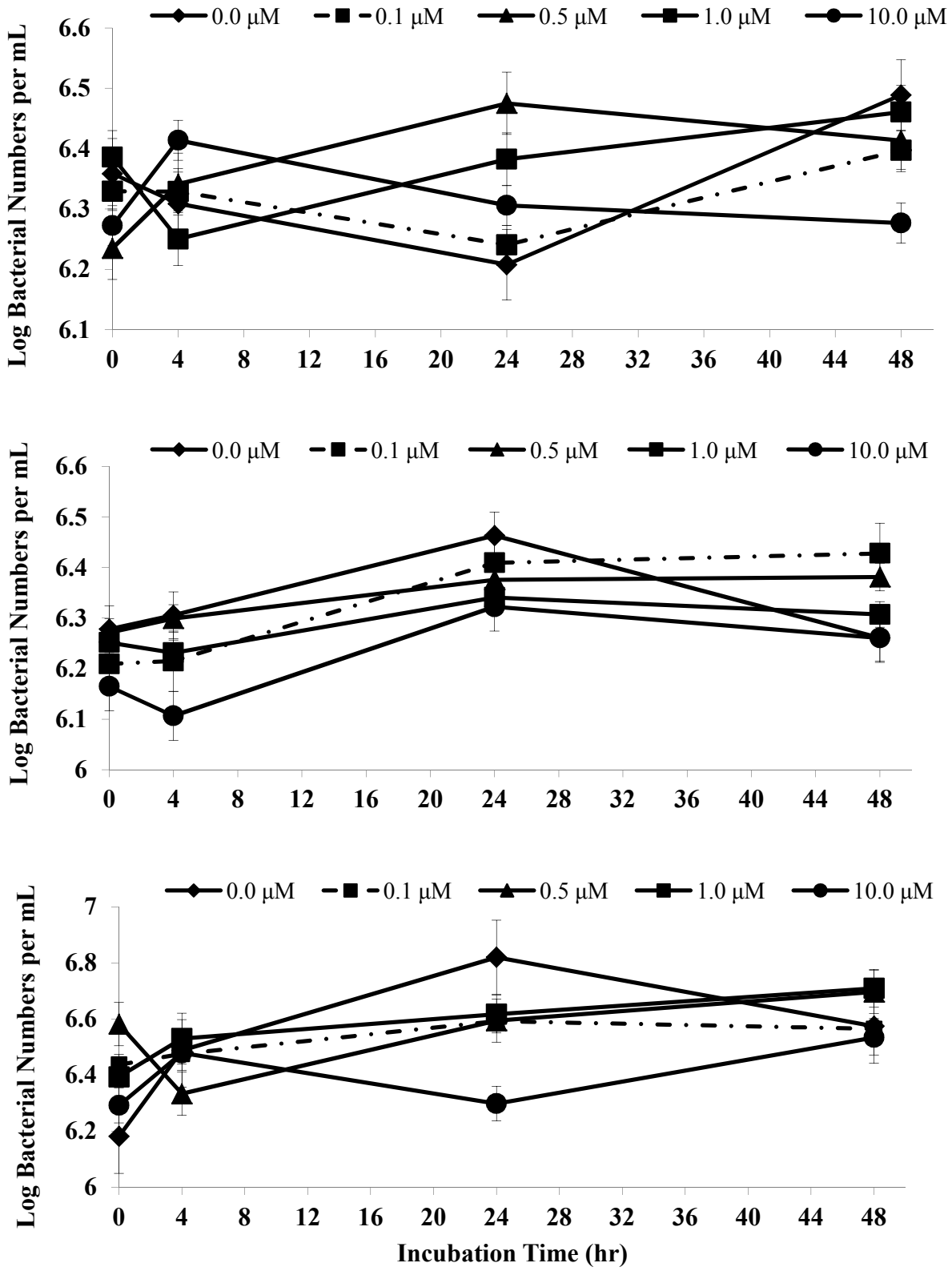
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Log Bacterial Numbers per mL
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0 4 8 12 16 20 24 28 32 36 40 44 48
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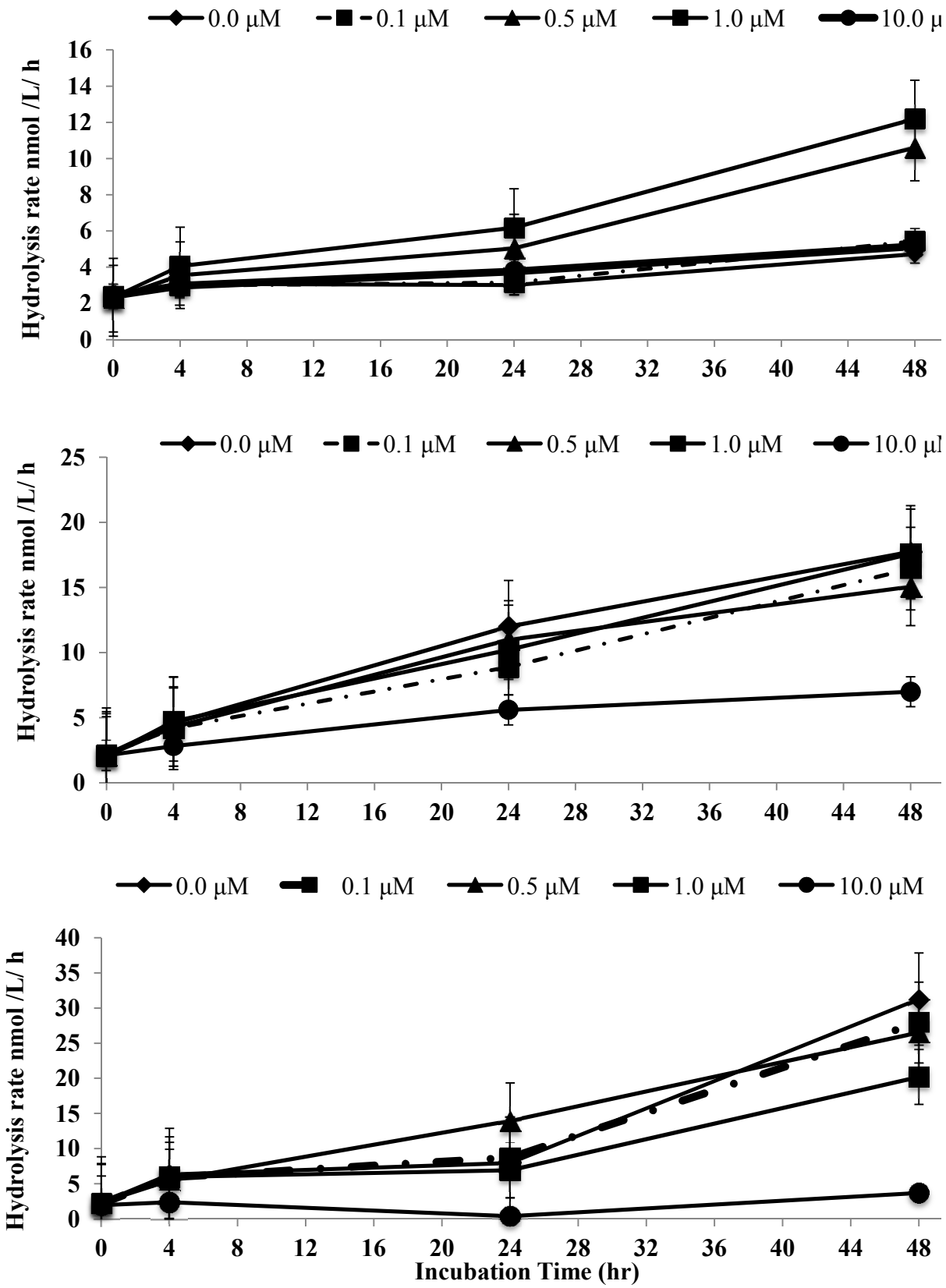
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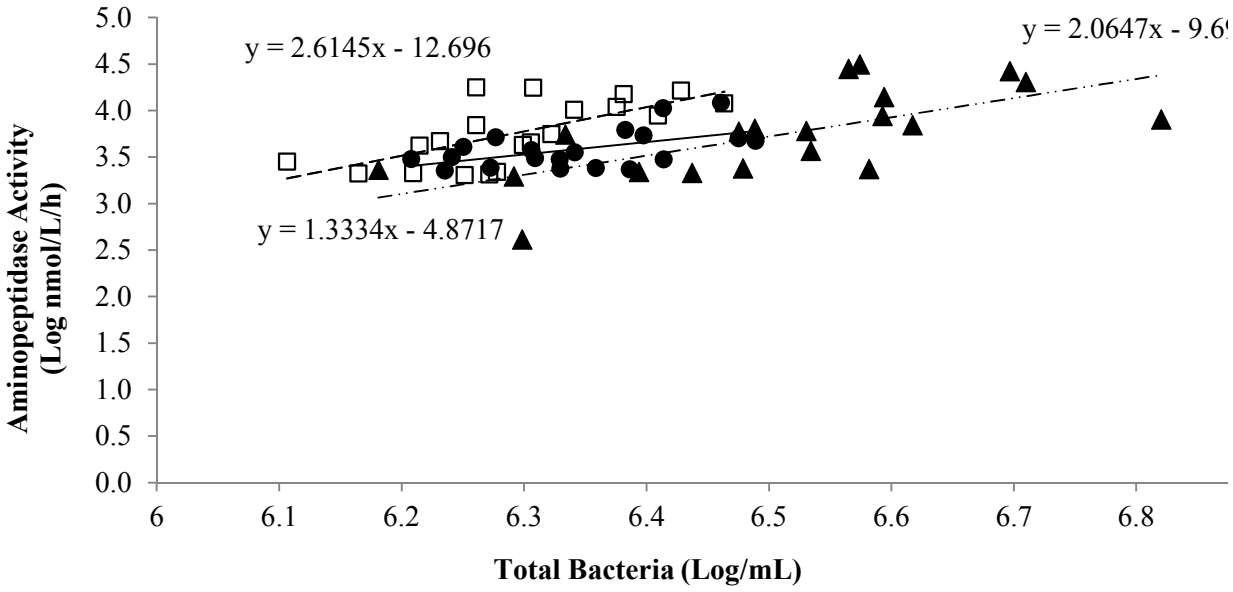
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Aminopeptidase Activity (Log nmol/L/h)
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995

6.9

Table 1. Water Chemistry Characteristics at the three study sites i.e. Kalamazoo River, Rice Creek and Huron River. Values are presented as means of 3 replicates ± SE

Date	Sites	Temp ^{0}C	Conduct mS/cm	DO %	pH	ORP	Chlorine mg/L	Nitrate mg/L	Sulfate mg/L	TBC * 10^6/mL
May	**KR**	13.05±0.01	0.54±0.00	98.10±2.63	7.02±0.00	23.70±1.27	21.15±0.09	8.29±0.31	33.32±0.05	1.08±0.08
	RC	17.12±0.04	0.64±0.03	73.07±17.17	7.80±0.08	-169.7±32.12	23.04±0.24	7.95±0.22	57.85±0.13	1.36±0.12
	HR	16.96±0.02	0.63±0.00	99.70±4.35	7.36±0.03	22.93±4.46	76.42±0.15	6.36±0.02	29.62±0.09	1.71±0.20
June	**KR**	17.93±0.01	0.51±0.00	80.87±2.61	7.28±0.03	14.53±3.06	18.58±0.06	7.43±0.04	30.64±0.15	2.28±0.76
	RC	16.41±0.00	0.59±0.00	77.17±2.77	7.59±0.01	23.20±3.31	21.44±0.16	12.02±0.34	68.23±0.11	1.90±0.24
	HR	21.35±0.03	0.69±0.01	139.25±26.85	7.74±0.07	23.67±6.27	86.19±0.27	5.96±0.02	30.34±0.06	1.52±0.25

Table 2. Results of analysis of variance (ANOVA) on bacterial populations and aminopeptidase activities in response to various zinc concentrations over time

		KR		RC		HR	
Source	df	*F*	*p*	*F*	*p*	*F*	*p*
Bacterial Abundance							
Concentration	4	1.76	0.146	1.77	0.14	2.41	0.56
Time	4	3.58	0.018	14.24	<0.0001	9.92	<0.0001
Concentration*Time	12	1.15	0.336	1.03	0.435	1.43	0.171
Aminopeptidase Activity							
Concentration	4	3.04	0.023	4.48	0.003	14.83	<0.0001
Time	4	33.10	<0.0001	44.80	<0.0001	80.62	<0.0001
Concentration*Time	12	2.33	0.014	1.5	0.145	5.54	<0.0001

Table 3. Results of Post-hoc tests on pair-wise comparison of bacterial numbers and aminopeptidase activities to various zinc concentrations using Bonferroni test. Significant level set at 0.05. NS = not significant

Pairwise Comparisons	*p*		
	KR	**RC**	**HR**
	Bacterial Abundance		
Concentrations (μM)			
0 versus 0.1	NS	NS	NS
0 versus 0.5	NS	NS	NS
0 versus 1.0	NS	NS	NS
0 versus 10	NS	NS	NS
0.1 versus 0.5	NS	NS	NS
0.1 versus 1.0	NS	NS	NS
0.1 versus 10	NS	NS	NS
0.5 versus 1.0	NS	NS	NS
0.5 versus 10	NS	NS	NS
1.0 versus 10	NS	NS	NS
	Aminopeptidase Activity		
Concentrations (μM)			
0 versus 0.1	NS	NS	NS
0 versus 0.5	NS	NS	NS
0 versus 1.0	NS	NS	NS
0 versus 10	NS	0.017	<0.0001
0.1 versus 0.5	NS	NS	NS
0.1 versus 1.0	NS	NS	NS
0.1 versus 10	NS	0.018	<0.0001
0.5 versus 1.0	NS	NS	NS
0.5 versus 10	NS	NS	<0.0001
1.0 versus 10	NS	0.002	<0.0001

Table 4. Results of Post-hoc tests on pair-wise comparison of mean bacterial numbers and aminopeptidase activities over incubation time using Bonferroni test. Significant level set at 0.05. NS = not significant

Pairwise Comparisons	*p*		
	KR	**RC**	**HR**
	Bacterial Abundance		
Incubation Time (h)			
0 versus 4	NS	NS	NS
0 versus 24	NS	0.004	0.018
0 versus 48	NS	0.003	<0.0001
4 versus 24	NS	<0.0001	NS
4 versus 48	0.003	<0.0001	<0.0001
24 versus 48	0.024	NS	NS
	Aminopeptidase Activity		
Incubation Time (h)			
0 versus 4	NS	NS	NS
0 versus 24	<0.0001	<0.0001	<0.0001
0 versus 48	<0.0001	<0.0001	<0.0001
4 versus 24	0.002	<0.0001	NS
4 versus 48	<0.0001	<0.0001	<0.0001
24 versus 48	<0.0001	NS	<0.0001