BIOLOGICAL INDICATORS OF TOXIC STRESS IN WETLAND SEDIMENTS

by

Subhomita Ghosh Roy

A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
in Biological Sciences

at
The University of Wisconsin-Milwaukee
May 2020

ABSTRACT

BIOLOGICAL INDICATORS OF TOXIC STRESS IN WETLAND SEDIMENTS

by

Subhomita Ghosh Roy

The University of Wisconsin- Milwaukee, 2020
Under the Supervision of Timothy Ehlinger Ph.D.

Rapid population growth has created problems in meeting the goals of the Clean Water Act (CWA) "*to restore and maintain the integrity of the nation's waters*". Approaches for monitoring and analysis have increasingly focused on identifying "biological response signatures" that can characterize the complex patterns of ecological responses to stress occurring across levels of biological, spatial and temporal organization. One productive area of research has employed integrated indices of chemical risk, ecotoxicological risk and ecological risk to assess the impact of human activity across disturbance gradients such as urbanization. Selecting relevant metrics for use in constructing multimetric index requires identifying bioindicator organisms across different trophic levels with capacities to detect signals from anthropogenic disturbances.

This study explored the efficacy of a suite of higher plant ecotoxicological assays and sediment bacterial taxonomic community metrics for use as indicators in ecological risk assessment along a gradient of urbanization. The study was conducted in the Pike River watershed (Racine, Wisconsin USA) in six wetlands selected across a gradient of dominant land use types (agricultural, commercial, residential, undeveloped and industrial). Field measurements were taken and sediment samples collected from 2015 through 2017.

MicroBioTest Phytotoxkit TM ecotoxicological assays, based on growth inhibition of three plants (*Sinapis, Sorghum* and *Lepidium*) were used to assess sediment toxicity. Likewise, bacterial taxonomical diversity metrics identified with 16S rRNA gene sequences were used to assess of the bacterial community assemblage of sediments. The Phytotox™ and bacterial community metrics were analyzed in relation to pollutant stress, measured by field concentrations of metals (Ag, As, Cd, Hg, Ni, Pb and Zn) in sediments, concentrations of nitrate and phosphate in the water, and predicted pollutant loadings calculated from surrounding landuse. Additionally, a laboratory microcosm experiment was conducted in 2017 that examined the effects of manipulated pollutant levels of phosphate, nitrate, copper and lead on both PhytoTox™ and bacterial community metrics.

Analysis of results from the field study and microcosm experiments indicate that PhytoTox™ assays (*Sinapis alba*, *Sorghum saccharatum* and *Lepidium sativum*) and sediment bacterial taxonomical diversity from16S rRNA gene sequences are responsive to variation in pollutant loadings and concentrations of metals and nutrients. Statistical interactions and patterns of responses demonstrate that a combination of PhytoTox™ and bacterial taxonomic diversity metrics can serve as predictive bioindicators for ecological risk assessment in urbanizing water sheds. In particular, the response patterns of bacterial genera observed in the microcosm experiments suggest directions for future research and the potential for the development pollutant-specific bacterial indicators.

To
My Niece Antara
The sweetest gift I have ever received

TABLE OF CONTENTS

LIST OF FIGURES

Figure 1.9: Prediction profiles from ANOVA showing the effect of predicted total loading of Cd, Cu, Zn, Pb in Kg/year on the growth inhibitions of the bioindicator species *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. The blue-lined area in each profile represents the 95% prediction confidence interval for the response variable. The profiler is set to Cd at 1.26 kg/ year , Cu at 1.43 kg/ year , Pb at 1.88 kg/ year and Zn at 6.04 kg/ year in case of root growth inhibition and kg/ yr Cd at 1.25 kg/ year , Cu at 1.36 kg/ year , Pb at 1.88 kg/ year and Zn at 13.85 kg/ year in case of stem growth inhibition.

Figure 1.10: Prediction profiles from ANOVA showing the effects of metal components 1 and 2 (from factor analysis) on the growth inhibitions of the bioindicator species *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. The blue-lined area in each profile represents the 95% prediction confidence interval of the response variable. The profiler is set to metal component 1 at 1.0 and metal component 2 at 0.75 in case of root and stem growth inhibition.

Figure 1.11. Prediction profiles for the effects of metal factor 1 and metal factor 1 on Root inhibition. (A) Profile when metal component 2 is set to 1.5, and (B) Profile when metal component 2 is set to -1.0)

Figure 2.1A: The relative abundance of bacterial phyla identified in the collected wetland sediments during summer 2015 identified by 16S rRNA gene sequencing in wetland sites 1,3 and 4. Insufficient DNA was isolated from wetland 2, 5 and 6 during 2015. Phyla with an abundance of less than 1% of the total sample were not included in this figure.

Figure 2.1B: The relative abundance of bacterial phyla identified in the collected wetland sediments during fall 2016 identified by 16S rRNA gene sequencing in wetland sites 1,2 and 5. Insufficient DNA was isolated from wetland 3, 4 and 6 during fall 2016. Phyla with an abundance of less than 1% were not included in this figure.

Figure 2.1C: The relative abundance of bacterial phyla identified in the collected wetland sediments during summer 2017 identified by 16S rRNA gene sequencing in wetland sites 1, 2, 4, 5 and 6. Insufficient DNA was isolated from wetland 3 during summer 2017. Phyla with an abundance of less than 1% were not included in this figure.

Figure 2.2A: The relative abundance of bacterial genera identified in the collected wetland sediments during summer 2015 identified by 16S rRNA gene sequencing in wetland sites 1, 3, 4.. Insufficient DNA was isolated from wetland 2,5 and 6 during summer 2015. Genera with an abundance of less than 1% were not included in this figure.

Figure 2.2B: The relative abundance of bacterial genera identified in the collected wetland sediments during fall 2016 identified by 16S rRNA gene sequencing in wetland sites 1, 2 and 5. Insufficient DNA was isolated from wetland 3,4 and 6 during fall 2016. Genera with an abundance of less than 1% were not included in this figure.

Figure 2.2C: The relative abundance of bacterial genera identified in the collected wetland sediments during summer 2017 identified by 16S rRNA gene sequencing in wetland sites 1, 2, 4, 5 and 6. Genera with less than 1% of the relative abundance in the sediment samples were

removed from this analysis. Insufficient DNA was isolated from wetland 3 during summer 2017. Genera with an abundance of less than 1% were not included in this figure.

Figure 2.3: Scatterplot and line of fit for Shannon and Simpson diversity indices of phyla and genera identified by 16S rRNA gene sequencing in samples from wetland sites 1-6. Sequence numbers are shown as Log_{10} values.

Figure 2.4: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla level out of wetland sites 1-6 from predicted total Nitrate-Nitrite and Phosphate loading (mg/m_2/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate + nitrite at 7.25 kg/ year, phosphate at 22.51 kg/ year in case of Shannon diversity, nitrate+ nitrite at 8.43 kg/ year, phosphate at 24.68 kg/ year in case of Simpson diversity .

Figure 2.5: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from predicted total Nitrate-Nitrite and Phosphate loading (mg/m_2/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate + nitrite at 8.43 kg/ year, phosphate at 24.68 kg/ year in case of Shannon diversity, Simpson diversity.

Figure 2.6: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla out of wetland sites 1-6 from measured nitrate and phosphate concentration (mg/L) during summer 2017. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for median ambient conditions, with nitrate at 3.37mg/L, phosphate at 0.21 mg/L in case of Shannon diversity, 2.19 nitrate at mg/L, phosphate at 0.27 mg/L in case of Simpson diversity.

Figure 2.7: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from measured nitrate and phosphate concentration (mg/L) during summer 2017. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate at 2.72 mg/L, phosphate at 0.49 mg/L in case of Shannon diversity, 2.60 nitrate at mg/L, phosphate at 0.32 mg/L in case of Simpson diversity.

Figure 2.8: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla out of wetland sites 1-6 from predicted total metal (Cd, Cu, Zn and Pb) loadings kg/year. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for Cu at 1.04 kg/ year, Pb at 1.60 kg/ year, Zn at 4.78 kg/year, Cd at 0.23 kg/year in case of Shannon diversity, Cu at 1.17 kg/ year, Pb at 1.82 kg/ year, Zn at 5.43 kg/year, Cd at 0.34 kg/year in case of Simpson diversity.

Figure 2.9: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from predicted total metal (Cd, Cu, Zn and Pb) loadings (Kg/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for Cu at 1.17 kg/ year,

Figure 3.5: The root and stem growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* during the start, middle and end of the metal and nutrient microcosm experiments. This test included a native treatment where no metal or nutrient were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native (control) sediment. Bars show mean ± 1 SE. Multifactor ANOVA tables are located in Appendix 3.2.

Figure 3.6: The Shannon and Simpson diversity indices of phyla and genera during the start and end of the metal and nutrient microcosm experiment. This test included a native(control) treatment where no metal or nutrient were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native sediment. Bars show mean ± 1 SE. Multifactor ANOVA results are presented in Appendix 3.3.

Figure 3.7: Relationship between the increasing concentration of Pb (in mg/L) in the water of the metal microcosm and root inhibition of *Sorghum saccharatum* grown in the sediment of metal microcosm. All other statistically non-significant relationship between increasing concentration of Pb and Cu (in mg/L) in the water of the metal microcosms with root and stem inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* are explained in Table 3.3.

Figure 3.8: Relationship of the (A) Simpson diversity index of phyla with increasing concentration of Pb in the water, (B) Simpson diversity index of phyla with increasing concentration of Cu in the water, (C) Shannon diversity index of phyla, with increasing concentration of Cu in the water, (D) Shannon diversity index of genera with increasing concentration of Pb in the water, (E) Shannon diversity index of genera with increasing concentration of Cu in the water (F) Simpson diversity index of genera with increasing concentration of Pb in the water (G) Simpson diversity index of genera with increasing concentration of Cu in the water, in the water of the metal microcosm . All other statistically non-significant relationship between increasing concentration of Pb and Cu in the water of the metal microcosm with the Shannon and Simpson diversity indices of bacterial phyla and genera from the sediment of metal microcosms are explained in Table 3.3

Figure 3.9: Relationship between the stem inhibition of *Sinapis alba and* the increasing concentration of (A) Ag, (B) Ni, (C)Rb, stem inhibition of *Sorghum saccharatum* with increasing concentration of (D) Ag, root inhibition of *Sinapis alba and* the increasing concentration of (E) As, (F) Cd, root inhibition of *Sorghum saccharatum* with increasing concentration of (G) Cd, (H) Ni and root growth inhibition of *Lepidium sativum* with increasing concentration of (I) As. All metals were detected in the sediment of metal microcosm in ppm. All other statistically non-significant relationship between increasing concentration (ppm) of detected sediment metals are shown in Table 3.4.

Figure 3.10: Relationship of the Simpson diversity index of phyla with increasing concentration of (A) As, (B) Zn, Shannon diversity index of phyla with increasing concentration of (C) Cd, (D) Fe, (E) Hg, (F) Zn, Simpson diversity index of phyla with increasing concentration of (G) Zn. All metals

were detected in the sediment of metal microcosm in ppm. All other statistically non-significant relationship between increasing concentration (ppm) of detected sediment metals of the metal microcosm and the Shannon and Simpson diversity indices and total number of individuals of bacterial phyla and genera from the sediment of metal microcosm. are explained in Table 3.5.

Figure 3.11: Relationship between the (A) root inhibition of *Sorghum saccharatum* (B) stem inhibition of *Sorghum saccharatum* with increasing concentration of nitrate. All metals were detected in the water of the water microcosm in mg/L. All other statistically non-significant relationship between increasing concentration of nitrate (in mg/L) in the water of the nutrient microcosm with the stem, root inhibition of *Sorghum saccharatum* and the diversity indices of bacterial phyla and genera from the sediment of nutrient microcosm. are explained in Table 3.6.

Figure 3.12: The concentration of Pb, Cu in mg/L in metal microcosm and nitrate and phosphate in mg/L in nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metal and nutrient were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

Figure 3.13: The concentration of sediment metal detected (ppm) in metal microcosm and in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE

Figure 3.14: The root and stem growth inhibition of *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum* in metal and nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

Figure 3.15: The Shannon and Simpson diversity indices identified as phyla and genera in metal and nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

Figure 3.16: Hierarchical cluster analysis of the bacterial genera detected (by 16S rRNA gene sequences) in the sediments of the treatments of wetland site 1 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15), control treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment, A low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high concentrations.

Figure 3.17: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 2 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15), control treatment of the metal microcosm experiment of the native sediment with no metals added, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment. . Adequate DNA was not found in the high treatment (with metals (Pb and Cu) added in lower concentration in the water to the native sediment) in in the water to the native sediment and autoclaved sediment with metals added in high and low concentrations.

Figure 3.18: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 5 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15) treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment, A low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high concentrations.

Figure 3.19: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 6 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15) treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high and low concentrations.

Figure 3.20: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 1 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

Figure 3.21: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 2 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

Figure 3.22: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences)detected in the sediments of the treatments of wetland site 5 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

Figure 3.23: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 6 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low and high concentrations.

Figure 3.24: Box plot showing the variation of temperature and DO (mg/L) in the metal microcosm from start (day 0) to end of the experiment (Day 15).

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my advisory committee: Dr. Timothy Ehlinger, Dr. Charles Wimpee, Dr. John Berges, Dr. Erica Young and Dr. Neil O'Reilly. Dr. Ehlinger not only inspired me about the field of Wetland Ecology but also supported me in all the difficult times I had here, professional or personal. Without his help and assistance and passion this project would not have been a reality. Dr. Wimpee and Dr. Young helped in forming my research questions and technical design of the study, including a big part of the lab work was done in their labs. Dr. Berges assisted me with a significant part of the writings whenever I needed it. Dr. O'Reilly helped find a lot of significant resources for developing the study. Thank you for all your time and help in all these difficult years.

I would also like to thank my fellow Fish Ecology students: Andy McGuire (Ph.D) and Lorenzo Backhaus. Andy has assisted me significantly during the field works over the years, which would be not possible without him. Lorenzo helped me in the Ecotoxicological experiments. This project would have been incomplete without the support of Village of Mount pleasant (Racine, WI). I thank you for all your help and support.

This degree could never be possible, neither I would have come to a foreign country to fulfill my dreams without the support from my father Asis Ghosh Roy, my mother Uma Ghosh Roy, my sister Moumita Ghosh Roy, my uncle Dr. N Sinha and my little niece Antara Sarkar. Thanks also to my friends for all your help and support throughout these difficult times.

Chapter 1: Effects of land use and pollution loadings on ecotoxicological assays in constructed wetlands

Introduction:

The establishment of water quality standards as the mechanism for categorizing water bodies and tracking progress toward the attainment of the goals of the Clean Water Act (CWA) "*to restore and maintain the integrity of the nation's waters*" (EPA 2012) has challenged the science of environmental monitoring to bridge the social-ecological domains of designated uses (e.g. fishable-swimmable) with the interconnected biogeochemical cycles affecting protective numerical criteria (e.g. phosphate standards) in order to inform antidegradation policies developed in the complex political arena (e.g. agricultural buffer widths) (Glicksman and Batzel 2010). To this end, it became imperative to incorporate diverse disciplinary perspectives in the selection of metrics and indicators for use in monitoring programs (Cairns and Pratt 1993).

The U.S. Environmental Protection Agency (USEPA) initially promoted the use of monitoring strategies that integrated metrics of water quality parameters, whole-effluent toxicity testing, and ambient biological assays (Karr 1993). This "3-legged stool" approach has proven to be limited in its capacity to characterize ecological integrity across diverse environmental context (Karr 1995, Yoder and Rankin 1995a, 1995b). In order to develop more effective and robust monitoring strategies, the use of biological assays and bioindicators has increased steadily (Karr 1993, 1995). Concurrent with the increase in available tools and data, analytical approaches have increasingly focused on detecting "biological response signatures" (Yoder and Rankin 1995a) as a way to characterize the complex patterns of ecological responses to stress occurring across levels of biological, spatial and temporal organization (Cairns and Pratt 1993).

Supporting this trend has been research on how multiple biological criteria can be used for assessing the impact of human activity on biological indicators across disturbance gradients (Clapcott et al. 2012, Decker et al. 2017). In United States urbanization is one of the major reason that impacts the surface water quality(Wang and Lyons 2003) correlated with increasing impervious surface area, increasing stormwater runoff into local streams, rivers, lakes and wetlands (EPA 2003a). Higher rates of runoff carry increased concentrations and loadings of nutrient and heavy metal pollutants contributing to deterioration in the water quality of the streams, rivers, lakes and wetlands (Foley et al. 2005) (Figure 1.1). Increased public awareness of the interconnections between changes in landcover and surface water quality contributed to the passing of the US Clean Water Act (CWA) in 1972, establishing quality standards for surface waters and setting limits for the discharge of pollutants and excess nutrients (Carey and Hochmuth 2012, EPA 2012).

Rivers and their contributing watersheds provide a diverse array of ecosystem goods and services (Wilson and Carpenter 1999) spanning from hydropower, agricultural irrigation, transportation and waste assimilation to fisheries, tourism and recreation. Managing for shared uses and the inevitable conflicts that arise as trade-offs are balanced is a central challenge for the 21st century (Tallis et al. 2008)**.**This creates complex challenges for the implementation of the CWA for environmental management that arise from the need to make predictions regarding the potential social, economic, and ecological impacts of a proposed activity (e.g. sighting a landfill) on ecosystem goods and services (Munns et al. 2016) .These challenges are compounded by the reality that decisions must often be formed on the basis incomplete information and inherent uncertainty, which gave rise to the field of ecological risk assessment (Suter 2006).Ecological risk assessment (ERA) has been defined as ‘‘the practice of determining the nature and

likelihood of effects of anthropic actions on animals, plants, and the environment'' (SETAC 1997). Different frameworks aimed at managing ERA studies have been developed in several countries (i.e., United States, Canada, European Union) and applied to environmental decision-making (Jardine et al. 2003).

Approaches have been developed to assess ecological risk that employ a triad approach (Dagnino et al. 2008) using an integrated index of chemical risk, ecotoxicological risk and ecological risk. These multi-metric indices incorporate data from multiple monitoring efforts conducted at different spatial and temporal scales which are then compared to data collected at the target sites (Suter 2001).Probabilistic methods have been collectively referred to as "*weight-of -evidence approaches*" for the integration of environmental data to asses ecological risk (Dagnino et al. 2008).

Selection of relevant metrics that comprise a multimetric index is not a simple process if it is to serve both in detecting change and predicting ecological risk (Schoolmaster et al. 2012). One must consider bioindicators from across different trophic levels and their capacity to detect signals from anthropogenic disturbances (Decker et al. 2017). Research has shown that the use of multiple groups of organisms increases the potential to gather the information necessary to develop a robust understanding of impacts on ecological integrity (Brown et al. 2009, Waite 2014). By incorporating patterns of covariation among diverse bioindicators in developing multimetric indicators, ecological risk assessment approaches can provide a robust tool for measuring impacts on ecological integrity. (Yoder and Rankin 1995a) used the term "biological response signatures" to describe the variety of ways that multimetric indicators in aquatic ecosystems may respond to different types of environmental stressors. Their work reframes the

question away from looking for distinct cause-effect relationships towards identifying signals of response amidst the complex noise of potential causes (Clapcott et al. 2012, 2014).

Studies have shown repeatedly that multimetric indicators of ecological integrity comprised of water quality, macroinvertebrates and fish communities are significantly more effective as risk assessment endpoints indices compared to non-integrative measure (Clapcott et al. 2014). Such multi-level approaches that employ diverse metrics can also provide effective methods for monitoring contaminant exposure levels and the environmental adverse effects into individual warning situations (Chapman 1990).

Ecological Risk Assessment approaches have been used extensively for both monitoring the effects of development (*ex post* impact assessment) and predicting the likely effects of proposed projects (*ex ante* impact assessment). To this end, constructed wetlands have been used extensively to address water quantity and quality problems and mitigate the environmental impacts of historical urbanization and minimize the impact of new construction (Tixier et al. 2012). In addition to capturing sediment and pollutants that flow off surrounding landscapes (Kadlec and Wallace 2009), constructed stormwater wetlands can play a critical role managing nutrients generated from agricultural and urban runoff (Manios et al. 2009, Scholz and Hedmark 2010, Beutel et al. 2014). In addition, constructed wetlands have been shown to be effective in reducing heavy metal contamination generated from industrial sources (Khan et al. 2009, Knox et al. 2010, Sahu 2014).

Biomonitoring is measuring and evaluating the conditions of a living system (Karr and Chu 1999) . Since the passage of CWA, biomonitoring has become an essential component for monitoring the ecological integrity and condition of watersheds (Karr and Chu 1999) and bioindicators developed to serve as tools for assessing attainment of and adherence to water

quality standards (Yoder and Rankin 1995a) . Bioindicators detect signals across diverse temporal and spatial scales and provides integrated assessment of level of environmental impact on watershed integrity and (Kovacs 1992, Karr and Chu 1999, Dellinger et al. 2014). Various forms of bioindicators have been used to predict ecosystem integrity in wetlands. For example, vegetation, invertebrates, fishes, birds, algae, amphibians and microorganisms have been used for bioindicator studies in wetlands (Sims et al. 2013). Bioindicators developed for wetland sediments have been shown to be particularly sensitive in detecting ecological changes in watersheds (Ke et al. 2015, Aylagas et al. 2017) and for conducting sediment risk assessments from pollutants such as metals or nutrients (Chapman 1995, Jensen 2011, Dellinger et al. 2014).

This present chapter investigates the efficacy of the Phytotoxkit™ (Microbiotest Inc 2015) ecotoxicological assay with plants *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba* as bioindicators of sediment toxicity among wetlands with varying land uses and associated pollutant (nutrient and metals) measurements. PhytoToxKits™ measure the growth inhibition of the indicator plants and have been shown to be effective in detecting toxic hazards in sediments in reservoirs and urban canals subjected to varying levels of marked nutrients and heavy metal such as Cd, Cr, Cu, Mn, Ni, Pb, Zn contamination (Czerniawska-Kusza, I. and Kusza 2010) (Czerniawska-Kusza et al. 2006). These Phytotoxkits™ provide low-cost, relatively easy assays to administer and have great potential for use for routine evaluations as bioindicators (Persoone and Vangheluwe 2000, Sims et al. 2013).

This present chapter will address two questions regarding the application of PhytoToxKits™ for use as bioindicators. First, does variation in growth inhibition of PhytoTox™ ecotoxicological assays (*Sorghum saccharatum, Lepidium sativum* and *Sinapis alba)* correlate with variation in pollution-related stressors, either as loadings estimates that enter

wetlands from their surrounding watersheds or as measured concentrations within the wetlands (i.e. *ex post* impact indicators for monitoring)? Second, can PhytoTox™ ecotoxicological assays serve as predictive bioindicators of pollution loadings wetlands (i.e. *ex ante* impact bioindicators for ecological risk assessment)?

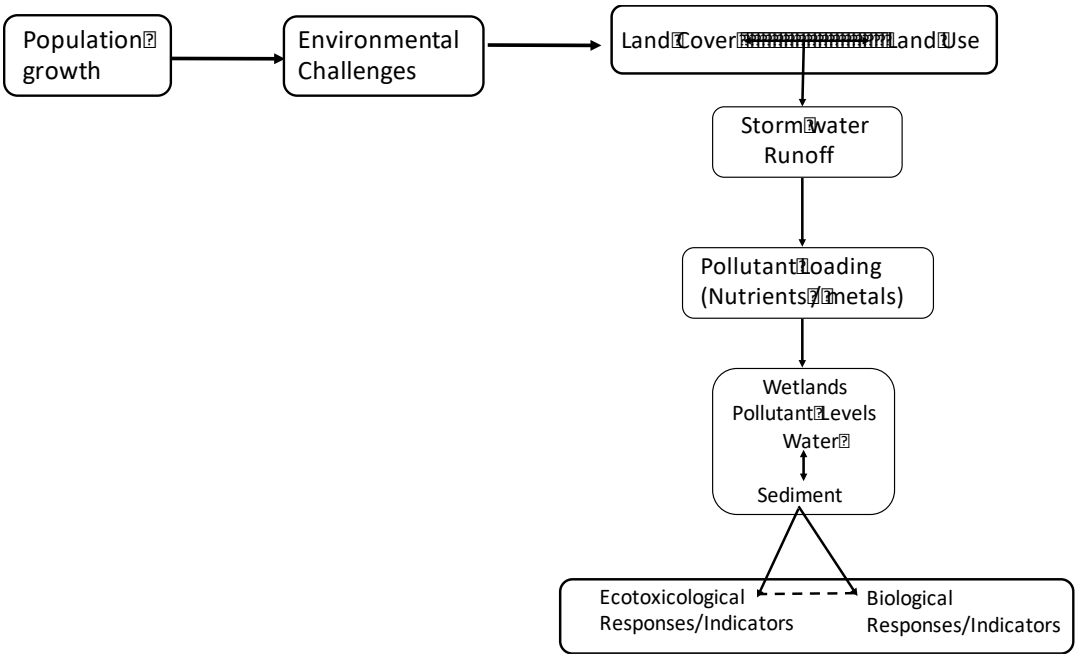


Figure 1.1: Hierarchy of factors affecting the investigation of landcover and land use on ecotoxicological and bacteriological bioindicators in wetland sediments.

Methods:

Study system, Land use and site characteristics:

This present study was conducted in Pike River watershed (Racine County, Wisconsin USA) utilizing a series of stormwater wetlands that were constructed between 2001 and 2008 as structural features in a flood-control plan implemented by the Village of Mount Pleasant. The plan included significant modifications in channel morphology, the creation of riparian wetland-pond systems, and the installation of fish habitat along an 8 km stretch of river (Crispell-Synder 1997, Ehlinger et al. 2002, Ehlinger and DeThorne 2004). The wetlands were excavated to receive runoff from adjacent catchments that comprised from a combination of agricultural, commercial, residential, undeveloped, and industrial land uses (Crispell-Synder 1997, Ehlinger et al. 2002, Ehlinger and DeThorne 2004). Six individual wetlands were selected for this study to capture a gradient of dominant land cover types (Figure 1.2). The catchment area and percent land uses (residential, commercial, industrial, agricultural and undeveloped) for each wetland were determined from (SEWRPC 2010) and are shown in Table 1.1 together with mean water quality characteristics for each wetland measured during summer 2017.

Table 1.1: Wetland site, water quality characteristics and organic matter (OM) percent monitored in ten separate days between June - August 2017 of wetland sites 1-6 in the Pike river watershed.

	Land Cover (Percent of Watershed)					
Wetland Site	Watershed Area (m^2)	Percent Residen -tial	Percent Commer -cial	Percent Indus -trial	Percent Agricu -ltural	Percent Undeve -loped
1	1044534.1	11.0	15.1	12.1	61.6	0.0
2	3341812.4	42.3	0.0	0.0	57.5	0.0
3	2674587.1	41.8	0.0	0.0	58.2	0.0
4	28773.2	58.9	6.0	0.0	35.2	0.0
5	4937165.0	15.7	14.2	20.8	0.0	49.3
6	7200013.8	0.0	72.2	20.2	0.0	7.2

Water and Sediment Characteristics					
Median Temperature (^0C)	Median pH	Median Specific conductance (mS/cm)	Median Dissolved Oxygen (%)	Median Dissolved Oxygen (mg/L)	Median Organic Matter percent
21.5	7.6	870.0	105.9	9.2	8.1
21.2	7.7	634.0	101.8	8.7	13.3
20.2	7.2	701.0	79.2	6.9	17.0
19.9	7.2	907.0	48.1	3.8	8.4
21.4	7.7	974.5	88.6	7.8	3.8
21.8	7.1	1395.5	81.5	7.1	14.0

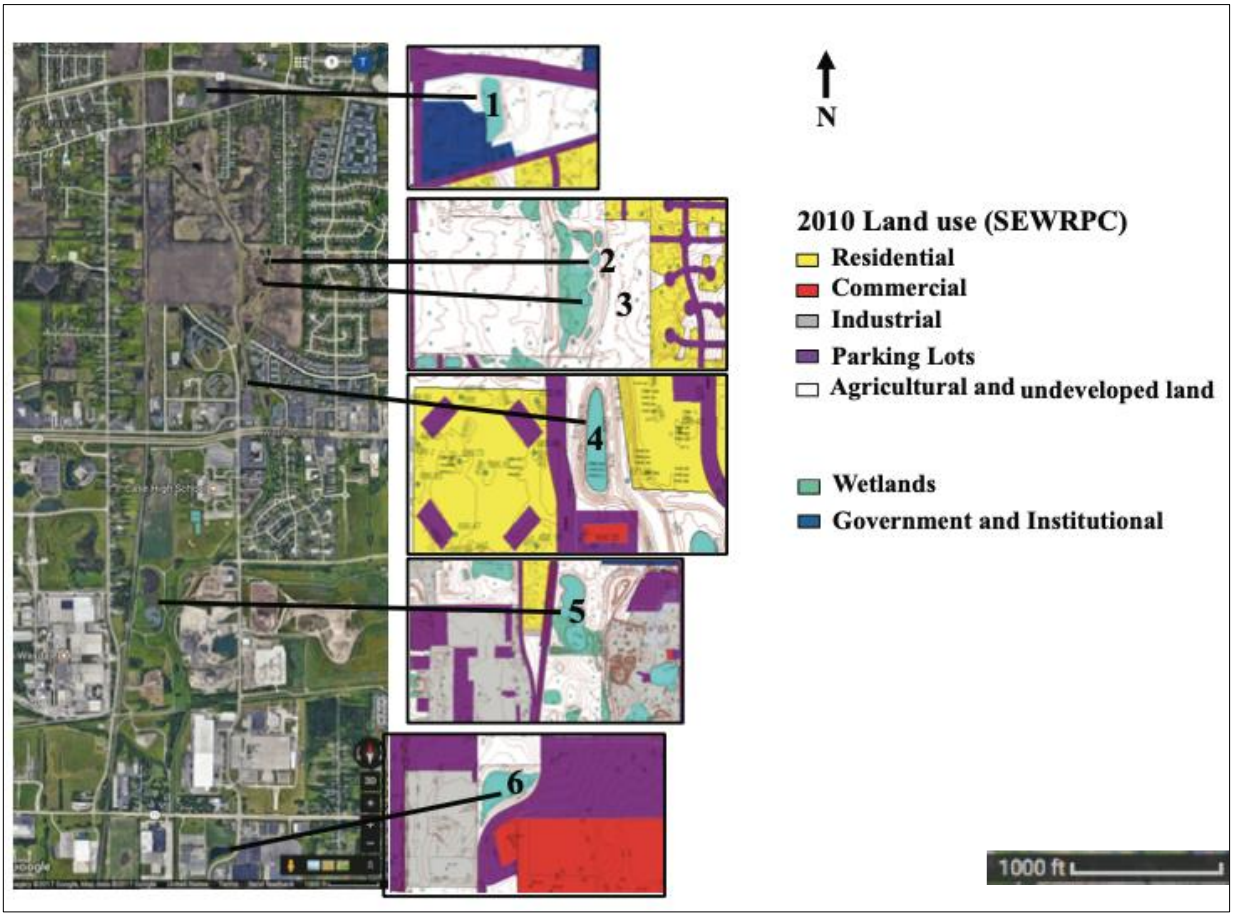


Figure 1.2: Aerial photograph of the Pike River North Branch (Google 2015) showing locations (42^0 43' N and 87^0 52' W) of study wetlands and surrounding 2010 land use (SEWRPC 2010).

Pollution loading estimates:

Pollutant loadings into wetland sites were estimated based upon the calculated land uses draining into each wetland. The percent land use measurements for each category within the area draining into each wetland site was provided in Source Load and Management Model or SLAMM (Pitt and Voorhees 2000), run by Village of Mount Pleasant, Racine County Wisconsin, 2011 during construction of these wetlands (Figure 1.3, Figure 1.4). By default, agricultural lands were incorporated into the undeveloped lands category in SLAMM, due to its use as an urban planning model (Crispell-Synder 1997, Pitt and Voorhees 2000). Therefore, land classifications were manually re-coded to agricultural land uses by cross comparison with the 2010 SEWRPC land cover data (SEWRPC 2010). All land use measurements were converted from acres as provide by SLAMM to square meters. The percent land uses were calculated with respect to the total area of the land cover draining into the wetlands. Values for each of the land uses categories (residential, industrial, commercial, undeveloped and agricultural) are the summation of the source subcategories (e.g. roofs, street area, parking, driveways, sidewalks and landscaped area) (Figure 1.3, Figure 1.4) (Pitt and Voorhees 1995).

Predictions for pollutant loadings (nitrate+nitrite , phosphate, Zn, Pb, Cu and Cd) were estimated using the geometric mean of values measured from studies reported in the literature (Pitt and Bozeman 1982, Bannerman et al. 1983, 1993) (Denver Regional Council of Governments 1983, Pitt and McLean 1986, Novotny 2003) by the source area subcategories (e.g. roofs, street area, parking, driveways, sidewalks and landscaped area) of each land use category (residential, industrial, commercial, undeveloped and agricultural) (Table 1.2). Due to inadequacy of data the loading estimates of nitrate and metals like Ag, As, Hg and Ni could not be calculated. Then the total pollutant loadings in Kg/year were calculated by multiplying the

pollutant loading estimates from the literature by the source area (m^2) subcategories (e.g. roofs, street area, parking, driveways, sidewalks and landscaped area) of each land use category (residential, industrial, commercial, undeveloped and agricultural) in a year. This produces the total pollutant loading at each wetland site by land use category (residential, industrial, commercial, undeveloped and agricultural) in a year. These calculated loadings are shown in Figure 1.4 and Table 1.2.

Table 1.2: Predicted area-weighted loading and total loadings of nutrients and metals for wetland sites 1-6 based upon land use and watershed area.

		Area-Weighted Loadings (mg/m2/year)					
Wetland Site	Watershed Area (m^2)	Nitrate-Nitrite	Phosphate	Cd	Cu	Pb	Zn
1	1044534	0.210	1.060	0.004	0.040	0.114	0.215
2	3341812	0.190	0.985	0.007	0.030	0.051	0.153
3	2674587	0.180	1.040	0.007	0.040	0.052	0.173
4	28773	0.310	0.950	0.005	0.020	0.071	0.180
5	4937165	0.410	0.941	0.028	0.050	0.080	0.240
6	7200014	0.390	0.712	0.004	0.050	0.052	0.199
		Total Loading from Watershed (Kg/year)					
Wetland Site	Watershed Area (m^2)	Nitrate-Nitrite	Phosphate	Cd	Cu	Pb	Zn
1	1044534	2.194	11.074	0.044	0.418	1.187	2.241
2	3341812	6.349	32.926	0.220	1.003	1.690	5.126
3	2674587	4.814	27.807	0.194	1.070	1.385	4.618
4	28773	0.089	0.273	0.001	0.006	0.021	0.052
5	4937165	20.242	46.434	1.364	2.469	3.963	11.870
6	7200014	28.080	51.265	0.272	3.600	3.737	14.321

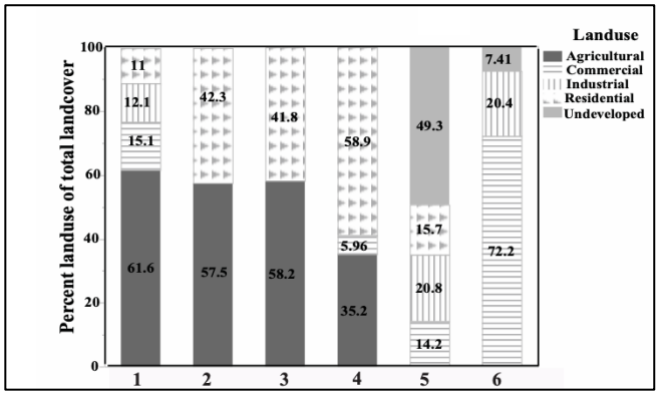


Figure 1.3: Percentage land use types for the drainage basins for the 6 constructed wetlands used in this study. Land use data from SEWRPC (2010) were accessed through the Racine County Map Server website. http://racinecounty.maps.arcgis.com.

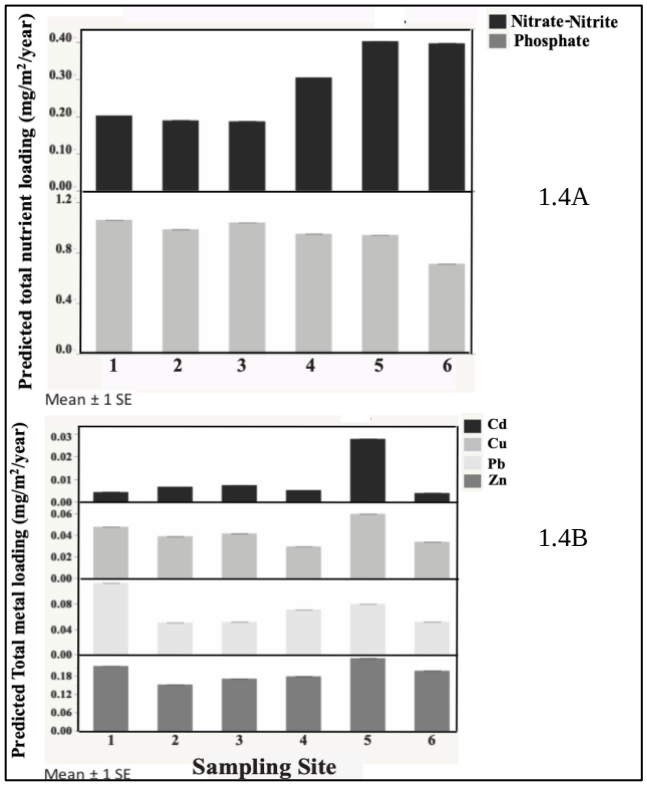


Figure 1.4: (A) Predicted model of total nutrient (nitrate-nitrite and phosphate) area-weighted loadings (Kg/year) from land use runoffs for wetland sites 1-6. (B) Predicted model of total metal (Cd, Cu, Zn, Pb) area-weighted loadings (Kg/year) from land use runoffs for wetland sites 1-6.

Sediment sampling collection, water quality monitoring:

Sediment sampling: Sediment samples were collected from the wetland sites 1-6 during summer 2015, fall 2016 and summer 2017. During summer 2015, a core sampler (5 x 50 cm) was used to collect sediment samples to a depth of 10-15 cm from top surface layer at three locations (two at both shorelines and the third one at the middle zone) for each wetland site. Sediments were homogenized in the field and were divided into two replicates, yielding two samples per site and were stored on ice in 1-liter Nalgene™ bottles for transport to the laboratory. At summer 2015, total 12 samples were collected. Based upon results from the Fall 2015 samples, the sediment collection process was modified in 2016 by using an Ekman dredge grab sampler (15 x 15 x 25 cm) to gather a greater amount of sediment from the upper surface layer, without compressing the sediment samples (Katich et al. 2012). At each wetland site, samples were collected at two different locations from the edge zone in fall 2016. Sediments were homogenized in the field and were divided into three replicates, yielding three samples per site and stored on ice in 1-liter Nalgene™ bottles for transport to the laboratory. During fall 2016, total 18 samples were collected. During summer 2017, two edge and two middle zone samples were collected. This process yielded four samples per site in 2017. Samples were stored in Nalgene 1 liter bottles on ice before transport to laboratory. During summer 2017, total 24 samples were collected. Hence, there were total 54 sediment samples from all sampling times. At the laboratory, samples were stored in separate replicates as collected at -80^0C for use in bacterial analysis (see Chapter 2) and -25^0C for eco-toxicological studies and sediment characterization of percent organic matter and analysis of metals using X-ray fluorescence (XRF).

Water quality parameters, wetland depth profile and sediment percent organic matter of each site were measured as site characteristics during summer 2017. Water quality parameters were measured using multi-parameter YSI 6600 sondes that recorded pH, temperature, dissolved oxygen, specific conductance, and turbidity(YSI 2020). Water quality characteristics were monitored in twelve separate days between June - August 2017 of wetland sites 1-6 in the Pike river watershed.

Water samples for nutrient analysis were collected during summer (in twelve separate days between June – August) 2017 of wetland sites 1-6 in the Pike river watershed, same days of water quality monitoring. These water samples were collected using inverted 1L Mason™ jars at the same locations where sonde data (water quality) were recorded. These water samples were collected using inverted 1L Mason™ jars. Water samples were stored on ice, transported back to the laboratory for analysis within 24 hours of sample collection.

Organic content of sediments was determined by measuring the loss of weight upon ignition (Storer 1984). Crucibles were weighed prior to the addition of 10-15 grams of sediment from each sample. The samples were then oven-dried (at 80^0C) overnight and then re-weighed, after which the sediment samples were ignited to ash at 500^0C using a muffle furnace (an additional hour required to allow the oven to reach the desired temperature). Upon cooling at room temperature between 25-30^0C the samples were weighed again and the weight of the crucibles were taken off from this post-ignition weight in grams. The percent organic matter was calculated for each sample by taking the difference of weight between oven-dried and the ashed sediments without the crucible weight, using the following formula (Storer 1984):

Ecotoxicological Assays:

Ecotoxicological tests were carried out following the standard operational procedures for Phytotoxkit™ (Microbiotest Inc 2015) using three plant species: monocot *Sorghum saccharatum*, dicot *Lepidium sativum* and *Sinapis alba*. Control and test sediments were added and saturated with distilled water in PVC test plates (21 x 15.5 x 0.8 cm). Filter paper was placed on top of each of the control and test sediments and ten seeds of the same plant were placed on the filter paper in one row and at equal distance from each other. This was repeated with all the three seed species. Plates were incubated at 25◦C in darkness for 72 hours. Digital images were taken of all the plates, and stem and root lengths were measured using Image J™ software (Schneider et al. 2012). The proportion of root and stem length inhibition of the test sample plants were calculated relative to the control plant growth to generate growth inhibition indices. One phytotoxkit test ™ combined the growth inhibition test plates for *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba*. Hence with 54 sediment samples each test had 162 test plates (54 x3). The same test was repeated twice yielding 310 sediment plates. Some sediment collected were not enough to do two tests.

Nutrient and metal measurement in wetland sites :

Water nutrients and sediment heavy metals: Water samples for nutrient analyses were collected using inverted 1L glass jars at the same locations and depths where sonde data were recorded. Water samples were stored on ice and transported back to the laboratory for analysis within 24 hours of sample collection. Nitrate and phosphate were analyzed with HACH DR 2800™ spectrophotometer using nitrate (powder pillow test kit – Cadmium reduction method) and phosphate test kit (powder pillow test kit –Ascorbic acid method). For nitrate test kit , the cadmium reduction method with a detection range of 0.3 - 30.0 mg/L NO_3^-–N was used

(Drummond and Maher 1995, Hach 2019). For phosphate kit, the PhosVer 3 (Ascorbic Acid) method was used with a detection range of 0.02-2.50 mg/L PO_4^{3-} (Hach 2019).

Sediment: The presence of heavy metals (Ag, Hg, Pb, As, Ni, Zn and Cd) in the sediments was estimated using X-ray fluorescence (XRF), a widely used technology for the detection of metals in soils and sediments (Baranowski et al. 2002, Kenna et al. 2011, Díaz Rizo et al. 2014). Sediment samples were dried at 60-80°C until a constant weight is obtained. Large rocks, organic debris, twigs, leaves roots were removed. Dried samples were homogenized using a mechanical homogenizer and then were turned into a ~5 g pellets of approximately 25 mm diameter and 5 mm height using a 25 metric ton press pellet. XRF analyses were conducted using Bruker Tracer III-V+ p-spectrophotometer (Bruker 2017) using the red filter settings. This setting allows x-rays from 14 to 40 KeV to reach the sample, which are better for analyses of higher Z elements such as heavy metals (EPA 2007, Díaz Rizo et al. 2014, DiScenza and Keimowitz 2014).

Calibrations were performed using National Institute of Standards and Technology (NIST 2013) Standard reference materials or SRMs containing certified amounts of the targeted metals in soil or sediments. The Standard reference materials or SRMs were obtained from NIST. The XRF signal intensity was plotted against the value of each of the SRM to construct the calibration curves. Blank samples composed of chemically pure Silica homogenized and formed into pellets were used to check the cross-contamination or other interferences. All the analyses were performed in a sample cup under the Si-Pin detector of the Bruker Tracer III-V+ p-spectrophotometer (EPA 2007, Díaz Rizo et al. 2014, DiScenza and Keimowitz 2014, Bruker 2017) and 3 readings (in ppm) were taken for each sample (Zięba-Palus, J., Kunicki 2006).

Sediment metal and ecotoxicological indicators:

To validate the process of using X-ray fluorescence (XRF) to detect the presence of metals in the sediments at ppm level we used this technology at a study in India. A large portion of India textile dye industry exists in the informal economy, dumping their wastewater without treatment into nearby land and water. The goal of this study was to use the Phytotoxkit™ ecotoxicological assays (Microbiotest Inc 2015) using three plant species: monocot *Sorghum saccharatum*, dicot *Lepidium sativum* and *Sinapis alba* to characterize the extent of toxic stress coming to the adjacent ecosystems by dumping informal dye industry-waste directly in soil at two different locations in India.. This wastewater has the potential for creating toxic impacts as dyes are contaminated with heavy metals like copper (Cu), zinc (Zn), lead (Pb), etc. We were able to detect Sr (Strontium), Zn (Zinc), Co (Cobalt), Pb (Lead), Zr (Zirconium), Fe (Iron), Rb (Rubidium), Ti (Titanium), Mo (Molybdenum), Mn (Manganese), V (Vanadium) and Cu (Copper) at ppm level. For example Pb was detected at the range of 0-20 ppm, Zn at the level of 0-40 ppm, Cu at 0-7 ppm. The commercial dyes were also observed to have metals like Fe, Cu. Rb, Sr, Zr. Mo, Pb and Au (Gold). With Pb was observed to be present in the range of 0-200 ppm, Cu in 0-600 ppm in the commercial dyes. Using the standard operational procedure of the Phytotoxkit, Microbiotest™, proportion root and stem growth inhibition of the bioindicator plants (*Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba*) was measured in dye contaminated test soil with respect to control soils for each plant. Our study clearly showed that metals concentration as detected in the dye-contaminated soil, resulted in growth inhibition in the bioindicator plants. Suggesting that in combination toxic can cause an impact on the ecosystem (Ghosh Roy et al. 2019) (Appendix A).

Data Analyses:

Data distributions were examined for normality and were transformed as necessary to meet assumptions of statistical tests. Count and length data were transformed using a log transformation (log10 (X + 1) while proportional data were transformed using an arcsine transformation (Sokal and Rohlf 1981) prior to statistical analyses conducted using JMP® 14 (SAS 2019).

Effect of Nutrients on Growth Inhibition: Multifactor Analysis of variance (ANOVA) was used to examine the effects of pollutants (nutrients and metals) and seed species on growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum*. ANOVA for the effects of nutrients on growth inhibition, the growth inhibitions were entered as dependent response variables with nutrient concentration and seed species as independent variables. Since *in-situ* nutrient data were collected only in 2017, effect of measured nutrient concentrations on the growth inhibition could only be tested for one year.

Land use was assumed to not have changed significantly over the course of the study, and as such the ANOVA tests for the effect of predicted loadings from the surrounding land use on stem and root growth inhibitions (the dependent response variable) included loadings and seed species and year as independent variables.

Effect of Sediment Metals on Growth Inhibition: Factor analysis was conducted using the log transformed concentrations of the metals (Ag, Zn, As, Cd, Ni, Pb and Hg) measured from the wetland sediments in ppm. Factor Analysis was conducted using from JMP® 14 (SAS 2019) with maximum likelihood and varimax rotation method based on correlation matrix. ANOVA for the effect of measured metal pollution on growth inhibition was conducted using the metal factor component scores, seed species and year as the independent factors in the model.

ANOVA tests for the effect of predicted loading of individual (Cd, Cu, Pb, Zn) metals from the surrounding land use on growth inhibition (the dependent response variable) included loadings and seed species and year as independent variables.

Examination of Effect Interactions: Prediction profiles were used together with multi-factor models in JMP® 14 (SAS 2019) to help examine how values of independent factors (either nutrients or metals) interact to influence growth inhibition a complex set of criteria. Prediction profiler uses the patterns of variation from ANOVA to visualize how response parameters (i.e. stem or root growth inhibition) change as the levels of individual factors are changed, and to understand the interactions between the pollutant concentrations. For example, in a nutrient analysis, prediction profiler would predict the effect of growth inhibition of individual species the when the nitrate concentration is high or low and the same time how the growth inhibition changes with respect to the phosphate concentration (SAS 2019). If there are interaction effects in the model, the prediction traces can shift their slope as the values of other terms are manipulated.

Finally, forward stepping multiple regression was used to determine a best fit model for the combined predictive linear relationships between pollutants (nutrients and metals) and growth inhibition of the ecotoxicological bioindicators species.

Results:

Ecotoxicological bioindicators:

Proportion root and stem growth inhibition values calculated relative to growth in control sediments (clean silica sand) so that positive values indicate inhibition (i.e. reduced growth = inhibition) whereas negative values indicate growth facilitation (i.e. increased growth = facilitation). For *Lepidium sativum*, root inhibition in ranged from -1.5 to +1.5 and stem

inhibition ranged from -0.75 to +1.25. For *Sinapis alba*, root inhibition varied from -1.5 to +1.25 and stem inhibition ranged from -1 to +1.25 (Figure 1.5B). For *Sorghum saccharatum*, the proportion root inhibition ranged from -1.5 to +1.25 and stem inhibition ranged from -3.5 to +1.5. Responses varied among wetland sites and between years (Figure 1.6). *Sorghum* exhibited consistently higher growth inhibition for both root and stem across the study compared to the other two bioindicator species (Figure 1.6). Wetland 1 exhibited consistently the lowest inhibition (highest facilitation) values for *Lepidium sativum* and *Sinapis alba*, whereas wetlands 3 & 4 exhibited higher inhibition (Figure 1.6).

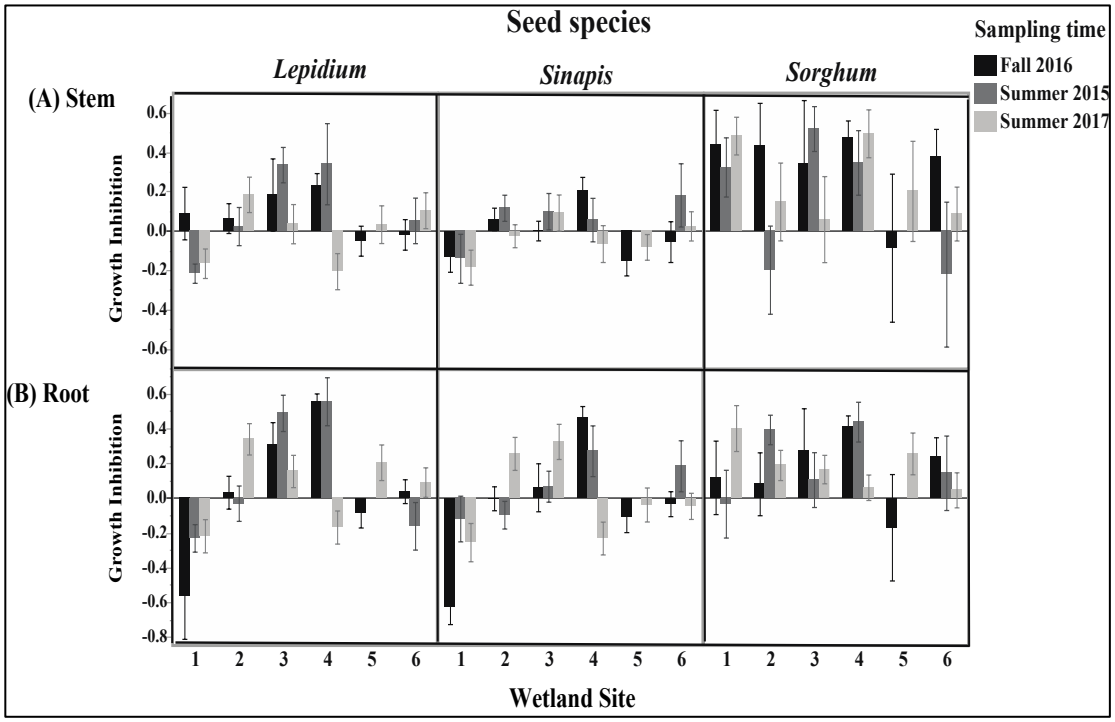


Figure 1.5: Variation of growth inhibition of the bioindicator plant species *(Lepidium sativum, Sinapis alba, Sorghum saccharatum)* and wetland sites (1-6) in three sampling dates of Fall 2016, Summer 2015 and 2017 seasons. (A) Stem Inhibition and (B) Root Inhibition. Bars show mean ± 1 SE.

Nutrient concentration range:

The Nitrate concentration ranged from 0 to 11 mg/L across all sampling sites during summer 2017 and the phosphate concentration ranged from 0 to 1.8 mg/L across all sampling site water during summer 2017. In the wetland site (1-6) sediment. concentration of silver (Ag) ranged from 8-13 ppm, Arsenic (As) was from 0-4 ppm, Cadmium (Cd) was from 1.4 – 2.6 ppm, Mercury (Hg) was from 0.25 – 2.75 ppm, Nickel (Ni) was from 12 – 21 ppm, Lead (Pb) was from 0.001 – 0.0035 ppm, Zinc (Zn) was from 5-40 ppm.

Factor analysis with measured metals:

Factor analysis was conducted with the metal concentrations resulting in two linear components: Component 1 with high positive loadings for Ag, Zn, As, Cd, Ni concentration, and Component 2 with a positive loading for Pb and negative loading for Hg (Figure 1.9). The concentration of Pb loaded positive on component 2 whereas Hg concentration loaded negatively along the component 2 axis (Figure 1.9), suggesting that these metals were negatively associated with each other in sediment samples.

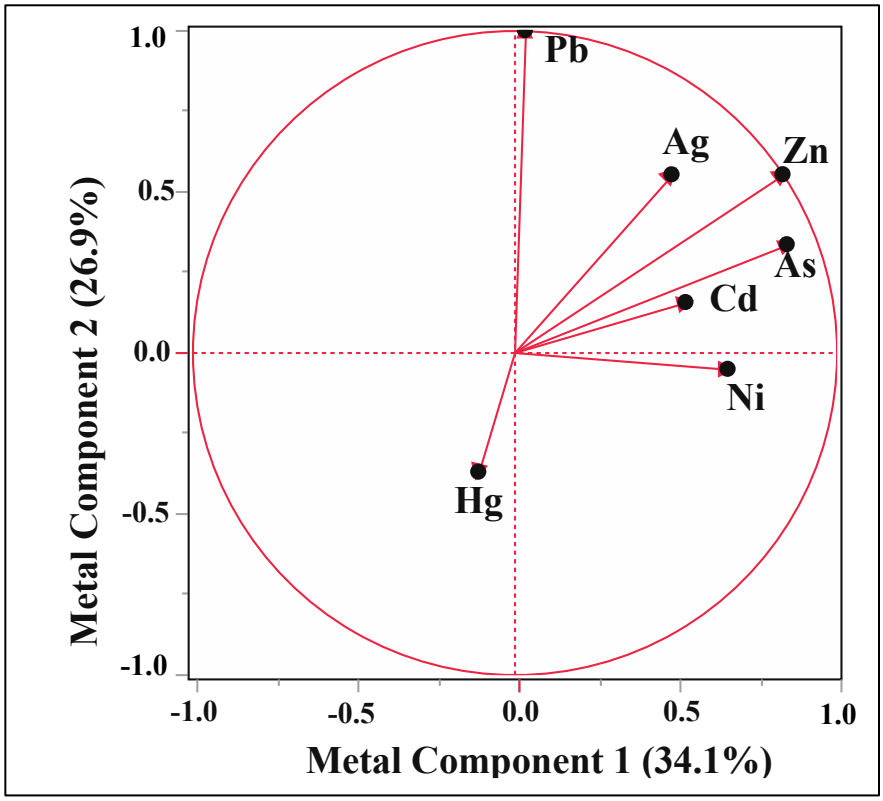


Figure 1.6: Component loadings from multivariate Factor Analysis using JMP® 14 (SAS 2019) Analysis was conducted on the correlations matrix using maximum likelihood and varimax rotation method. The detected metals by XRF (Ag, Zn, As, Cd, Ni, Pb and Hg in ppm) were factored into two linear components (Component 1 and 2) which represented 61% of the total variation. Data were from sediments collected from wetland sites 1-6 sampled during 2015-2017. The plot shows rotated factor loadings relative to each component in the multivariate space.

Ecotoxicological bioindicator Responses to Pollution Stress:

Nutrient Effects:

For predicted nutrient loading ANOVA models the dependent variables were the growth inhibitions and the x variables (effects) were predicted total nutrient loadings and seed species. This model initially considered year as an independent variable but as no significant effect of this variable was observed, the year effect was not considered in the final model (Figure 1.10). This final model detected no statistically significant effects of predicted nutrient loadings or the seed species on the root growth inhibition of *Lepidium sativum, Sinapis alba, Sorghum saccharatum* (Figure 1.10, p-values: seed species = 0.5024, Nitrate+nitrite loading = 0.4916, phosphate loading = 0.8761, nutrient interaction = 0.9162). Although with increase in the nitrate+nitrite and phosphate loading decrease in the root growth inhibition was observed. There were significant results for stem growth inhibition. There were significant effect of the seed species ($p< 0.0001$) with highest inhibition in *Sorghum saccharatum*, nitrate+nitrite loading (P= 0.0041) and the nutrient interaction (P= 0.0116) on stem growth inhibition of *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. But the effect of phosphate loading was not significant for stem growth inhibition (P=0.0898) (Figure 1.10). Stem growth inhibition was observed to be decreasing with nitrate+nitrite loading but increasing with phosphate loading (Figure 1.10).

Response patterns were different with respect to *in situ* nutrients measured directly in the wetlands (Figure 1.11) where root inhibition was significantly affected by phosphate (P= 0.0207) and its interaction with nitrate (P= 0.0190) (Figure 1.11). In this case, increased phosphate levels were related to increased root inhibition (Figure 1.11). However, the effects of nitrate concentration (P= 0.0743) and seed species (P= 0.4924) were not significant (Figure 1.11) Stem inhibition exhibited no significant relationship to variation in measured nitrate concentration (P= 0.4060), phosphate concentration (P= 0.2304), the nutrient interaction (P = 0.2859 or seed species (P= 0.0551) (Figure 1.11).

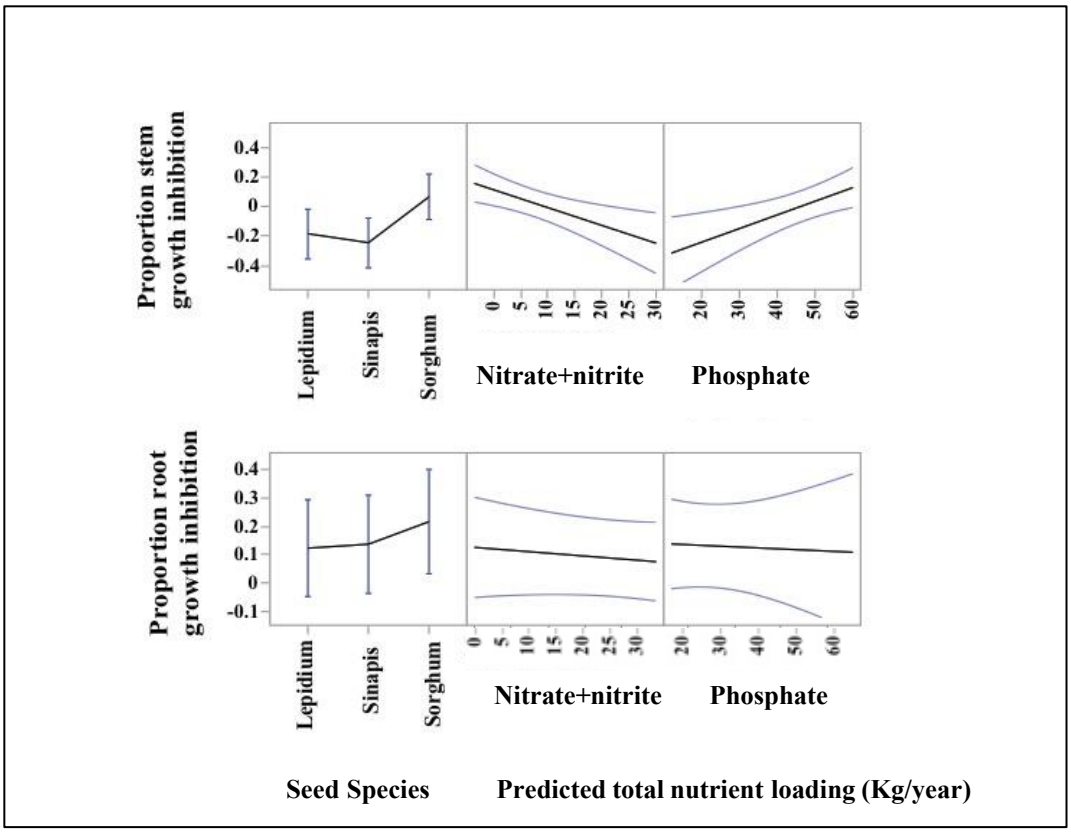


Figure 1.7: Prediction profiles from ANOVA showing the effects of seed species and the predicted total loadings of total nitrate+nitrite and phosphate (Kg/year) on the growth inhibitions of stems and roots for the bioindicator species *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate+nitrite at 1.96 kg/ year, phosphate at 26.23 kg/ year in case of root growth inhibition and nitrate+nitrite at 10.91 kg/ year, phosphate at 26.23 kg/ year in case of stem growth inhibition.

.

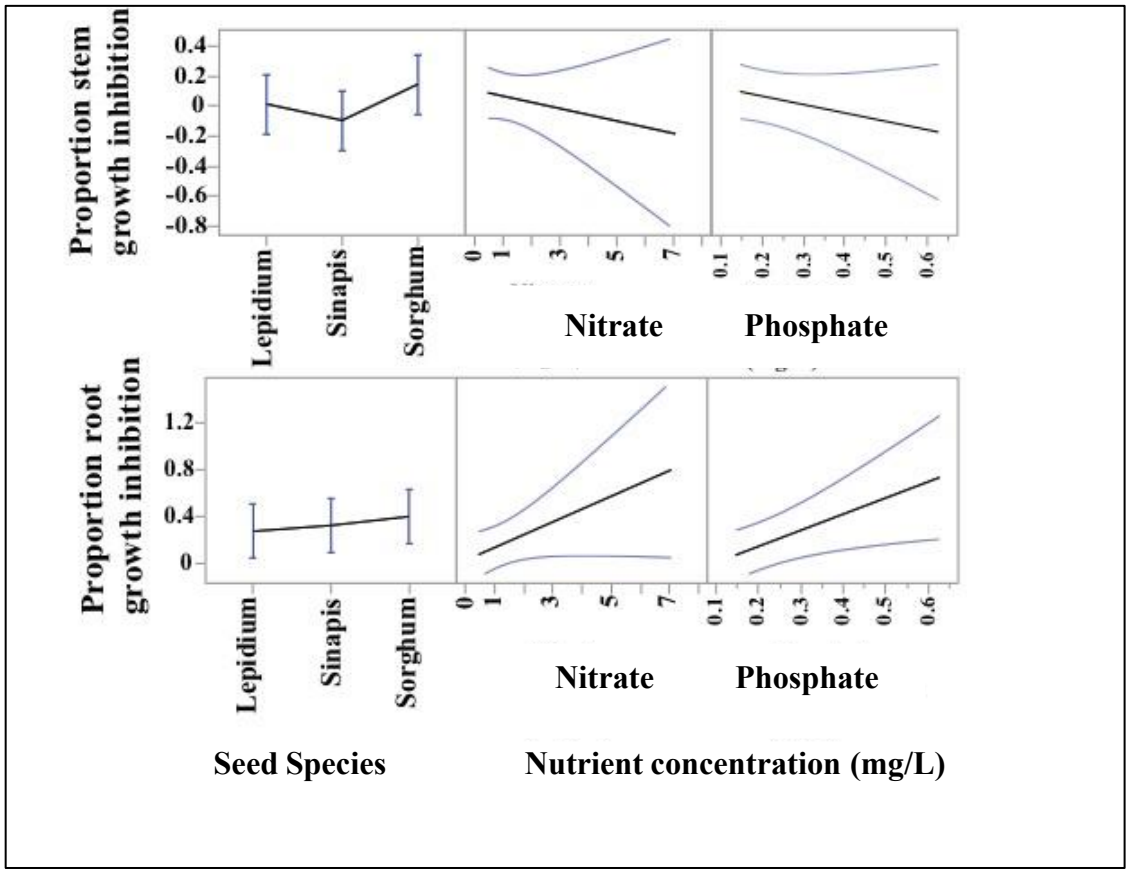


Figure 1.8: Prediction profiles from ANOVA showing the effects of seed species and nitrate and phosphate concentrations (mg/L) measured in wetlands on the growth inhibitions of the bioindicator species *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum*. The blue-lined area in each profile represents the 95% confidence prediction interval of the y (continuous) variable). The profiler is set to nitrate at 2.27 mg/L and phosphate at 0.29 mg/L in case of root and stem growth inhibition.

Metal Effects

For predicted metal loading ANOVA models the dependent variables were the growth inhibitions and the x variables (effects) were predicted total metal loadings and seed species. This model initially considered year as an independent variable but as no significant effect of this variable was observed, the year effect was not considered in the final model (Figure 1.12). The effects of heavy metal loadings predicted by land cover on root inhibition were not statistically significant except for Pb (Figure 1.12). P-values for root inhibition: seed species = 0.4359, Cd loading = 0.3064, Cu loading = 0.9990. Pb loading = 0.0168, Zn loading = 0.6119, metal loading interactions = 0.4625). Decreased root inhibition (i.e. facilitated root growth) was associated with increase in Pb loading. Likewise the effects of heavy metal loadings predicted by land cover on stem inhibition were not significant (P-values for effect on stem inhibition: Cd loading = 0.3167, Cu loading = 0.6489, Pb loading = 0.1512, Zn loading = 0.9076, metal loading interaction = 0.4629). Seed species responded differently to predicted metal loadings. For stem inhibition the effect of seed species effect was significant ($P < .0001$) with highest inhibition observed in *Sorghum saccharatum*.

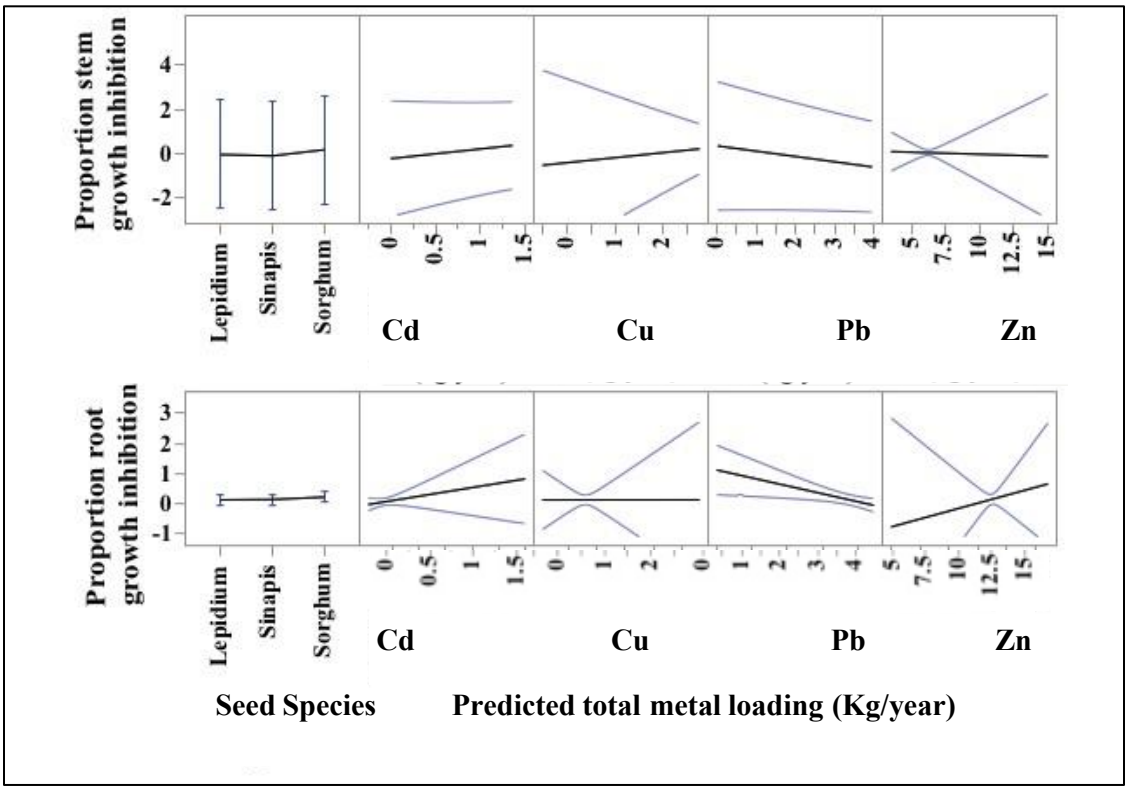


Figure 1.9: Prediction profiles from ANOVA showing the effect of predicted total loading of Cd, Cu, Zn, Pb in Kg/year on the growth inhibitions of the bioindicator species *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. The blue-lined area in each profile represents the 95% prediction confidence interval for the response variable. The profiler is set to Cd at 1.26 kg/ year , Cu at 1.43 kg/ year , Pb at 1.88 kg/ year and Zn at 6.04 kg/ year in case of root growth inhibition and kg/ yr Cd at 1.25 kg/ year , Cu at 1.36 kg/ year , Pb at 1.88 kg/ year and Zn at 13.85 kg/ year in case of stem growth inhibition.

The effects metal concentrations measured on ecotoxicological bioindicator growth inhibition parameters were examined through ANOVA using component scores calculated from the Factor Analysis (described above) as independent variables: Component 1 with high positive loadings for Ag, Zn, As, Cd, Ni concentration, and component 2 with a positive loading for Pb and negative loading for Hg (Figure 1.9). Neither of the metal components had a statistically significant effect on stem growth inhibition (Figure 1.13). P values metal component 1 = 0.3511, component 2 = 0.8892, interaction = 0.5697) . However, seed species did respond differently (p=0.0002), with highest inhibition observed in *Sorghum saccharatum*.

By contrast, metal component 2 showed significant effect on root inhibition ((P= 0.0026 Figure 1.13), indicating an interaction between Pb and Hg on root growth inhibition. An increase in component 2 is associated with a both a higher Pb concentration and lower Hg concentration, which is associated with decreased root growth inhibition (i.e. growth facilitation, Figure 1.13). Stated in the reverse, the combination of decreasing Pb and increasing Hg are associated with increased root inhibition (Figure 1.13). The effect of seed species on root inhibition was not significant (P = 0.4391). There was a suggestion of an effect of metal component 1 on root inhibition but it was not statistically signification (p = 0.0969), potentially due to a statistical interaction between the two metal components (p = 0.0508)

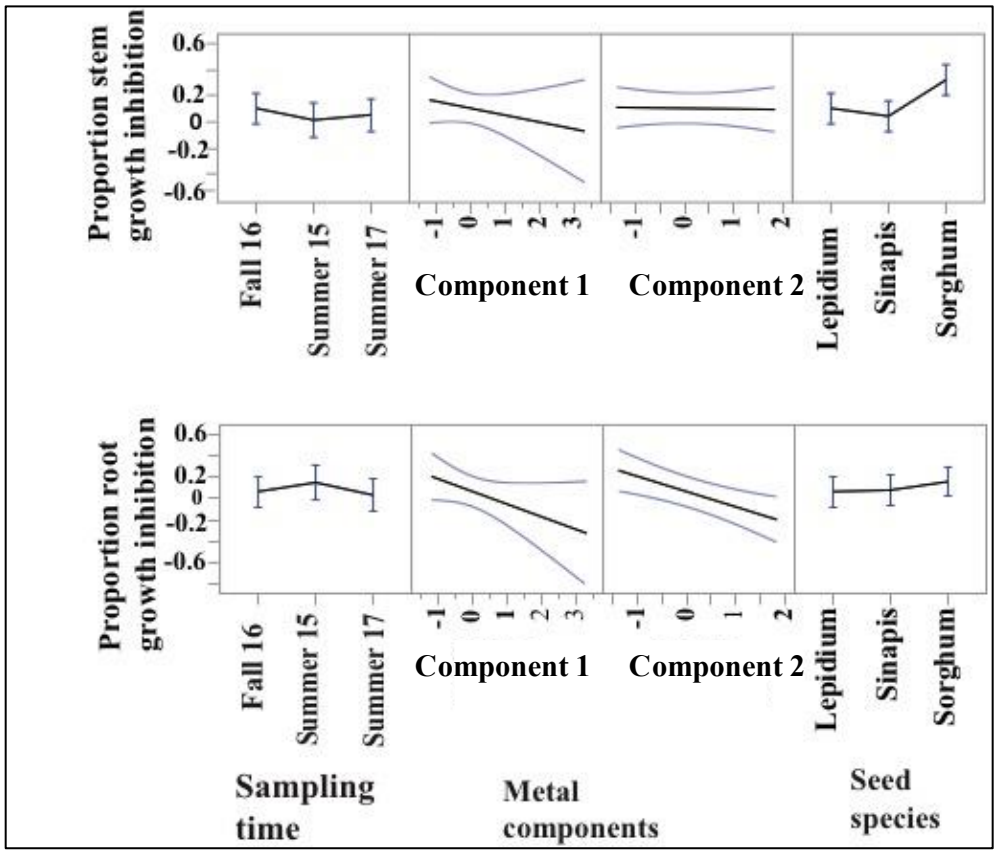


Figure 1.10: Prediction profiles from ANOVA showing the effects of metal components 1 and 2 (from factor analysis) on the growth inhibitions of the bioindicator species *Lepidium sativum, Sinapis alba, Sorghum saccharatum*. The blue-lined area in each profile represents the 95% confidence interval for the prediction of the response variable. The profiler is set to metal component 1 at 1.0 and metal component 2 at 0.75 in case of root and stem growth inhibition.

Stepwise Regression:

The capacity for nutrients (nitrate and phosphate) and metals (Hg and Pb) to predict ecotoxicological bioindicators was assessed directly using stepwise multiple regression. Each indicator species was run separately for nutrient (2017 data only) and metal (2015-2017 data) and best fit models using forward stepping algorithm are presented in Table 1.3. The indicator data was also used by the year along with the pollutants.

For stem inhibition, *Lepidium sativum* exhibited no significant response to variation in nutrients or metals (Table 1.3). *Sinapis alba* stem inhibition had a positive relationship with nitrate but a negative relationship with phosphate with total of R_2 0.84 Table 1.3. Conversely, *Sorghum sachharatum* stem inhibition exhibited a negative relationship with nitrate but a positive relationship with phosphate but also had a positive relationship with Pb and Hg with overall R_2 of 0.96 (Table 1.3).

With respect to root inhibition, *Lepidium sativum* growth inhibition was associated positively with nitrate and negatively with Pb and Hg with overall R_2 of 0.82 (Table 1.3). *Sinapis alba* root inhibition was negatively associated with Pb and Hg with overall R_2 of 0.55 (Table 1.3). For *Sorghum sachharatum* root inhibition had a negative estimated relationship with Pb with overall R_2 of 0.39 (Table 1.3).

Table 1.3: Stepwise regression with the estimates of relationship between nutrient (nitrate and phosphate concentration in mg/L and metal (Hg, Pb) concentration in ppm with stem and root growth inhibition of *Lepidium sativum*, *Sinapis alba* and *Sorghum sachharatum* in wetland sites 1-6 along with appropriate R_2 and P values. The blank spaces revealed no estimates of relationship.

	Seed Species	Nitrate	Phosphate	Pb	Hg	R_2
(A)Stem Inhibition						
	Lepidium					
	Sinapis	0.31 (P=0.0656)	-1.96 (P=0.0286)			0.84
	Sorghum	-1.77 (P=0.1249)	1.88 (P=0.2417)	998.83 (P=0.1879)	4.80 (P=0.1548)	0.96
(B)Root Inhibition						
	Seed Species	Nitrate	Phosphate	Pb	Hg	R_2
	Lepidium	2.12 (P=0.0998)		-1559.8 (P=0.1216)	-6.75 (P=0.1310)	0.82
	Sinapis			-1011 (P=0.1962)	-2.46 (P=0.1196)	0.55
	Sorghum			-234.54 (P=0.0031)		0.39

Discussion:

This study was designed to explore the potential of PhytoTox™ ecotoxicological tests to serve as possible bioindicators for pollution loading from surrounding land uses for wetland ponds located in urbanizing watersheds. The present study investigates the efficacy of the Phytotoxkit™ (Microbiotest Inc 2015) ecotoxicological assay with plants *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba* as bioindicators of sediment toxicity among wetlands with varying land uses and associated pollutant (nutrient and metals) measurements. Previous studies have demonstrated utility of ecotoxicological assays as practical and dependable tools to detect hazards in polluted ecosystems (Czerniawska-Kusza et al. 2006, Czerniawska-Kusza, I., & Kusza 2010). With increasing phosphate concentration in mg/L increased root inhibition was observed (Figure 1.11) but an opposite trend relationship was observed with the model predictive loadings. With increase in the nitrate+nitrite and phosphate loading a trend of decrease in the root growth inhibition was observed. With regard to metal pollution, analysis using factor analysis revealed significant effects of associated groups of metals (component 2: Pb and Hg) (Figure 1.13). The combination of decreased Pb and increased Hg are associated with increased root inhibition (Figure 1.13). This is also supported in the regression analysis suggesting a negative estimated relationship between Pb and root growth inhibitions of growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* (Table 1.3). Even the predictive model suggested that for root growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* there could be decrease in inhibition with increase in Pb (Figure 1.11). All these facts suggest that the concentration at which Pb was detected (in ppm) in these wetland sites (1-

6) did not affect these three plant bioindicator species of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* negatively.

Mercury is most often released into the environment from industrial pollution, mining, fossil fuel burning, and is a chemical of concern (Heidenreich et al. 2001, Manikandan et al. 2015). Experiments have shown that Hg causes reduced growth in the root and stem of seedlings (Manikandan et al. 2015). Reduced growth happens due the production of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide causing damage in the cell membranes, chloroplast pigments and nucleic acids (Baker et al. 2001, Manikandan et al. 2015). The factor analysis revealed that the combination of decreased Pb and increased Hg are associated with increased root inhibition (Figure 1.13). But regression analysis suggested that root growth inhibition of *Lepidium sativum* and *Sinapis alba* decreased in association with increased Hg with a negative estimated relationship and no relationship with *Sorghum saccharatum* (Figure 1.13). This suggests that the metal factor analysis was not able to clearly depict the individual interplay among species with the detected Hg concentration (Table 1.3). This clearly shows with the help of two different statistical test a much more robust risk assessment can be done.

The regression analyses for each ecotoxicological bioindicator species also revealed complex relationship between inhibition and facilitation when nutrients were analyzed in conjunction with metal pollutants (Table 1.3). For example, *Sinapis alba* stem inhibition had a positive relationship with nitrate but a negative relationship with phosphate with total of R_2 0.84 (Table 1.3). Conversely, *Sorghum sachharatum* stem inhibition exhibited a negative relationship with nitrate but a positive relationship with phosphate but also had a positive relationship with Pb and Hg with overall R_2 of 0.96 (Table 1.3). With respect to root inhibition, *Lepidium*

sativum growth inhibition was associated positively with nitrate and negatively with Pb and Hg with overall R_2 of 0.82 (Table 1.3). *Sinapis alba* root inhibition was negatively associated with Pb and Hg with overall R_2 of 0.55 (Table 1.3). For *Sorghum sachharatum* root inhibition had a negative estimated relationship with Pb with overall R_2 of 0.39 (Table 1.3).

Agricultural and residential land uses both produce runoffs rich in nutrients such as phosphate and nitrate due to the presence of fertilizers and pesticides applied to lawns, gardens and agricultural fields. These fertilizers and pesticides especially when rich in nutrients affect plant growth (Altieri and Nicholls 2003, Chen et al. 2004, Scheirs and De Bruyn 2004, López-Luna et al. 2009, Pang et al. 2010, Liu et al. 2014a, Zhawar et al. 2014, Shukla et al. 2017). Our results that indicate that high phosphate be associated with higher growth inhibition was surprising (Figure 1.11) since phosphorus deficiency reportedly causes growth inhibition in plants (Morgan and Connolly 2013).This suggests that there may be interactions between the loadings of nutrients (e.g. from fertilizers) and loadings of metals associated with these pesticides. This became more clear in the regression analysis, where phosphate did not have a significant association with root growth inhibitions of *Lepidium sativum, Sinapis alba* and *Sorghum sachharatum* when analyzed individually, so phosphate individually did not create a negative impact (Table 1.3).

In component 1 in the factor analysis had positive loadings for an array of metals Ni, Cd, As, and Zn (Figure 1.9), which had a modest impact on growth inhibition (Figure 1.13). Our previous studies performed on textile dye contaminated soils in India (Ghosh Roy et al. 2019), also have shown how combination of metals present in a soil ecosystem can affect bioindicator species of *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba*. Th results in the present

chapter are consistent with a pattern of metals interacting to have combined toxic effect on bioindicator plant species (Ghosh Roy et al. 2019).

The reasons contributing to the differing responses by the different ecotoxicological bioindicator species is grounds for further study. Herbicides and metals are well-known to affect growth and development of *Sorghum saccharatum* (López-Luna et al. 2009, Gerik et al. 2010) as in this present chapter . In comparison however, *Sinapis alba* and *Lepidium sativum* frequently exhibited negative inhibition (facilitation) for root and stem growth in this present chapter.

The predicted loadings were a constant measure over a period of time (a year) and was used as a reference point in comparison to the instantaneous nutrient and metal concentration measurements. The significant effects from the measured pollutants such as phosphate concentration and the predicted phosphate loadings did not agree with each other but the predictive Pb loadings and the measure Pb did agree with each other as stated before. Mercury(Hg) was not in the predictive model as adequate data on Hg was not available.

One of the challenges for monitoring for environmental impacts in terms of Clean Water Act is to identify and develop indictors that can capture and integrate the effects of pollutants or stressors across various (sometimes mis-matched) spatial and temporal scales. Chronic stressors such as baseline nutrient loading from agricultural fields provide fundamentally different signals to detect compared to acute events such as a manure spill or pesticide application whose detection by direct chemical measurement may be missed between monitoring sessions. To this point, multimetric indicators must be constructed to include an array of biological sub-metrics that can detect biological responses to human activities across robust spatial-temporal scales (Karr and Chu 1999). The situation is made more complicated by the fact that interactions

among different stressors in nature may result in complex response patterns that can result in the interpretation of the patterns detected being very context dependent.

Prediction profiles provide a useful tool for visualizing the complexity of interactions among pollutants and help in understanding why a single relationship for a single indicator is not sufficient in characterizing biological response signature. An example demonstrating the interaction effect of metal factors on root inhibition is shown in Figure 1.14. The top half shows the expected relationship between root inhibition and Metal component 1 when Metal component 2 is set to a value of 1.5 which would indicate high levels of lead and low mercury (Figure 1.14 A). In this case, the prediction is that one would expect to see at most a small positive effect if any of increasing levels of metal component 1 on root inhibition. By contrast, the prediction profile shown in Figure 1.14B illustrates the predicted changes in root inhibition relative to changing levels of metal component 1 when the level of metal component 2 is held to -1.0 (Low lead with high mercury). In this situational circumstance, the slope of the relationship between root inhibition and metal component 1 is negative, where increasing levels of metal component 1 are predicted to result in lower levels of root inhibition (Figure 1.14B).

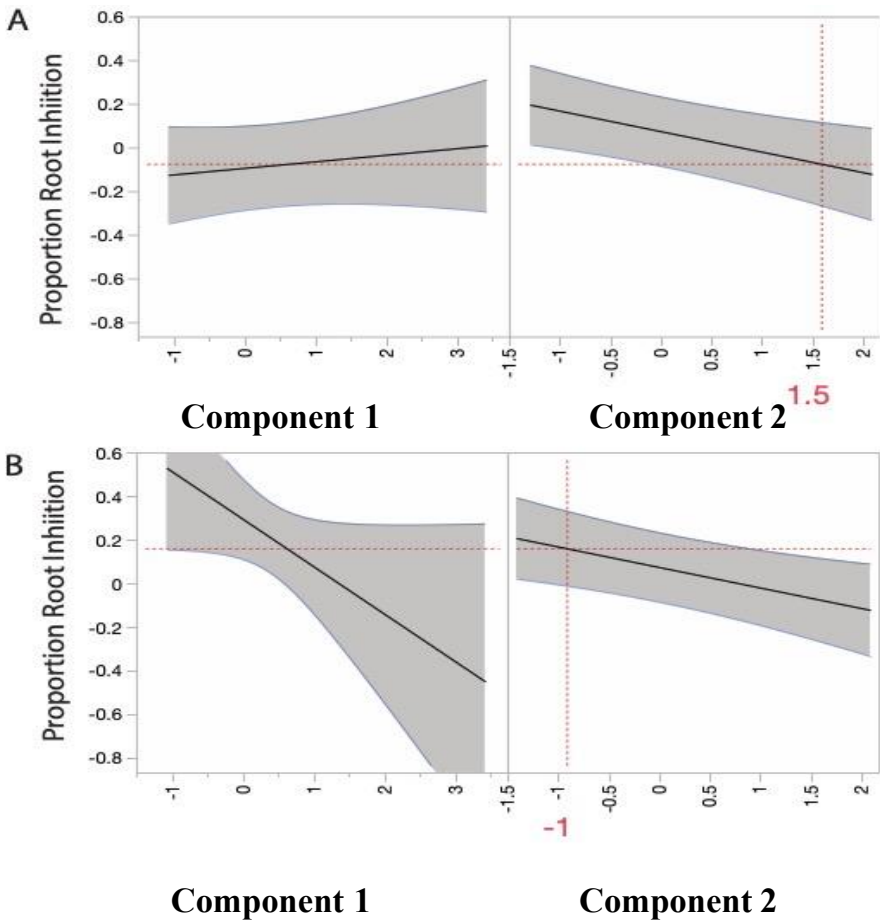


Figure 1.11. Prediction profiles for the effects of metal factor 1 and metal factor 1 on Root inhibition. (A) Profile when metal component 2 is set to 1.5, and (B) Profile when metal component 2 is set to -1.0 (high mercury and low lead)

The results form this chapter demonstrate the interactions among complex mixture of nutrients and metals in wetland systems, which can produce relationships that run counter to the predictions made by considering individual pollutants independently (Alia et al. 2015). But overall the selected plant ecotoxicological bioindicators for this present chapter provide signals of stress from watershed pollutants draining into the wetland sites. A major character of a biological sub-metrics that it should be able to detect biological responses to human activities across different scales (Karr and Chu 1999), these ecotoxicological bioindicators demonstrated evidence of stress across different spatial scales of six different wetlands. When it comes ecological risk assessment, approaches have been developed to assess ecological risk that employ a triad approach (Dagnino et al. 2008) with an integrated index of chemical ecotoxicological and ecological risk. We estimated the chemical risk (nutrient and metals) with the use of plant ecotoxicological bioindicators. Our results detected correlation in the ecotoxicological bioindicators with watershed pollutants that was measured and predicted.

Use of multimetric indices is a common approach for assessing ecological risk assessment (Karr and Chu 1999). Selection of relevant metrics that comprise a multimetric index is not a simple process if it is to serve both in detecting change and predicting ecological risk (Schoolmaster et al. 2012). Bioindicators from across different trophic levels with capacity to detect signals from anthropogenic disturbances must be considered as a candidate metrics in a multimetric index (Decker et al. 2017). Research has shown that the use of multiple groups of organisms increases the potential to gather the information necessary to develop a robust understanding of impacts on ecological integrity (Brown et al. 2009, Waite 2014). For this

reason, the next chapter will assess the efficiency of bacteria as ecological indicators for risk assessment.

Conclusion:

The present chapter investigates the efficacy of the Phytotoxkit™ (Microbiotest Inc 2015) ecotoxicological assay with plants *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba* as bioindicators of sediment toxicity among wetlands with varying land uses and associated pollutant (nutrient and metals) measurements. Results demonstrate that growth inhibitions of *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba* had significant correlations with concentrations of Pb and Hg in sediments and phosphate concentration in water, which are consistent with ecotoxicological risk presented by the varying landuse patterns in the surroundings sub-watersheds.

Bibliography

Ahmed, A. M., E. Lyautey, C. Bonnineau, A. Dabrin, and S. Pesce. 2018. Environmental concentrations of copper, alone or in mixture with arsenic, can impact river sediment microbial community structure and functions. Frontiers in Microbiology 9:1–13.

Ahn, C., P. Gillevet, and M. Sikaroodi. 2007. Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading. Ecological Indicators 7:852=863.

Alia, N., K. Sardar, M. Said, K. Salma, A. Sadia, S. Sadaf, A. Toqeer, and S. Miklas. 2015. Toxicity and bioaccumulation of heavy metals in spinach (Spinacia oleracea) grown in a controlled environment. International Journal of Environmental Research and Public Health 12:7400–7416.

Alori, E. T., B. R. Glick, and O. O. Babalola. 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Frontiers in Microbiology 8:1–8.

Altieri, M. A., and C. I. Nicholls. 2003. Soil fertility management and insect pests: Harmonizing soil and plant health in agroecosystems. Soil and Tillage Research 72:203–211.

Altimira, F., C. Yǎez, G. Bravo, M. González, L. A. Rojas, and M. Seeger. 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. BMC Microbiology 12.

An, F., Z. Diao, and J. Lv. 2018. Microbial diversity and community structure in agricultural soils suffering from 4 years of Pb contamination. Canadian Journal of Microbiology 64:305–316.

Andreazza, R., S. Pieniz, B. C. Okeke, and F. A. O. Camargo. 2011. Evaluation of copper resistant bacteria from vineyard soils and mining waste for copper biosorption. Brazilian Journal of Microbiology 42:66–74.

Artursson, V., R. D. Finlay, and J. K. Jansson. 2006. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol. 8:1–10.

Ayangbenro, A. S., O. O. Bababola, and O. S. Aremu. 2019. Bioflocculant production and heavy metal sorption by metal resistant bacterial isolates from gold mining soil. Chemosphere 231:113–120.

Aylagas, E., Á. Borja, M. Tangherlini, A. Dell'Anno, C. Corinaldesi, C. T. Michell, X. Irigoien, R. Danovaro, and N. Rodríguez-Ezpeleta. 2017. A bacterial community-based index to assess the ecological status of estuarine and coastal environments. Marine Pollution Bulletin 114:679–688.

Azarbad, H., C. A. M. Van Gestel, M. Niklińska, R. Laskowski, W. F. M. Röling, and N. M. Van Straalen. 2016. Resilience of soil microbial communities to metals and additional stressors: DNA-based approaches for assessing "stress-on-stress" responses. International Journal of Molecular Sciences 17:1–20.

Azarbad, H., M. Niklińska, C. A. M. Van Gestel, N. M. Van Straalen, W. F. M. Röling, and R. Laskowski. 2013. Microbial community structure and functioning along metal pollution gradients. Environmental Toxicology and Chemistry 32:1992–2002.

Babalola, O. O., and B. R. Glick. 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. Journal of Food, Agriculture and Environment 10:540–549.

Bachand, P., and A. Horne. 2000. Denitrification in constructed free-water surface wetlands: II.

Effects of vegetation and temperature. Ecol. Eng. 14(1–2):17–32.
Baker, A. J. M., S. P. McGrath, R. D. Reeves, and J. A. C. Smith. 2001. Metal hyperaccumulator plants: a review of the ecology and physiology of biochemical resource for phytoremediation of metal-polluted soils. Phytoremediation of Contaminated Soil and Water:85–107.
Bannerman, R., K. Baun, P. Bohn, and D. Graczyk. 1983. Evaluation of Urban Nonpoint Source Pollution Management in Milwaukee County, Wisconsin. Chicago,IL.
Bannerman, R. T., D. W. Owens, R. B. Dodds, and N. J. Hornewer. 1993. Sources of pollutants in wisconson stormwater. Water Science and Technology 28:241–259.
Baranowski, R., A. Rybak, and I. Baranowska. 2002. Speciation Analysis of Elements in Soil Samples by XRF. Polish Journal of Environmental Studies 11:473–482.
Begon, M., J. L. Harper, C. R. Townsend, and M. Begon. 1996. Ecology: Individuals, populations, and communities. 3rd edition. Blackwell Science, Oxford, UK.
Behrends, L. L. 2007. Use of aquatic plants for removal of nitrate-nitrogen in subsurface flow constructed wetlands.
Berg, J., K. K. Brandt, W. A. Al-Soud, P. E. Holm, L. H. Hansen, S. J. Sørensen, and O. Nybroe. 2012. Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term cu exposure. Applied and Environmental Microbiology 78:7438–7446.
Beutel, M., M. Morgan, J. Erlenmeyer, and E. Brouillard. 2014. Phosphorus Removal in a Surface-Flow Constructed Wetland Treating Agricultural Runoff. Journal Of Environment Quality 43:1071.
Bhattacharyya, P. N., and D. K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327–1350.
Bodelier, P., and S. Dedysh. 2013. Microbiology of wetlands. Frontiers In Microbiology 4.
Boyd, E. S., and T. Barkay. 2012. The mercury resistance operon: From an origin in a geothermal environment to an efficient detoxification machine. Frontiers in Microbiology 3:1–13.
Brown, L. R., T. F. Cuffney, J. F. Coles, F. Fitzpatrick, G. McMahon, J. Steuer, A. H. Bell, and J. T. May. 2009. Urban streams across the USA: Lessons learned from studies in 9 metropolitan areas. Journal of the North American Benthological Society 28:1051–1069.
Bruker. 2017. No Title.
Burbach, K., J. Seifert, D. H. Pieper, and A. Camarinha-Silva. 2016. Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. MicrobiologyOpen 5:70–82.
Busnardo, M., R. Gersberg, R. Langis, T. Sinicrope, and J. Zedler. 1992. Nitrogen and phosphorus removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:287–307.
Cai, L., G. Liu, C. Rensing, and G. Wang. 2009. Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. BMC Microbiology 9:1–11.
Cairns, J. J., and J. R. Pratt. 1993. A history of biological monitoring using benthic macroinvertebrates, in D.M. Rosenberg and V.H. Resh (Eds.). Pages 10–27 Freshwater Biomonitoring and Benthic Macroinvrtebrates. Chapman and Hall, New York.
Calheiros, C. S. C., A. Teixeira, C. Pires, A. R. Franco, A. F. Duque, L. F. C. Crispim, S. C. Moura, and P. M. L. Castro. 2010. Bacterial community dynamics in horizontal flow

constructed wetlands with different plants for high salinity industrial wastewater polishing. Water Research 44:5032–5038.
Carey, R., and G. J. Hochmuth. 2012. Regulatory and Resource Management Practices for Urban Watersheds: The Florida Experience. Hort Technology 22:418–429.
Castellano-Hinojosa, A., D. Correa-Galeote, P. Carrillo, E. J. Bedmar, and J. M. Medina-Sánchez. 2017. Denitrification and biodiversity of denitrifiers in a High-Mountain Mediterranean Lake. Frontiers in Microbiology 8:1911.
Chakravorty, S., D. Helb, M. Burday, and N. Connell. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69:330–339.
Al Chami, Z., N. Amer, L. Al Bitar, and I. Cavoski. 2015. Potential use of Sorghum bicolor and Carthamus tinctorius in phytoremediation of nickel, lead and zinc. International Journal of Environmental Science and Technology 12:3957–3970.
Chapman, D. P. M. 1995. Do sediment toxicity tests require field validation? Environmental Toxicological and Chemistry 14:1451-1453.
Chapman, P. M. 1990. The sediment quality triad approach to determining pollution-induced degradation. Science of The Total Environment 97–98:815–825.
Chen, B., J. Liu, Z. Wang, L. Dong, J. Fan, and J. Qu. 2011. Remediation of Pb-Resistant Bacteria to Pb Polluted Soil. Journal of Environmental Protection 02:130–141.
Chen, B. M., Z. H. Wang, S. X. Li, G. X. Wang, H. X. Song, and X. N. Wang. 2004. Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables. Plant Science 167:635–643.
Chen, J., F. He, X. Zhang, X. Sun, J. Zheng, and J. Zheng. 2014. Heavy metal pollution decreases microbial abundance, diversity and activity within particle-size fractions of a paddy soil. FEMS Microbiology Ecology 87:164–181.
Chen, J., J. Li, H. Zhang, W. Shi, and Y. Liu. 2019. Bacterial heavy-metal and antibiotic resistance genes in a copper tailing dam area in northern China. Frontiers in Microbiology 10:1–12.
Chen, Y., Y. Jiang, H. Huang, L. Mou, J. Ru, J. Zhao, and S. Xiao. 2018. Long-term and high-concentration heavy-metal contamination strongly influences the microbiome and functional genes in Yellow River sediments. Science of the Total Environment 637–638:1400–1412.
Clapcott, J. E., K. J. Collier, R. G. Death, E. O. Goodwin, J. S. Harding, D. Kelly, J. R. Leathwick, and R. G. Young. 2012. Quantifying relationships between land-use gradients and structural and functional indicators of stream ecological integrity. Freshwater Biology 57:74–90.
Clapcott, J. E., E. O. Goodwin, R. G. Young, and D. J. Kelly. 2014. Une approche multimétrique pour prédire l'intégrité écologique des cours d'eau en Nouvelle-Zélande. Knowledge and Management of Aquatic Ecosystems.
Crispell-Synder, I. 1997. Executive summary of WDNR facilitation presentation for Pike River improvements by Mount Pleasant Storm Water Drainage District No. 1.
Cui, J., J. J. Wang, J. Xu, C. H. Xu, and X. N. Xu. 2017. Changes in soil bacterial communities in an evergreen broad-leaved forest in east China following 4 years of nitrogen addition. J. Soils Sediments 17.
Czerniawska-Kusza, I., & Kusza, G. 2010. The potential of the Phytotoxkit microbiotest for hazard evaluation of sediments in eutrophic freshwater ecosystems. Environmental

Monitoring And Assessment. 179:113–121.
Czerniawska-Kusza, I., T. Ciesielczuk, G. Kusza, and A. Cichoń. 2006. Comparison of the Phytotoxkit microbiotest and chemical variables for toxicity evaluation of sediments. ENVIRONMENTAL TOXICOLOGY 21:367–372.
Dagnino, A., S. Sforzini, F. Dondero, S. Fenoglio, E. Bona, J. Jensen, and A. Viarengo. 2008. A "Weight-of-Evidence" approach for the integration of environmental "Triad" data to assess ecological risk and biological vulnerability. Integrated Environmental Assessment and Management 4:314–326.
David, P., R. S. Raj, R. Linda, and S. B. Rhema. 2014. NoMolecular characterization of phosphate solubilizing bacteria (PSB) and plant growth promoting rhizobacteria (PGPR) from pristine soils. Int. J. Innov. Sci. Eng. Technol. 1:317–324.
Decker, K. L., A. Pocewicz, S. Harju, M. Holloran, M. M. Fink, T. P. Toombs, and D. B. Johnston. 2017. Landscape disturbance models consistently explain variation in ecological integrity across large landscapes. Ecosphere 8.
Dellinger, M., M. Carvan, R. Klingler, J. McGraw, and T. Ehlinger. 2014. An Exploratory Analysis of Stream Teratogenicity and Human Health Using Zebrafish Whole-Sediment Toxicity Test. Challenges 5:75–97.
Denver Regional Council of Governments. 1983. Urban Runoff Quality in the Denver (Colorado) Region. Washington, DC.
Díaz Rizo, O., A. Gelen Rudnikas, R. D. Lavin Pérez, G. Arencibia Caraballo, and K. D´Alessandro Rodríguez. 2014. XRF analysis of sediments from Nuevitas Bay (Cuba): assessment of current heavy metal contamination. Nucleus:11–14.
Ding, G.-C., Y. M. Piceno, H. Heuer, N. Weinert, A. B. Dohrmann, A. Carrillo, G. L. Andersen, T. Castellanos, C. C. Tebbe, and K. Smalla. 2013. Changes of Soil Bacterial Diversity as a Consequence of Agricultural Land Use in a Semi-Arid Ecosystem. PLoS ONE 8:e59497.
Ding, X., X. J. Peng, B. S. Jin, M. Xiao, J. K. Chen, B. Li, C. M. Fang, and M. Nie. 2015. Spatial distribution of bacterial communities driven by multiple environmental factors in a beach wetland of the largest freshwater lake in China. Frontiers in Microbiology 6:1–9.
Ding, Z., J. Wu, A. You, B. Huang, and C. Cao. 2017. Effects of heavy metals on soil microbial community structure and diversity in the rice (Oryza sativa L. subsp. Japonica, Food Crops Institute of Jiangsu Academy of Agricultural Sciences) rhizosphere. Soil Science and Plant Nutrition 63:75–83.
DiScenza, D. J., and A. R. Keimowitz. 2014. Calibration and Evaluation of an X-Ray Fluorescence Method for the Determination of Lead and Arsenic in Soils. Journal of Environmental Analytical Chemistry 01:1–3.
Drummond, L., and W. Maher. 1995. Chimica acta. Analytica Chemica Acta 302:69–74.
Ehlinger, T., and L. DeThorne. 2004. onitoring of stream habitat and aquatic biotic integrity - Pike River North and South Branches, Racine and Kenosha Counties, Wisconsin, Interim Report.
Ehlinger, T., L. DeThorne, and B. Berner. 2002. Monitoring of stream habitat and aquatic biotic integrity - Pike River North and South Branches, Racine and Kenosha Counties, Wisconsin.
Elder, G. J. 2016. OUP accepted manuscript. Journal Of Tropical Pediatrics:5–8.
EPA. 1985. U.S. Environmental Protection Agency. Freshwater Wetlands for Wastewater Management Handbook. EPA Region IV Atlanta, GA EPA. 904:9-85–135.
EPA. 1986. Ambient Water Quality Criteria for Dissolved Oxygen.
EPA. 2003a. Protecting water quality from urban runoff.

EPA. 2003b. Protecting Water Quality from Urban Runoff. United States Environmental Protection Agency:EPA-841-F-03-003.
EPA. 2007. FIELD PORTABLE X-RAY FLUORESCENCE SPECTROMETRY FOR THE DETERMINATION OF ELEMENTAL CONCENTRATIONS IN SOIL AND SEDIMENT.
EPA. 2012. Summary of the Clean Water Act.
Faoro, H., A. C. Alves, E. M. Souza, L. U. Rigo, L. M. Cruz, S. M. Al-Janabi, R. A. Monteiro, V. A. Baura, and F. O. Pedrosa. 2010. Influence of soil characteristics on the diversity of bacteria in the southern brazilian atlantic forest. Applied and Environmental Microbiology 76:4744–4749.
Fargašová, A. 2001. Phytotoxic effects of Cd, Zn, Pb, Cu and Fe on Sinapis alba L. seedlings and their accumulation in roots and shoots.
Feng, G., T. Xie, X. Wang, J. Bai, L. Tang, H. Zhao, W. Wei, M. Wang, and Y. Zhao. 2018. Metagenomic analysis of microbial community and function involved in cd-contaminated soil. BMC Microbiology 18:1–13.
Figueiredo, N. L. L., J. Canário, A. Duarte, M. L. Serralheiro, and C. Carvalho. 2014. Isolation and characterization of mercury-resistant bacteria from sediments of tagus estuary (Portugal): Implications for environmental and human health risk assessment. Journal of Toxicology and Environmental Health - Part A: Current Issues 77:155–168.
Foley, J. A., R. DeFries, G. P. Asner, C. Barford, G. Bonan, S. R. Carpenter, F. S. Chapin, M. T. Coe, G. C. Daily, H. K. Gibbs, J. H. Helkowski, T. Holloway, E. A. Howard, C. J. Kucharik, C. Monfreda, J. A. Patz, I. C. Prentice, N. Ramankutty, and P. K. Snyder. 2005. Global consequences of land use. Science 309:570.
Freedman, Z. B., K. J. Romanowicz, R. A. Upchurch, and D. R. Zak. 2015. Differential responses of total and active soil microbial communities to long- term experimental N deposition. Soil Biol. Biochem 90:275–282.
Frossard, A., J. Donhauser, A. Mestrot, S. Gygax, E. Bååth, and B. Frey. 2018. Long- and short-term effects of mercury pollution on the soil microbiome. Soil Biology and Biochemistry 120:191–199.
Gadd, G. M. 1992. Heavy metal pollutants: environments and biotechnological aspects. Encycloped. Academic Press Inc., CA.
Gerardi, M. H. 2006. Chapter 1 Wastewater Microorgansims. Page (M. H. Gererdi, Ed.).
Gerik, T., B. Bean, and R. Vanderlip. 2010. Sorghum Growth and Development. Texas. Texas A & M University System.
Gersberg, R. M., B. V. Elkins, S. R. Lyon, and C. R. Goldman. 1986. Role of aquatic plants in wastewater treatment by artificial wetlands. Water Res. 20:363–367.
Ghosh Roy, S. 2020. Biological indicators of toxic stress in wetland sediments. University of Wisconsin Milwaukee.
Ghosh Roy, S., T. J. Ehlinger, and M. R. Jablonski. 2019. EFFECT OF METALS ON GROWTH INHIBITONS OF SINAPIS , SORGHUM AND LEPIDIUM IN TEXTILE DYE WASTE CONTAMINATED SOIL , INDIA 619:608–619.
Gikas, P., E. Ranieri, and G. Tchobanoglous. 2013. Removal of iron, chromium and lead from waste water by horizontal subsurface flow constructed wetlands. Journal of Chemical Technology and Biotechnology 88:1906–1912.
Glibert, P., C. Heil, D. Hollander, M. Revilla, A. Hoare, J. Alexander, and S. Murasko. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial

bloom in Florida Bay. Marine Ecology Progress Serie 280:73-83.
Glicksman, R. L., and M. R. Batzel. 2010. Science, politics, law, and the arc of the Clean Water Act: the role of assumptions in the adoption of a pollution control landmark. Washington University Journal of Law & Policy, Washington University, School of Law 32:99.
Groffman, P., G. Hanson, E. Kiviat, and G. Stevens. 1996. Variation in Microbial Biomass and Activity in Four Different Wetland Types. . Soil Science Society Of America Journal 60:622.
Gummersheimer, B. S., and T. Giblin. 2003. Identification of Lead Resistant Bacteria from a Heavily Contaminated Site Author (s): Beth S . Gummersheimer and Tara Giblin Published by : Beta Beta Beta Biological Society Stable URL : https://www.jstor.org/stable/4608669 Identification of lead resis. jstor 74:48–54.
Gurave, N. A., V. V Korde, S. S. Dhas, and M. Disale. 2015. Isolation and identification of heavy metal resistant bacteria from petroleum soil of Loni, Ahmednagar. European Journal of Experimental Biology 5:6–11.
Hach. 2019. Phosphorus, Reactive (Orthophosphate), 8048.
He, L. Y., Y. F. Zhang, H. Y. Ma, L. N. Su, Z. J. Chen, Q. Y. Wang, M. Qian, and X. F. Sheng. 2010. Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. Applied Soil Ecology 44:49–55.
Heidenreich, B., K. Mayer, H. J. Sandermann, and D. Ernst. 2001. Mercury-induced genes in Arabidopsis thaliana: identification of induced genes upon long-term mercuric ion exposure. Plant, Cell and Environment 24:1227–1234.
Hemme, C. L., Y. Deng, T. J. Gentry, M. W. Fields, L. Wu, S. Barua, K. Barry, S. G. Tringe, D. B. Watson, Z. He, T. C. Hazen, J. M. Tiedje, E. M. Rubin, and J. Zhou. 2010. Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. ISME Journal 4:660–672.
Hermans, S. M., H. L. Buckley, B. S. Case, F. Curran-Cournane, M. Taylor, and G. Lear. 2016. Bacteria as emerging indicators of soil condition. Applied and Environmental Microbiology:AEM.02826–16.
Hill, T. C. J., K. A. Walsh, J. A. Harris, and B. F. Moffett. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiology Ecology 43:1–11.
Hughes, J., and B. Bohannan. 2004. Application of ecological diversity statistics in microbial ecology. Molecular Microbial Ecology Manual, Second Edition 7.01:1321-1344.
Irawati, W., Patricia, Y. Soraya, and A. hugo Baskoro. 2012. A Study on Mercury-Resistant Bacteria Isolated from a Gold Mine in Pongkor Village, Bogor, Indonesia. HAYATI Journal of Biosciences 19:197–200.
Istina, I. N., H. Widiastuti, B. Joy, and M. Antralina. 2015. Phosphate- solubilizing microbe from Saprists peat soil and their potency to enhance oil palm growth and P uptake. Proc. Food Sci. 3:426–435.
Janda, J., and S. Abbott. 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal Of Clinical Microbiology 45:2761–2764.
Jardine, C., S. Hrudey, J. Shortreed, L. Craig, D. Krewski, C. Furga, and S. McColl. 2003. Risk Management Frameworks for Human Health and Environmental Risks. J Toxicol Environ Health B Crit Rev. 6:569–720.
Jarosławiecka, A., and Z. Piotrowska-Seget. 2014. Lead resistance in micro-organisms. Microbiology (United Kingdom) 160:12–25.

Jasper, J. T., Z. L. Jones, J. O. Sharp, and D. L. Sedlak. 2014. Nitrate removal in shallow, Open-water treatment wetlands. Environmental Science and Technology 48:11512–11520.
Jebara, S. H., S. Abdelkerim, I. C. Fatnassi, M. Chiboub, O. Saadani, and M. Jebara. 2015. Identification of effective Pb resistant bacteria isolated from Lens culinaris growing in lead contaminated soils. Journal of Basic Microbiology 55:346–353.
Jensen, J. 2011. Sediment toxicity and the recoveryof biological integrity in a restored stream channel. Milwaukee. University of Wisconsin Milwaukee.
Jiang, X.-T., X. Peng, G.-H. Deng, H.-F. Sheng, Y. Wang, & Zhou, H.-W., and N. F.-Y. Tam. 2013. Illumina Sequencing of 16S rRNA Tag Revealed Spatial Variations of Bacterial Communities in a Mangrove Wetland. Environmental Microbiology 66:96–104.
Kadlec, R. H. 2016. Large constructed wetlands for phosphorus control: A review. Water (Switzerland) 8.
Kadlec, R., and S. Wallace. 2009. Treatment Wetlands. CRC Press Taylor & Francis Group, LLC.
Kaduková, J., O. Velgosová, A. Mrazíková, R. Marcinčáková, and E. Tkáčová. 2015. Assessment of Biologically Synthesized Ag Nanoparticles Toxicity Against E. coli, Staphylococcus aureus, Parachlorella kessleri and Sinapis alba. Nova Biotechnologica et Chimica 14:69–77.
Kafilzadeh, F., R. Afrough, H. Johari, and Y. Tahery. 2012. Range determination for resistance / tolerance and growth kinetic of indigenous bacteria isolated from lead contaminated soils near gas stations (Iran). European Journal of Experimental Biology 2:62–69.
Kang, S., J. D. Van Nostrand, H. L. Gough, Z. He, T. C. Hazen, D. A. Stahl, and J. Zhou. 2013. Functional gene array-based analysis of microbial communities in heavy metals-contaminated lake sediments. FEMS Microbiology Ecology 86:200–214.
Karr, J. R. 1993. Measuring Biological integrity: lesions from stream, in S. Woodley, J. Kay and G. Francis (Eds.). Page Ecological Integrity and Management of Ecosystems. St. Lucie Press, Delray, FL.
Karr, J. R. 1995. Preticting Aquatic ecosystems: clean water is not enough, in W.S Davis and T.P. Simon(Eds.). Pages 7–13 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
Karr, J. R., and E. W. Chu. 1999. No Title. Page Restoring Life in Running Waters. Island Press, Washington D.C., Covelo California.
Katich, J. T., M. Nelly, and J. Scott. 2012. Sediment Sampling Guide and Methodologies. : Ohio EPA Sediment Sampling Guide. Environmental Protection Agency.
Ke, X., C. Wang, D. Jing, Y. Zhang, and H. Zhang. 2015. Assessing water quality by ratio of the number of dominant bacterium species between surface/subsurface sediments in Haihe River Basin. Marine Pollution Bulletin 98:267-273.
Kenna, T. C., F. O. Nitsche, M. M. Herron, B. J. Mailloux, D. Peteet, S. Sritrairat, E. Sands, and J. Baumgarten. 2011. Evaluation and calibration of a Field Portable X-Ray Fluorescence spectrometer for quantitative analysis of siliciclastic soils and sediments. Journal of Analytical Atomic Spectrometry 26:395.
Khan, S., I. Ahmad, M. Shah, S. Rehman, and A. Khaliq. 2009. Use of constructed wetland for the removal of heavy metals from industrial wastewater. Journal Of Environmental Management. 90:3451-3457.
Knight, B., S. P. McGrath, and A. M. Chaudri. 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium,

copper, or zinc. Appl. Environ. Microbiol 63:39–43.
Knox, A., E. Nelson, N. Halverson, and J. Gladden. 2010. Long-Term Performance of a Constructed Wetland for Metal Removal. Soil And Sediment Contamination. 19:667-685.
Knox, A. S., D. L. Dunn, M. H. Paller, E. A. Nelson, W. L. Specht, and J. C. Seaman. 2006. Assessment of contaminant retention in constructed wetland sediments. Engineering in Life Sciences 6:31–36.
Konopka, A., T. Zakharova, M. Bischoff, L. Oliver, C. Nakatsu, and R. F. Turco. 1999. Microbial biomass and activity in lead-contaminated soil. Applied and Environmental Microbiology 65:2256–2259.
Kou, S., G. Vincent, E. Gonzalez, F. E. Pitre, M. Labrecque, and N. J. B. Brereton. 2018. The response of a 16S ribosomal RNA gene fragment amplified community to lead, zinc, and copper pollution in a Shanghai field trial. Frontiers in Microbiology 9:1–23.
Kovacs, M. 1992. Biological indicators of environmental pollution. Page (M Kovacs (ed.), Ed.). New York: Ellis Horwood.
Kowalczyk, A., M. Wilińska, M. Chyc, M. Bojko, and D. Latowski. 2016. Isolation, screening and identification of mercury resistant bacteria from mercury contaminated soil. E3S Web of Conferences 10.
Krachler, M., R. Alvarez-Sarandes, and G. Rasmussen. 2016. High-Resolution Inductively Coupled Plasma Optical Emission Spectrometry for 234U/238Pu Age Dating of Plutonium Materials and Comparison to Sector Field Inductively Coupled Plasma Mass Spectrometry. Analytical Chemistry 88:8862–8869.
Kulakovskaya, T. 2014. Phosphorus storage in Microorganisms: Diversity and Evolutionary Insight. Biochemistry & Physiology: Open Access 04:1–4.
Kunito, T., K. Nagaoka, N. Tada, K. Senoo, H. Oyaizu, S. Matsumoto, and K. Saeki. 1997. Characterization of cu-resistant bacterial communities in cu-contaminated soils. Soil Science and Plant Nutrition 43:709–717.
Lakshmi, K. V. N. S., C. Sasikala, V. V. Ramana, E. V. V. Ramaprasad, and C. V. Ramana. 2011. Rhodovulum phaeolacus sp. nov. a phototrophic alphaproteobacterium isolated from a brown pond. Journal of General and Applied Microbiology 57:145–151.
Lee, W. M., J. Il Kwak, and Y. J. An. 2012. Effect of silver nanoparticles in crop plants Phaseolus radiatus and Sorghum bicolor: Media effect on phytotoxicity. Chemosphere 86:491–499.
Li, J., B. Li, Y. Zhou, J. Xu, and J. Zhao. 2011. A rapid DNA extraction method for PCR amplification from wetland soils. Letters in Applied Microbiology 52:626–633.
Li, L. G., Y. Xia, and T. Zhang. 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME Journal 11:651–662.
Ligi, T., K. Oopkaup, M. Truu, J.-K. Preem, H. Nõlvak, W. J. Mitsch, and J. Truu. 2014. Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16SrRNA amplicon sequencing. Ecological Engineering 72:56-66.
Liu, C. W., Y. Sung, B. C. Chen, and H. Y. Lai. 2014a. Effects of nitrogen fertilizers on the growth and nitrate content of lettuce (Lactuca sativa L.). International Journal of Environmental Research and Public Health 11:4427–4440.
Liu, K., M. Sun, M. Ye, H. Chao, Y. Zhao, B. Xia, W. Jiao, Y. Feng, X. Zheng, M. Liu, J. Jiao, and F. Hu. 2019. Coexistence and association between heavy metals, tetracycline and corresponding resistance genes in vermicomposts originating from different substrates.

Environmental Pollution 244:28–37.
Liu, Y., J. Zhang, L. Zhao, X. Zhang, and S. Xie. 2014b. Spatial distribution of bacterial communities in high-altitude freshwater wetland sediment. Limnology 15:249–256.
López-Luna, J., M. González-Chávez, F. Esparza-García, and R. Rodríguez-Vázquez. 2009. Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. Journal of Hazardous Materials 163:829–834.
Magurran, A. 2004. Measuring Biologcial Diversity. Page Blackwell Publishing.
Mamta, R. P., V. Pathania, A. Gulati, B. Singh, R. K. Bhanwra, and R. Tewari. 2010. timulatory effect of phosphate-solubilizing bacteria on plant growth, stevioside and rebaudioside-A contents of Stevia rebaudiana Bertoni. Appl. Soil Ecol. 46:222–229.
Manasi, N. Rajesh, and V. Rajesh. 2016. Evaluation of the genetic basis of heavy metal resistance in an isolate from electronic industry effluent. Journal of Genetic Engineering and Biotechnology 14:177–180.
Manikandan, R., S. V. Sahi, and P. Venkatachalam. 2015. Impact assessment of mercury accumulation and biochemical and molecular response of Mentha arvensis: A potential hyperaccumulator plant. Scientific World Journal 2015.
Manios, T., M. Fountoulakis, and A. Karathanasis. 2009. Construction Simplicity and Cost as Selection Criteria Between Two Types of Constructed Wetlands Treating Highway Runoff. Environmental Management 43:908–920.
Margesin, R., and F. Schinner. 1996. Bacterial heavy metal-tolerance - Extreme resistance to nickel in Arthrobacter spp. strains. Journal of Basic Microbiology 36:269–282.
Matraszek, R., B. Hawrylak-Nowak, M. Chwil, S. Chwil, and M. Rudaś. 2017. Effect of the Interaction of Nickel Stress and Sulphur Supplementation on the Content and Accumulation of Macronutrients in White Mustard (Sinapis Alba L.). Electronic Journal of Polish Agricultural Universities 20.
Messer, T., M. Burchell, F. Birgand, S. Broome, and G. Chescheir. 2017. Nitrate removal potential of restored wetlands loaded with agricultural drainage water: A mesocosm scale experimental approach. Ecological Engineering 106:541–554.
Microbiotest Inc. 2015. Standard Operational Procedure, Phytotoxkit. Seed germination and early growth microbiotest with higher plants.
Mitsch, W. J., and J. G. Gosselink. 2000. Wetlands. Third. John Wiley & Sons., NY.
Morgan, J. B., and E. L. Connolly. 2013. Plant-Soil Interactions : Nutrient Uptake Aa Plant Acquisition of Nutrients : Direct Uptake from the Soil. Nature Education Knowledge 4(8):1–6.
Morris, S. J., and C. B. Blackwood. 2015. he ecology of the soil biota and their function. Pages 273–309 Soil Microbiology, Ecology and Biochemistry. 4th Edn. E. A. Eldor (Cambridge, MA: Academic Press),.
MP Biomedicals. 2017. Fast DNA TM spin kit.
Müller, A. K., K. Westergaard, S. Christensen, and S. J. Sørensen. 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiology Ecology 36:11–19.
Munns, W. R., A. W. Rea, G. W. Suter, L. Martin, L. Blake-Hedges, T. Crk, C. Davis, G. Ferreira, S. Jordan, M. Mahoney, and M. G. Barron. 2016. Ecosystem services as assessment endpoints for ecological risk assessment. Integrated Environmental Assessment and Management 12:522–528.

Naguib, M., A. Khairalla, A. El-Gendy, and W. Elkhatib. 2019. Isolation and Characterization of mercury-resistant bacteria from wastewater sources in Egypt. Canadian Journal of Microbiology 65:308–321.

Naz, N., H. K. Young, N. Ahmed, and G. M. Gadd. 2005. Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. Applied and Environmental Microbiology 71:4610–4618.

Nie, Y., M. Wang, W. Zhang, Z. Ni, Y. Hashidoko, and W. Shen. 2018. Ammonium nitrogen content is a dominant predictor of bacterial community composition in an acidic forest soil with exogenous nitrogen enrichment. Sci. Total Environ. 624:407–415.

Nies, D. H. 1999. Microbial heavy-metal resistance. Applied Microbiology and Biotechnology 51:730–750.

Nikinmaa, M. 2014. An Introduction to Aquatic Toxicology. Academic Press; 1 edition (July 24, 2014).

NIST. 2013. National Institute of Standards and Technology.

Novotny, V. 2003. Water Quality - Diffuse Pollution and Watershed Management. Second. John Wiley & Sons.

Novotny, V., T. Ehlinger, A. Bartošová, E. Manolakos, and N. O' Reilly. 2005. Developing Risk Propagation Model for Estimating Ecological Responses of Streams to Anthropogenic Watershed Stresses and Stream Modifications. Center for Urban Environmental Studies Publications.

Olson, B. H., and I. Thornton. 1982. The resistance patterns to metals of bacterial population in contaminated land. J. Soil Sci. 33:271–277.

Owens, P. L., R. C. HInkle, and R. G. Best. 1989. Low-Energy Wastewater Recycling Through Wetland Ecosystem: Copper and Zinc in Wetland Microcosm. Page Freshwater wetlands and wildlife. Oak Ridge, Tenesse.

Pal, C., J. Bengtsson-Palme, E. Kristiansson, and D. G. J. Larsson. 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16:1–14.

Pang, J., M. H. Ryan, M. Tibbett, G. R. Cawthray, K. H. M. Siddique, M. D. A. Bolland, M. D. Denton, and H. Lambers. 2010. Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus supply. Plant and Soil 331:241–255.

Pei, Y., Z. Yu, J. Ji, A. Khan, and X. Li. 2018. Microbial community structure and function indicate the severity of chromium contamination of the Yellow River. Frontiers in Microbiology 9:1–12.

Persoone, G., and M. Vangheluwe. 2000. Toxicity determination of the sediments of the river Seine in France by application of a bat- tery of microbiotests. Page (D. C. W. Persoone G, Janssen C, Ed.). New Microbiotests for Routine Toxicity Screening and Biomonitoring, Kluwer Academic, New York.

Pitt, R., and M. Bozeman. 1982. Sources of Urban Runoff Pollution and Its Effects on an Urban Creek. Cincinnati, OH.

Pitt, R., and J. McLean. 1986. Toronto Area Watershed Management Strategy Study. Humber River Pilot Watershed Project. Ontario Ministry of the Environment. Toronto, Ontario.

Pitt, R., and J. Voorhees. 1995. Source loading and management model (SLAMM). Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels March 30 – April 2, 1993.

Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. Ohio.:225-243.
Pitt, R., and J. Voorhees. 2000. The Source Loading and Management Model (SLAMM), A Water Quality Management Planning Model for Urban Stormwater Runoff.
Postma, J., E. H. Nijhuis, and E. Someus. 2010. Selection of phosphorus solubilizing bacteria with biocontrol potential for growth in phosphorus rich animal bone charcoal. Appl. Soil Ecol. 46:464–469.
Prabhakaran, P., Mu. A. Ashraf, and W. S. Aqma. 2015. Microbial Stress Response to Heavy Metal in the Environment. RSC Advances 5:54667.
Ramond, J.-B., P. J. Welz, D. A. Cowan, and S. G. Burton. 2012. Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up. Research in Microbiology 163:28-35.
Ranjard, L., A. Richaume, L. Jocteur-Monrozier, and S. Nazaret. 1997. Response of soil bacteria to Hg(II) in relation to soil characteristics and cell location. FEMS Microbiology Ecology 24:321–331.
Reilly, J. F., A. J. Horne, and C. D. Miller. 2000. Nitrate removal from a drinking water supply with large free-surface constructed wetlands prior to groundwater recharge. Ecol. Eng. 14(1–2):33-47.
Risso, C., J. Sun, K. Zhuang, R. Mahadevan, R. DeBoy, W. Ismail, S. Shrivastava, H. Huot, S. Kothari, S. Daugherty, O. Bui, C. H. Schilling, D. R. Lovley, and B. A. Methé. 2009. Genome-scale comparison and constraint-based metabolic reconstruction of the facultative anaerobic Fe(III)-reducer Rhodoferax ferrireducens. BMC Genomics 10:447.
Roane, T. M., and S. T. Kellogg. 1996. Characterization of bacterial communities in heavy metal contaminated soils. Canadian Journal of Microbiology 42:593–603.
Rüegg, S. R., L. R. Nielsen, S. C. Buttigieg, M. Santa, M. Aragrande, M. Canali, T. Ehlinger, I. Chantziaras, E. Boriani, M. Radeski, M. Bruce, K. Queenan, and B. Häsler. 2018. A systems approach to evaluate One Health initiatives. Frontiers in Veterinary Science 5:1–18.
Ryan, R. P., D. J. Ryan, and D. N. Dowling. 2005. Multiple metal resistant transferable phenotypes in bacteria as indicators of soil contamination with heavy metals. Journal of Soils and Sediments 5:95–100.
S. Sengar, R., S. Gupta, M. Gautam, A. Sharma, and K. Sengar. 2008. Occurrence, Uptake, Accumulation and Physiological Responses of Nickel in Plants and its Effects on Environment. Research Journal of Phytochemistry 2:44–60.
Sabry, S. A., H. A. Ghozlan, and D. M. Abou-Zeid. 1997. Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. Journal of Applied Microbiology 82:245–252.
Sahu, O. 2014. Reduction of Heavy Metals from Waste Water by Wetland. International Letters Of Natural Sciences 12:35–43.
Santo, C. E., P. V. Morais, and G. Grass. 2010. Isolation and characterization of bacteria resistant to metallic copper surfaces. Applied and Environmental Microbiology 76:1341–1348.
SAS. 2019. JMP®, Version <14>.
Scheirs, J., and L. De Bruyn. 2004. Excess of nutrients results in plant stress and decreased grass miner performance. Entomologia Experimentalis et Applicata 113:109–116.
Schloss, P. D. 2009. Introducing mothur: Open-source, platform-independent, community-

supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541.
Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature methods 9:671–675.
Scholz, M., and Å. Hedmark. 2010. Constructed Wetlands Treating Runoff Contaminated with Nutrients. Water, Air, And Soil Pollution 205:323–332.
Schoolmaster, D. R., J. B. Grace, and E. W. Schweiger. 2012. A general theory of multimetric indices and their properties. Methods in Ecology and Evolution 3:773–781.
SETAC. 1997. Ecological risk assessment. Technical issue paper:4.
SEWRPC. 2010. Racine County Mapbook.
Shange, R., E. Haugabrooks, R. Ankumah, A. Ibekwe, R. Smith, and S. Dowd. 2013. Assessing the Diversity and Composition of Bacterial Communities across a Wetland, Transition, Upland Gradient in Macon County Alabama. Diversity 5:461–478.
Shannon, R. D., O. P. Flite, and M. S. Hunter. 2000. Subsurface flow constructed wetland performance at a Pennsylvania campground and conference center. J. Environ. Qual. 29:2029-2036.
Shukla, D., C. A. Rinehart, and S. V. Sahi. 2017. Comprehensive study of excess phosphate response reveals ethylene mediated signaling that negatively regulates plant growth and development. Scientific Reports 7:1–16.
Sims, A., Y. Zhang, S. Gajaraj, P. Brown, and Z. Hu. 2013. Toward the development of microbial indicators for wetland assessment. Water Research 47:1711–1725.
Sinicorpe, T. L., R. Langis, R. M. Gersberg, M. J. Busnardo, and J. B. Zedler. 1992. Metal removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:309–322.
Smith, M. S., and K. Zimmerman. 1981. Nitrous Oxide Production by Nondenitrifying Soil Nitrate Reducers1. Soil Science Society of America Journal 45:865.
Sobolev, D., and M. Begonia. 2008. Effects of heavy metal contamination upon soil microbes: Lead-induced changes in general and Denitrifying microbial communities as evidenced by molecular markers. International Journal of Environmental Research and Public Health. 5:450–456.
Sokal, R., and J. Rohlf. 1981. Biometry. Second. W.H. Freeman and Company, San Fransisco.
Soon, Y. K., B. T.E., and J. R. Moyer. 1980. Land application of chemically treated sewage sludge III. Effects on soil and plant heavy metal content. J. Environ. Qual. 9:497–504.
Stoeva, M. K., S. Aris-Brosou, J. Chételat, H. Hintelmann, P. Pelletier, and A. J. Poulain. 2014. Microbial community structure in lake and Wetland sediments from a high arctic polar desert revealed by targeted Transcriptomics. PLoS ONE 9:e89531.
Storer, D. A. 1984. A simple high sample volume ashing procedure for determination of soil organic matter. Communications in Soil Science and Plant Analysis 15:759–772.
Suter, G. . 2001. Applicability of indicator monitoring to ecological risk assessment. Ecological Indicators 1:101–112.
Suter, G. I. 2006. Ecological Risk Assessment. CRC Press.
Szoboszlay, M., A. Dohrmann, C. Poeplau, A. Don, and C. Tebbe. 2017. Impact of land use change and soil organic carbon quality on microbial diversity in soils across Europe. FEMS Microbiol Ecol.
Tajini, F., M. Trabelsi, and J. J. Drevon. 2012. Combined inoculation with Glomus intraradices and Rhizobium tropici CIAT899 increases phosphorus use efficiency for symbiotic nitrogen

fixation in common bean (Phaseolus vulgaris L.). Saudi J. Biol. Sci. 19:157–163.
Tallis, H., P. Kareiva, M. Marvier, A. Chang, and A. H. Mwinyi. 2008. Practical Conservation and Economic Development. Proceedings of the National Academy of Sciences 105:9457–9464.
Thomas, P. R., P. Glover, and T. Kalaroopan. 1995. An evaluation of pollutant removal from secondary treated sewage effluent using a constructed wetland system. Water Sci. Tech. 32:87-93.
Tipayno, S. C., J. Truu, S. Samaddar, M. Truu, J. K. Preem, K. Oopkaup, M. Espenberg, P. Chatterjee, Y. Kang, K. Kim, and T. Sa. 2018. The bacterial community structure and functional profile in the heavy metal contaminated paddy soils, surrounding a nonferrous smelter in South Korea. Ecology and Evolution 8:6157–6168.
Tixier, G., Q. Rochfort, L. Grapentine, J. Marsalek, and M. Lafont. 2012. Spatial and seasonal toxicity in a stormwater management facility: Evidence obtained by adapting an integrated sediment quality assessment approach. Water Research 46:6671–6682.
Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol 5:240–245.
Trajanovska, S., M. L. Britz, and M. Bhave. 1997. Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead contaminated site. Biodegradation 8:113–124.
Tremblay, J., K. Singh, A. Fern, E. S. Kirton, S. He, T. Woyke, J. Lee, F. Chen, J. L. Dangl, and S. G. Tringe. 2015. Primer and platform effects on 16S rRNA tag sequencing. Frontiers in Microbiology 6:1–15.
Umar, S., N. Gauba, N. A. Anjum, and T. O. Siddiqi. 2013. Arsenic toxicity in garden cress (Lepidium sativum Linn.): Significance of potassium nutrition. Environmental Science and Pollution Research 20:6039–6049.
USEPA. 2016. The Sources and Solutions: Agriculture (online) https://www.epa.gov/nutrientpollution/sources-and-solutions-agriculture:2017–2019.
USGS, U. S. G. S. 2017. Nitrogen and Water. U.S. Department of the Interior.
Vamerali, T., M. Bandiera, and G. Mosca. 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environmental Chemistry Letters 8:1–17.
Vasileiadis, S., E. Puglisi, M. Trevisan, K. G. Scheckel, K. A. Langdon, M. J. McLaughlin, E. Lombi, and E. Donner. 2015. Changes in soil bacterial communities and diversity in response to long-term silver exposure. FEMS Microbiology Ecology 91:1–11.
Vymazal, J. 2007. Removal of nutrients in various types of constructed wetlands. Science of the Total Environment 380:48–65.
Waite, I. R. 2014. Agricultural disturbance response models for invertebrate and algal metrics from streams at two spatial scales within the U.S. Citation metadata. Hydrobiologia 726:285–303.
Walker, C. W., and R. D. Shannon. 2006. Nitrate and phosphate removal effects of compost amendments in wetland mesocosms. American Society of Agricultural and Biological Engineers 49:1773–1778.
Wan, D., Z. Han, J. Yang, G. Yang, and X. Liu. 2016. Heavy metal pollution in settled dust associated with different urban functional areas in a heavily air-polluted city in North China. International Journal of Environmental Research and Public Health 13.
Wang, H., C. W. Marshall, M. Cheng, H. Xu, L. Hu, X. Yang, and T. Zheng. 2017. Changes in land use driven by urbanization impact nitrogen cycling and the microbial community

composition in soils. Scientific Reports 7.
Wang, L., and J. Lyons. 2003. Wang2003.pdf. Page 227 Biological Response Signatures: Indiactor Patterns Using Aquatic Communities. CRC Press.
Wang, L., J. Lyons, and P. Kanehl. 2001. Impacts of Urbanization on Stream Habitat and Fish across Multiple Spatial Scales. Environmental Management. Environmental Management 28:255–266.
Wang, N. F., T. Zhang, X. Yang, S. Wang, Y. Yu, L. L. Dong, Y. D. Guo, Y. X. Ma, and J. Ye Zang. 2016. Diversity and composition of bacterial community in soils and lake sediments from an arctic lake area. Frontiers in Microbiology 7:1–9.
Wang, Q., C. Wang, W. W. Yu, A. Turak, D. Chen, Y. Huang, J. Ao, Y. Jiang, and Z. Huang. 2018. Effects of nitrogen and phosphorus inputs on soil bacterial abundance, diversity, and community composition in chinese fir plantations. Frontiers in Microbiology 9:1–10.
Wang, Y., H. F. Sheng, Y. He, J. Y. Wu, Y. X. Jiang, N. F. Y. Tam, and H. W. Zhou. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. Applied and Environmental Microbiology 78:8264–8271.
Ward, M. H. 2009. Too much of a good thing? Nitrate from nitrogen fertilizers and cancer. Reviews on Environmental Health 24:357–363.
Webb, J. M.-S. 1998. Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. Journal of Applied Microbiology 84:240-248.
Wilson, M. A., and S. R. Carpenter. 1999. Economic valuation of freshwater ecosystem services in the United States: 1971-1997. Ecological Applications 9:772–783.
Wohl, D. L., S. Arora, and R. Gladstone. 2004. Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. Ecology 85:1534–1540.
Xiao, H., S. Zhang, J. Zhai, Q. He, A. Mels, K. Ning, and J. Liu. 2013. Retention and distribution of Cu, Pb, Cr, and Zn in a full-scale hybrid constructed wetland receiving municipal sewage. Water Science and Technology 67:2257–2264.
Xie, Y., J. Fan, W. Zhu, E. Amombo, Y. Lou, L. Chen, and J. Fu. 2016. Effect of heavy metals pollution on soil microbial diversity and Bermudagrass genetic variation. Frontiers in Plant Science 7.
Yazdankhah, S., E. Skjerve, and Y. Wasteson. 2018. Antimicrobial resistance due to the content of potentially toxic metals in soil and fertilizing products. Microbial Ecology in Health and Disease 29:1548248.
Yoder, C., and E. Rankin. 1995a. Biological response signatures and the area degradation value:new tools for interpreting multi-metric data, in W.S. Davis and T.P. Simons(Eds). Pages 263–286 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
Yoder, C., and E. Rankin. 1995b. BIological criteria program development and implementation in Ohio in W.S. Ohio and T.P.Simon (Eds.). Pages 109–144 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
YSI. 2020. YSI a xylem brand.
Yu, J., X. Lv, B. Ma, F. Qu, K. Ning, and H. Wu. 2014. A meta-analysis of the bacterial and archaeal diversity observed in wetland soils. Salt Marshes: Ecosystem, Vegetation and Restoration Strategies 2014:13–32.
Yu, Y., H. Wang, J. Liu, Q. Wang, T. Shen, W. Guo, and R. Wang. 2012. Shifts in microbial

community function and structure along the successional gradient of coastal wetlands in Yellow River Estuary. European Journal of Soil Biology 49:12–21.
Zancheta, A. C. F., C. A. De Abreu, F. C. B. Zambrosi, N. de M. Erismann, and A. M. M. A. Lagôa. 2015. Cadmium Accumulation by Jack-Bean and Sorghum in Hydroponic Culture. International Journal of Phytoremediation 17:298–303.
Zhang, D., J. Luo, Z. Lee, Y. Maspolim, R. Gersberg, and Y. Liu. 2016. Characterization of bacterial communities in wetland mesocosms receiving pharmaceutical-enriched wastewater. Ecological Engineering 90:215–224.
Zhang, M. J., F. K. Huang, G. Y. Wang, X. Y. Liu, J. K. Wen, X. S. Zhang, Y. S. Huang, and Y. Xia. 2017. Geographic distribution of cadmium and its interaction with the microbial community in the Longjiang River: Risk evaluation after a shocking pollution accident. Scientific Reports 7:1–12.
Zhang, W. hui, Z. Huang, L. yan He, and X. fang Sheng. 2012. Assessment of bacterial communities and characterization of lead-resistant bacteria in the rhizosphere soils of metal-tolerant Chenopodium ambrosioides grown on lead-zinc mine tailings. Chemosphere 87:1171–1178.
Zhao, J., X. Zhao, L. Chao, W. Zhang, T. You, and J. Zhang. 2014a. Diversity change of microbial communities responding to zinc and arsenic pollution in a river of northeastern china. Journal of Zhejiang University SCIENCE B 15:670–680.
Zhao, K., P. Penttinen, X. Zhang, X. Ao, M. Liu, X. Yu, and Q. Chen. 2014b. Maize rhizosphere in Sichuan, China, hosts plant growth promoting Burkholderia cepacia with phosphate solubilizing and antifungal abilities. Microbiological Research 169:76–82.
Zhawar, V. K., N. Kaur, and A. K. Gupta. 2014. Effect of increasing concentration of inorganic phosphate (Pi) and pyrophosphate (PPi) on growth and Phosphatases of germinating chickpea seeds. Journal of Plant Nutrition 37:2195–2207.
Zhu, F., L. Qu, X. Hong, and X. Sun. 2011. Isolation and characterization of a phosphate-solubilizing halophilic bacterium Kushneria sp. YCWA18 from Daqiao saltern on the coast of yellow sea of China. Evidence-based Complementary and Alternative Medicine 2011.
Zięba-Palus, J., Kunicki, M. 2006. Application of the micro-fTIR spectroscopy, Raman spectroscopy and XRF method examination of inks. Forensic Science International 158:164–172.

Chapter 2: The effects of land use on bacterial assays in constructed wetland sediments

Introduction:

Several approaches have been taken to meet the goal of the Clean Water Act (CWA). Which is "*to restore and maintain the integrity of the nation's waters*" (EPA 2012) . For example the U.S. Environmental Protection Agency (USEPA) initially proposed the use of "3-legged stool" that integrates metrics of water quality parameters, whole-effluent toxicity testing, and ambient biological assays (Karr 1993). Later on, the use of biological assays and bioindicators increased (Karr 1993, 1995). These assays focused on "biological response signatures" (Yoder and Rankin 1995a) that can characterize complicated interactions of ecological responses to stress from biological, spatial and temporal scales (Cairns and Pratt 1993). Research increasingly started to focus on biological criteria to understand impact of anthropogenic disturbances on ecosystems (Clapcott et al. 2012, Decker et al. 2017). In the United States, due to urban encroachment and increasing impervious surface area a significant amount of stormwater runoff drains into local streams, rivers, lakes and wetlands impacting the quality of surface waters (Figure 1.1) (Wang et al. 2001, EPA 2003a). Balancing ecological integrity with the needs of increasing population (Figure 1.1) is a challenge to the goals of CWA (Tallis et al. 2008). To meet this complex challenge Ecological risk assessment (ERA) became necessary. ERA is ''the practice of determining the nature and likelihood of effects of anthropic actions on animals, plants, and the environment'' (SETAC 1997). Integrated index with triad approach (Dagnino et al. 2008) of chemical risk, ecotoxicological risk and ecological risk has been taken towards ERA. At the same time, metric selection is very important for a mutimetric index as metrics should detect change and predict ecological risk (Schoolmaster et al. 2012). In this regard, the use of bioindicators across different trophic levels to detect signals of pollution coming from anthropogenic disturbances across different spatial and temporal scales started to

increase. (Decker et al. 2017). Different groups of organisms as bioindicators increase the potential to develop a robust understanding of impacts on ecological integrity (Brown et al. 2009, Waite 2014). The terms "biological response signatures" has been used (Yoder and Rankin 1995a) to describe the variety of ways that multimetric bioindicators in aquatic ecosystems possibly can respond to several kinds of environmental stressors. This kind of approach also refrains from looking for distinct cause-effect relationships but towards identifying signals of response within the complex noise of pollutants (Clapcott et al. 2012, 2014).

Ecological Risk Assessment approaches have been used for extensively for both monitoring the effects of post development and predicting the possible effects of a proposed projects .To this end, as discussed in chapter 1, constructed wetlands have been used extensively to address water quantity and quality problems and mitigate the environmental impacts of urbanization (Tixier et al. 2012). Constructed stormwater wetlands capture sediment and pollutants from surrounding landscapes (Kadlec and Wallace 2009), manage nutrients generated from agricultural and urban runoff (Manios et al. 2009, Scholz and Hedmark 2010, Beutel et al. 2014) and can remediate heavy metal contaminations from industrial land use runoffs (Khan et al. 2009, Knox et al. 2010, Sahu 2014).

Biomonitoring is measuring and evaluating the conditions of a living system (Karr and Chu 1999). Biomonitoring is an essential component for monitoring the ecological integrity and condition of watersheds (Karr and Chu 1999) and bioindicators have been developed to serve as tools for assessing aquatic ecosystem integrity across diverse temporal and spatial scales (Yoder and Rankin 1995a) . Various forms of bioindicators including communities of plants invertebrates, fishes, birds, algae, amphibians and microorganisms have been used as bioindicator studies in wetlands (Sims et al. 2013). These wetland bioindicators has proven to

effective to detect ecological changes in watersheds (Ke et al. 2015, Aylagas et al. 2017) and for conducting sediment risk assessments in relation to pollutants like nutrients and metals (Chapman 1995, Jensen 2011, Dellinger et al. 2014).

Wetlands have biogeochemical cycles with a combination of chemical transformations and transport processes. In wetlands, transformation of elements such as nitrogen, phosphate, sulfur, iron, manganese and carbon occurs in anaerobic environment and is highly mediated by anaerobic microbial populations (Mitsch and Gosselink 2000). Chemicals like nutrients (such as nitrate and phosphate) and heavy metals (Pb, Hg, Cu, Ni, Cd, Zn, Ag, As) are hydrologically transported to wetlands through surface flow, precipitation, groundwater and tides (Mitsch and Gosselink 2000). Microbes play important role in recycling and removal of these elements too (Knox et al. 2010, Bodelier and Dedysh 2013). In spite of these vital ecosystem services provided by microbial communities, wetland health-indices have largely ignored of microbial components (Sims et al. 2013).

Bacterial communities are excellent candidates for bioindicator development, because they are highly influenced both by external toxins such as nutrient loading. Over the years many studies have shown how land cover affects soil bacterial communities and various land uses and its associated input of nutrients and metals pollutants (Wang et al. 2012, Ding et al. 2013, Szoboszlay et al. 2017, Zhang et al. 2017). The input of nutrients recycles fast due to active aerobes and anaerobes making these systems highly productive (Groffman et al. 1996, Bodelier and Dedysh 2013). Cyanobacterial blooms are often associated with highest concentrations of water quality indicating nitrogen and phosphate loading (Glibert et al. 2004). Bacteria are known to be instrumental in the removal of metal contamination (Knox et al. 2010). Studies have also shown that with increasing metal concentrations, substantial change can take place in microbial

diversity (Sobolev and Begonia 2008, Xie et al. 2016). Heavy metals like Zinc (Zn) and Arsenic (As), Mercury (Hg), Lead (Pb) may impact the community negatively, decreasing the number or diversity (Müller et al. 2001, Zhao et al. 2014a, An et al. 2018, Frossard et al. 2018). These negative impacts as a results of heavy metal contamination might happen even at low concentrations of the metals (Gummersheimer and Giblin 2003).

Recent studies have illustrated the value of incorporating bacterial assemblage data in monitoring the ecological status of coastal estuarine (Aylagas et al. 2017) and freshwater (Ke et al. 2015) environments. These studies developed indices that utilized 16S rRNA diversity as a surrogate for bacterial taxonomic composition, and demonstrated patterns relating bacterial community assemblages with ecosystem integrity. With the aid of molecular tool like 16S rRNA gene sequences, bacterial communities can be characterized relatively quickly and in detail (Janda and Abbott 2007) and researchers are able to identify the dominant taxa in wetland sediment using molecular tools (Calheiros et al. 2010, Shange et al. 2013, Ligi et al. 2014).

This present chapter investigates the efficacy of the sediment taxonomical diversity and number (based on communities identified with 16S rRNA gene sequences) of the bacterial community assemblage in constructed wetlands as bioindicators of sediment toxicity among wetlands with varying land uses and associated pollutant (nutrient and metals) measurements.

This present chapter will address two questions regarding the application of the sediment taxonomical diversity of the bacterial community assemblage as bioindicators. First, does variation of sediment taxonomical diversity of the bacterial community assemblage correlate with variation in pollution-related stressors, either as loadings entering wetlands from their surrounding watersheds or ambient concentrations within the wetlands (i.e. *ex post* impact indicators for monitoring)? Second, can sediment taxonomical diversity of the bacterial

community assemblage serve as predictive bioindicators of pollution loadings wetlands (i.e. *ex ante* impact bioindicators for ecological risk assessment)?

Methods:

Study location and sampling protocol:

This present study was conducted in Pike River watershed (Racine County, Wisconsin USA) in six individual wetlands sites (Figure 1.2). The details of the study site are described in chapter 1. Sediment samples were collected from 6 wetland sites 1-6 during summer 2015, fall 2016 and summer 2017. The sediment sampling and storage procedure has been described in chapter 1.

Pollution loading estimates*:*

The pollutant loadings from surrounding watersheds were predicted for nitrate+ nitrite, Phosphorus, Zn, Pb, Cu and Cd in Kg /year using a model as described in chapter 1 (Table 1.2).

Water Quality parameters and Percent organic matter

Water quality parameters, wetland depth profile and sediment percent organic matter of each site were measured during summer 2017 as described in chapter 1.

Bacterial community structure:

DNA was extracted using 0.8 g of each of the collected sediment samples with Fast DNA™ spin kit for soil (Li et al. 2011, Burbach et al. 2016, MP Biomedicals 2017). The standard protocol provided from the instruction manual were followed. This method yields DNA 70µl in DES solution. After extraction DNA was stored at -20°C for immediate next steps. About 2µl (of 70µl) of the extracted DNA was quantified using a Nanodrop 1000 Spectrophotometer, Thermo Fischer Scientific (Shange et al. 2013).

PCRs were conducted using 1µl of the supernatant and 19µl of GoTaq master mix

(Promega) using the manufacturer's recommended protocol. GoTaq includes the necessary Taq polymerase, nucleotides and the buffer for the PCR cycle. Primers that were used to amplify variable region of the 16S rRNA gene from genomic DNA were - 16S V3, 16S V4-V6, 16S11F - 907R and 16S 515F – 1512 R (Chakravorty et al. 2007, Liu et al. 2014b, Tremblay et al. 2015).After PCR, the DNA was electrophoresed in 1% Agarose gel for size comparison with a size marker ladder.

For the next steps, 10µl - 20µl of the extracted DNA was sent to the University of Wisconsin- Madison Biotechnology Centre where library preparation and sequencing of the v3-v4 region in 16S bacterial rRNA gene were performed using Illumina Next-Generation Sequencing (Jiang et al. 2013) . The sequences were retrieved electronically. Bio-informatics analyses were done using the software mothur (v1.36.1). This set of analyses uses SILVA database (Release Version 128) for sequence alignment and for taxonomy it uses Greengenes Reference Taxonomy (Version13_8_99) (Schloss 2009). Mothur processes the sequences through a couple of quality control measures. The sequences were screened to remove any sequence with ambiguous bases anything longer than 464 bp. After which unique sequences were identified, removing the duplicates. Then the sequences were aligned as per SILVA database (Release Version 128). To run this step the start and end of the alignment was specified. After this the sequences were counted, filtered and pre-clustered (splitting the sequences by group and sort them by abundance and list from most to least abundant and identify sequences that are within 2 nt of each other). After this chimera.vsearch was performed that removes the chimeric sequences. Then finally the sequences were classified using Greengenes Reference Taxonomy (Version13_8_99) (Schloss 2009).

Nutrient and metal measurement in wetland sites:

Water samples for nutrient analysis were collected during summer (between June – August) 2017) of wetland sites 1-6 in the Pike river watershed. The details of water sampling, storage and nutrient (nitrate and phosphate) concentration measurement in mg/L has been described in chapter 1.

The concentrations of heavy metals (Ag, Hg, Pb, As, Ni, Zn and Cd) in the sediments were estimated using X-ray fluorescence (XRF).The measurement and calibration procedure has been described in chapter 1. The metals were detected in ppm.

Data Analyses:

Bacterial taxonomical diversity was calculated using Shannon and Simpson diversity indices for both phyla and genera in case of each sediment sample collected and sequenced using JMP® 14 (SAS 2019).

Effect of Nutrients: Multifactor general linear models analysis of variance (SAS 2019) was used to examine the effects of pollutants as independent variables (concentrations of nutrients and metals) on bacterial indicators as dependent response variables (Shannon and Simpson diversity indices of phyla and genera). The effect of measured nutrient concentrations on the bacterial indicators could only be tested for 2017, the one year when measured nutrient data were collected. Land use was assumed to not have changed significantly over the course of the study, and as such the ANOVA tests for the effect of predicted loadings from the surrounding land use on bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) included year as an independent factor.

Effect of Sediment Metals: ANOVA tests for the effect of predicted pollution loadings of individual metals (Cu, Pb, Zn and Cd) from the surrounding land use on bacterial indicators were

tested, where the dependent response variables were the bacterial indicators and the independent variables (effects) were predicted total nutrient (nitrate + nitrite and phosphate) loadings and year. Factor analysis was conducted in a prior study (see Chapter 1) using the log transformed concentrations of the metals (Ag, Zn, As, Cd, Ni, Pb and Hg) measured from the wetland sediments using from JMP® 14 (SAS 2019). ANOVA for the effects of metal pollution on bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) was conducted using the metal factor component scores and year as the independent factors in the model. In addition, forward stepping multiple regression was used to estimate a best fit model for the combined predictive linear relationships between concentrations of the highest loading metal pollutants from the factor analysis (Hg, Pb, Cd, As) and bacterial indicators.

Examination of Effect Interactions: Prediction profiles were used together with multi-factor models in JMP® 14 (SAS 2019) to help examine how values of independent factors (either nutrients or metals) interact to influence bacterial indicators. Prediction profiles use the patterns of variation from ANOVA to visualize how response parameters change as the levels of individual factors are changed, helping to understand the interaction between the pollutants. For example, in a nutrient analysis, prediction profiler visualizes the changes in bacterial indicators (e.g. Shannon diversity) when the concentrations of nitrate and phosphate are increased or decreased (SAS 2019) The effects of interactions between factors can be observed when he prediction traces shift their slope as the values of other terms are manipulated.

Results:

Relative abundance of bacterial community

The sequence alignment and taxonomical analysis were conducted using SILVA database Release Version 128 and Greengenes Reference Taxonomy Version 13_8_99. Taxonomic

profiles were determined up to and including the lowest level of classification for each sediment sample from each wetland site. A total of 67,503 sequences were identified. The lowest number of sequences that was identified in a sample was 115, while the highest was 16,005. At the broadest level, 70 unique phyla were identified (Table 2.1A), while at the narrowest level, 924 unique genera identified (Table 2.1B). Among the total number of sequences detected proteobacteria was the most abundant phylum (38.57%) (Table 2.1). These unique types of phyla and genera as identified were used to calculate the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) across all the wetland sample as described in Figure 2.

Table 2.1: (A) Number of sequences and the percent of total for each detected phylum across all wetland sites, samples and 3 years of the study
(B)Number of unique type of phyla and genera detected across all wetland sites, samples and 3 years of the study

(A) Phyla detected	**Number of sequences for each phyla**	**Percent of total**
Proteobacteria	26037	40.04
Bacteroidetes	6472	9.95
Chloroflexi	5173	7.96
Firmicutes	4719	7.26
Actinobacteria	4497	6.92
Planctomycetes	3327	5.12
Acidobacteria	2548	3.92
Verrucomicrobia	2096	3.22
OD1	1032	1.59
Spirochaetes	996	1.53
Chlorobi	781	1.20
Gemmatimonadetes	612	0.94
Armatimonadetes	568	0.87
Chlamydiae	528	0.81
Cyanobacteria	504	0.78
Nitrospirae	447	0.69
OP8	377	0.58

Elusimicrobia	360	0.55
TM6	358	0.55
TM7	344	0.53
BRC1	336	0.52
GN02	273	0.42
Fibrobacteres	198	0.30
OP3	191	0.29
Chlamydiae	188	0.29
OP11	177	0.27
AC1	163	0.25
WS6	155	0.24
NKB19	141	0.22
GN04	139	0.21
Lentisphaerae	121	0.19
WS3	95	0.15
WS4	91	0.14
SR1	80	0.12
WS2	76	0.12
LCP-89	68	0.10
NC10	59	0.09
Tenericutes	56	0.09
KSB3	53	0.08
Fusobacteria	48	0.07
SC4	47	0.07
TA06	44	0.07
WS5	41	0.06
ZB3	34	0.05
OP1	31	0.05
FCPU426	30	0.05
Caldiserica	29	0.04
Thermi	28	0.04
Caldithrix	26	0.04
LD1	21	0.03
GAL15	21	0.03
OC31	21	0.03
Synergistetes	20	0.03
GOUTA4	17	0.03
WPS-2	17	0.03
H-178	16	0.02
Kazan-3B-28	15	0.02

WS1	11	0.02
AD3	10	0.02
MAT-CR-M4-B07	9	0.01
TPD-58	9	0.01
MVS-104	8	0.01
Thermotogae	8	0.01
OP9	7	0.01
SBR1093	5	0.01
Caldithrix	5	0.01
FBP	4	0.01
WWE1	1	0.00
PAUC34f	1	0.00
Poribacteria	1	0.00
Total number of sequences of all phyla	65021	100.00
Type of unique Taxa identified as phyla or genera	**number**	
phyla	70	
genera	924	

The most common phyla found from collected surface layer (10 cm from top) of wetland sediments are shown in Figures 2.1A-C. For summer 2015, the most common phyla included: Proteobacteria, Actinobacteria, Acidobacteria, Choloroflexi, Plancomycetes, Bacteroidetes (Figure 2.1A). For fall 2016, the most abundant phyla were Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, Bacteroidetes and Gemmatimonadates (Figure 2.1B). The most abundant phyla during summer 2017 included Proteobacteria, Choloroflexi, Bacteroidetes, Actinobacteria, Acidobacteria, Verrucomicrobia, Firmicutes, Chlorobi and Cyanobacteria (Figure 2.1C).

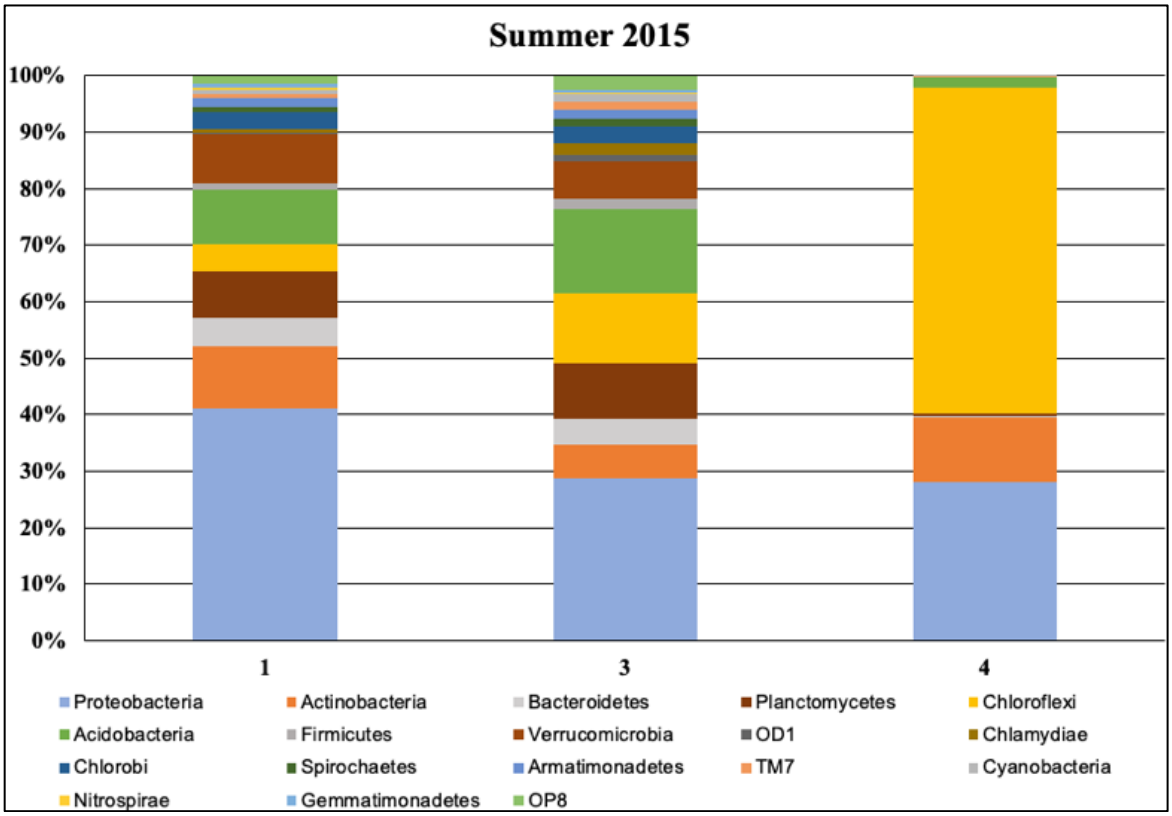


Figure 2.1A: The relative abundance of bacterial phyla identified in the collected wetland sediments during summer 2015 identified by 16S rRNA gene sequencing in wetland sites 1,3 and 4. Insufficient DNA was isolated from wetland 2, 5 and 6 during 2015. Phyla with an abundance of less than 1% of the total sample were not included in this figure.

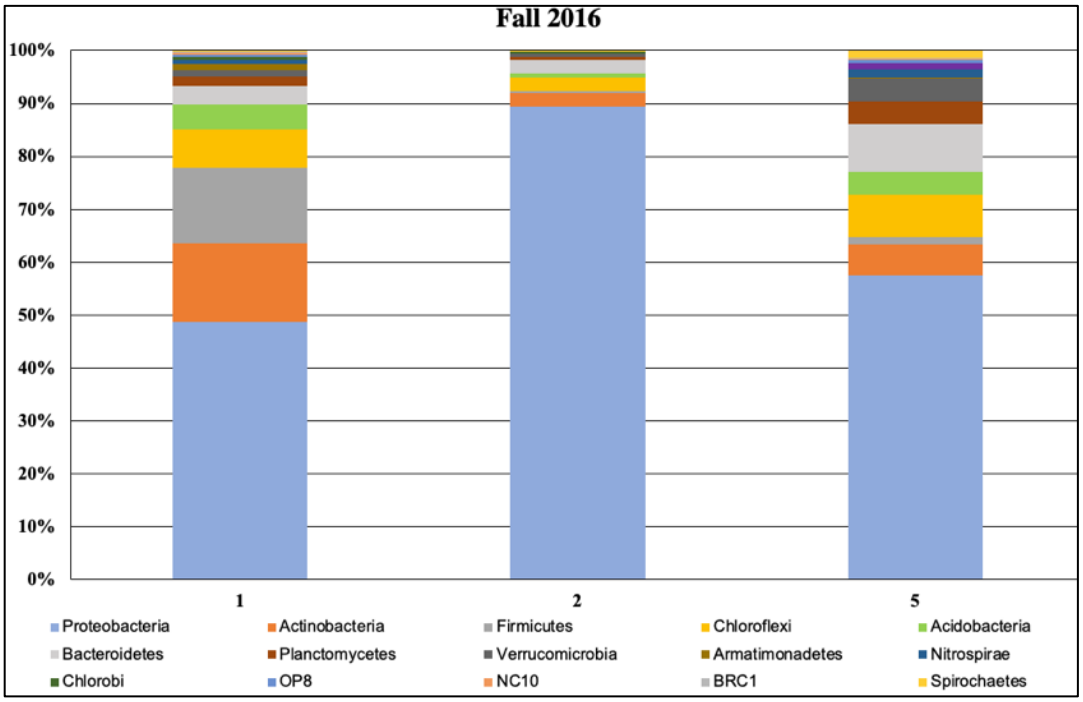


Figure 2.1B: The relative abundance of bacterial phyla identified in the collected wetland sediments during fall 2016 identified by 16S rRNA gene sequencing in wetland sites 1,2 and 5. Insufficient DNA was isolated from wetland 3, 4 and 6 during fall 2016. Phyla with an abundance of less than 1% were not included in this figure.

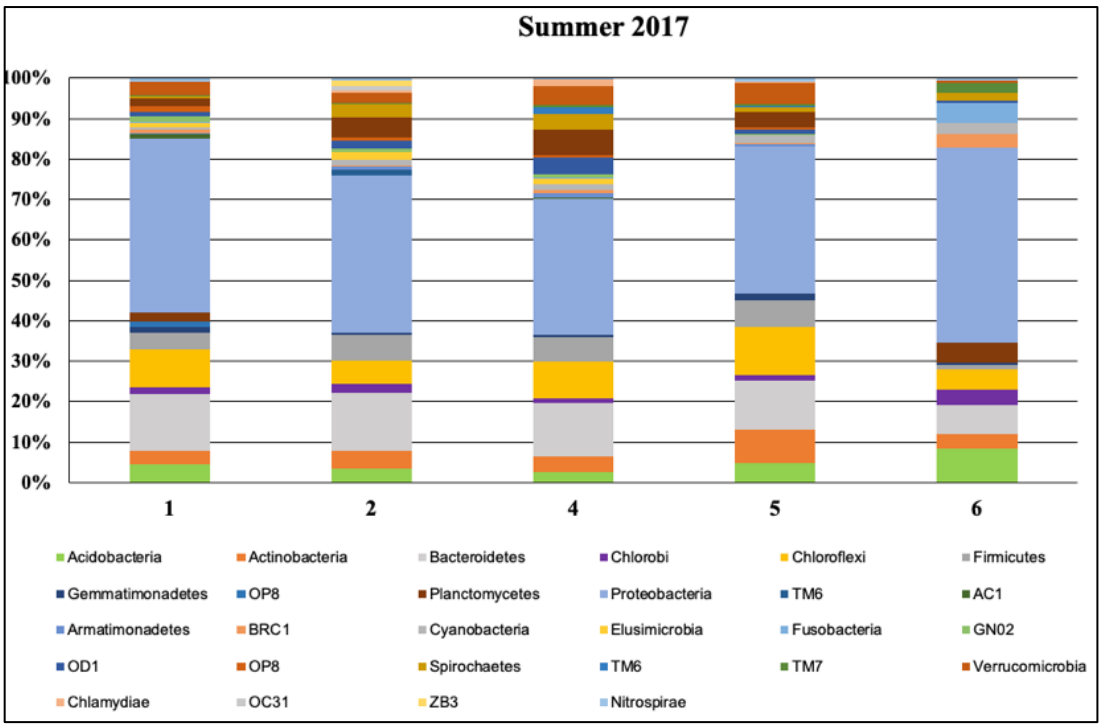


Figure 2.1C: The relative abundance of bacterial phyla identified in the collected wetland sediments during summer 2017 identified by 16S rRNA gene sequencing in wetland sites 1, 2, 4, 5 and 6. Insufficient DNA was isolated from wetland 3 during summer 2017. Phyla with an abundance of less than 1% were not included in this figure.

The dominant genera detected are shown in Figures 2.2A-C. During summer 2015, the most abundant genera were: *Bacillus, Pseudomonas, Streptococcus, Bdellovibrio, Flavobacterium, Clostridium, Treponema, Thiobacillus, Plancomycetes, Gemmata* (Figure 2.2A). During fall 2016, the most abundant genera were: *Thiobacillu, Sphingomonas, Pseudomonas, Pelomonas, Herbaspirillium, Geobacter, Gaiella, Flavobacterium, Clostridium* and *Ralstonia* (Figure 2.2B). During summer 2017, the most abundant genera were: *Thiobacillus, Sphingomonas, SJA-88, Rhodobacter, Pseudomonas, Pelomonas, LCD-6, Hyphomicrobium, Crenothrix, Clostridium, Methylotene*

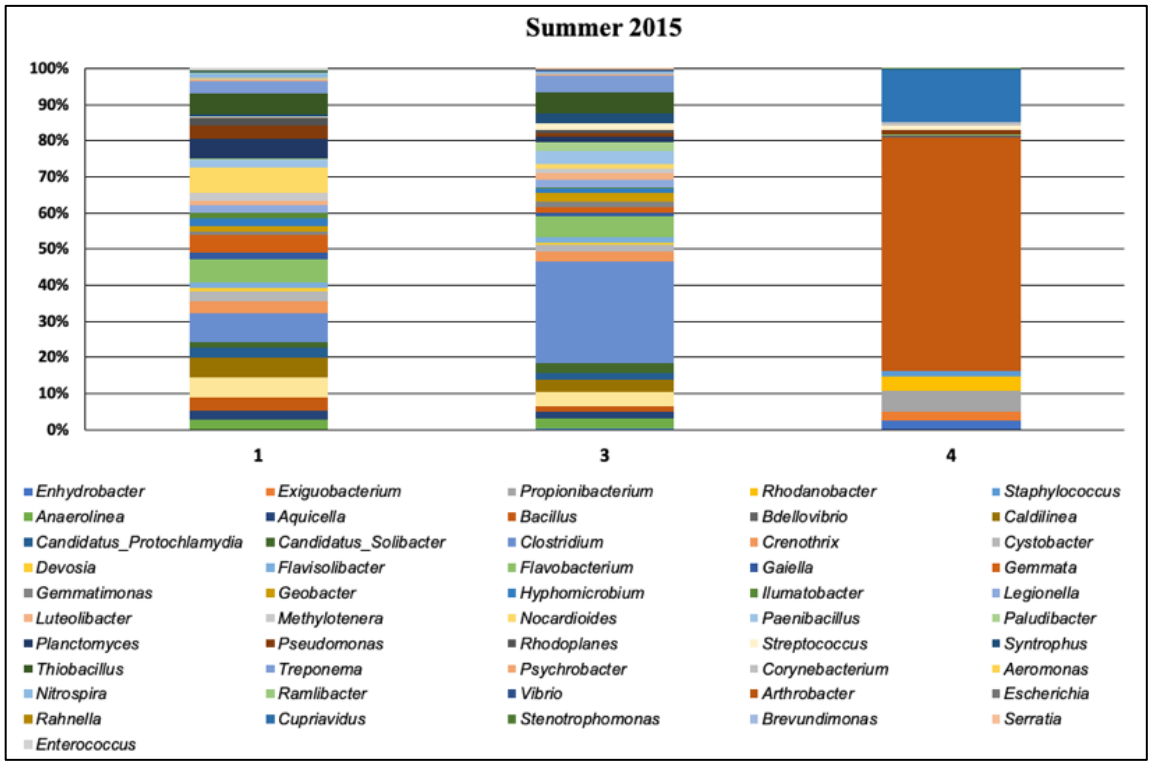


Figure 2.2A: The relative abundance of bacterial genera identified in the collected wetland sediments during summer 2015 identified by 16S rRNA gene sequencing in wetland sites 1, 3, 4.. Insufficient DNA was isolated from wetland 2,5 and 6 during summer 2015. Genera with an abundance of less than 1% were not included in this figure.

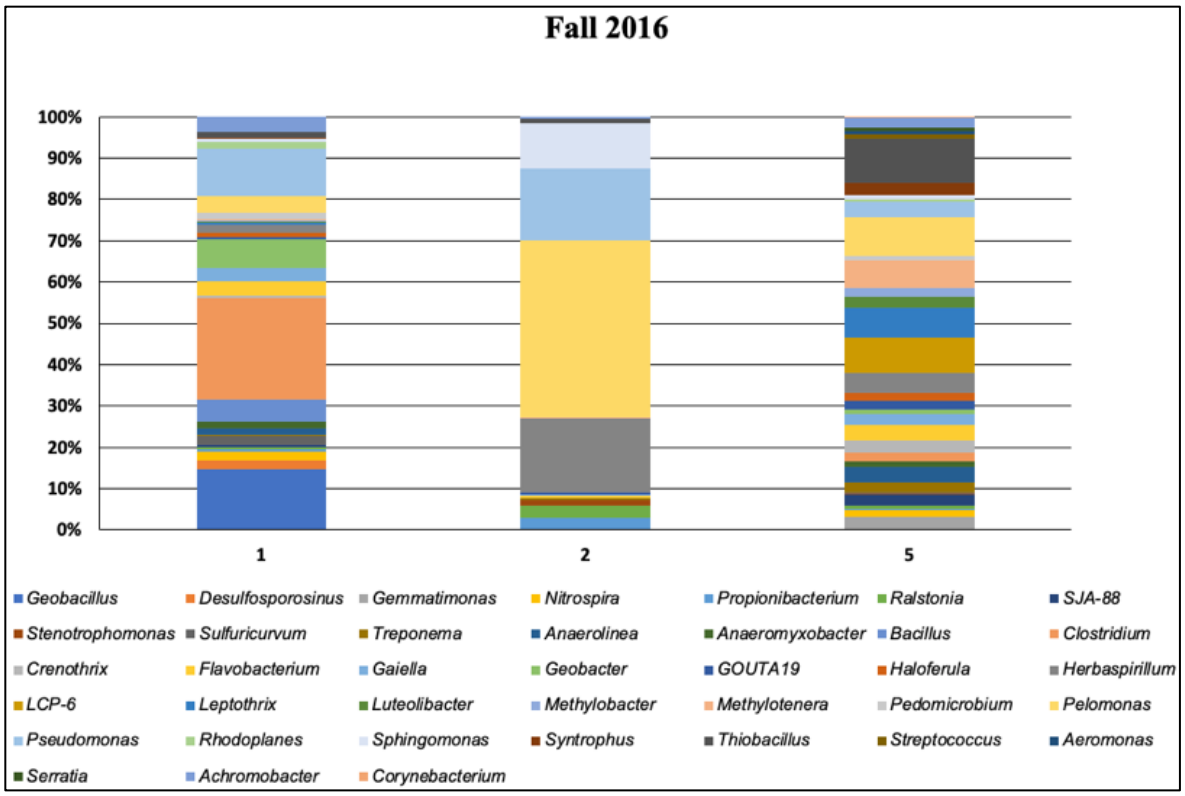


Figure 2.2B: The relative abundance of bacterial genera identified in the collected wetland sediments during fall 2016 identified by 16S rRNA gene sequencing in wetland sites 1, 2 and 5. Insufficient DNA was isolated from wetland 3,4 and 6 during fall 2016. Genera with an abundance of less than 1% were not included in this figure.

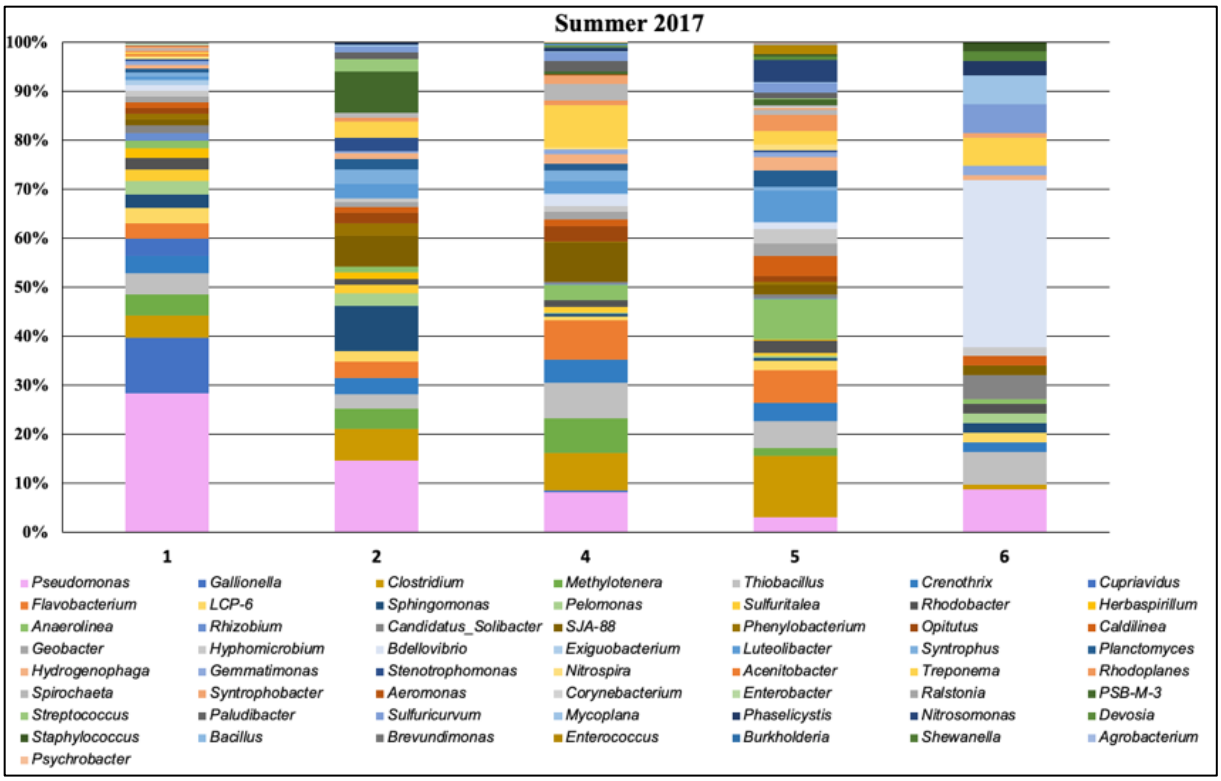


Figure 2.2C: The relative abundance of bacterial genera identified in the collected wetland sediments during summer 2017 identified by 16S rRNA gene sequencing in wetland sites 1, 2, 4, 5 and 6. Genera with less than 1% of the relative abundance in the sediment samples were removed from this analysis. Insufficient DNA was isolated from wetland 3 during summer 2017. Genera with an abundance of less than 1% were not included in this figure.

Diversity of bacterial indicators across the wetland sites

The Shannon diversity index of phyla ranged between 0.64 to 2.69 and the Simpson diversity index of phyla ranged from 0.22 to 4.04 in all the wetland sites 1-6 during all the sampling times. The Shannon diversity index of genera ranged between 1.30 to 5.18 and the Simpson diversity index of genera ranged from 0.64 to 0.99 in all the wetland sites 1-6 during all the sampling times.

Relationship between diversity indices and number of sequences: Potential bias in the diversity indices related to the number of 16S rRNA gene sequences retrieved from each of the wetland sites were shown. Results reveal slightly positive correlation between total number of sequences in the sample (x-axis) and both measures of diversity (y-axis) (Figure 2.3). The slope values for relationship between the total number of sequences and Shannon, Simpson diversity indices of phyla and genera was positive (Figure 2.3).

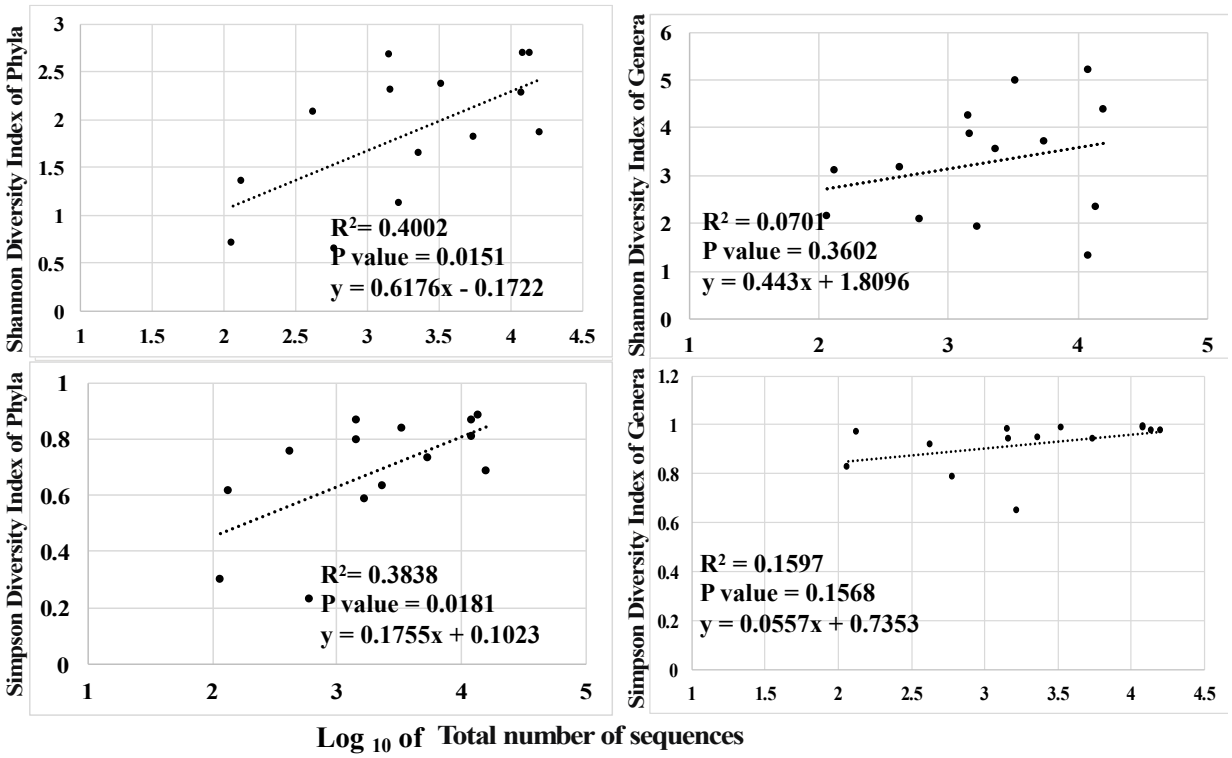


Figure 2.3: Scatterplot and line of fit for Shannon and Simpson diversity indices of phyla and genera identified by 16S rRNA gene sequencing in samples from wetland sites 1-6. Sequence numbers are shown as Log_{10} values.

Bacterial Indicator Responses to Pollution Stress

Nutrient Effects on Bacterial Indicators: The effects of nutrient pollution on bacterial indicators were examined using multifactor general linear model analysis of variance. Nutrient loadings predicted from land cover types showed no significant effects on phyla Shannon Diversity ($p = 0.1522$ for nitrate+nitrite, $p = 0.1397$ for phosphate, $p = 0.2102$ for interaction) (Figure 2.4). By contrast, predicted nutrient loadings were significantly related to phyla Simpson diversity for nitrate+nitrite ($p=0.0219$), phosphate ($p=0.0150$) and the interaction term ($p=0.0526$). With increasing predicted nitrate + nitrite loadings there was a significant increase in the Simpson diversity index but with increasing phosphate loadings there was a significant decrease in the Simpson diversity index (Figure 2.4).

A similar pattern was observed for Genera Shannon Diversity and Shannon Simpsons Index (Figure 2.5). Nutrient loadings predicted from landcover exhibited significant effects on Simpson diversity (for nitrate+nitrite $p = 0.0028$, for phosphate $p = 0.0043$, and nutrient interaction $p = 0.0028$). With increasing nitrate+nitrite loadings there was an increase in the Simpson diversity index, but with increasing phosphate loadings there was a decrease in the Simpson diversity index significantly (Figure 2.5). There were no significant effect for Shannon Diversity (Figure 2.5, p-value for nitrate+nitrite = 0.2580, p-value for phosphate loading = 0.2710, p-value of the nutrient interaction = 0.3997).

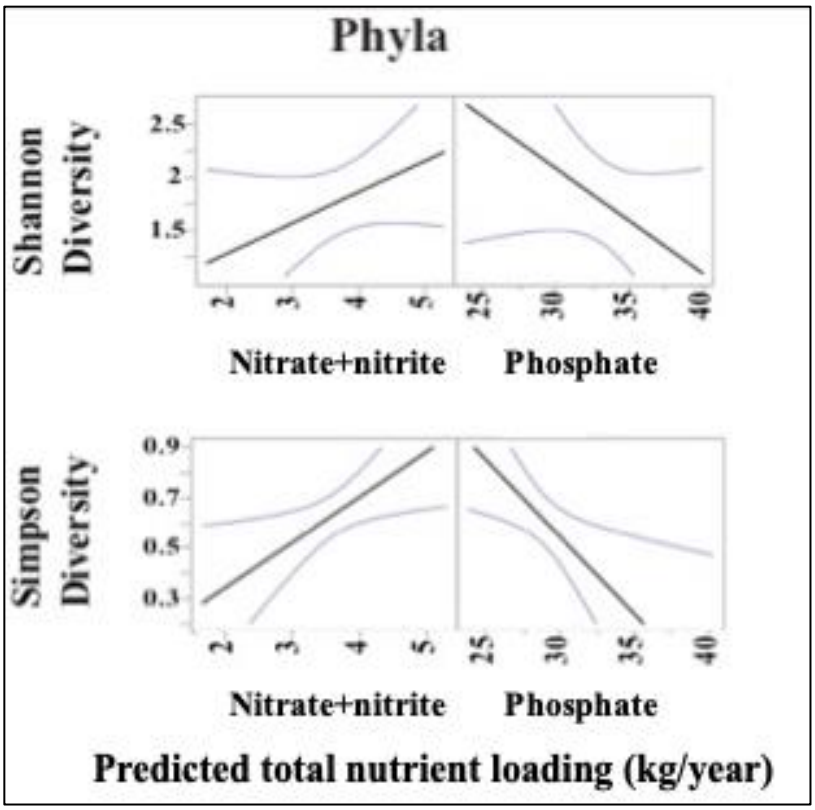


Figure 2.4: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla level out of wetland sites 1-6 from predicted total Nitrate-Nitrite and Phosphate loading (mg/m2/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate + nitrite at 7.25 kg/ year, phosphate at 22.51 kg/ year in case of Shannon diversity, nitrate+ nitrite at 8.43 kg/ year, phosphate at 24.68 kg/ year in case of Simpson diversity .

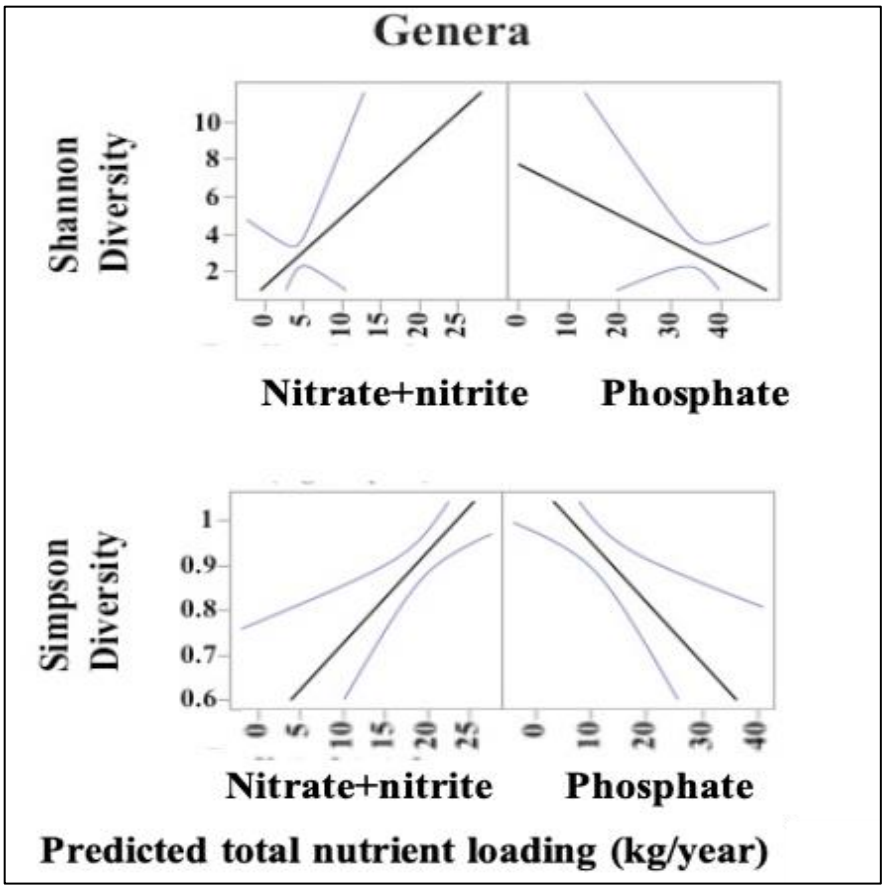


Figure 2.5: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from predicted total Nitrate-Nitrite and Phosphate loading (mg/m2/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate + nitrite at 8.43 kg/ year, phosphate at 24.68 kg/ year in case of Shannon diversity, Simpson diversity.

The effects of measured nutrient concentrations in the wetlands on bacterial diversity indices are shown in Figures 2.6 and 2.7. Shannon Diversity for phyla decreased significantly in relation to phosphate concentrations (Figure 2.6, $P<0.0001$) but not significantly for nitrate ($P=0.3649$) or nutrient interaction (The $p = 0.0559$) (Figure 2.6). Simpson diversity index of phyla was observed to be decreasing significantly with increasing nitrate ($P<0.0001$) and phosphate concentration ($P=0.0006$) with a strong interaction ($p <0.0001$) (Figure 2.6).

For bacterial genera, Shannon diversity index of genera was significantly related to concentrations of phosphate ($P=0.0266$) but not for nitrate ($P= 0.7033$), or for nutrient interactions ($p= 0.3604$) (Figure 2.7). Simpson diversity index of genera was observed to decrease with increasing nitrate ($P = 0.0215$) with no effect of phosphate ($P = 0.1673$) but with a strong interaction ($p = 0.0087$) (Figure 2.7).

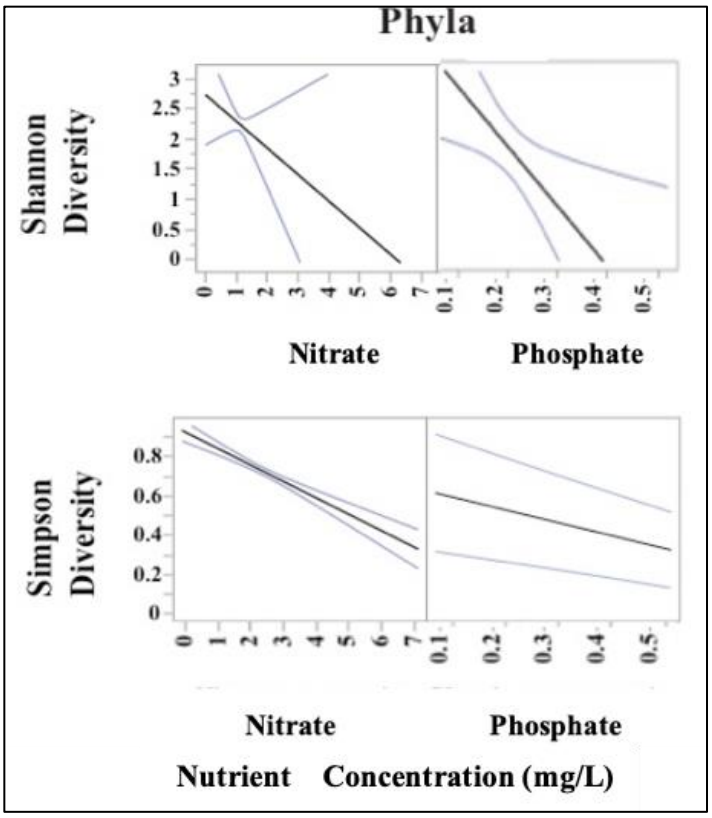


Figure 2.6: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla out of wetland sites 1-6 from measured nitrate and phosphate concentration (mg/L) during summer 2017. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for median ambient conditions, with nitrate at 3.37mg/L, phosphate at 0.21 mg/L in case of Shannon diversity, 2.19 nitrate at mg/L, phosphate at 0.27 mg/L in case of Simpson diversity.

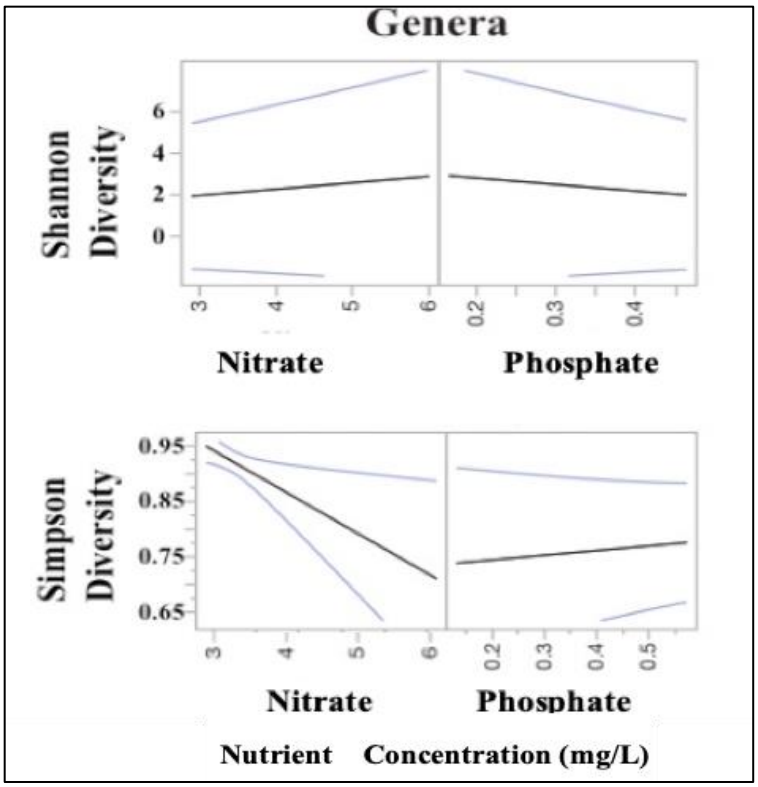


Figure 2.7: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from measured nitrate and phosphate concentration (mg/L) during summer 2017. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for nitrate at 2.72 mg/L, phosphate at 0.49 mg/L in case of Shannon diversity, 2.60 nitrate at mg/L, phosphate at 0.32 mg/L in case of Simpson diversity.

Metal Effects

ANOVA tests for the effect of predicted loadings of individual (Cu, Pb, Zn and Cd) metals from the surrounding land use on bacterial indicators were tested, where the dependent response variables were the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) and the independent variables (effects) were predicted total metal loadings.

Shannon diversity of phyla increased with increasing predicted loadings for Cu (P=0.0002), Pb (p=0.0045) and Cd (P=0.0045) loadings, the but decreased in relationship with Zn (P=0.0003). The effects of the metals did interact in a statistically significant manner (p =0.0179, (Figure 2.8). The Simpson diversity of phyla increased Cu (P <0.0001), Pb (P <0.0001) and Cd (P=0.0031) loadings, but decreases with Zn (P <0.0001), together with a significant interaction among metals (p=0.0209) (Figure 2.8).

No significant effect on Shannon diversity of genera were detected for calculated loadings of Cu (P=0.6620), Pb (P=0.9003), Zn (P=0.5828) and Cd (P=0.2932) (Figure 2.9). Simpson diversity of genera increased in relation to loadings of Cu (P=0.0098), Pb (P=0.0018) and Cd (P=0.0520), the but decreased with Zn (P=0.0045) loadings. Statistical interaction among metal loadings was marginally significant (p = 0.0559, Figure 2.9).

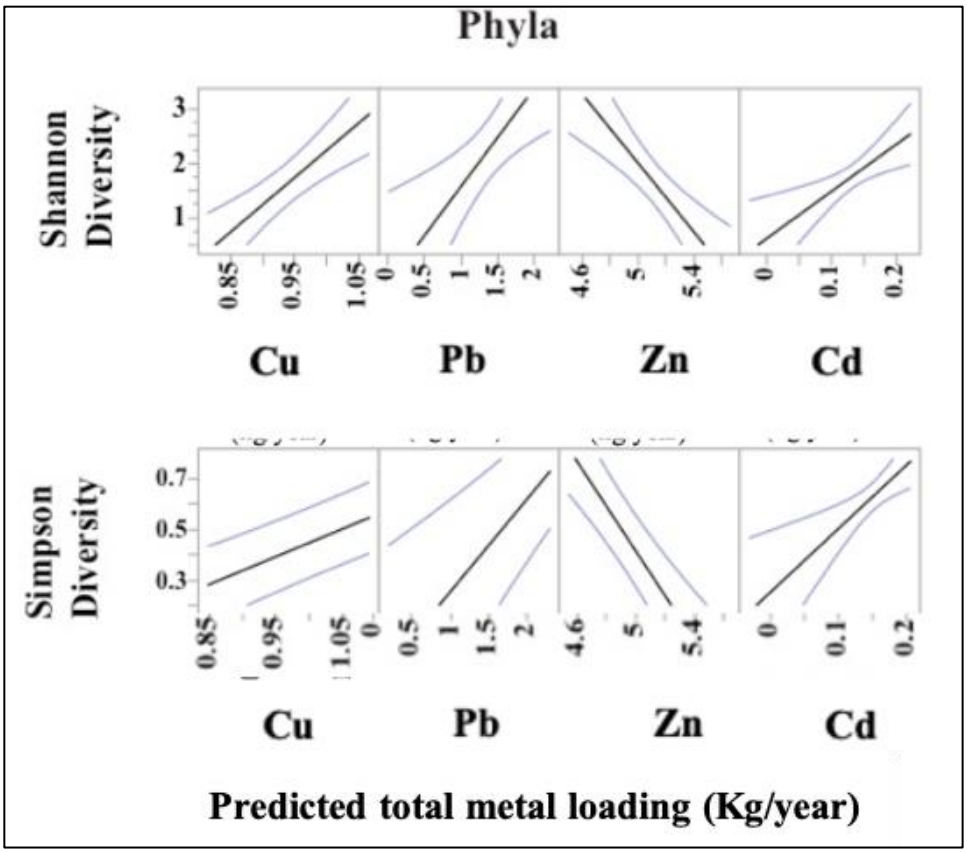


Figure 2.8: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as phyla out of wetland sites 1-6 from predicted total metal (Cd, Cu, Zn and Pb) loadings kg/year. The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for Cu at 1.04 kg/ year, Pb at 1.60 kg/ year, Zn at 4.78 kg/year, Cd at 0.23 kg/year in case of Shannon diversity, Cu at 1.17 kg/ year, Pb at 1.82 kg/ year, Zn at 5.43 kg/year, Cd at 0.34 kg/year in case of Simpson diversity.

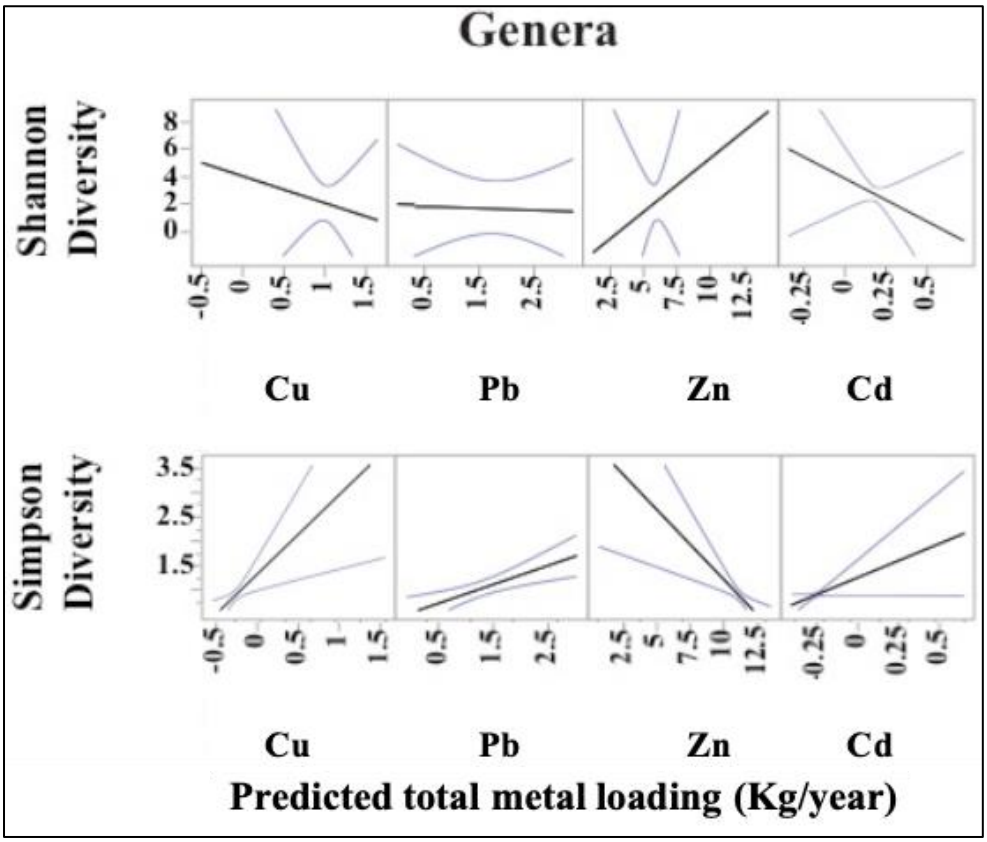


Figure 2.9: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from predicted total metal (Cd, Cu, Zn and Pb) loadings (Kg/year). The blue-lined area in each profile represents the 95% confidence prediction interval of the response variable. The profiler is set for Cu at 1.17 kg/ year, Pb at 1.82 kg/ year, Zn at 5.43 kg/year, Cd at 0.34 kg/year in case of Shannon diversity, Simpson diversity.

The effects of measured metal concentrations measured in situ on bacterial diversity indicators were analyzed by ANOVA. Using metal principal component factor identified in a prior study (Chapter 1), metal component 1 (characterized as increased concentrations of As, Ag, Cd, Ni, Zn in ppm) had no significant effect on Shannon diversity index of phyla (P=0.2024), Simpson diversity index of phyla (P=0.1826) (Figure 2.10 and 2.11). By contrast, an increase in of metal component 2 (interpreted as associated higher Pb and lower Hg concentration) was associated with increases in the Shannon diversity index of phyla (P<0.0001), the Simpson diversity index of phyla (P<0.0001) (Figure 2.11). The interaction effect of metal component 1 and 2 has a significant effect on Shannon diversity index of phyla (P=0.0003) (Figure 2.10).

Metal component 1 (interpreted as associated with increases in concentrations of As, Ag, Cd, Ni, Zn in ppm) did have a significant effect on Shannon diversity index of genera (P=0.0413), but not on the Simpson diversity index of genera (P=0.0945) (Figure 2.11). With increase in metal component 1, Shannon diversity index of genera decreased (Figure 2.11).

Simpson diversity index of genera (p<.0001) increased in relation to the increase in metal component 2 (interpreted as associated higher Pb and lower Hg concentration), but not significantly related to the Shannon diversity index of genera (P=0.5952) (Figure 2.11). The statistical interactions among metal component 1 and 2 were also statistically significant on Simpson diversity (P<.0001) but not on Shannon diversity of genera (P=0.072).

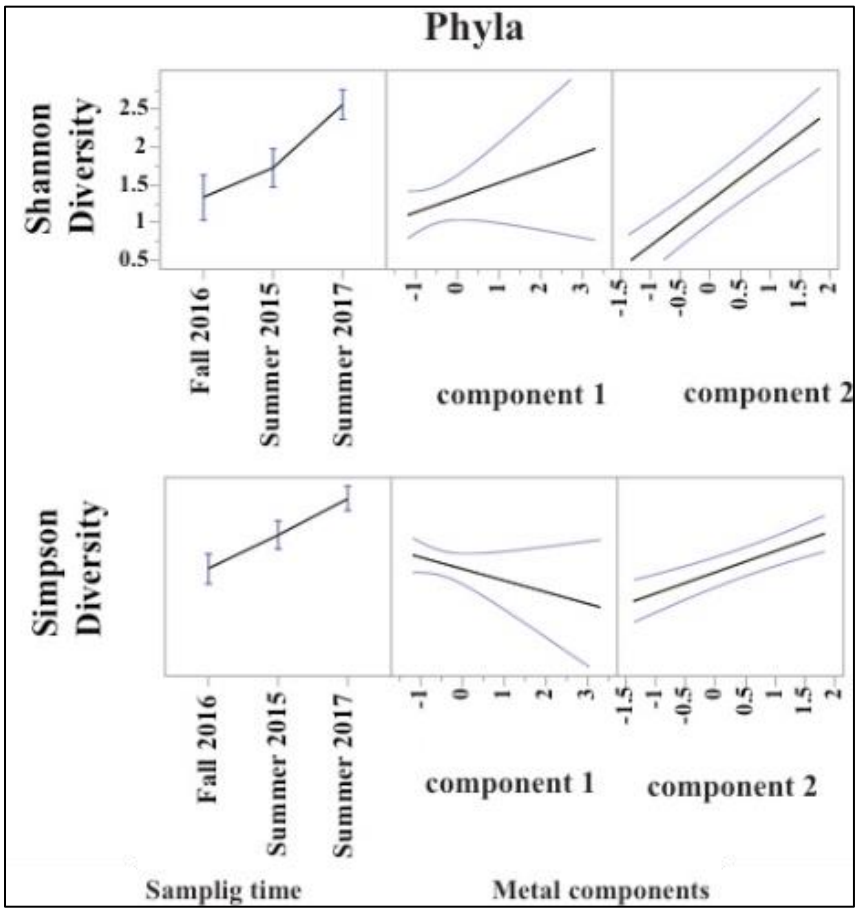


Figure 2.10: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified to the level of phyla from wetland sites 1-6 from three sampling times of fall 2016, summer 2015 and 2017 and metal component 1 and 2 (from principal component analysis). The blue-lined area in each profile represents the 95% prediction confidence interval of the response variable. The profiler is set to metal component 1 at 0.026 and metal component 2 at 0.075 in case of Shannon diversity, metal component 1 at 1.38 and metal component 2 at 0.18 in case of Simpson diversity.

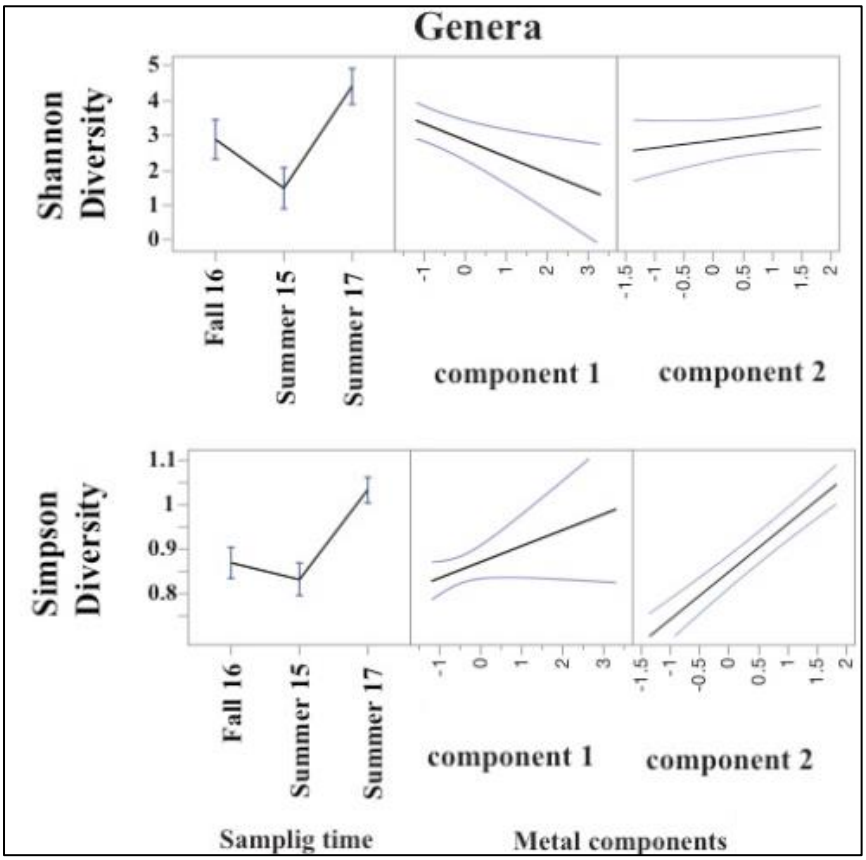


Figure 2.11: Prediction profiles from ANOVA showing the effect on the Shannon and Simpson diversity indices identified as genera out of wetland sites 1-6 from three sampling dates of fall 2016, summer 2015 and 2017 seasons and metal component 1 and 2. The blue-lined area in each profile represents the 95% prediction confidence interval of the response variable. The profiler is set to metal component 1 at 0.65 and metal component 2 is set to 0.18 in case of Shannon diversity, Simpson diversity.

Forward stepping multiple regression was employed to assess the best-fit metal predictors of bacterial indicators. Pb, Hg, As and Zn concentrations were selected for multiple regression analysis due to their high loadings in the metal factor analysis (See Figure 1.9). Shannon diversity index of phyla was associated positively with Hg and Pb (Table 2.2, $R_2 = 0.40$) (Table 2.2). The Shannon diversity index of bacterial genera was associated positively with Hg and As and negatively with Pb ($R_2 = 0.52$, Table 2.2). The Simpson diversity index was associated positively Hg and Pb ($R_2 = 0.27$, Table 2.2).

Table 2.2: Stepwise regression with the estimates of relationship between metals concentrations with the highest loadings from factor analysis (As, Zn, Hg, Pb) regressed against Shannon diversity index, Simpson diversity index and total number of identified phyla and genera in wetland sites 1-6., including p-values and total R_2 for the regression.

	As	**Zn**	**Hg**	**Pb**	**R_2**
(A) Phyla Shannon diversity index	--	—	3.22 (P=0.00185)	1395.34 (P=0.00057)	0.40
(B) Phyla Simpson diversity index	—	—	—	—	—
	As	**Zn**	**Hg**	**Pb**	**R_2**
(C) Genera Shannon diversity index	5.53 (P=0.00022)	—	11.0024 (P=0.00001)	-1845.24 (P=0.0066)	0.52
(D) Genera Simpson diversity index	--	--	0.36 (P=0.01618)	161.35 (P=0.00595)	0.27

Discussion:

The objective of this study was to determine if variation in taxonomical diversity of sediment bacterial community assemblages identified by 16S rRNA measured from wetland sediments correlates with predicated loads of pollution-related stressors such as nutrients and metals entering into the wetlands from surrounding land uses and/or concentrations of nutrient or metal measured within the wetlands. If so, could sediment taxonomical diversity of the bacterial community assemblage serve as predictive bioindicators of pollution loadings into the wetlands?

Analysis of data collected in this study from 6 wetlands across 3 years, demonstrates that bacterial community assemblages are impacted by and highly correlate with the pollutant stress measured heavy metals such as Pb and Hg, and phosphate.

Measures of Community Composition: Results from this study are consistent with other published studies that applied 16Sr RNA to wetland sediments, which also found common phyla to be Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Acidobateria, Chloroflexi, Gemmatimonadetes, Chlorobi, Cyanobacteria and Verrucomicrobia (Calheiros et al. 2010, Shange et al. 2013, Ligi et al. 2014, Zhang et al. 2016). Similarly our results are consistent with other published studies that revealed common genera to be *Bacillus, Pseudomonas, Clostridium, Thiobacillus, Plancomycetes* and *Rhodobacter* in wetland soils or sediments (Wang et al. 2012, Ding et al. 2015, Zhang et al. 2017).

The Shannon and Simpson diversity indices have been used extensively for characterizing bacterial communities (Calheiros et al. 2010, Shange et al. 2013, Stoeva et al. 2014, Zhang et al. 2016), and each provides somewhat different information in regard to community composition. For example, the Shannon Index is more sensitive to taxonomic richness, resulting in identifying each unique OTU adding to the index value evenness (Hughes and Bohannan 2004). By

comparison, the Simpson Index is weighted towards the most abundant OTU of the sample (Begon et al. 1996) and is less sensitive to taxonomic richness (Hughes and Bohannan 2004), although both indices can vary by taxonomic level of hierarchy (Begon et al. 1996). Also, when using indices to characterize differences in community composition it is advisable to include multiple indices (Hughes and Bohannan 2004). In this study, the wide range of variation of the indices for both phyla and genera across all the wetland sites demonstrated differences in bacterial community assemblages – a primary consideration when selecting good sub-metrics for indicators (Karr and Chu 1999). In addition, the use of multiple indices also avoids the biasness specifically towards richness or abundance of the identified OTUs among the taxonomical profiles of the wetland sites.

With respect to the Shannon diversity index of phyla, it is important to recognize the relationship between number of sequences detected influences the diversity indices. In this study, the correlations between total number of sequences in the sample and Shannon diversity index and Simpson diversity index were significant for phyla, but not so for genera (Figure 2.3). The highest R_2 value was 0.40, between total number of sequences in the sample (x-axis) and Shannon diversity index of phyla (y-axis). This suggests that the number of sequences found in the wetland samples affected the phyla diversity more than genera. But, generally, R_2 value ranges from 0 to 1.0, (Sokal and Rohlf 1981) hence even the highest R_2 between total number of sequences in the sample and Shannon diversity index of phyla as stated, was not high. So the effect between total number of sequences in the sample on phyla diversity was not very high. Generally, Shannon diversity indices ranges between 1.5 and 3.5, Simpson diversity indices ranges from 0 to 1(Magurran 2004). Shannon diversity index was on the lower range (Shannon diversity index of phyla between 0.15 to 3.0 and Shannon diversity index of genera between 1 to

5.5: Figure 2.3) was less compared to these standards, this was possibly driven by the fact that some of the wetland samples had low number of sequences due to poor DNA yield and this affected the Shannon diversity index overall compared to Simpson diversity index. Other studies have also shown that Shannon diversity index of phyla and genera in soil and wetland sediment have been reported to range between 3.57 and 5.38 (Faoro et al. 2010, Shange et al. 2013), which was also observed in these wetland samples in the higher range (Figure 2.3). Simpson diversity index of phyla and genera ranged from 0.002-0.037 in soils and wetland sediments in other literatures (Hill et al. 2003, Yu et al. 2014). The value detected in the wetland sites of this study was much higher compared to the literatures (Figure 2.3) . Proving the efficiency of Simpson diversity more (Figure 2.3) compared to Shannon while detecting wetland sediment bacterial diversity.

Relationships with Nutrient Pollution: Soil microorganisms are one of the main drivers of nutrient cycling in ecosystems (Artursson et al. 2006, Morris and Blackwood 2015, Wang et al. 2018) and are excellent candidates for indicator development (Groffman et al. 1996, Glibert et al. 2004, Bodelier and Dedysh 2013). Fertilizers and pesticides from surrounding agricultural lands are rich in phosphate and nitrate, serving as a source of nutrients that may runoff into the wetland sites (Ward 2009, USEPA 2016).

This study detected an array of relationships between nutrients and sediment bacterial communities. For example, Simpson diversity of phyla, and both Shannon and Simpson diversity of genera were positively associated with increasing nitrate+nitrite loadings predicted from land use (Figures 2.4 and 2.5). By contrast, the Simpson diversity of phyla and genera were observed to decrease with increasing measured nitrate concentration (Figure 2.6 and 2.7). This discrepancy between the effects of predicted total nitrate-nitrite and the measured nitrate

concentration on the bacterial composition is consistent with the literature, which suggests that responses of soil bacterial communities to nitrogen addition are highly variable among different ecosystems (Freedman et al. 2015, Cui et al. 2017, Nie et al. 2018, Wang et al. 2018). But as the measured nitrate concentration was performed in the filed in contrast to the predicted nitrate+nitrite loading that was based on other studies (see chapter 1), we could consider the effect on the bacterial indicators by measured concentrations of nitrate to be more precise and effective in the context of the study. In this case, there are opposite relationships between community composition indices and the predicted loading models (nitrate+nitrite) and the measured concentrations (nitrate). The spatial-temporal sampling regime of this study did not allow for sorting out this relationship further, but it is likely that these relationships are affected by interplay with other pollutants such as phosphorus and thus we see reduction in bacterial diversity when the nutrients were measured.

Phosphate loading predicted from land use had a significant negative association with Simpson diversity index for phyla and genera, but no measurable effect on Shannon diversity of phyla and genera (Figure 2.4 and 2.5). Similarly, higher phosphate concentrations measured were associated with decreased Shannon diversity for phyla and genera, Simpson diversity of phyla (Figure 2.6 and 2.7). Hence for data collected during summer 2017, both the measured phosphate concentration and predicted phosphate loading models showed a similar pattern where higher phosphorus was associate with less diverse bacterial community composition.

The wetland sites are rich in agricultural and residential land uses. Both these land uses produce runoffs rich in nutrients such as phosphate and nitrate due to the presence of fertilizers and pesticides applied to agricultural lands, lawns and gardens .This suggests possible interactions between the loadings of nutrients (e.g. from fertilizers) and loadings of other

pollutants such as heavy metals (Hg and Pb as observed in chapter 1) associated with these pesticides causing less bacterial diversity. Other studies have also shown that phosphate can cause reduction in diversity and microbial biodiversity in soils or sediments is often associated with a complex interaction of multiple factors such presence of several other pollutants (Faoro et al. 2010).

Relationships with Metal Pollution: Studies have shown substantial change can take place in microbial diversity in response to increasing metal concentrations (Sobolev and Begonia 2008, Xie et al. 2016). Heavy metals can result in the extinction of non-resistant microorganisms (Zhao et al. 2014a) as well. Most heavy metals cause toxic effects to bacterial cells (Gadd 1992, Trajanovska et al. 1997, Wan et al. 2016). Several studies have found bacterial communities at phyla and genera level to be correlated with higher levels of metal contamination (An et al. 2018), and bacteria have been shown to be instrumental in the remediation of metal contamination (Knox et al. 2010).

The loading of metals predicted from surrounding land use had significant effect on the Shannon and Simpson diversity indices of phyla and genera (Figure 2.8 and 2.9). Shannon diversity index of phyla and Simpson diversity index of genera were associated with higher loadings of Pb. It was also observed that higher scores for metal component 2 (interpreted as increasing Pb and decreasing Hg as measured) were associated with higher Shannon diversity index of phyla, Simpson index of phyla and genera (Figure 2.10 and 2.11). With Pb there was consistency of effect patterns between predicted loadings and the measured concentrations. But examination of metal component 2 also suggest that Hg concentrations are negatively associated the relationship of bacterial diversity with Shannon Index, Simpson Index of phyla and genera (Figure 2.10 and 2.11). Studies indicate that metals are often unevenly distributed in the soil structure (Ranjard et al. 1997) and most heavy metals including Hg can cause toxic effects to

bacterial cells at low concentrations (Gadd 1992, Trajanovska et al. 1997). As such, the interaction terms of several factors such as pollutants and sediment characters like pH (Faoro et al. 2010) could be due in part to the variability of the responses of the bacterial indicators to the metals within the samples, which was the case of effect on metal component 2 on the bacterial indicators.

The multiple regression analyses of bacterial response to Hg and Pb concentrations show a strong interactions between the two metals. Multiple regression analysis showed that Pb concentration had a significant positive relationship with Shannon diversity index of phyla and Simpson diversity index of genera (Table 2.2). Also, Hg concentration had a significant positive estimated relationship with Shannon diversity index of phyla and genera, Simpson diversity index of genera (Table 2.2). Stepwise multiple regression provided a best-fit model where Pb and Hg concentration (ppm) also had mostly significant positive estimated relationship with Shannon diversity index of phyla and genera, Simpson diversity index of genera when analyzed concurrently with As and Zn (Table 2.2). Sometimes bacterial communities also can develop metal resistance when exposed to metals for a longer duration, which result in the positive relationship of the bacterial indicators with concentrations of metals such as Pb and Hg (Gummersheimer and Giblin 2003, Chen et al. 2011, Irawati et al. 2012, Zhang et al. 2012, Figueiredo et al. 2014, Jarosławiecka and Piotrowska-Seget 2014, Jebara et al. 2015, Kowalczyk et al. 2016, Naguib et al. 2019). The metal component analysis revealed the effect of interactions of metals like Pb and Hg on bacterial indicators while the regression analysis showed how the metals (Pb and Hg) affected the bacterial indicators when analyzed individually (but concurrently with metal like As and Zn).

Chapter 1 demonstrated how prediction profiles can provide a useful tool for visualizing the complexity of interactions among watershed pollutants such as nutrients and metals. This present chapter investigates and establishes that the variation in taxonomical diversity (Shannon and Simpson of phyla and genera) of sediment bacterial community assemblages identified by 16S rRNA gene measured from wetland sediments highly correlates with variation in predicted pollution-related stressors(such as nutrients and metals) entering into the wetlands from surrounding land uses and/or measured concentrations and nutrient or metal pollutants measured within the wetlands. This chapter also establishes the fact that sediment taxonomical diversity of the bacterial community assemblage serve as predictive bioindicators of pollution loadings into the wetlands in particular to nutrients like phosphate and nitrate and heavy metals such as Pb and Hg.

Research has shown how land cover change various land uses and its associated input of nutrients and metals pollutants affects soil bacterial communities (Yu et al. 2012, Ding et al. 2013, Szoboszlay et al. 2017, Wang et al. 2017). With the help of molecular tool like 16S rRNA gene sequences, bacterial communities can be characterized relatively quickly and in detail (Janda and Abbott 2007) and thus also can be used as predictors of pollutants.

Conclusion:

This present chapter investigated the potential for bacterial to serve as indicators for risk assessment analysis from watershed pollutants in these constructed wetland ecosystems. The bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) have the potential to serve as an indicator to pollutant stress such as nutrients and metals. The previous chapter explored the similar potential for the ecotoxicological plant indicators *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum)*. The third and final chapter builds upon these field

results by investigating how these indicators work when exposed to continued stress over a period applied within a controlled experimental wetland microcosm, and examines whether these ecotoxicological plant and bacterial indicators are suitable candidate metrics to be included as part of a multimetric risk assessment index for wetland ecosystems.

The third chapter of this dissertation seeks to identify the presence of specific "guilds" of bacterial taxa within the identified (by 16s rRNA) bacterial community that could possibly serve as more specific bioindicators of risk assessment. A limitation of the current studies presented here is that taxonomic identification was conducted only up to genus level but not up to species level. However, the statistical patterns suggest that there is enough predictive signal to warrant further study.

When it comes ecological risk assessment, approaches have been developed to assess ecological risk via triad approach (Dagnino et al. 2008) using an integrated index of chemical risk, ecotoxicological risk and ecological risk. The research presented in this current chapter estimated risk using a bacterial ecological indicator, whereas the chemical risk was assessed in Chapter 1 using Phytotox™ plant ecotoxicological bioindicators. In combination, the correlations and interactions among the ecotoxicological and bacterial bioindicators demonstrate that there is sufficient signal detection capacity for these indicators to be considered as metrics for indicator development. To address this issue, the next chapter will use experimental microcosms to calibrate these responses by manipulating nutrient and metal pollution and following indicator responses.

Bibliography

Ahmed, A. M., E. Lyautey, C. Bonnineau, A. Dabrin, and S. Pesce. 2018. Environmental concentrations of copper, alone or in mixture with arsenic, can impact river sediment microbial community structure and functions. Frontiers in Microbiology 9:1–13.

Ahn, C., P. Gillevet, and M. Sikaroodi. 2007. Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading. Ecological Indicators 7:852=863.

Alia, N., K. Sardar, M. Said, K. Salma, A. Sadia, S. Sadaf, A. Toqeer, and S. Miklas. 2015. Toxicity and bioaccumulation of heavy metals in spinach (Spinacia oleracea) grown in a controlled environment. International Journal of Environmental Research and Public Health 12:7400–7416.

Alori, E. T., B. R. Glick, and O. O. Babalola. 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Frontiers in Microbiology 8:1–8.

Altieri, M. A., and C. I. Nicholls. 2003. Soil fertility management and insect pests: Harmonizing soil and plant health in agroecosystems. Soil and Tillage Research 72:203–211.

Altimira, F., C. Yǎez, G. Bravo, M. González, L. A. Rojas, and M. Seeger. 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. BMC Microbiology 12.

An, F., Z. Diao, and J. Lv. 2018. Microbial diversity and community structure in agricultural soils suffering from 4 years of Pb contamination. Canadian Journal of Microbiology 64:305–316.

Andreazza, R., S. Pieniz, B. C. Okeke, and F. A. O. Camargo. 2011. Evaluation of copper resistant bacteria from vineyard soils and mining waste for copper biosorption. Brazilian Journal of Microbiology 42:66–74.

Artursson, V., R. D. Finlay, and J. K. Jansson. 2006. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol. 8:1–10.

Ayangbenro, A. S., O. O. Bababola, and O. S. Aremu. 2019. Bioflocculant production and heavy metal sorption by metal resistant bacterial isolates from gold mining soil. Chemosphere 231:113–120.

Aylagas, E., Á. Borja, M. Tangherlini, A. Dell'Anno, C. Corinaldesi, C. T. Michell, X. Irigoien, R. Danovaro, and N. Rodríguez-Ezpeleta. 2017. A bacterial community-based index to assess the ecological status of estuarine and coastal environments. Marine Pollution Bulletin 114:679–688.

Azarbad, H., C. A. M. Van Gestel, M. Niklińska, R. Laskowski, W. F. M. Röling, and N. M. Van Straalen. 2016. Resilience of soil microbial communities to metals and additional stressors: DNA-based approaches for assessing "stress-on-stress" responses. International Journal of Molecular Sciences 17:1–20.

Azarbad, H., M. Niklińska, C. A. M. Van Gestel, N. M. Van Straalen, W. F. M. Röling, and R. Laskowski. 2013. Microbial community structure and functioning along metal pollution gradients. Environmental Toxicology and Chemistry 32:1992–2002.

Babalola, O. O., and B. R. Glick. 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. Journal of Food, Agriculture and Environment 10:540–549.

Bachand, P., and A. Horne. 2000. Denitrification in constructed free-water surface wetlands: II.

Effects of vegetation and temperature. Ecol. Eng. 14(1–2):17–32.
Baker, A. J. M., S. P. McGrath, R. D. Reeves, and J. A. C. Smith. 2001. Metal hyperaccumulator plants: a review of the ecology and physiology of biochemical resource for phytoremediation of metal-polluted soils. Phytoremediation of Contaminated Soil and Water:85–107.
Bannerman, R., K. Baun, P. Bohn, and D. Graczyk. 1983. Evaluation of Urban Nonpoint Source Pollution Management in Milwaukee County, Wisconsin. Chicago,IL.
Bannerman, R. T., D. W. Owens, R. B. Dodds, and N. J. Hornewer. 1993. Sources of pollutants in wisconson stormwater. Water Science and Technology 28:241–259.
Baranowski, R., A. Rybak, and I. Baranowska. 2002. Speciation Analysis of Elements in Soil Samples by XRF. Polish Journal of Environmental Studies 11:473–482.
Begon, M., J. L. Harper, C. R. Townsend, and M. Begon. 1996. Ecology: Individuals, populations, and communities. 3rd edition. Blackwell Science, Oxford, UK.
Behrends, L. L. 2007. Use of aquatic plants for removal of nitrate-nitrogen in subsurface flow constructed wetlands.
Berg, J., K. K. Brandt, W. A. Al-Soud, P. E. Holm, L. H. Hansen, S. J. Sørensen, and O. Nybroe. 2012. Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term cu exposure. Applied and Environmental Microbiology 78:7438–7446.
Beutel, M., M. Morgan, J. Erlenmeyer, and E. Brouillard. 2014. Phosphorus Removal in a Surface-Flow Constructed Wetland Treating Agricultural Runoff. Journal Of Environment Quality 43:1071.
Bhattacharyya, P. N., and D. K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327–1350.
Bodelier, P., and S. Dedysh. 2013. Microbiology of wetlands. Frontiers In Microbiology 4.
Boyd, E. S., and T. Barkay. 2012. The mercury resistance operon: From an origin in a geothermal environment to an efficient detoxification machine. Frontiers in Microbiology 3:1–13.
Brown, L. R., T. F. Cuffney, J. F. Coles, F. Fitzpatrick, G. McMahon, J. Steuer, A. H. Bell, and J. T. May. 2009. Urban streams across the USA: Lessons learned from studies in 9 metropolitan areas. Journal of the North American Benthological Society 28:1051–1069.
Bruker. 2017. No Title.
Burbach, K., J. Seifert, D. H. Pieper, and A. Camarinha-Silva. 2016. Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. MicrobiologyOpen 5:70–82.
Busnardo, M., R. Gersberg, R. Langis, T. Sinicrope, and J. Zedler. 1992. Nitrogen and phosphorus removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:287–307.
Cai, L., G. Liu, C. Rensing, and G. Wang. 2009. Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. BMC Microbiology 9:1–11.
Cairns, J. J., and J. R. Pratt. 1993. A history of biological monitoring using benthic macroinvertebrates, in D.M. Rosenberg and V.H. Resh (Eds.). Pages 10–27 Freshwater Biomonitoring and Benthic Macroinvrtebrates. Chapman and Hall, New York.
Calheiros, C. S. C., A. Teixeira, C. Pires, A. R. Franco, A. F. Duque, L. F. C. Crispim, S. C. Moura, and P. M. L. Castro. 2010. Bacterial community dynamics in horizontal flow

constructed wetlands with different plants for high salinity industrial wastewater polishing. Water Research 44:5032–5038.
Carey, R., and G. J. Hochmuth. 2012. Regulatory and Resource Management Practices for Urban Watersheds: The Florida Experience. Hort Technology 22:418–429.
Castellano-Hinojosa, A., D. Correa-Galeote, P. Carrillo, E. J. Bedmar, and J. M. Medina-Sánchez. 2017. Denitrification and biodiversity of denitrifiers in a High-Mountain Mediterranean Lake. Frontiers in Microbiology 8:1911.
Chakravorty, S., D. Helb, M. Burday, and N. Connell. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69:330–339.
Al Chami, Z., N. Amer, L. Al Bitar, and I. Cavoski. 2015. Potential use of Sorghum bicolor and Carthamus tinctorius in phytoremediation of nickel, lead and zinc. International Journal of Environmental Science and Technology 12:3957–3970.
Chapman, D. P. M. 1995. Do sediment toxicity tests require field validation? Environmental Toxicological and Chemistry 14:1451-1453.
Chapman, P. M. 1990. The sediment quality triad approach to determining pollution-induced degradation. Science of The Total Environment 97–98:815–825.
Chen, B., J. Liu, Z. Wang, L. Dong, J. Fan, and J. Qu. 2011. Remediation of Pb-Resistant Bacteria to Pb Polluted Soil. Journal of Environmental Protection 02:130–141.
Chen, B. M., Z. H. Wang, S. X. Li, G. X. Wang, H. X. Song, and X. N. Wang. 2004. Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables. Plant Science 167:635–643.
Chen, J., F. He, X. Zhang, X. Sun, J. Zheng, and J. Zheng. 2014. Heavy metal pollution decreases microbial abundance, diversity and activity within particle-size fractions of a paddy soil. FEMS Microbiology Ecology 87:164–181.
Chen, J., J. Li, H. Zhang, W. Shi, and Y. Liu. 2019. Bacterial heavy-metal and antibiotic resistance genes in a copper tailing dam area in northern China. Frontiers in Microbiology 10:1–12.
Chen, Y., Y. Jiang, H. Huang, L. Mou, J. Ru, J. Zhao, and S. Xiao. 2018. Long-term and high-concentration heavy-metal contamination strongly influences the microbiome and functional genes in Yellow River sediments. Science of the Total Environment 637–638:1400–1412.
Clapcott, J. E., K. J. Collier, R. G. Death, E. O. Goodwin, J. S. Harding, D. Kelly, J. R. Leathwick, and R. G. Young. 2012. Quantifying relationships between land-use gradients and structural and functional indicators of stream ecological integrity. Freshwater Biology 57:74–90.
Clapcott, J. E., E. O. Goodwin, R. G. Young, and D. J. Kelly. 2014. Une approche multimétrique pour prédire l'intégrité écologique des cours d'eau en Nouvelle-Zélande. Knowledge and Management of Aquatic Ecosystems.
Crispell-Synder, I. 1997. Executive summary of WDNR facilitation presentation for Pike River improvements by Mount Pleasant Storm Water Drainage District No. 1.
Cui, J., J. J. Wang, J. Xu, C. H. Xu, and X. N. Xu. 2017. Changes in soil bacterial communities in an evergreen broad-leaved forest in east China following 4 years of nitrogen addition. J. Soils Sediments 17.
Czerniawska-Kusza, I., & Kusza, G. 2010. The potential of the Phytotoxkit microbiotest for hazard evaluation of sediments in eutrophic freshwater ecosystems. Environmental

Monitoring And Assessment. 179:113–121.
Czerniawska-Kusza, I., T. Ciesielczuk, G. Kusza, and A. Cichoń. 2006. Comparison of the Phytotoxkit microbiotest and chemical variables for toxicity evaluation of sediments. ENVIRONMENTAL TOXICOLOGY 21:367–372.
Dagnino, A., S. Sforzini, F. Dondero, S. Fenoglio, E. Bona, J. Jensen, and A. Viarengo. 2008. A "Weight-of-Evidence" approach for the integration of environmental "Triad" data to assess ecological risk and biological vulnerability. Integrated Environmental Assessment and Management 4:314–326.
David, P., R. S. Raj, R. Linda, and S. B. Rhema. 2014. NoMolecular characterization of phosphate solubilizing bacteria (PSB) and plant growth promoting rhizobacteria (PGPR) from pristine soils. Int. J. Innov. Sci. Eng. Technol. 1:317–324.
Decker, K. L., A. Pocewicz, S. Harju, M. Holloran, M. M. Fink, T. P. Toombs, and D. B. Johnston. 2017. Landscape disturbance models consistently explain variation in ecological integrity across large landscapes. Ecosphere 8.
Dellinger, M., M. Carvan, R. Klingler, J. McGraw, and T. Ehlinger. 2014. An Exploratory Analysis of Stream Teratogenicity and Human Health Using Zebrafish Whole-Sediment Toxicity Test. Challenges 5:75–97.
Denver Regional Council of Governments. 1983. Urban Runoff Quality in the Denver (Colorado) Region. Washington, DC.
Díaz Rizo, O., A. Gelen Rudnikas, R. D. Lavin Pérez, G. Arencibia Caraballo, and K. D´Alessandro Rodríguez. 2014. XRF analysis of sediments from Nuevitas Bay (Cuba): assessment of current heavy metal contamination. Nucleus:11–14.
Ding, G.-C., Y. M. Piceno, H. Heuer, N. Weinert, A. B. Dohrmann, A. Carrillo, G. L. Andersen, T. Castellanos, C. C. Tebbe, and K. Smalla. 2013. Changes of Soil Bacterial Diversity as a Consequence of Agricultural Land Use in a Semi-Arid Ecosystem. PLoS ONE 8:e59497.
Ding, X., X. J. Peng, B. S. Jin, M. Xiao, J. K. Chen, B. Li, C. M. Fang, and M. Nie. 2015. Spatial distribution of bacterial communities driven by multiple environmental factors in a beach wetland of the largest freshwater lake in China. Frontiers in Microbiology 6:1–9.
Ding, Z., J. Wu, A. You, B. Huang, and C. Cao. 2017. Effects of heavy metals on soil microbial community structure and diversity in the rice (Oryza sativa L. subsp. Japonica, Food Crops Institute of Jiangsu Academy of Agricultural Sciences) rhizosphere. Soil Science and Plant Nutrition 63:75–83.
DiScenza, D. J., and A. R. Keimowitz. 2014. Calibration and Evaluation of an X-Ray Fluorescence Method for the Determination of Lead and Arsenic in Soils. Journal of Environmental Analytical Chemistry 01:1–3.
Drummond, L., and W. Maher. 1995. Chimica acta. Analytica Chemica Acta 302:69–74.
Ehlinger, T., and L. DeThorne. 2004. onitoring of stream habitat and aquatic biotic integrity - Pike River North and South Branches, Racine and Kenosha Counties, Wisconsin, Interim Report.
Ehlinger, T., L. DeThorne, and B. Berner. 2002. Monitoring of stream habitat and aquatic biotic integrity - Pike River North and South Branches, Racine and Kenosha Counties, Wisconsin.
Elder, G. J. 2016. OUP accepted manuscript. Journal Of Tropical Pediatrics:5–8.
EPA. 1985. U.S. Environmental Protection Agency. Freshwater Wetlands for Wastewater Management Handbook. EPA Region IV Atlanta, GA EPA. 904:9-85–135.
EPA. 1986. Ambient Water Quality Criteria for Dissolved Oxygen.
EPA. 2003a. Protecting water quality from urban runoff.

EPA. 2003b. Protecting Water Quality from Urban Runoff. United States Environmental Protection Agency:EPA-841-F-03-003.
EPA. 2007. FIELD PORTABLE X-RAY FLUORESCENCE SPECTROMETRY FOR THE DETERMINATION OF ELEMENTAL CONCENTRATIONS IN SOIL AND SEDIMENT.
EPA. 2012. Summary of the Clean Water Act.
Faoro, H., A. C. Alves, E. M. Souza, L. U. Rigo, L. M. Cruz, S. M. Al-Janabi, R. A. Monteiro, V. A. Baura, and F. O. Pedrosa. 2010. Influence of soil characteristics on the diversity of bacteria in the southern brazilian atlantic forest. Applied and Environmental Microbiology 76:4744–4749.
Fargašová, A. 2001. Phytotoxic effects of Cd, Zn, Pb, Cu and Fe on Sinapis alba L. seedlings and their accumulation in roots and shoots.
Feng, G., T. Xie, X. Wang, J. Bai, L. Tang, H. Zhao, W. Wei, M. Wang, and Y. Zhao. 2018. Metagenomic analysis of microbial community and function involved in cd-contaminated soil. BMC Microbiology 18:1–13.
Figueiredo, N. L. L., J. Canário, A. Duarte, M. L. Serralheiro, and C. Carvalho. 2014. Isolation and characterization of mercury-resistant bacteria from sediments of tagus estuary (Portugal): Implications for environmental and human health risk assessment. Journal of Toxicology and Environmental Health - Part A: Current Issues 77:155–168.
Foley, J. A., R. DeFries, G. P. Asner, C. Barford, G. Bonan, S. R. Carpenter, F. S. Chapin, M. T. Coe, G. C. Daily, H. K. Gibbs, J. H. Helkowski, T. Holloway, E. A. Howard, C. J. Kucharik, C. Monfreda, J. A. Patz, I. C. Prentice, N. Ramankutty, and P. K. Snyder. 2005. Global consequences of land use. Science 309:570.
Freedman, Z. B., K. J. Romanowicz, R. A. Upchurch, and D. R. Zak. 2015. Differential responses of total and active soil microbial communities to long- term experimental N deposition. Soil Biol. Biochem 90:275–282.
Frossard, A., J. Donhauser, A. Mestrot, S. Gygax, E. Bååth, and B. Frey. 2018. Long- and short-term effects of mercury pollution on the soil microbiome. Soil Biology and Biochemistry 120:191–199.
Gadd, G. M. 1992. Heavy metal pollutants: environments and biotechnological aspects. Encycloped. Academic Press Inc., CA.
Gerardi, M. H. 2006. Chapter 1 Wastewater Microorgansims. Page (M. H. Gererdi, Ed.).
Gerik, T., B. Bean, and R. Vanderlip. 2010. Sorghum Growth and Development. Texas. Texas A & M University System.
Gersberg, R. M., B. V. Elkins, S. R. Lyon, and C. R. Goldman. 1986. Role of aquatic plants in wastewater treatment by artificial wetlands. Water Res. 20:363–367.
Ghosh Roy, S. 2020. Biological indicators of toxic stress in wetland sediments. University of Wisconsin Milwaukee.
Ghosh Roy, S., T. J. Ehlinger, and M. R. Jablonski. 2019. EFFECT OF METALS ON GROWTH INHIBITONS OF SINAPIS , SORGHUM AND LEPIDIUM IN TEXTILE DYE WASTE CONTAMINATED SOIL , INDIA 619:608–619.
Gikas, P., E. Ranieri, and G. Tchobanoglous. 2013. Removal of iron, chromium and lead from waste water by horizontal subsurface flow constructed wetlands. Journal of Chemical Technology and Biotechnology 88:1906–1912.
Glibert, P., C. Heil, D. Hollander, M. Revilla, A. Hoare, J. Alexander, and S. Murasko. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial

bloom in Florida Bay. Marine Ecology Progress Serie 280:73-83.
Glicksman, R. L., and M. R. Batzel. 2010. Science, politics, law, and the arc of the Clean Water Act: the role of assumptions in the adoption of a pollution control landmark. Washington University Journal of Law & Policy, Washington University, School of Law 32:99.
Groffman, P., G. Hanson, E. Kiviat, and G. Stevens. 1996. Variation in Microbial Biomass and Activity in Four Different Wetland Types. . Soil Science Society Of America Journal 60:622.
Gummersheimer, B. S., and T. Giblin. 2003. Identification of Lead Resistant Bacteria from a Heavily Contaminated Site Author (s): Beth S . Gummersheimer and Tara Giblin Published by : Beta Beta Beta Biological Society Stable URL : https://www.jstor.org/stable/4608669 Identification of lead resis. jstor 74:48–54.
Gurave, N. A., V. V Korde, S. S. Dhas, and M. Disale. 2015. Isolation and identification of heavy metal resistant bacteria from petroleum soil of Loni, Ahmednagar. European Journal of Experimental Biology 5:6–11.
Hach. 2019. Phosphorus, Reactive (Orthophosphate), 8048.
He, L. Y., Y. F. Zhang, H. Y. Ma, L. N. Su, Z. J. Chen, Q. Y. Wang, M. Qian, and X. F. Sheng. 2010. Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. Applied Soil Ecology 44:49–55.
Heidenreich, B., K. Mayer, H. J. Sandermann, and D. Ernst. 2001. Mercury-induced genes in Arabidopsis thaliana: identification of induced genes upon long-term mercuric ion exposure. Plant, Cell and Environment 24:1227–1234.
Hemme, C. L., Y. Deng, T. J. Gentry, M. W. Fields, L. Wu, S. Barua, K. Barry, S. G. Tringe, D. B. Watson, Z. He, T. C. Hazen, J. M. Tiedje, E. M. Rubin, and J. Zhou. 2010. Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. ISME Journal 4:660–672.
Hermans, S. M., H. L. Buckley, B. S. Case, F. Curran-Cournane, M. Taylor, and G. Lear. 2016. Bacteria as emerging indicators of soil condition. Applied and Environmental Microbiology:AEM.02826–16.
Hill, T. C. J., K. A. Walsh, J. A. Harris, and B. F. Moffett. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiology Ecology 43:1–11.
Hughes, J., and B. Bohannan. 2004. Application of ecological diversity statistics in microbial ecology. Molecular Microbial Ecology Manual, Second Edition 7.01:1321-1344.
Irawati, W., Patricia, Y. Soraya, and A. hugo Baskoro. 2012. A Study on Mercury-Resistant Bacteria Isolated from a Gold Mine in Pongkor Village, Bogor, Indonesia. HAYATI Journal of Biosciences 19:197–200.
Istina, I. N., H. Widiastuti, B. Joy, and M. Antralina. 2015. Phosphate- solubilizing microbe from Saprists peat soil and their potency to enhance oil palm growth and P uptake. Proc. Food Sci. 3:426–435.
Janda, J., and S. Abbott. 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal Of Clinical Microbiology 45:2761–2764.
Jardine, C., S. Hrudey, J. Shortreed, L. Craig, D. Krewski, C. Furga, and S. McColl. 2003. Risk Management Frameworks for Human Health and Environmental Risks. J Toxicol Environ Health B Crit Rev. 6:569–720.
Jarosławiecka, A., and Z. Piotrowska-Seget. 2014. Lead resistance in micro-organisms. Microbiology (United Kingdom) 160:12–25.

Jasper, J. T., Z. L. Jones, J. O. Sharp, and D. L. Sedlak. 2014. Nitrate removal in shallow, Open-water treatment wetlands. Environmental Science and Technology 48:11512–11520.
Jebara, S. H., S. Abdelkerim, I. C. Fatnassi, M. Chiboub, O. Saadani, and M. Jebara. 2015. Identification of effective Pb resistant bacteria isolated from Lens culinaris growing in lead contaminated soils. Journal of Basic Microbiology 55:346–353.
Jensen, J. 2011. Sediment toxicity and the recoveryof biological integrity in a restored stream channel. Milwaukee. University of Wisconsin Milwaukee.
Jiang, X.-T., X. Peng, G.-H. Deng, H.-F. Sheng, Y. Wang, & Zhou, H.-W., and N. F.-Y. Tam. 2013. Illumina Sequencing of 16S rRNA Tag Revealed Spatial Variations of Bacterial Communities in a Mangrove Wetland. Environmental Microbiology 66:96–104.
Kadlec, R. H. 2016. Large constructed wetlands for phosphorus control: A review. Water (Switzerland) 8.
Kadlec, R., and S. Wallace. 2009. Treatment Wetlands. CRC Press Taylor & Francis Group, LLC.
Kaduková, J., O. Velgosová, A. Mrazíková, R. Marcinčáková, and E. Tkáčová. 2015. Assessment of Biologically Synthesized Ag Nanoparticles Toxicity Against E. coli, Staphylococcus aureus, Parachlorella kessleri and Sinapis alba. Nova Biotechnologica et Chimica 14:69–77.
Kafilzadeh, F., R. Afrough, H. Johari, and Y. Tahery. 2012. Range determination for resistance / tolerance and growth kinetic of indigenous bacteria isolated from lead contaminated soils near gas stations (Iran). European Journal of Experimental Biology 2:62–69.
Kang, S., J. D. Van Nostrand, H. L. Gough, Z. He, T. C. Hazen, D. A. Stahl, and J. Zhou. 2013. Functional gene array-based analysis of microbial communities in heavy metals-contaminated lake sediments. FEMS Microbiology Ecology 86:200–214.
Karr, J. R. 1993. Measuring Biological integrity: lesions from stream, in S. Woodley, J. Kay and G. Francis (Eds.). Page Ecological Integrity and Management of Ecosystems. St. Lucie Press, Delray, FL.
Karr, J. R. 1995. Preticting Aquatic ecosystems: clean water is not enough, in W.S Davis and T.P. Simon(Eds.). Pages 7–13 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
Karr, J. R., and E. W. Chu. 1999. No Title. Page Restoring Life in Running Waters. Island Press, Washington D.C., Covelo California.
Katich, J. T., M. Nelly, and J. Scott. 2012. Sediment Sampling Guide and Methodologies. : Ohio EPA Sediment Sampling Guide. Environmental Protection Agency.
Ke, X., C. Wang, D. Jing, Y. Zhang, and H. Zhang. 2015. Assessing water quality by ratio of the number of dominant bacterium species between surface/subsurface sediments in Haihe River Basin. Marine Pollution Bulletin 98:267-273.
Kenna, T. C., F. O. Nitsche, M. M. Herron, B. J. Mailloux, D. Peteet, S. Sritrairat, E. Sands, and J. Baumgarten. 2011. Evaluation and calibration of a Field Portable X-Ray Fluorescence spectrometer for quantitative analysis of siliciclastic soils and sediments. Journal of Analytical Atomic Spectrometry 26:395.
Khan, S., I. Ahmad, M. Shah, S. Rehman, and A. Khaliq. 2009. Use of constructed wetland for the removal of heavy metals from industrial wastewater. Journal Of Environmental Management. 90:3451-3457.
Knight, B., S. P. McGrath, and A. M. Chaudri. 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium,

copper, or zinc. Appl. Environ. Microbiol 63:39–43.
Knox, A., E. Nelson, N. Halverson, and J. Gladden. 2010. Long-Term Performance of a Constructed Wetland for Metal Removal. Soil And Sediment Contamination. 19:667-685.
Knox, A. S., D. L. Dunn, M. H. Paller, E. A. Nelson, W. L. Specht, and J. C. Seaman. 2006. Assessment of contaminant retention in constructed wetland sediments. Engineering in Life Sciences 6:31–36.
Konopka, A., T. Zakharova, M. Bischoff, L. Oliver, C. Nakatsu, and R. F. Turco. 1999. Microbial biomass and activity in lead-contaminated soil. Applied and Environmental Microbiology 65:2256–2259.
Kou, S., G. Vincent, E. Gonzalez, F. E. Pitre, M. Labrecque, and N. J. B. Brereton. 2018. The response of a 16S ribosomal RNA gene fragment amplified community to lead, zinc, and copper pollution in a Shanghai field trial. Frontiers in Microbiology 9:1–23.
Kovacs, M. 1992. Biological indicators of environmental pollution. Page (M Kovacs (ed.), Ed.). New York: Ellis Horwood.
Kowalczyk, A., M. Wilińska, M. Chyc, M. Bojko, and D. Latowski. 2016. Isolation, screening and identification of mercury resistant bacteria from mercury contaminated soil. E3S Web of Conferences 10.
Krachler, M., R. Alvarez-Sarandes, and G. Rasmussen. 2016. High-Resolution Inductively Coupled Plasma Optical Emission Spectrometry for 234U/238Pu Age Dating of Plutonium Materials and Comparison to Sector Field Inductively Coupled Plasma Mass Spectrometry. Analytical Chemistry 88:8862–8869.
Kulakovskaya, T. 2014. Phosphorus storage in Microorganisms: Diversity and Evolutionary Insight. Biochemistry & Physiology: Open Access 04:1–4.
Kunito, T., K. Nagaoka, N. Tada, K. Senoo, H. Oyaizu, S. Matsumoto, and K. Saeki. 1997. Characterization of cu-resistant bacterial communities in cu-contaminated soils. Soil Science and Plant Nutrition 43:709–717.
Lakshmi, K. V. N. S., C. Sasikala, V. V. Ramana, E. V. V. Ramaprasad, and C. V. Ramana. 2011. Rhodovulum phaeolacus sp. nov. a phototrophic alphaproteobacterium isolated from a brown pond. Journal of General and Applied Microbiology 57:145–151.
Lee, W. M., J. Il Kwak, and Y. J. An. 2012. Effect of silver nanoparticles in crop plants Phaseolus radiatus and Sorghum bicolor: Media effect on phytotoxicity. Chemosphere 86:491–499.
Li, J., B. Li, Y. Zhou, J. Xu, and J. Zhao. 2011. A rapid DNA extraction method for PCR amplification from wetland soils. Letters in Applied Microbiology 52:626–633.
Li, L. G., Y. Xia, and T. Zhang. 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME Journal 11:651–662.
Ligi, T., K. Oopkaup, M. Truu, J.-K. Preem, H. Nõlvak, W. J. Mitsch, and J. Truu. 2014. Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16SrRNA amplicon sequencing. Ecological Engineering 72:56-66.
Liu, C. W., Y. Sung, B. C. Chen, and H. Y. Lai. 2014a. Effects of nitrogen fertilizers on the growth and nitrate content of lettuce (Lactuca sativa L.). International Journal of Environmental Research and Public Health 11:4427–4440.
Liu, K., M. Sun, M. Ye, H. Chao, Y. Zhao, B. Xia, W. Jiao, Y. Feng, X. Zheng, M. Liu, J. Jiao, and F. Hu. 2019. Coexistence and association between heavy metals, tetracycline and corresponding resistance genes in vermicomposts originating from different substrates.

Environmental Pollution 244:28–37.
Liu, Y., J. Zhang, L. Zhao, X. Zhang, and S. Xie. 2014b. Spatial distribution of bacterial communities in high-altitude freshwater wetland sediment. Limnology 15:249–256.
López-Luna, J., M. González-Chávez, F. Esparza-García, and R. Rodríguez-Vázquez. 2009. Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. Journal of Hazardous Materials 163:829–834.
Magurran, A. 2004. Measuring Biologcial Diversity. Page Blackwell Publishing.
Mamta, R. P., V. Pathania, A. Gulati, B. Singh, R. K. Bhanwra, and R. Tewari. 2010. timulatory effect of phosphate-solubilizing bacteria on plant growth, stevioside and rebaudioside-A contents of Stevia rebaudiana Bertoni. Appl. Soil Ecol. 46:222–229.
Manasi, N. Rajesh, and V. Rajesh. 2016. Evaluation of the genetic basis of heavy metal resistance in an isolate from electronic industry effluent. Journal of Genetic Engineering and Biotechnology 14:177–180.
Manikandan, R., S. V. Sahi, and P. Venkatachalam. 2015. Impact assessment of mercury accumulation and biochemical and molecular response of Mentha arvensis: A potential hyperaccumulator plant. Scientific World Journal 2015.
Manios, T., M. Fountoulakis, and A. Karathanasis. 2009. Construction Simplicity and Cost as Selection Criteria Between Two Types of Constructed Wetlands Treating Highway Runoff. Environmental Management 43:908–920.
Margesin, R., and F. Schinner. 1996. Bacterial heavy metal-tolerance - Extreme resistance to nickel in Arthrobacter spp. strains. Journal of Basic Microbiology 36:269–282.
Matraszek, R., B. Hawrylak-Nowak, M. Chwil, S. Chwil, and M. Rudaś. 2017. Effect of the Interaction of Nickel Stress and Sulphur Supplementation on the Content and Accumulation of Macronutrients in White Mustard (Sinapis Alba L.). Electronic Journal of Polish Agricultural Universities 20.
Messer, T., M. Burchell, F. Birgand, S. Broome, and G. Chescheir. 2017. Nitrate removal potential of restored wetlands loaded with agricultural drainage water: A mesocosm scale experimental approach. Ecological Engineering 106:541–554.
Microbiotest Inc. 2015. Standard Operational Procedure, Phytotoxkit. Seed germination and early growth microbiotest with higher plants.
Mitsch, W. J., and J. G. Gosselink. 2000. Wetlands. Third. John Wiley & Sons., NY.
Morgan, J. B., and E. L. Connolly. 2013. Plant-Soil Interactions : Nutrient Uptake Aa Plant Acquisition of Nutrients : Direct Uptake from the Soil. Nature Education Knowledge 4(8):1–6.
Morris, S. J., and C. B. Blackwood. 2015. he ecology of the soil biota and their function. Pages 273–309 Soil Microbiology, Ecology and Biochemistry. 4th Edn. E. A. Eldor (Cambridge, MA: Academic Press),.
MP Biomedicals. 2017. Fast DNA TM spin kit.
Müller, A. K., K. Westergaard, S. Christensen, and S. J. Sørensen. 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiology Ecology 36:11–19.
Munns, W. R., A. W. Rea, G. W. Suter, L. Martin, L. Blake-Hedges, T. Crk, C. Davis, G. Ferreira, S. Jordan, M. Mahoney, and M. G. Barron. 2016. Ecosystem services as assessment endpoints for ecological risk assessment. Integrated Environmental Assessment and Management 12:522–528.

Naguib, M., A. Khairalla, A. El-Gendy, and W. Elkhatib. 2019. Isolation and Characterization of mercury-resistant bacteria from wastewater sources in Egypt. Canadian Journal of Microbiology 65:308–321.
Naz, N., H. K. Young, N. Ahmed, and G. M. Gadd. 2005. Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. Applied and Environmental Microbiology 71:4610–4618.
Nie, Y., M. Wang, W. Zhang, Z. Ni, Y. Hashidoko, and W. Shen. 2018. Ammonium nitrogen content is a dominant predictor of bacterial community composition in an acidic forest soil with exogenous nitrogen enrichment. Sci. Total Environ. 624:407–415.
Nies, D. H. 1999. Microbial heavy-metal resistance. Applied Microbiology and Biotechnology 51:730–750.
Nikinmaa, M. 2014. An Introduction to Aquatic Toxicology. Academic Press; 1 edition (July 24, 2014).
NIST. 2013. National Institute of Standards and Technology.
Novotny, V. 2003. Water Quality - Diffuse Pollution and Watershed Management. Second. John Wiley & Sons.
Novotny, V., T. Ehlinger, A. Bartošová, E. Manolakos, and N. O' Reilly. 2005. Developing Risk Propagation Model for Estimating Ecological Responses of Streams to Anthropogenic Watershed Stresses and Stream Modifications. Center for Urban Environmental Studies Publications.
Olson, B. H., and I. Thornton. 1982. The resistance patterns to metals of bacterial population in contaminated land. J. Soil Sci. 33:271–277.
Owens, P. L., R. C. HInkle, and R. G. Best. 1989. Low-Energy Wastewater Recycling Through Wetland Ecosystem: Copper and Zinc in Wetland Microcosm. Page Freshwater wetlands and wildlife. Oak Ridge, Tenesse.
Pal, C., J. Bengtsson-Palme, E. Kristiansson, and D. G. J. Larsson. 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16:1–14.
Pang, J., M. H. Ryan, M. Tibbett, G. R. Cawthray, K. H. M. Siddique, M. D. A. Bolland, M. D. Denton, and H. Lambers. 2010. Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus supply. Plant and Soil 331:241–255.
Pei, Y., Z. Yu, J. Ji, A. Khan, and X. Li. 2018. Microbial community structure and function indicate the severity of chromium contamination of the Yellow River. Frontiers in Microbiology 9:1–12.
Persoone, G., and M. Vangheluwe. 2000. Toxicity determination of the sediments of the river Seine in France by application of a bat- tery of microbiotests. Page (D. C. W. Persoone G, Janssen C, Ed.). New Microbiotests for Routine Toxicity Screening and Biomonitoring, Kluwer Academic, New York.
Pitt, R., and M. Bozeman. 1982. Sources of Urban Runoff Pollution and Its Effects on an Urban Creek. Cincinnati, OH.
Pitt, R., and J. McLean. 1986. Toronto Area Watershed Management Strategy Study. Humber River Pilot Watershed Project. Ontario Ministry of the Environment. Toronto, Ontario.
Pitt, R., and J. Voorhees. 1995. Source loading and management model (SLAMM). Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels March 30 – April 2, 1993.

Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. Ohio.:225-243.
Pitt, R., and J. Voorhees. 2000. The Source Loading and Management Model (SLAMM), A Water Quality Management Planning Model for Urban Stormwater Runoff.
Postma, J., E. H. Nijhuis, and E. Someus. 2010. Selection of phosphorus solubilizing bacteria with biocontrol potential for growth in phosphorus rich animal bone charcoal. Appl. Soil Ecol. 46:464–469.
Prabhakaran, P., Mu. A. Ashraf, and W. S. Aqma. 2015. Microbial Stress Response to Heavy Metal in the Environment. RSC Advances 5:54667.
Ramond, J.-B., P. J. Welz, D. A. Cowan, and S. G. Burton. 2012. Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up. Research in Microbiology 163:28-35.
Ranjard, L., A. Richaume, L. Jocteur-Monrozier, and S. Nazaret. 1997. Response of soil bacteria to Hg(II) in relation to soil characteristics and cell location. FEMS Microbiology Ecology 24:321–331.
Reilly, J. F., A. J. Horne, and C. D. Miller. 2000. Nitrate removal from a drinking water supply with large free-surface constructed wetlands prior to groundwater recharge. Ecol. Eng. 14(1–2):33-47.
Risso, C., J. Sun, K. Zhuang, R. Mahadevan, R. DeBoy, W. Ismail, S. Shrivastava, H. Huot, S. Kothari, S. Daugherty, O. Bui, C. H. Schilling, D. R. Lovley, and B. A. Methé. 2009. Genome-scale comparison and constraint-based metabolic reconstruction of the facultative anaerobic Fe(III)-reducer Rhodoferax ferrireducens. BMC Genomics 10:447.
Roane, T. M., and S. T. Kellogg. 1996. Characterization of bacterial communities in heavy metal contaminated soils. Canadian Journal of Microbiology 42:593–603.
Rüegg, S. R., L. R. Nielsen, S. C. Buttigieg, M. Santa, M. Aragrande, M. Canali, T. Ehlinger, I. Chantziaras, E. Boriani, M. Radeski, M. Bruce, K. Queenan, and B. Häsler. 2018. A systems approach to evaluate One Health initiatives. Frontiers in Veterinary Science 5:1–18.
Ryan, R. P., D. J. Ryan, and D. N. Dowling. 2005. Multiple metal resistant transferable phenotypes in bacteria as indicators of soil contamination with heavy metals. Journal of Soils and Sediments 5:95–100.
S. Sengar, R., S. Gupta, M. Gautam, A. Sharma, and K. Sengar. 2008. Occurrence, Uptake, Accumulation and Physiological Responses of Nickel in Plants and its Effects on Environment. Research Journal of Phytochemistry 2:44–60.
Sabry, S. A., H. A. Ghozlan, and D. M. Abou-Zeid. 1997. Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. Journal of Applied Microbiology 82:245–252.
Sahu, O. 2014. Reduction of Heavy Metals from Waste Water by Wetland. International Letters Of Natural Sciences 12:35–43.
Santo, C. E., P. V. Morais, and G. Grass. 2010. Isolation and characterization of bacteria resistant to metallic copper surfaces. Applied and Environmental Microbiology 76:1341–1348.
SAS. 2019. JMP®, Version <14>.
Scheirs, J., and L. De Bruyn. 2004. Excess of nutrients results in plant stress and decreased grass miner performance. Entomologia Experimentalis et Applicata 113:109–116.
Schloss, P. D. 2009. Introducing mothur: Open-source, platform-independent, community-

supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541.
Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature methods 9:671–675.
Scholz, M., and Å. Hedmark. 2010. Constructed Wetlands Treating Runoff Contaminated with Nutrients. Water, Air, And Soil Pollution 205:323–332.
Schoolmaster, D. R., J. B. Grace, and E. W. Schweiger. 2012. A general theory of multimetric indices and their properties. Methods in Ecology and Evolution 3:773–781.
SETAC. 1997. Ecological risk assessment. Technical issue paper:4.
SEWRPC. 2010. Racine County Mapbook.
Shange, R., E. Haugabrooks, R. Ankumah, A. Ibekwe, R. Smith, and S. Dowd. 2013. Assessing the Diversity and Composition of Bacterial Communities across a Wetland, Transition, Upland Gradient in Macon County Alabama. Diversity 5:461–478.
Shannon, R. D., O. P. Flite, and M. S. Hunter. 2000. Subsurface flow constructed wetland performance at a Pennsylvania campground and conference center. J. Environ. Qual. 29:2029-2036.
Shukla, D., C. A. Rinehart, and S. V. Sahi. 2017. Comprehensive study of excess phosphate response reveals ethylene mediated signaling that negatively regulates plant growth and development. Scientific Reports 7:1–16.
Sims, A., Y. Zhang, S. Gajaraj, P. Brown, and Z. Hu. 2013. Toward the development of microbial indicators for wetland assessment. Water Research 47:1711–1725.
Sinicorpe, T. L., R. Langis, R. M. Gersberg, M. J. Busnardo, and J. B. Zedler. 1992. Metal removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:309–322.
Smith, M. S., and K. Zimmerman. 1981. Nitrous Oxide Production by Nondenitrifying Soil Nitrate Reducers1. Soil Science Society of America Journal 45:865.
Sobolev, D., and M. Begonia. 2008. Effects of heavy metal contamination upon soil microbes: Lead-induced changes in general and Denitrifying microbial communities as evidenced by molecular markers. International Journal of Environmental Research and Public Health. 5:450–456.
Sokal, R., and J. Rohlf. 1981. Biometry. Second. W.H. Freeman and Company, San Fransisco.
Soon, Y. K., B. T.E., and J. R. Moyer. 1980. Land application of chemically treated sewage sludge III. Effects on soil and plant heavy metal content. J. Environ. Qual. 9:497–504.
Stoeva, M. K., S. Aris-Brosou, J. Chételat, H. Hintelmann, P. Pelletier, and A. J. Poulain. 2014. Microbial community structure in lake and Wetland sediments from a high arctic polar desert revealed by targeted Transcriptomics. PLoS ONE 9:e89531.
Storer, D. A. 1984. A simple high sample volume ashing procedure for determination of soil organic matter. Communications in Soil Science and Plant Analysis 15:759–772.
Suter, G. . 2001. Applicability of indicator monitoring to ecological risk assessment. Ecological Indicators 1:101–112.
Suter, G. I. 2006. Ecological Risk Assessment. CRC Press.
Szoboszlay, M., A. Dohrmann, C. Poeplau, A. Don, and C. Tebbe. 2017. Impact of land use change and soil organic carbon quality on microbial diversity in soils across Europe. FEMS Microbiol Ecol.
Tajini, F., M. Trabelsi, and J. J. Drevon. 2012. Combined inoculation with Glomus intraradices and Rhizobium tropici CIAT899 increases phosphorus use efficiency for symbiotic nitrogen

fixation in common bean (Phaseolus vulgaris L.). Saudi J. Biol. Sci. 19:157–163.
Tallis, H., P. Kareiva, M. Marvier, A. Chang, and A. H. Mwinyi. 2008. Practical Conservation and Economic Development. Proceedings of the National Academy of Sciences 105:9457–9464.
Thomas, P. R., P. Glover, and T. Kalaroopan. 1995. An evaluation of pollutant removal from secondary treated sewage effluent using a constructed wetland system. Water Sci. Tech. 32:87-93.
Tipayno, S. C., J. Truu, S. Samaddar, M. Truu, J. K. Preem, K. Oopkaup, M. Espenberg, P. Chatterjee, Y. Kang, K. Kim, and T. Sa. 2018. The bacterial community structure and functional profile in the heavy metal contaminated paddy soils, surrounding a nonferrous smelter in South Korea. Ecology and Evolution 8:6157–6168.
Tixier, G., Q. Rochfort, L. Grapentine, J. Marsalek, and M. Lafont. 2012. Spatial and seasonal toxicity in a stormwater management facility: Evidence obtained by adapting an integrated sediment quality assessment approach. Water Research 46:6671–6682.
Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol 5:240–245.
Trajanovska, S., M. L. Britz, and M. Bhave. 1997. Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead contaminated site. Biodegradation 8:113–124.
Tremblay, J., K. Singh, A. Fern, E. S. Kirton, S. He, T. Woyke, J. Lee, F. Chen, J. L. Dangl, and S. G. Tringe. 2015. Primer and platform effects on 16S rRNA tag sequencing. Frontiers in Microbiology 6:1–15.
Umar, S., N. Gauba, N. A. Anjum, and T. O. Siddiqi. 2013. Arsenic toxicity in garden cress (Lepidium sativum Linn.): Significance of potassium nutrition. Environmental Science and Pollution Research 20:6039–6049.
USEPA. 2016. The Sources and Solutions: Agriculture (online) https://www.epa.gov/nutrientpollution/sources-and-solutions-agriculture:2017–2019.
USGS, U. S. G. S. 2017. Nitrogen and Water. U.S. Department of the Interior.
Vamerali, T., M. Bandiera, and G. Mosca. 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environmental Chemistry Letters 8:1–17.
Vasileiadis, S., E. Puglisi, M. Trevisan, K. G. Scheckel, K. A. Langdon, M. J. McLaughlin, E. Lombi, and E. Donner. 2015. Changes in soil bacterial communities and diversity in response to long-term silver exposure. FEMS Microbiology Ecology 91:1–11.
Vymazal, J. 2007. Removal of nutrients in various types of constructed wetlands. Science of the Total Environment 380:48–65.
Waite, I. R. 2014. Agricultural disturbance response models for invertebrate and algal metrics from streams at two spatial scales within the U.S. Citation metadata. Hydrobiologia 726:285–303.
Walker, C. W., and R. D. Shannon. 2006. Nitrate and phosphate removal effects of compost amendments in wetland mesocosms. American Society of Agricultural and Biological Engineers 49:1773–1778.
Wan, D., Z. Han, J. Yang, G. Yang, and X. Liu. 2016. Heavy metal pollution in settled dust associated with different urban functional areas in a heavily air-polluted city in North China. International Journal of Environmental Research and Public Health 13.
Wang, H., C. W. Marshall, M. Cheng, H. Xu, L. Hu, X. Yang, and T. Zheng. 2017. Changes in land use driven by urbanization impact nitrogen cycling and the microbial community

composition in soils. Scientific Reports 7.
Wang, L., and J. Lyons. 2003. Wang2003.pdf. Page 227 Biological Response Signatures: Indiactor Patterns Using Aquatic Communities. CRC Press.
Wang, L., J. Lyons, and P. Kanehl. 2001. Impacts of Urbanization on Stream Habitat and Fish across Multiple Spatial Scales. Environmental Management. Environmental Management 28:255–266.
Wang, N. F., T. Zhang, X. Yang, S. Wang, Y. Yu, L. L. Dong, Y. D. Guo, Y. X. Ma, and J. Ye Zang. 2016. Diversity and composition of bacterial community in soils and lake sediments from an arctic lake area. Frontiers in Microbiology 7:1–9.
Wang, Q., C. Wang, W. W. Yu, A. Turak, D. Chen, Y. Huang, J. Ao, Y. Jiang, and Z. Huang. 2018. Effects of nitrogen and phosphorus inputs on soil bacterial abundance, diversity, and community composition in chinese fir plantations. Frontiers in Microbiology 9:1–10.
Wang, Y., H. F. Sheng, Y. He, J. Y. Wu, Y. X. Jiang, N. F. Y. Tam, and H. W. Zhou. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. Applied and Environmental Microbiology 78:8264–8271.
Ward, M. H. 2009. Too much of a good thing? Nitrate from nitrogen fertilizers and cancer. Reviews on Environmental Health 24:357–363.
Webb, J. M.-S. 1998. Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. Journal of Applied Microbiology 84:240-248.
Wilson, M. A., and S. R. Carpenter. 1999. Economic valuation of freshwater ecosystem services in the United States: 1971-1997. Ecological Applications 9:772–783.
Wohl, D. L., S. Arora, and R. Gladstone. 2004. Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. Ecology 85:1534–1540.
Xiao, H., S. Zhang, J. Zhai, Q. He, A. Mels, K. Ning, and J. Liu. 2013. Retention and distribution of Cu, Pb, Cr, and Zn in a full-scale hybrid constructed wetland receiving municipal sewage. Water Science and Technology 67:2257–2264.
Xie, Y., J. Fan, W. Zhu, E. Amombo, Y. Lou, L. Chen, and J. Fu. 2016. Effect of heavy metals pollution on soil microbial diversity and Bermudagrass genetic variation. Frontiers in Plant Science 7.
Yazdankhah, S., E. Skjerve, and Y. Wasteson. 2018. Antimicrobial resistance due to the content of potentially toxic metals in soil and fertilizing products. Microbial Ecology in Health and Disease 29:1548248.
Yoder, C., and E. Rankin. 1995a. Biological response signatures and the area degradation value:new tools for interpreting multi-metric data, in W.S. Davis and T.P. Simons(Eds). Pages 263–286 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
Yoder, C., and E. Rankin. 1995b. BIological criteria program development and implementation in Ohio in W.S. Ohio and T.P.Simon (Eds.). Pages 109–144 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
YSI. 2020. YSI a xylem brand.
Yu, J., X. Lv, B. Ma, F. Qu, K. Ning, and H. Wu. 2014. A meta-analysis of the bacterial and archaeal diversity observed in wetland soils. Salt Marshes: Ecosystem, Vegetation and Restoration Strategies 2014:13–32.
Yu, Y., H. Wang, J. Liu, Q. Wang, T. Shen, W. Guo, and R. Wang. 2012. Shifts in microbial

community function and structure along the successional gradient of coastal wetlands in Yellow River Estuary. European Journal of Soil Biology 49:12–21.
Zancheta, A. C. F., C. A. De Abreu, F. C. B. Zambrosi, N. de M. Erismann, and A. M. M. A. Lagôa. 2015. Cadmium Accumulation by Jack-Bean and Sorghum in Hydroponic Culture. International Journal of Phytoremediation 17:298–303.
Zhang, D., J. Luo, Z. Lee, Y. Maspolim, R. Gersberg, and Y. Liu. 2016. Characterization of bacterial communities in wetland mesocosms receiving pharmaceutical-enriched wastewater. Ecological Engineering 90:215–224.
Zhang, M. J., F. K. Huang, G. Y. Wang, X. Y. Liu, J. K. Wen, X. S. Zhang, Y. S. Huang, and Y. Xia. 2017. Geographic distribution of cadmium and its interaction with the microbial community in the Longjiang River: Risk evaluation after a shocking pollution accident. Scientific Reports 7:1–12.
Zhang, W. hui, Z. Huang, L. yan He, and X. fang Sheng. 2012. Assessment of bacterial communities and characterization of lead-resistant bacteria in the rhizosphere soils of metal-tolerant Chenopodium ambrosioides grown on lead-zinc mine tailings. Chemosphere 87:1171–1178.
Zhao, J., X. Zhao, L. Chao, W. Zhang, T. You, and J. Zhang. 2014a. Diversity change of microbial communities responding to zinc and arsenic pollution in a river of northeastern china. Journal of Zhejiang University SCIENCE B 15:670–680.
Zhao, K., P. Penttinen, X. Zhang, X. Ao, M. Liu, X. Yu, and Q. Chen. 2014b. Maize rhizosphere in Sichuan, China, hosts plant growth promoting Burkholderia cepacia with phosphate solubilizing and antifungal abilities. Microbiological Research 169:76–82.
Zhawar, V. K., N. Kaur, and A. K. Gupta. 2014. Effect of increasing concentration of inorganic phosphate (Pi) and pyrophosphate (PPi) on growth and Phosphatases of germinating chickpea seeds. Journal of Plant Nutrition 37:2195–2207.
Zhu, F., L. Qu, X. Hong, and X. Sun. 2011. Isolation and characterization of a phosphate-solubilizing halophilic bacterium Kushneria sp. YCWA18 from Daqiao saltern on the coast of yellow sea of China. Evidence-based Complementary and Alternative Medicine 2011.
Zięba-Palus, J., Kunicki, M. 2006. Application of the micro-fTIR spectroscopy, Raman spectroscopy and XRF method examination of inks. Forensic Science International 158:164–172.

Chapter 3: Responses of biological indicators to pollutant loading in experimental wetland microcosms

Introduction:

Increasing urbanization in the United States has contributed to to higher runoff rates into surface waters, generating more pollutant loadings for nutrients and heavy metals carried in the in the stormwater runoff (EPA 2003, Wang and Lyons 2003). This in turn has strong negative impacts affecting the ecological integrity of aquatic ecosystems (Foley et al. 2005) (Figure 3.1). Public awareness of the severe declines in water quality across the United States contributed the passing of the US Clean Water Act (CWA) in 1972, which established quality standards and anti-degradation plans to limit discharge of pollution into surface waters (Carey and Hochmuth 2012, EPA 2012).

The field of ecological risk assessment arose to meet the goals of Clean Water Act (Wilson and Carpenter 1999, Tallis et al. 2008, Munns et al. 2016) as a methodology for making decisions in the face of incomplete or imperfect information regarding compels risk factors (Suter 2006) such as pollutant stress propagating through environment (Figure 3.1) (Foley et al. 2005).

A triad approach (Dagnino et al. 2008) using an integrated multimetric index of chemical risk, ecotoxicological risk and ecological risk have been applied to assess ecological risk. For metrics of a multimetric index bioindicators from several trophic levels should be incorporated to detect signals from anthropogenic disturbances (Decker et al. 2017), to make the ecological risk assessment more robust. In this regard, Yoder and Rankin (Yoder and Rankin 1995) used the term “biological response signatures” to characterize the various ways that the individual metrics of multimetric indicators may respond to different kinds of environmental stressors.

Rather than establishing distinct cause-effect relationships between a pollutant and a response metric, the objective of this research area is to identify signals provided by responses within the complex noise of multiple interacting pollutants (Clapcott et al. 2012, 2014).

Biomonitoring has been defined as the process of measuring and evaluating the conditions of a living system using bioindicators, so that a dynamic picture of environmental conditions can be portrayed (Karr and Chu 1999). In wetland, the goal is to identify bioindicators for pollutants like heavy metals and nutrients (Chapman 1995) that can be used for risks assessment to monitor for ecological changes in watersheds (Ke et al. 2015, Aylagas et al. 2017). To this end, ecotoxicological assays have been used extensively as bioindicators to assess the ecological status of aquatic sediments (Jensen 2011, Dellinger et al. 2014). The Phytotox™ assays *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum*, (Microbiotest Inc 2015) were chosen in this study to undf nutrients and metals loading from surrounding land uses (Czerniawska-Kusza et al. 2006, Czerniawska-Kusza, I., & Kusza 2010). Potential bacterial indicators including taxonomical diversity and community structure were identified in the previous chapters of this dissertation, and were included in this study.

Over the years many studies have shown how changing land use along with associated pollutant input of pollutants affect soil bacterial communities (Yu et al. 2012, Ding et al. 2013, Szoboszlay et al. 2017, Wang et al. 2017). Interest in identifying indicator species from wetland bacterial communities in the natural environment is high (Nikinmaa 2014) because their role in maintaining ecosystem stability and resilience after contamination, nutrient cycling efficiencies, and biodiversity sustainability (Torsvik and Ovreas 2002, Wohl et al. 2004).

In this regard, microcosm-scale experimental wetlands are useful tools for the study of wetland ecosystems and have been widely used to examine the fate and transport of pollutants

(Zhang et al. 2016, Messer et al. 2017). Experimental microcosms allow controlled treatment manipulations that are not possible in natural systems (Ramond et al. 2012) and allows for the manipulation of pollutant types (e.g. nutrient and metals) and loading rates. Numerous studies have demonstrated that sediment microbiology influences the reduction in the levels metals and nutrients in wetlands (Webb 1998, Vymazal 2007).

This chapter used controlled microcosms consisting of sediments collected from wetlands across a gradient of land use types in an urbanizing watershed to characterize how bacterial and ecotoxicological bioindicators respond to various levels of pollution stress. The questions asked include:

1. Do the responses of sediment ecotoxicological bioindicators (*Lepidium sativum, Sinapis alba and Sorghum saccharatum)* and sediment bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) correlate with manipulated changes in the concentration of nutrients (nitrate, phosphate) and metals (Cu, Pb) added to the water of experimental microcosms?
2. Are there specific assemblages of identified bacterial taxa that can potentially serve as indicators of the induced pollution to the water of experimental microcosms and used as *ex ante* impact indicators for ecological risk assessment?

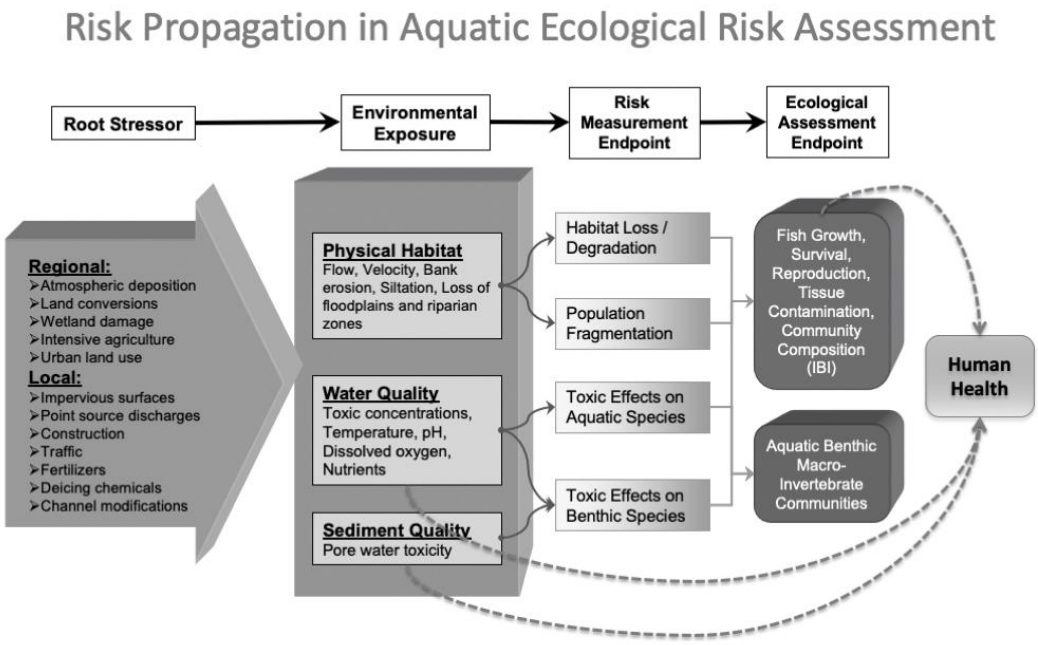


Figure 3.1: Risk propagation model for watershed-based aquatic ecological risk assessment. Root stressors act on a global, regional or local scale. Drivers of change create exposures to risk facors. Risk Probabilities are associated with exposures. Impact Endpoints reflect measures of system-related goods and services of value to society (Rüegg et al. 2018) modified from (Novotny et al. 2005).

Methods:

Sediment samples were collected from four wetland sites in the Pike River watershed during summer 2017 for the microcosm study, two sites from sub-watersheds watersheds dominated by agricultural land use (Wetlands 1 and 2) and two sites from sub-watersheds draining lands dominated by commercial & industrial land uses (See Chapter 1, Figure 1.2 and 1.3). An Ekman dredge grab sampler (15 x 15 x 25 cm) was used to collect six samples per site and at two separate zones (three samples from each zone) of each wetland site. The samples were homogenized by zone, yielding two samples per wetland site. The sediment samples were collected and stored in 4.73-liter plastic containers, transported to the laboratory and stored in a cold room at 0-2$_0$ C.

Microcosms were constructed from 4.73-liter plastic containers (22 cm diameter and 18 cm height). Sediments were placed into the microcosms to a level of 5 cm from the bottom of the microcosm with the water added to a level rising 10 cm from the bottom (Busnardo et al. 1992, Ahn et al. 2007). The volumes of the added sediments (sample and control) in the microcosms were approximately 1900 cm$_3$, with the total volume (water and sediment) approximately 3800 cm$_3$. Microcosms were placed in a controlled growth chamber with a temperature between 25$_0$C and 30$_0$C.

Two different series of microcosm experiments were conducted (Figure 3.2), one examining effects of metal pollutants (Figure 3.2: Metal Treatments) and the other examining effects of nutrient pollutants (Figure 3.2: Nutrient Treatments). Both treatment types followed similar designs, with nutrients or metals added on day 0, followed by sequential 7-day cycles between water changes where pollutants were re-entered into the system by changing the water in the microcosms.

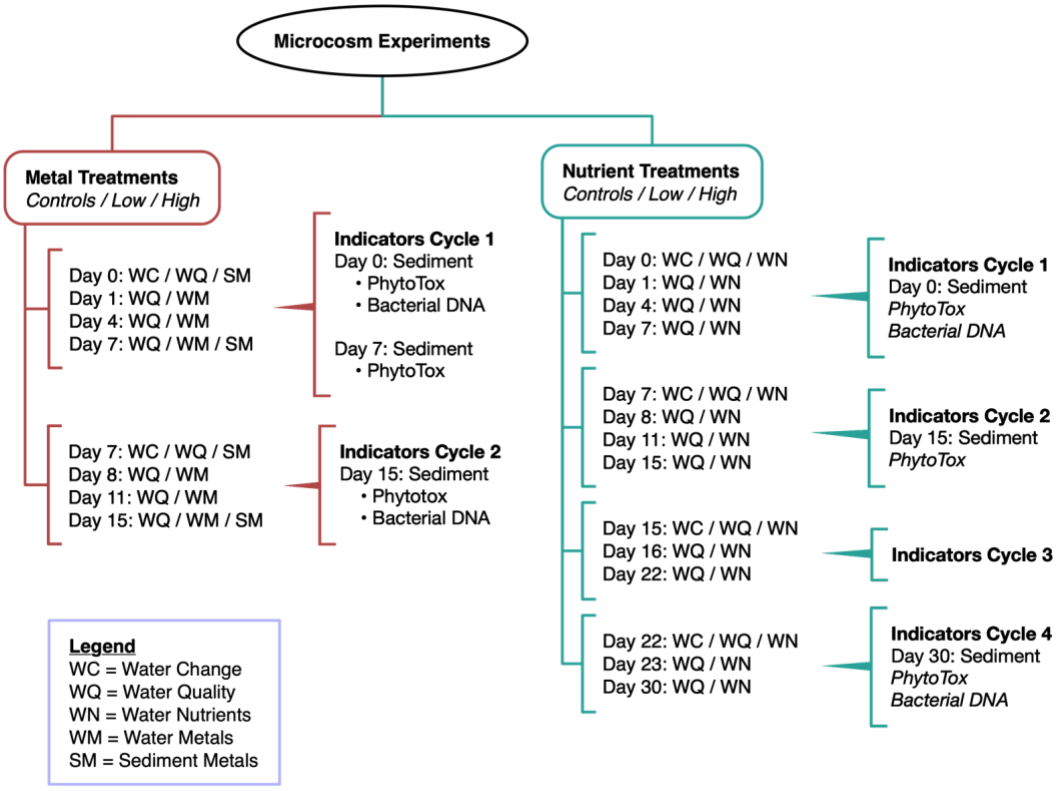


Figure 3.2: Experimental design and sampling schema for wetland sediments microcosm experiments that manipulated loadings of pollutant metals (metal treatments) and pollutant nutrients (nutrient treatments). Experiments in the Metal Treatments ran for 15 days with 2 seven-day cycles. Experiments in the Nutrient Treatments ran for 30 days with 4 seven-day cycles.

Table 3.1: Metal and nutrient microcosm experiment design with sediment treatment type for each wetland (1,2, 5 and 6) in native and autoclaved sediments. Experiments were conducted for 15 days utilizing sediments collected from wetlands 1, 2, 5, and 6.

(A) Metal microcosm					
		Sediment Treatment Type			
		Native sediment		Autoclaved sediment	
Metal Added to Water	**Control**	**low concentration**	**high concentration**	**low concentration**	**high concentration**
Cu as CuSO4	0 mg/L	0.05 mg/L	0.15 mg/L	0.05 mg/L	0.15 mg/L:
Pb as PbNO3	0 mg/L	0.1 mg/L	0.3 mg/L	0.1 mg/L	0.3 mg/L
(B) Nutrient microcosm					
		Sediment Treatment Type			
		Native sediment		Autoclaved sediment	
Nutrient Added to Water	**Control**	**low concentration**	**high concentration**	**low concentration**	**high concentration**
Nitrate as KNO3	0 mg/L	5.0 mg/L	15.0 mg/L	5.0 mg/L	15.0 mg/L:
Phosphate as Na2HPO4	0 mg/L	1.0 mg/L	3.0 mg/L	1.0 mg/L	3.0 mg/L

Metal microcosm treatments:

In the metal microcosm treatments, metals in the form of lead ($PbNO_3$) and Copper ($CuSO_4$) were dissolved in RO water to produce the desired concentrations (Table 3.1) and poured over the sediments (Owens et al. 1989, Busnardo et al. 1992, Sinicorpe et al. 1992, Gikas et al. 2013, Elder 2016). The compounds and the concentrations of Pb and Cu selected were adapted from the published literature (Busnardo et al. 1992, Sinicorpe et al. 1992, Ahn et al. 2007, Behrends 2007, Messer et al. 2017)

There were two levels of treatments: low-levels (Pb and Cu applied at 0.05 and 0.1 mg/L) respectively) and high-levels (Pb and Cu applied at 0.15 and 0.3 mg/L) respectively) in combination with controls without any added metals (Table 3.1). For each wetland site in this experiment, one control consisted of no metals added in the microcosm containing native sediment from the wetland. A second control consisted of no metals added to wetland sediments that had been autoclaved for sterilization at 121^0C for 30 minutes and at 15 psi (Table 3.1). This procedure was repeated for sediments from each of the 4 wetland sites (1, 2, 5 and 6) from watersheds with different land uses.

In week 1, at day 0 the metals (Pb and Cu) were added as per the treatments in low or high concentration or none for the control (Figure 3.2, Table 3.1). Following the addition of pollutants at day 0, measurements were taken for Pb and Cu concentration in water and water quality characters (pH, temperature, dissolved oxygen, specific conductance, and turbidity) were taken. On days 1, 4, 7, 8, 11 and 15 for Pb and Cu concentration in water and water quality characters were measured as well. At day 7 in the microcosm water was decanted down to the level of the sediment layer and replaced with water containing the same initial treatment concentrations of the pollutants (WC in Figure 3.2, Table 3.1). The retention time of 7 days was

selected based on the commonly recommended design for retention time of water in artificial wetlands (marshes and ponds) wastewater treatment systems as 4-12 days suggested by EPA (EPA 1985) .

Sediment samples were collected from the microcosms (control and experimental) on days 0, 7 and 15 (end of cycles 1 and 2), to identify the response of the predictive ecotoxicological indicators of the induced pollutant (metal) loadings (Figure 3.2). In addition, sediment samples collected on day 0 and 15 were used to identify the response of the bacterial DNA indicators (bacterial community structure and diversity of phyla and genera) to the induced pollutant (metal) loadings (Figure 3.2).

Within the metal microcosm treatments, concentrations of Pb and Cu in water (WM in Figure 3.2) were measured using ICP-MS (Inductively coupled plasma mass spectrophotometry) technology. Analysis was conducted in laboratories at the UW-Milwaukee School of Freshwater Sciences utilizing a Thermo Scientific Element 2 High Resolution Sector field ICP-MS following methods of Krachler et al. (Krachler et al. 2016).

Concentrations of metals in the sediments of each microcosm (SM in Figure 3.2) were analyzed using XRF following the detailed methods presented in chapter 1 of this dissertation (Baranowski et al. 2002, Zięba-Palus, J., Kunicki 2006, EPA 2007, Kenna et al. 2011, Díaz Rizo et al. 2014, DiScenza and Keimowitz 2014, Bruker 2017). Water quality parameters (WQ in Figure 3.2, including pH, temperature, dissolved oxygen, specific conductance, and turbidity) were measured using YSI 6600EDS™ multi parameter sondes (YSI 2020) .

Sediment ecotoxicological bioindicators were assessed using Phytotoxkit™ (Microbiotest Inc 2015) standard operational procedures following the methods details in chapter 1 of this

dissertation (Ghosh Roy 2020). The indicator plants included - *Sorghum saccharatum*, *Lepidium sativum and Sinapis alba*.

Bacterial DNA was extracted using DNA$_{TM}$ spin kit for soil (Li et al. 2011, Burbach et al. 2016, MP Biomedicals 2017) following the standard protocol provided from the instruction manual. The detailed methods is explained in chapter 2 of this dissertation (Ghosh Roy 2020).

Nutrient microcosms:

In the nutrient microcosm treatments, nutrients in the form of phosphate (Na_2HPO_4) and nitrate (KNO_3) were dissolved in RO water to produce the desired concentrations (Table 3.1) and poured over the sediments (Busnardo et al. 1992, Sinicorpe et al. 1992, Ahn et al. 2007, Behrends 2007, Messer et al. 2017). There were two levels of treatments: low-levels (nitrate was applied at 5 mg/L and for phosphate at 1 mg/L) and high-levels (nitrate was applied at 15 mg/L and for phosphate at 3 mg/L) in combination with controls without any added nutrients (Table 3.1). For each wetland site in this experiment, one control consisted of no nutrients added in the microcosm containing native sediment from the wetland. A second control consisted of n nutrients added to wetland sediments that had been autoclaved for sterilization at 121^0C for 30 minutes and at 15 psi (Table 3.1).

Following the addition of nutrients (day 0), measurements were taken in the microcosms for nitrate and phosphate concentration in water and water quality characters, which were then repeated on days 1, 4, 7, 8, 11,15,16, 22, 23 and 30. Experimental water treatments for each microcosm were recharged each week (days 7, 15 and 22), by decanting water down to the level of the sediment layer and replacing with water containing the initial treatment concentrations of the pollutants (Table 3.1). The retention time of 7 days was selected based on the commonly

recommended design for retention time of water in artificial wetlands (marshes and ponds) wastewater treatment systems as 4-12 days suggested by EPA (EPA 1985).

At day 0, day 15 and day 30 (end of cycle 4), sediment samples were collected from the microcosms (control and experimental) to identify the response of the predictive indicators (eco-toxicological) to the induced pollutant (nutrient) loadings using the same methods as described above for the metal microcosms. At day 0 and day 30 (end of cycle 4), sediment samples were collected from the microcosms (control and experimental) to identify the response of the predictive indicators (bacterial community structure and diversity of phyla and genera) to the induced pollutant (nutrient) loadings using the same methods as described above for the metal microcosms.

Nitrate and phosphate were analyzed with HACH DR 2800TM spectrophotometer using powder pillow test kit – Cadmium reduction method for nitrate (in mg/L) and powder pillow test kit –Ascorbic acid method for phosphate (in mg/L) as described in details in chapter 1.

Sediment ecotoxicological bioindicators were assessed using PhytotoxkitTM (Microbiotest Inc 2015) as described in the previous section of metal microcosm and chapter 1 of this dissertation. Bacterial DNA was extracted using DNATM spin kit for soil (Li et al. 2011, Burbach et al. 2016, MP Biomedicals 2017) as described in the previous section of metal microcosm and chapter 2 of this dissertation.

Distributional properties were examined for all data collected from the microcosm experiments. When appropriate, concentrations and count data were log10 transformed and proportional data were arcsin transformed to address deviations from normality (Sokal and Rohlf 1981) prior to analysis. All statistical analyses were conducted using JMP™ Software Version 14.0 (SAS Institute 2020)

Data Analyses

The effect of time in each experiment as examined by comparing between measurements taken at the start, middle and end of each treatment using multifactor Analysis of Variance (ANOVA). Specific contrasts were made for changes in pollutant concentrations (metals or nutrients) among treatment levels and experimental timeline for water nutrients (WN), water metals (WM) and sediment metals (SM) (Figure 3.2) . Metal and nutrient concentrations were entered as dependent response variables with treatment level and experimental timeline as independent variables.

The effects of treatment level and experimental timeline on ecotoxicological bioindicators (growth inhibitions of *Sorghum saccharatum, Lepidium sativum and Sinapis alba*) and bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) were examined using multifactor analysis of variance (ANOVA), The indicator variables were entered as dependent response variables with treatment level and experimental timeline as independent variables. An additional analysis was conducted to examine the effect of autoclaving on sediments on the ecotoxicological and bacterial community indicators.

Forward stepping multiple regression was used to determine best fit models for the predictive linear relationships between concentrations of pollutants entered into the microcosms (metals or nutrients) and the measured ecotoxicological bioindicators *(*growth inhibitions of *Sorghum saccharatum, Lepidium sativum and Sinapis alba)* and bacterial community indicators (Shannon and Simpson diversity indices of phyla and genera).

Taxonomical profile of the sediment bacterial communities determined from sequencing of the 16S bacterial rRNA (v3-v4 region), identifying to the phylum and genus levels (Figure 3.2). Hierarchical cluster analysis was performed with all these identified communities in each

wetland site of each type of microcosm (metal and nutrient). Based upon visual examination of changes in clusters over the course of the experiments relative to their response to the pollutants added to the metal and nutrient microcosm treatments, bacterial genera were identified and categorized into specific categories based on tolerance level with in relation to added metal and nutrient to the microcosms .

Specific predictions

1. There will be reduction pollutant (Pb, Cu, nitrate and phosphate) in the water of metal and nutrient microcosms from beginning to the end of the experiments of microcosms.
2. There will be reduction in the growth inhibition responses of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* (ecotoxicological bioindicators) grown in the sediments of the metal and nutrient microcosms from beginning to the end of the experiment in metal and nutrient microcosms as the pollutants (Pb, Cu, nitrate and phosphate) are also reduced.
3. There will be increase in the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) from beginning to the end of the experiment metal and nutrient microcosms as the pollutants (Pb, Cu, nitrate and phosphate) are also reduced.
4. With the increase in the detected sediment metal concentration (in ppm) from beginning to the end the growth inhibition responses of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* (ecotoxicological bioindicators) will increase and bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) will decrease in the metal microcosm experiment.
5. The bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) and the pollutant concentration in mg/L (Pb, Cu, nitrate and phosphate) will be lower in the autoclaved sediments of the metal and nutrient microcosms but the growth inhibition

responses of the ecotoxicological bioindicators (*Lepidium sativum, Sinapis alba and Sorghum saccharatum)* will be higher.

6. There will be specific bacterial genera present in the sediments of the metal and nutrient microcosms which will be predictive indicators to the added pollutants (Pb, Cu, Nitrate and Phosphate) added in mg/L to the water of metal and nutrient microcosms.

Results:

Time and Treatment Effects on Metals and Nutrients

The distributions of measured concentrations of metals in the water and sediments, and nutrients in water are shown Appendix 3.7 and 3.8. Concentrations of Pb and Cu decreased from start to the end of the metal microcosm experiment, both in high and low level of treatment (Figure 3.1). Multifactor analysis of variance (ANOVA) indicated. The experimental timeline effect for the reduction of Pb concentration was significant ($P<.0001$) but not for Cu ($P= 0.3186$) (Figure 3.1). The effect of treatment level was not significant for both Pb ($P=0.6082$) or Cu ($P=0.5145$).

Concentration of nitrate and phosphate decreased from start to the end of the nutrient microcosm experiments, both in high and low level of treatment (Figure 3.3). Multifactor analysis of variance (ANOVA) indicated that the experimental timeline effect or the reduction of nutrient concentration from start end was statistically significant for nitrate ($P = 0.0036$) and phosphate ($P<.0001$) (Figure 3.3). The effect of treatment level was not statistically significant for nitrate ($P=0.9455$) or phosphate ($P=0.0890$) (Figure 3.3).

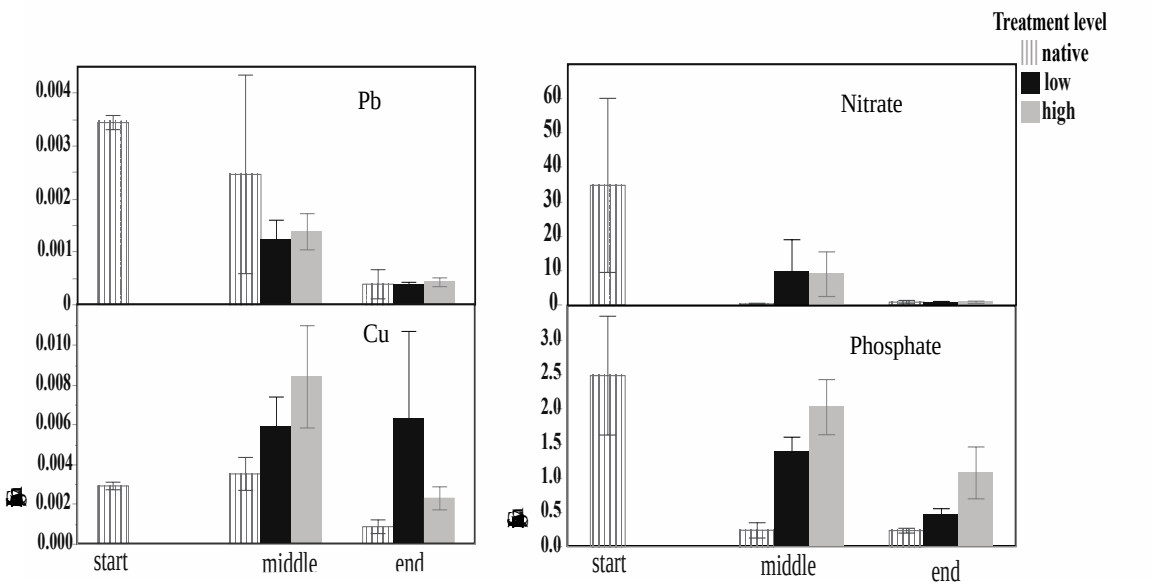


Figure 3.3: The concentration of Pb, Cu in mg/L in metal microcosm and nitrate and phosphate in mg/L in nutrient microcosm experiment during the start, middle and end of the microcosm experiment. This test included a native(control) treatment where no metals or nutrients were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native sediment. Bars show mean ± 1 SE. Multifactor ANOVA tables are located in Appendix 3.1.

In the Metals microcosms, the concentration of As decreased but Pb increased in the sediments from start to the end of the metal microcosm experiment (Figure 3.4). None of the other metals (Ag, Cd, Fe, Hg, Ni, Rb, Zn) showed any significant changes (Figure 3.4). Multifactor analysis of variance (ANOVA) showed that neither the effect of treatment level or experimental timeline was statistically significant on any of the detected metals. The experimental timeline effect was only significant for As ($P=0.0223$) (Figure 3.4).

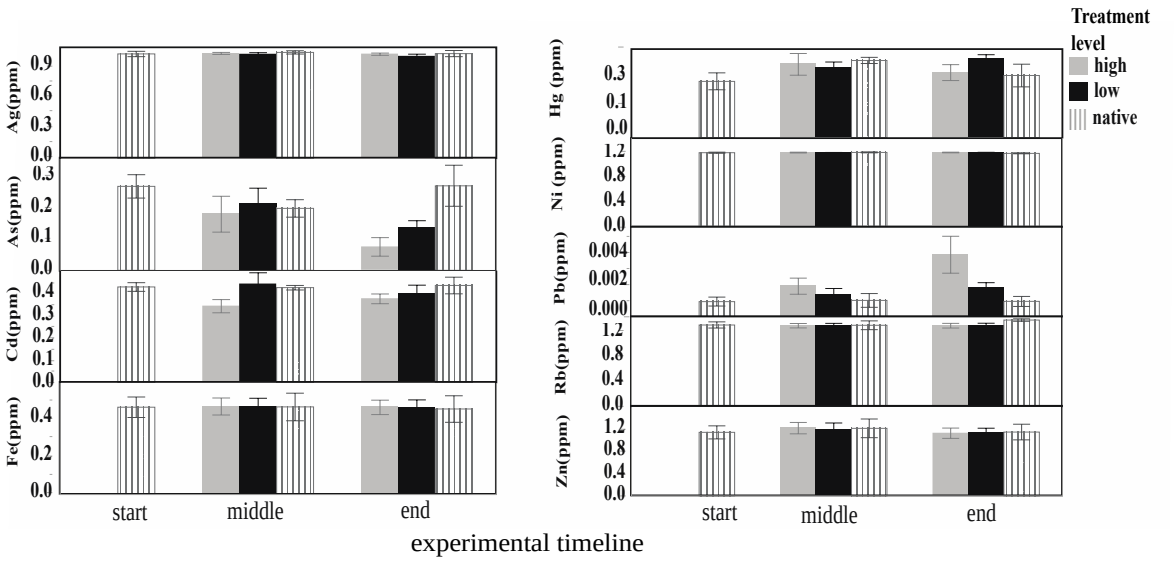


Figure 3.4: The concentration (in ppm) of detected sediment metals in metal microcosm during the start, middle and end of the microcosm experiments. This test included a native(control) treatment where no metal or nutrient were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native sediment. Bars show mean ± 1 SE. Multifactor ANOVA tables are located in Appendix 3.1.

Time and Treatment Effects on ecotoxicological Indicators

In metal microcosms, root inhibition of *Lepidium sativum* ranged from -0.41 (facilitation) to +0.62 (inhibition). *Sinapis alba* ranged from – 0.39 (facilitation) to +0.65 (inhibition). In *Sorghum saccharatum,* the root inhibition ranged from -0.35 (facilitation) to +0.56. The stem inhibition of *Lepidium sativum* ranged from -0.26 (facilitation) to +0.51, in *Sinapis alba* ranged from –0.44 (facilitation) to +0.61. In *Sorghum saccharatum,* the root inhibition ranged from -0.65 (facilitation) to +0.91. In nutrient microcosm, the root inhibition of *Lepidium sativum* ranged from -1.1 (facilitation) to +0.61, in *Sinapis alba* ranged from – 1.22 (facilitation) to +0.60. In *Sorghum saccharatum,* the root inhibition ranged from -0.49 (facilitation) to +0.56. The stem inhibition of *Lepidium sativum* ranged from -0.60 (facilitation) to +0.43, in *Sinapis alba* ranged from –0.49 (facilitation) to +0.51. In *Sorghum saccharatum*, the root inhibition ranged from -1.2 (facilitation) to +0.68.

The root inhibition of ecotoxicological indicators from microcosms across all wetland sediments were lower both in low and high treatment for the metals microcosms at the end of the experiment (day 15) compared to the day 0 or start (Figure 3.5). In the native (control) sediment, there was a clear trend of increase in the root inhibition of *Lepidium sativum* and *Sinapis alba* but decreased in *Sorghum saccharatum* from start to the end of the experiment compared to the treatment microcosms (Figure 3.5). Multifactor analysis of variance (ANOVA) showed that the effect of experimental timeline was statistically significant only on root growth inhibition of *Sorghum saccharatum* (P=0.0153). The effect of treatment level (high and low) was statistically significant only on root growth inhibition of *Sinapis alba* (P= 0.0487) (Figure 3.5).

Stem inhibition of *Lepidium sativum* and *Sorghum saccharatum* from microcosms of wetland sediments were mostly lower in low and high treatments for the metals microcosms treatments by the end of the experiment (day 15) compared to the start (day 0). Although the

stem inhibitions of *Sinapis alba* was highly variable and inhibition was increased from start to the end of the experiment in both high and low treatment level (Figure 3.5). In the native(control) sediment, there was a clear trend of decrease in the stem inhibition of *Lepidium sativum* and *Sorghum saccharatum* but increased in *Sinapis alba* from start to the end of the experiment compared to the treatment microcosms (Figure 3.5). However, multifactor analysis of variance (ANOVA) showed that the effect of experimental timeline and treatment level (high and low) on the stem growth inhibition of ecotoxicological indicators were not statistically significant (Figure 3.5).

In *Sinapis alba*, the root inhibition from microcosms of wetland sediments were increased at high treatment (of nitrate and phosphate applied in water in mg/L) at the end of the experiment (day 30) compared to the start or day 0 but were decreased in the lower treatment level (of nitrate and phosphate applied in water) at the end of the experiment (day 30) compared to the start or day 0. The root growth inhibition of *Sorghum saccharatum* from microcosms of wetland sites 1, 2, 5 and 6 sediments decreased in low and high treatments (of nitrate and phosphate applied in water of the microcosms at the end of the experiment (day 30) compared to the day 0 or start. (Figure 3.5).

The stem growth inhibition of *Sorghum saccharatum* from microcosms of wetland sites 1, 2, 5 and 6 sediments highly varied in high and low treatments (of nitrate and phosphate applied in water) of the microcosms at the end of the experiment (day 30) compared with the day 0 or start (Figure 3.5). The variation trend of *Lepidium sativum* root and stem inhibition and *Sinapis alba* stem inhibition from day 0 or start to the end of the experiment (day 30) was not clear (Figure 3.5). The multifactor analysis of variance (ANOVA) examined the effects of treatment level and experimental timeline (independent variable) to the root inhibitions

(dependent variable) tested during experiment. The effect of experimental timeline and treatment level on the stem and root growth inhibition of the ecotoxicological indicators was not statistically significant (Figure 3.5).

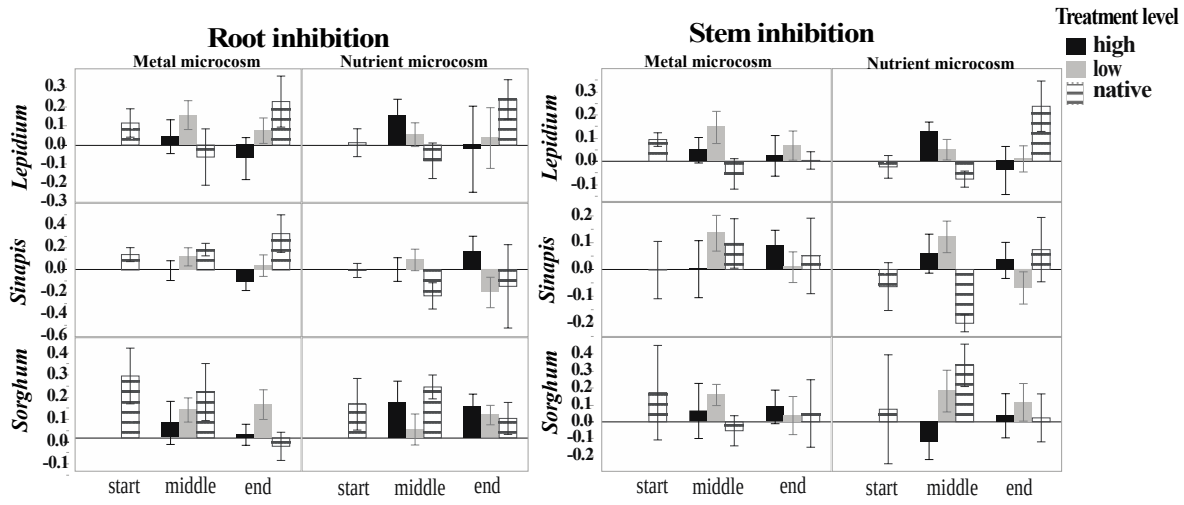


Figure 3.5: The root and stem growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* during the start, middle and end of the metal and nutrient microcosm experiments. This test included a native treatment where no metal or nutrient were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native (control) sediment. Bars show mean ± 1 SE. Multifactor ANOVA tables are located in Appendix 3.2.

Time and Treatment Effects on Bacterial Indicators

The sequence alignment and taxonomical analysis were conducted using SILVA database Release Version 128 and Greengenes Reference Taxonomy Version 13_8_99. Taxonomic profiles were determined up to and including the lowest level of classification for each sediment sample from each wetland site. A total of 175207 sequences were identified. At the broadest level, 70 unique phyla were identified (Table 3.2 A), while at the narrowest level 32,848 unique genera identified (Table 3.2 B). Among the total number of sequences detected proteobacteria was the most abundant phylum (26.23%) (Table 3.2). These unique types of phyla and genera as identified were used to calculate the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) across both nutrient and metal microcosms (Figure 3.6).

Table 3.2: (A) Number of sequences and the percent of total for each detected phylum in nutrient and metal microcosms. (B) Number of unique type of phyla and genera detected in nutrient and metal microcosms

(A) Phyla detected	**Number of sequences for each phyla**	**Percent of total**
Proteobacteria	51598	29.45%
Bacteroidetes	23252	13.27%
Chloroflexi	16236	9.27%
Planctomycetes	10013	5.71%
OD1	9477	5.41%
Firmicutes	7513	4.29%
Acidobacteria	7323	4.18%
Verrucomicrobia	6934	3.96%
Actinobacteria	5417	3.09%
Chlorobi	3561	2.03%
Spirochaetes	3428	1.96%
Armatimonadetes	2702	1.54%
Elusimicrobia	2148	1.23%
Cyanobacteria	2099	1.20%
GN02	2090	1.19%
Gemmatimonadetes	1654	0.94%
BRC1	1296	0.74%
Nitrospirae	1288	0.74%
Chlamydiae	1253	0.72%
TM7	1232	0.70%
OP11	1173	0.67%
TM6	1127	0.64%
OP8	1111	0.63%
Lentisphaerae	1048	0.60%
WS6	958	0.55%
OP3	939	0.54%
GN04	749	0.43%
NKB19	737	0.42%
AC1	635	0.36%
Fibrobacteres	529	0.30%
SR1	502	0.29%
WS2	454	0.26%
WS4	352	0.20%

LCP-89	346	0.20%
SC4	343	0.20%
WS5	340	0.19%
WS3	290	0.17%
KSB3	286	0.16%
TA06	249	0.14%
ZB3	223	0.13%
OP1	180	0.10%
Synergistetes	177	0.10%
Caldithrix	173	0.10%
GOUTA4	129	0.07%
LD1	126	0.07%
OC31	125	0.07%
Tenericutes	118	0.07%
Caldiserica	116	0.07%
FCPU426	111	0.06%
Fusobacteria	106	0.06%
WPS-2	100	0.06%
MVS-104	99	0.06%
NC10	92	0.05%
H-178	85	0.05%
Kazan-3B-28	85	0.05%
TPD-58	84	0.05%
Caldithrix	83	0.05%
Euryarchaeota	53	0.03%
Thermi	45	0.03%
FBP	43	0.02%
Thermotogae	37	0.02%
GAL15	34	0.02%
Parvarchaeota	30	0.02%
WS1	26	0.01%
MAT-CR-M4-B07	12	0.01%
WWE1	11	0.01%
Crenarchaeota	10	0.01%
OP9	9	0.01%
Poribacteria	2	0.00%
Hyd24-12	1	0.00%
Total Number of sequences	175207	100.00%

(B) Type of unique phyla and genera identified	number
Phyla	70
Genera	32848

Experimental Timeline and Treatment Effects – Metals Microcosms

The phyla Shannon diversity index in metal microcosm, ranged from 1.51 to 5.42, the genera Shannon diversity index ranged from 3.9 to 5.04. The phyla Simpson diversity index ranged from 2.98 to 9.78, the genera Simpson diversity Index ranged from 10.77 to 87.35. In nutrient microcosm, the phyla Shannon diversity Index ranged from 1.9 to 2.88, the genera Shannon diversity index ranged from 4.63 to 5.04. The phyla Simpson diversity index ranged from 3.63 to 9.78. The genera Simpson diversity Index ranged from 52.95 to 87.35.

The bacterial phyla and genera Shannon, Simpson diversity indices of the sediments from the metal microcosms decreased over time from the start to the end for both low and high treatments compared to controls (Figure 3.6). Multifactor analysis of variance (ANOVA) showed that the effect of experimental timeline was statistically significant on the Simpson diversity indices of phyla (P=0.0045) and genera (P=0.0314).

Experimental Timeline and Treatment Effects – Nutrient Microcosms

The bacterial phyla and genera Shannon, Simpson diversity indices of the sediments from nutrient microcosms treatments decreased across the time course of the experiment (Figure 3.6). Multifactor analysis of variance (ANOVA) detected that timeline was statistically significant on the Shannon (P=0.0104), Simpson (P=0.0004) diversity indices of phyla and Shannon (P=0.0013), Simpson (P=0.0418) diversity indices of genera, compared to controls. (Figure 3.6). However, there was no significant difference between high and low treatment levels (Figure 3.6).

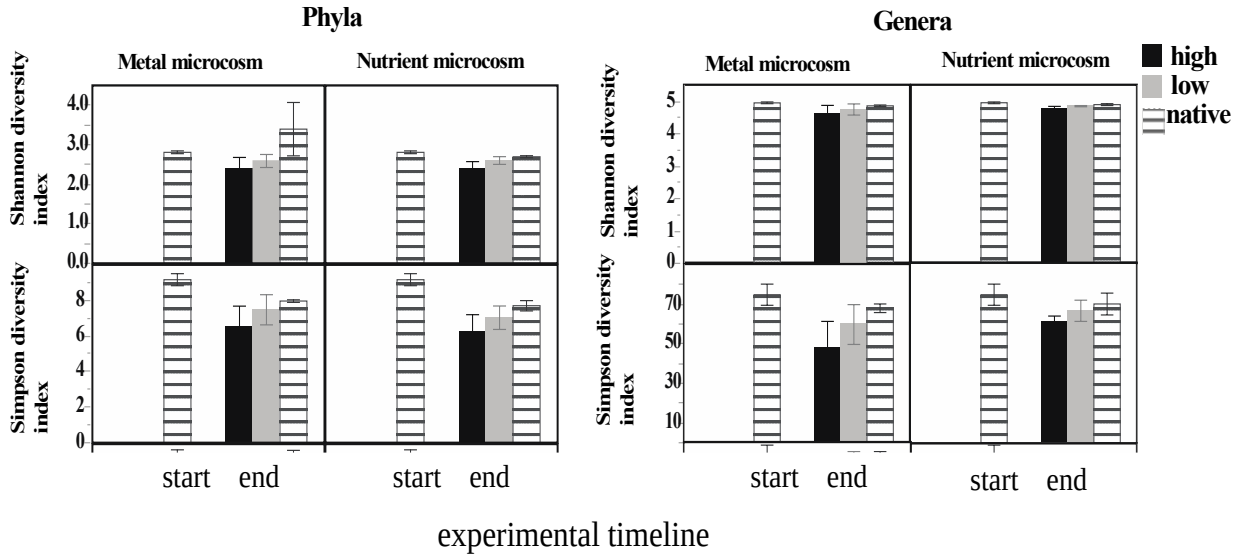


Figure 3.6: The Shannon and Simpson diversity indices of phyla and genera during the start and end of the metal and nutrient microcosm experiment. This test included a native(control) treatment where no metals or nutrients were added in water to the sediment followed by low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the native sediment. Bars show mean ± 1 SE. Multifactor ANOVA results are presented in Appendix 3.3.

Effects of Metal and Nutrient Pollutants on ecotoxicological and bacterial Indicators

Water Metals

Forward-stepping Multiple regression analyses were used to examine the predictive relationships between indicators and metal pollutant levels measured in the water. Best-fit Models are presented in Table 3.3. There were no significant effects of Pb and Cu concentrations measured in the water on the root and stem growth inhibition of ecotoxicological indicators in metal microcosm treatments with the exception increasing concentration of Pb having a positive impact on the root growth inhibition of *Sorghum saccharatum* (Figure 3.7, Table 3.3).

For bacterial indicators, the effect of increasing Cu concentration in the water of metal microcosms significantly decreased the Shannon, Simpson diversity indices of phyla and genera (Figure 3.8 and Table 3.3). With increasing Pb concentration there was significant increase in the Simpson diversity indices of phyla and Shannon diversity indices of genera, Simpson diversity indices of genera (Figure 3.8, Table 3.3).

Table 3.3: Multiple regression model with estimate (slope), combined R_2 and P value (significance) of relationship between root and stem growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum,* Shannon and Simpson diversity indices of phyla and genera and the concentration of Pb and Cu added (mg/L) in the water of metal microcosm.

	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00	0.082	0.0434	0.06
Concentration of Phosphate (mg/L) in water	0	NS		0	NS		0	NS	
	stem inhibition *Lepidium*			**stem inhibition *Sinapis***			**stem inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00	-0.3190	<.0001	0.26
Concentration of Phosphate (mg/L) in water	0	NS		0	NS		0	NS	

	Phyla Shannon diversity index			**Phyla Simpson diversity index**		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Pb (mg/L) in water	0	NS	0.16	2295	<.0001*	0.65
Concentration of Cu (mg/L) in water	-225.6	0.0422*		-1123.2	<.0001*	

	Genera Shannon diversity index			Genera Simpson diversity index		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Pb (mg/L) in water	254	<.0001*	0.64	10187	<.0001*	0.64
Concentration of Cu (mg/L) in water	-116	<.0001*		1797	<.0001*	

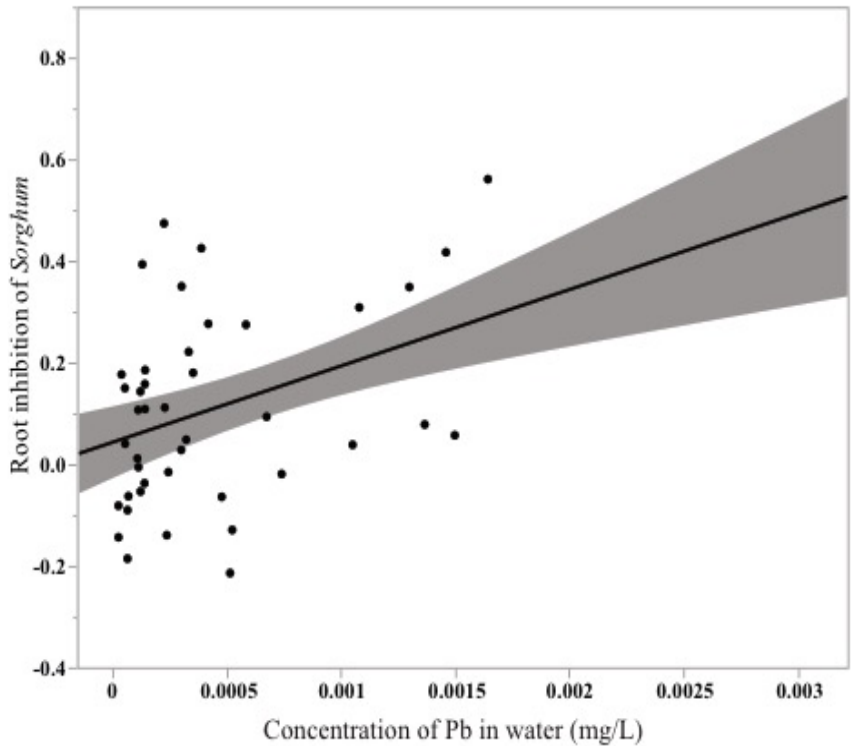


Figure 3.7: Relationship between the increasing concentration of Pb (in mg/L) in the water of the metal microcosm and root inhibition of *Sorghum saccharatum* grown in the sediment of metal microcosm. All other statistically non-significant relationship between increasing concentration of Pb and Cu (in mg/L) in the water of the metal microcosms with root and stem inhibition of *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum* are explained in Table 3.3.

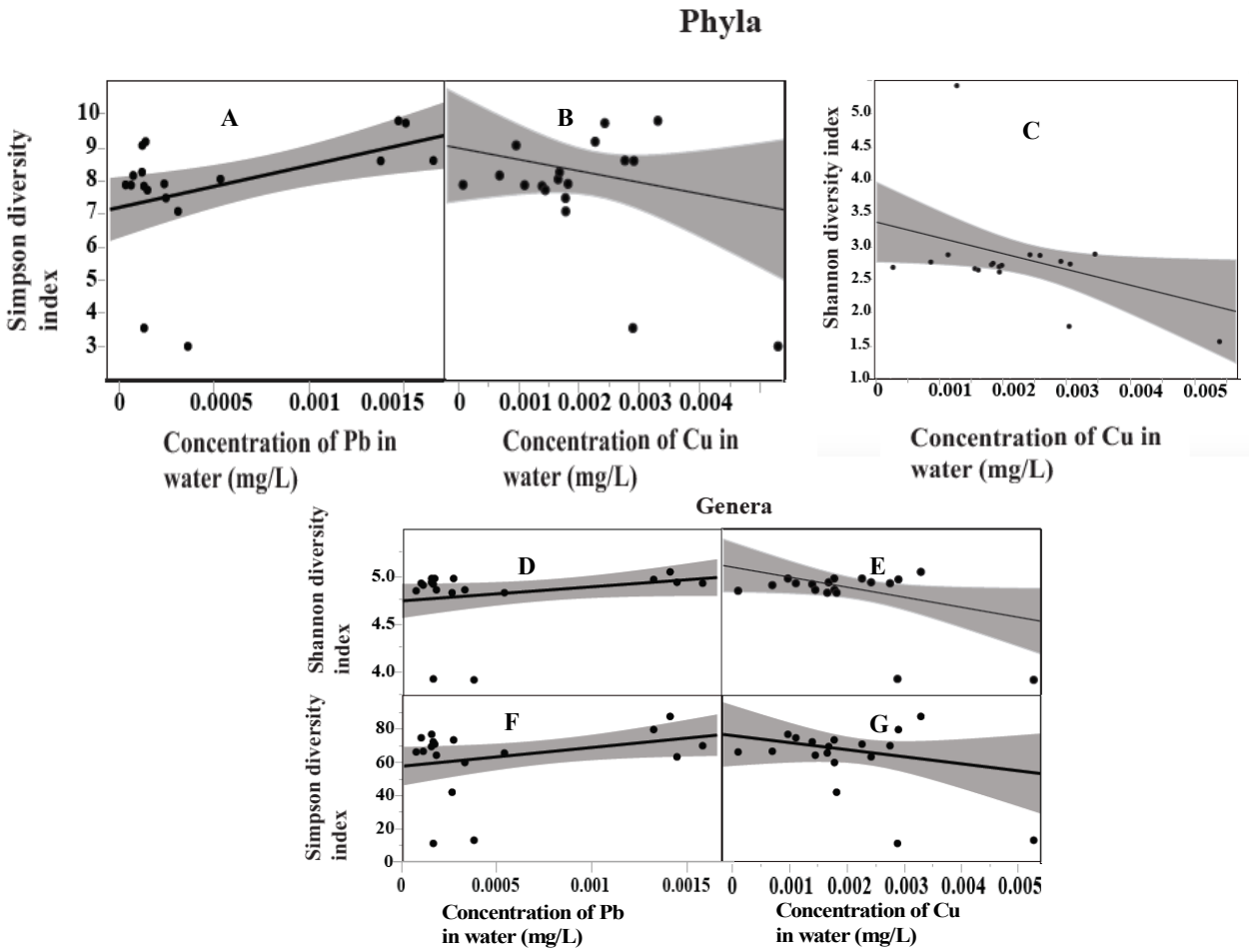


Figure 3.8: Relationship of the (A) Simpson diversity index of phyla with increasing concentration of Pb in the water, (B) Simpson diversity index of phyla with increasing concentration of Cu in the water, (C) Shannon diversity index of phyla, with increasing concentration of Cu in the water, (D) Shannon diversity index of genera with increasing concentration of Pb in the water, (E) Shannon diversity index of genera with increasing concentration of Cu in the water (F) Simpson diversity index of genera with increasing concentration of Pb in the water (G) Simpson diversity index of genera with increasing concentration of Cu in the water, in the water of the metal microcosm . All other statistically non-significant relationship between increasing concentration of Pb and Cu in the water of the metal microcosm with the Shannon and Simpson diversity indices of bacterial phyla and genera from the sediment of metal microcosms are explained in Table 3.3

Sediment Metals

Forward-stepping Multiple regression analyses were used to examine the predictive relationships between indicators and metal pollutants measure in the sediments. Best fit models are presented in Table 3.4. As, Cd, and Ni were observed to have effects on inhibition. Ag, Ni and Rb were detected to have effects on stem inhibition. (Table 3.4 and Figure 3.9)

Table 3.4: Multiple regression model with estimate (slope), combined R_2 and P value (significance) of relationship between root and stem growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum* and the metals detected (in ppm) in the sediments of metal microcosm.

	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Ag(ppm)	0	NS		0	NS		0	NS	
As(ppm)	0.7579	0.0184		0.6551	0.0276		-0.1003	NS	
Cd(ppm)	0	NS		0.8672	0.0314		1.0935	0.0114	
Fe(ppm)	0	NS		0	NS		0	NS	
Hg(ppm)	0	NS	0.13	0	NS	0.43	0	NS	0.24
Ni(ppm)	0	NS		0	NS		-5.3001	0.0150*	
Pb(ppm)	0	NS		-29.89	NS		0	NS	
Rb(ppm)	0	NS		0	NS		0	NS	
Zn(ppm)	0	NS		0	NS		0	NS	
	stem inhibition *Lepidium*			**stem inhibition *Sinapis***			**stem inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Ag(ppm)	0	NS		1.8448	0.0189		3.8784	0.0052	
As(ppm)	0	NS		0	NS		0	NS	
Cd(ppm)	0	NS		0	NS		0	NS	
Fe(ppm)	0	NS		0	NS		0	NS	
Hg(ppm)	0	NS	0.00	0	NS	0.22	0	NS	0.17
Ni(ppm)	0	NS		0	NS		0	NS	
Pb(ppm)	0	NS		0	NS		0	NS	
Rb(ppm)	0	NS		-0.7097	0.0386		0	NS	
Zn(ppm)	0	NS		0	NS		0	NS	

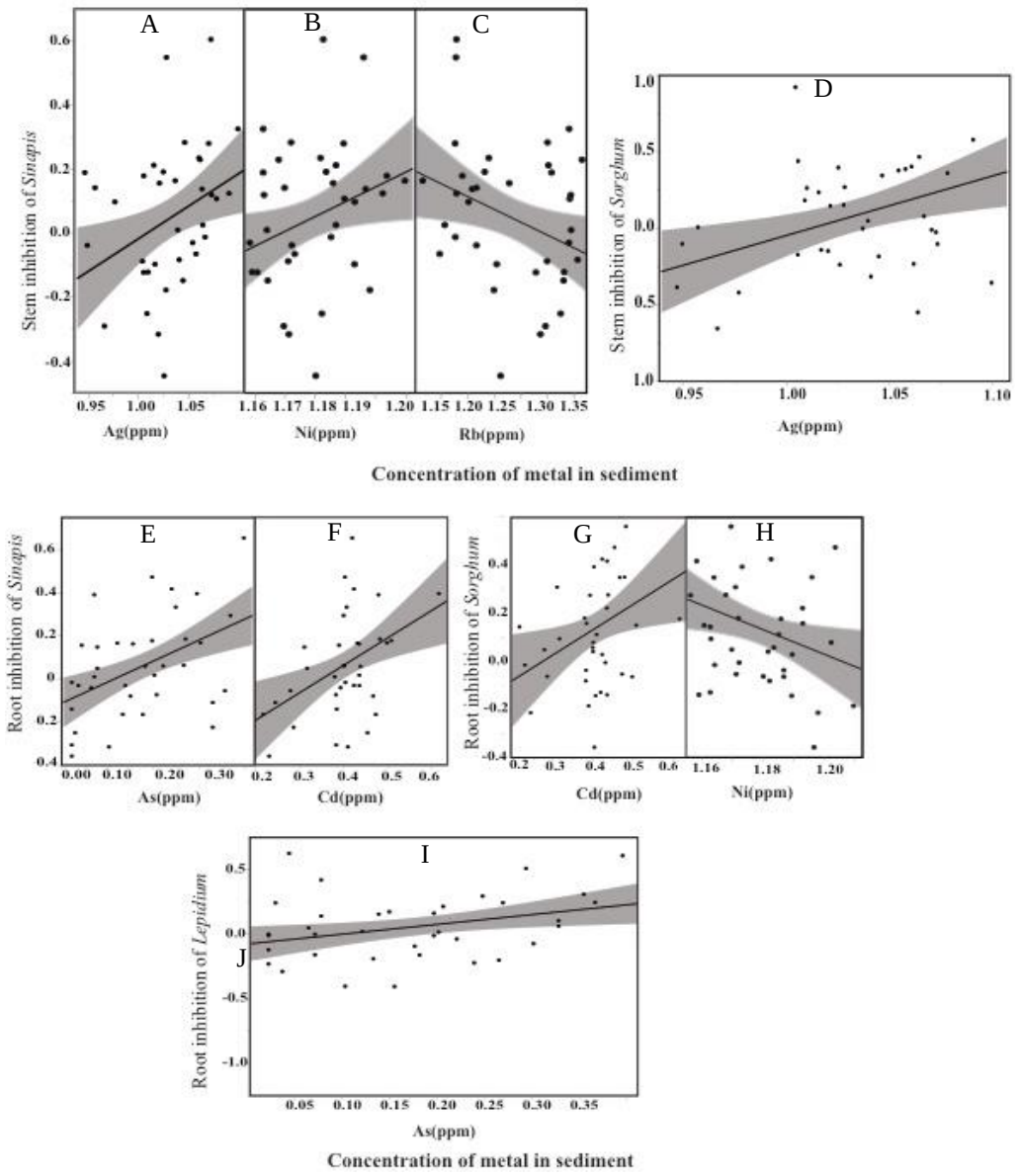


Figure 3.9: Relationship between the stem inhibition of *Sinapis alba and* the increasing concentration of (A) Ag, (B) Ni, (C)Rb, stem inhibition of *Sorghum saccharatum* with increasing concentration of (D) Ag, root inhibition of *Sinapis alba and* the increasing concentration of (E) As, (F) Cd, root inhibition of *Sorghum saccharatum* with increasing concentration of (G) Cd, (H) Ni and root growth inhibition of *Lepidium sativum* with increasing concentration of (I) As. All metals were detected in the sediment of metal microcosm in ppm. All other statistically non-significant relationship between increasing concentration (ppm) of detected sediment metals are shown in Table 3.4.

For the bacterial indicators, among the metals detected As, Cd, Fe, Hg, and Zn were shown to have effects on Phyla-based indicators Table 3.5. Shannon Diversity of Phyla had the greatest number of contributing metals in the best-fit model, with Cd, Zn positively contributing to Shannon diversity of phyla, while Fe and Hg contributed to a decrease in the Shannon diversity of phyla (Figure 3.10, Table 3.5). For Simpson Diversity of phyla , As and Zn contributed positively. For Genera, Zn concentration was associated with increased Simpson diversity of genera (Figure 3.10, Table 3.5). The R_2 values are also listed in Table 3.5.

Table 3.5: Multiple regression model with estimate (slope), combined R_2 and P value (significance) of relationship between Shannon and Simpson diversity indices of phyla and genera and the metals detected (in ppm) in the sediments of metal microcosm.

	Phyla Shannon diversity index			**Phyla Simpson diversity index**		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Ag(ppm)	0	NS		0	NS	
As(ppm)	0	NS		9.7619	0.0039	
Cd(ppm)	4.1667	0.0488		0	NS	
Fe(ppm)	-5.5585	0.0038		0	NS	
Hg(ppm)	-3.7311	0.0133	0.55	0	NS	0.44
Ni(ppm)	0	NS		0	NS	
Pb(ppm)	0	NS		0	NS	
Rb(ppm)	0	NS		-7.7248	0.0514	
Zn(ppm)	3.5775	0.0003		4.2563	0.0142	
	Genera Shannon diversity index			**Genera Simpson diversity index**		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Ag(ppm)	0	NS		0	NS	
As(ppm)	0	NS		0	NS	
Cd(ppm)	0	NS		0	NS	
Fe(ppm)	0	NS		0	NS	
Hg(ppm)	0	NS	0.00	0	NS	0.27
Ni(ppm)	0	NS		0	NS	
Pb(ppm)	0	NS		-5606	0.0616	
Rb(ppm)	0	NS		0	NS	
Zn(ppm)	0	NS		57.85	0.0118	

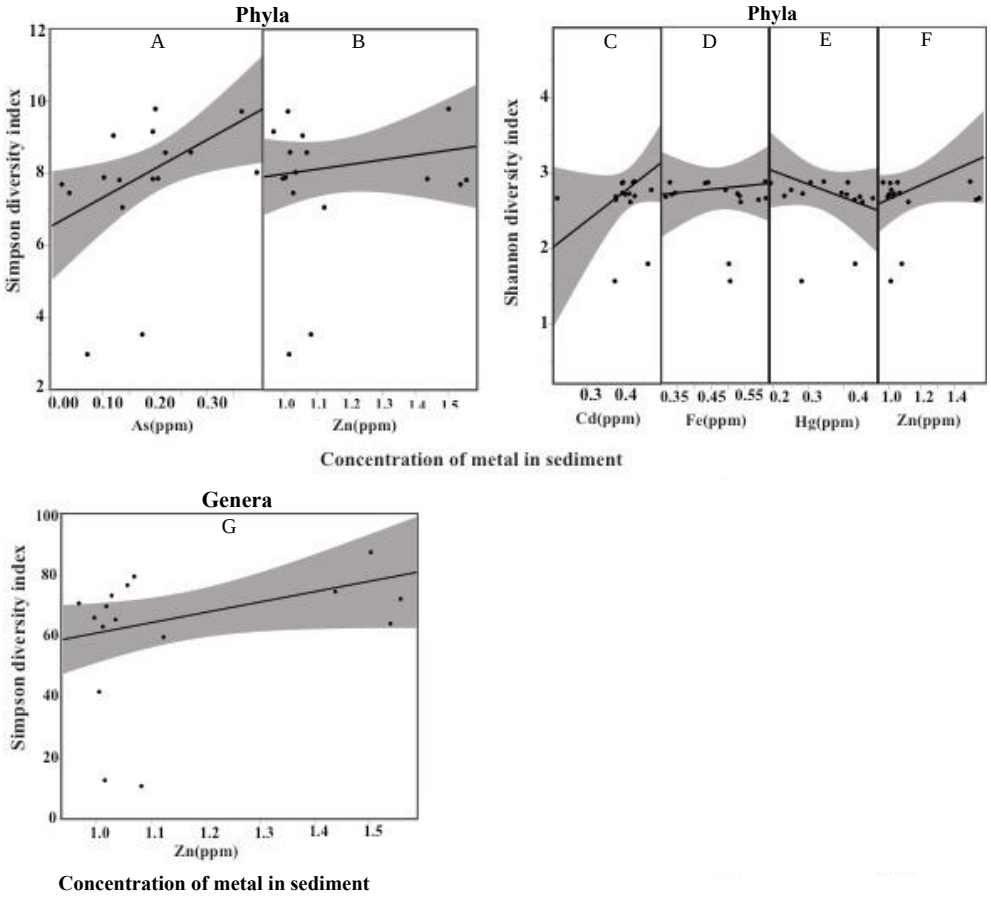


Figure 3.10: Relationship of the Simpson diversity index of phyla with increasing concentration of (A) As, (B) Zn, Shannon diversity index of phyla with increasing concentration of (C) Cd, (D) Fe, (E) Hg, (F) Zn, Simpson diversity index of phyla with increasing concentration of (G) Zn. All metals were detected in the sediment of metal microcosm in ppm. All other statistically non-significant relationship between increasing concentration (ppm) of detected sediment metals of the metal microcosm and the Shannon and Simpson diversity indices and total number of individuals of bacterial phyla and genera from the sediment of metal microcosm. are explained in Table 3.5.

Water Nutrients

Best fit models for forward-stepping multiple regressions for the effects of water nutrients on ecotoxicological and sediment bacterial community indicators are presented in Table 3.6. Nitrate was the only nutrient with significant effect on PhytoTox™ with *Sorghum saccharatum* root inhibition (positive) and stem inhibition (Figure 3.11, Table 3.6). There was no significant relationship with nutrients and the bacterial indicators (Table 3.6). The R_2 values are also listed in Table 3.6.

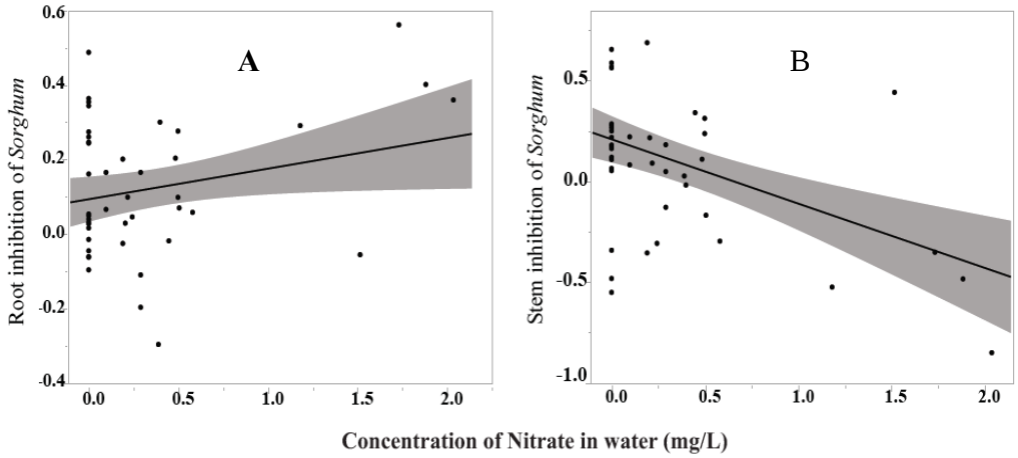


Figure 3.11: Relationship between the (A) root inhibition of *Sorghum saccharatum* (B) stem inhibition of *Sorghum saccharatum* with increasing concentration of nitrate. All metals were detected in the water of the water microcosm in mg/L. All other statistically non-significant relationship between increasing concentration of nitrate in the water of the nutrient microcosm with the stem, root inhibition of *Sorghum saccharatum* and the diversity indices of bacterial phyla and genera from the sediment of nutrient microcosm. are explained in Table 3.6.

Table 3.6: Multiple regression model with estimate (slope), combined R_2 and P value (significance) of relationship between root and stem growth inhibition of *Lepidium sativum, Sinapis alba* and *Sorghum saccharatum*, Shannon and Simpson diversity indices of phyla and genera and the concentration of nitrate and phosphate added (mg/L) in the water of nutrient microcosm.

	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00	0.082	0.0434	0.06
Concentration of Phosphate (mg/L) in water	0	NS		0	NS		0	NS	
	stem inhibition *Lepidium*			**stem inhibition *Sinapis***			**stem inhibition *Sorghum***		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00	-0.3190	<.0001	0.26
Concentration of Phosphate (mg/L) in water	0	NS		0	NS		0	NS	

	Phyla Shannon diversity index			**Phyla Simpson diversity index**		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00
Concentration of Phosphate (mg/L) in water	0	NS		0	NS	

	Genera Shannon diversity index			Genera Simpson diversity index		
Parameter	**Estimate**	**P value**	**R_2**	**Estimate**	**P value**	**R_2**
Concentration of Nitrate (mg/L) in water	0	NS	0.00	0	NS	0.00
Concentration of Phosphate (mg/L) in water	0	NS		0	NS	

Effect of Autoclaving Sediments

In metal microcosm, the concentrations of Pb, Cu were observed to be higher in the autoclaved sediments compared to non-autoclaved sediments, both in higher and lower level of the treatment. Multifactor analysis of variance (ANOVA) showed the effect of autoclaving the sediments was statistically significant on added Pb concentration (P=0.0270) (Figure 3.12). The concentration level of low and high have been listed in Table 3.1.

In metal microcosm, the levels of all the measured sediment metals, Ag, As, Cd, Fe, Hg, Ni, Pb, Rb, Zn were not observed to be different between autoclaved and non-autoclaved sediments (Figure 3.13).

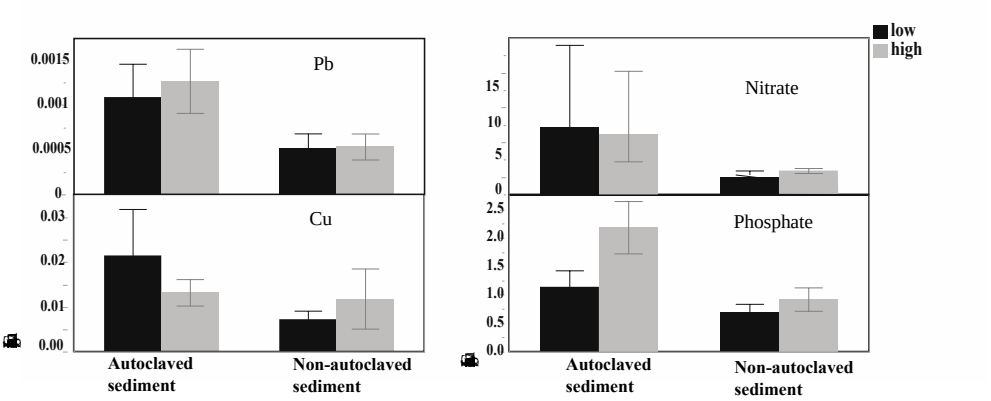


Figure 3.12: The concentration of Pb, Cu in mg/L in metal microcosm and nitrate and phosphate in mg/L in nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metal and nutrient were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

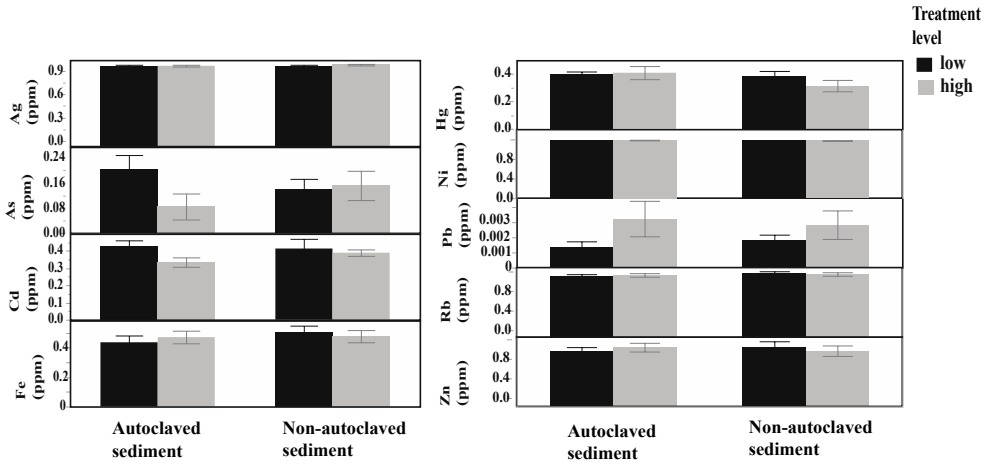


Figure 3.13: The concentration of sediment metal detected (ppm) in metal microcosm and in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE

In nutrient microcosm, the concentrations of nitrate and phosphate were observed to be higher in the autoclaved sediments compared to non-autoclaved sediments, both in higher and lower level of the treatment. Analysis of variance (ANOVA) showed that the effect of autoclaving the sediments was statistically significant on phosphate concentration (P= 0.0080) (Figure 3.14). The effect of autoclaving did not have a specific trend or significant effect of autoclaving on the stem and root growth inhibition of the ecotoxicological indicators in nutrient and metal microcosm (Figure 3.14).

Not surprisingly, Shannon and Simpson diversity indices of phyla and genera were higher in the non-autoclaved sediments of metal microcosms for both in low and high level of treatment (Figure 3.15). Analysis of variance (ANOVA) that autoclaving did have a significant effect on Simpson diversity of phyla (P= 0.0430) and Simpson diversity of genera (P=0.0418), Shannon diversity of index of phyla (P= 0.0176) and Shannon diversity of index of genera (P=0.0178) (Figure 3.15) of metal microcosm. The Shannon and Simpson diversity indices of phyla observed to higher in the non-autoclaved sediments of nutrient microcosms (Figure 3.15) for both in low and high level of treatment. The effect of autoclaving did have a significant effect on Shannon ($P<0.0001$) and Simpson (P=0.0004) diversity indices of phyla of nutrient microcosm. The Shannon diversity indices of genera observed to higher in the non-autoclaved sediments of nutrient microcosms for both in low and high level of treatment (Figure 3.15). The effect of autoclaving did have a significant effect on the Shannon diversity indices of genera (P=0.0045) of nutrient microcosm. In the multifactor analysis of variance (ANOVA) examined the effects of treatment level and autoclaving (independent variable) to the bacterial indicators (dependent variable) of the nutrient microcosm sediment.

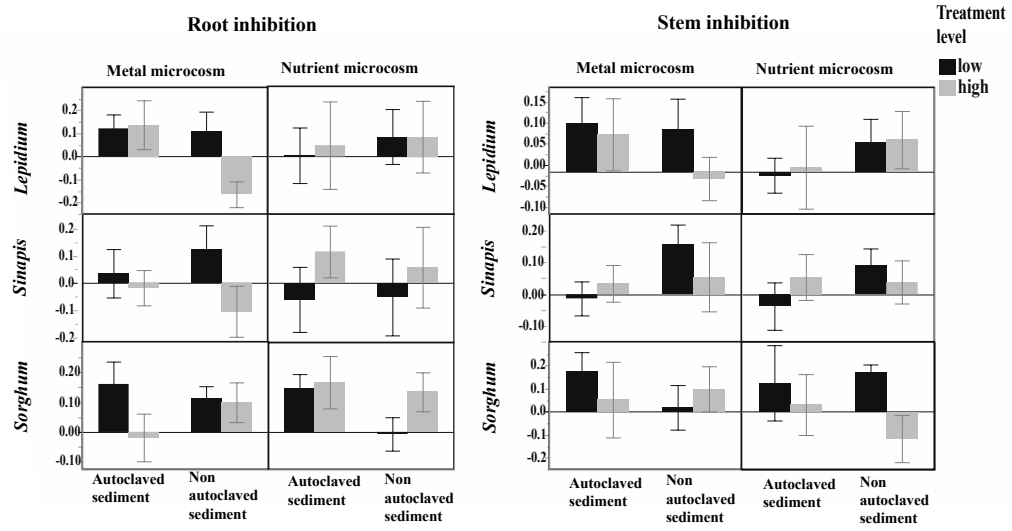


Figure 3.14: The root and stem growth inhibition of *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum* in metal and nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

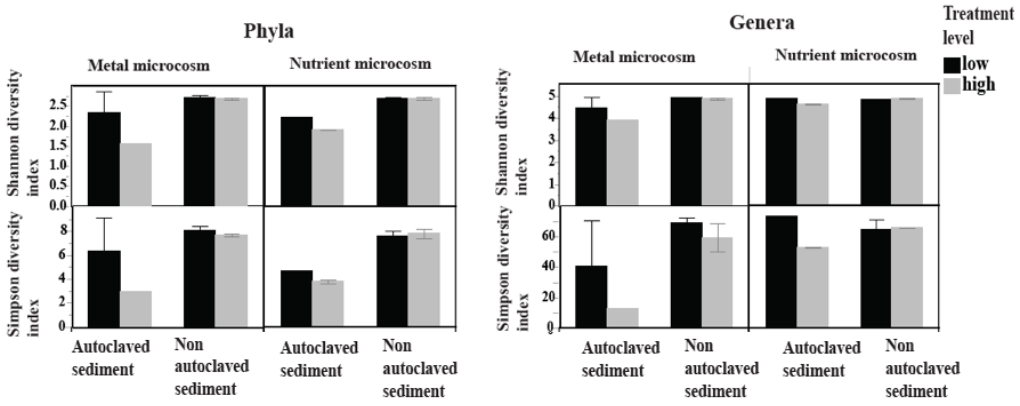


Figure 3.15: The Shannon and Simpson diversity indices identified as phyla and genera in metal and nutrient microcosm experiment in the autoclaved and nonautoclaved sediments. This test included low and high concentration treatments where the metals (Pb and Cu) and nutrients (nitrate and phosphate) were added in low and high concentration respectively in the water of the microcosm to the autoclaved sediment. Bars show mean ± 1 SE.

Predictive indicator categories based on key pollutants in field and microcosm study

Based on the response of the ecotoxicological and bacterial indicators, some key pollutants (Pb, Hg and Phosphate) were identified in chapter 1 and 2 field study. The added pollutants in the microcosm experiment in the present chapter were Pb, Hg , nitrate and phosphate. Based on the response of the bacterial indicators to the key pollutants a list of specific predictive bacterial genera were identified and categorized in terms of their function to the pollutants as described in Table 3.7. The specific categories are: Phosphorus solubilizing, Hg resistant and/or Hg bioremediating, Pb-resistant, remediating, precipitating, biomethylating, Denitrifiers and Cu resistant (Table 3.7). The identification of the bacterial genera was based on the taxonomical identification using 16S rRNA gene as described in the method section of chapter 2 and the present chapter. The identification of the specific predictive indicators categories in relation to the key pollutants were based on a literature survey. The specific predictive indicator categories and the list of literature survey is listed in Table 3.7.

Table 3.7: Pb-resistant, remediating, precipitating, biomethylating , Hg resistant and/or Hg bioremediating and phosphorus solubilizing genera detected identified based on literature survey in each wetland site (1-6) during summer 2015, fall 2016 and summer 2017 and from the microcosms built from sediments collected from wetland sites 1,2, 5 and 6 collected during summer 2017. Additionally Cu-resistant and Denitrifiers genera were also identified from the microcosms.

Genera - in wetland sites and microcosms		
Phosphorus solubilizing	**Hg resistant and/or Hg bioremediating**	**Pb-resistant, remediating, precipitating, biomethylating**
Bacillus, Pseudomonas, Agrobacterium	*Bacillus, Pseudomonas, Agrobacterium, Aeromonas, Vibrio, Serratia, Clostridium, Stenotrophomonas, Streptococcus*	*Bacillus, Pseudomonas, Agrobacterium, Aeromonas, Streptococcus*
Thiobacillus, Rhizobium		
Ralstonia, Flavobacterium, Enterobacter		*Ralstonia, Flavobacterium, Enterobacter*
	Enterococcus	*Ramlibacter,Shewanella, Psychrobacter,Staphylococcus Achromobacter,Corynebacterium, Arthrobacter,Nitrospira Exiguobacterium,Vibrio, Cupriavidus,Acinetobacter*
Mamta et al. 2010, Postma et al. 2010, Tajini et al. 2012, David et al. 2014, Zhao et al. 2014b,Istina et al. 2015, ori et al. 2017	Irawati et al. 2012, Maiti and Bhattacharyya 2013, Kowalczyk et al. 2016, Naguib et al. 2019	Gummersheimer and Giblin 2003, Chen et al. 2011, Kafilzadeh et al. 2012, Zhang et al. 2012, Jarosławiecka and Piotrowska-Seget 2014, Jebara et al. 2015, Jiang et al. 2017, An et al. 2018b, Luo et al. 2018, Ayangbenro et al. 2019

Genera in wetland microcosms	
Dentirifiers	**Cu resistant**
Bacillus, Enterobacter, Pseudomonas, Hyphomicrobium, Arthrobacter, Burkholderia, Rhizobium, Thiobacillus, Flavobacterium, Corynbacterium,Agrobacterium	*Clostridium, Pseudomonas, Bacillus, Arthrobacter*
Smith and Zimmerman 1981, Gerardi 2006, Castellano-Hinojosa et al. 2017)	Kunito et al. 1997, He et al. 2010, Santo et al. 2010, Andreazza et al. 2011, Altimira et al. 2012, Berg et al. 2012

Patterns of specific predictive bacterial indicators detected in the metal microcosm experiments

Taxonomical profile of the sediment bacterial communities was determined from sequencing of the 16S bacterial rRNA (v3-v4 region), identifying sequences to the phylum and genus levels (Figure 3.2). Hierarchical cluster analysis was performed with all these identified communities in each wetland site of each type of microcosm (metal and nutrient). Based upon visual examination of changes in clusters over the course of the experiments relative to their response to the pollutants added to the metal(Pb and Cu) and nutrient (nitrate and phosphate) microcosm treatments, bacterial genera were identified and categorized into groupings. These included: (1) Intolerant bacterial genera, were present at the start of an experiment but disappeared later in both the low and high treatments; (2) Sensitive bacterial genera, were absent in the high treatments of the metal microcosm by the end of the experiment compared to start of the experiment; and (3) Tolerant bacterial genera, were present at the start and end of both the low and high treatments.

Additional sub-types of tolerant genera were observed. Less tolerant bacterial genera decreased in abundance in the high treatments compared to the low treatment whereas highly tolerant bacterial genera increased in abundance in the high treatments compared to the low treatment. The bacterial genera that were not found at the start of the experiment but appeared later in the experiment were not included in the analysis as they do not provide any frame of reference for comparison and may have been introduced by contamination

In the wetland site 1 metal microcosm experiment, the intolerant (to Pb and Cu) bacterial genera were: *Exigobacterium* and *Psychrobacter*. The sensitive (to Pb and Cu) bacterial genera that were observed included: *Pedosphaera.* Among the tolerant bacterial genera, some less

tolerant (to Pb and Cu) genera were: *Bdellovibrio, Flavobacterium, Planctomyces, Rhodobacter, Spirochaeta, Aquicella, Bacillus, Pseudomonas* and more. Some highly tolerant (to Pb and Cu) genera were: *Clostridium, Treponema, Caldilinea, Blvii28, Thiobacillus, Rhodoplanes, Rhodoferax* and more. The entire list in described *in* Table 3.8, Figure 3.16.

In wetland site 2 microcosm experiment, the intolerant (to Pb and Cu) bacterial genus was *Chlorobium.* The sensitive (to Pb and Cu) bacterial genera were: *Gemmata and Sphingomonas.* Among the tolerant bacterial genera, Some less tolerant (to Pb and Cu) genera were: *Clostridium, Luteobacter, Treponema, Syntrophus, Bacteroides, Bdellovibrio, Flavobacterium* and more. Some highly tolerant (to Pb and Cu) genera were: *Anaerolinea, SJA-88, Geobacter, Caldilinea, Blvii28, LCP-6, Thiobacillus, Cystobacter, Aeromonas, Ralstonia* and more. The entire list in described *in* Table 3.8 and Figure 3.17.

In wetland site 5 microcosm experiment, the intolerant(to Pb and Cu) bacterial genera were: *Enterobacter, Corynebacterium* and *Chlorobium.* The sensitive(to Pb and Cu) bacterial genera were: *Balneimonas, Arthrobacter, Chlorobaculum, Aeromonas and Agrobacterium.* Among the tolerant bacterial genera, some less tolerant (to Pb and Cu) genera were: *Anaerolinea, SJA-88, Luteolibacter, Treponema, Thiobacillus, Cystobacter, Desulfococcus, Rhodoferax, Planctomyces, , Rhodobacter* and more. Some highly tolerant(to Pb and Cu) genera were: *Bdellovibrio, Flavobacterium, Clostridium, Geobacter, Blvii28, LCP-6, Rhodoplanes, Sulfuritalea, Hyphomicrobium, Methylotenera , Bacillus* and more. The entire list in described in Table 3.8 and Figure 3.18.

In wetland site 6 microcosm experiment, the intolerant (to Pb and Cu) bacterial genera were: *Aquicella, Methylobacterium, Microbacterium, Shewanella, Cupriavidus, Enterococcus, Rhizobium* and *Chlorobium*. The sensitive (to Pb and Cu) bacterial genera that was observed:

Nitrospira. Among the tolerant bacterial genera, some less tolerant (to Pb and Cu) genera were: *Clostridium, Anaerolinea, Luteolibacter, Treponema*, *Geobacter*, *Bacillus* and more. Some highly tolerant (to Pb and Cu) genera were: *Bdellovibrio, Flavobacterium, SJA-88, Caldilinea, Blvii28, LCP-6, Thiobacillus, Sulfuritalea,* and more. The entire list in described in Table 3.8 and Figure 3.19.

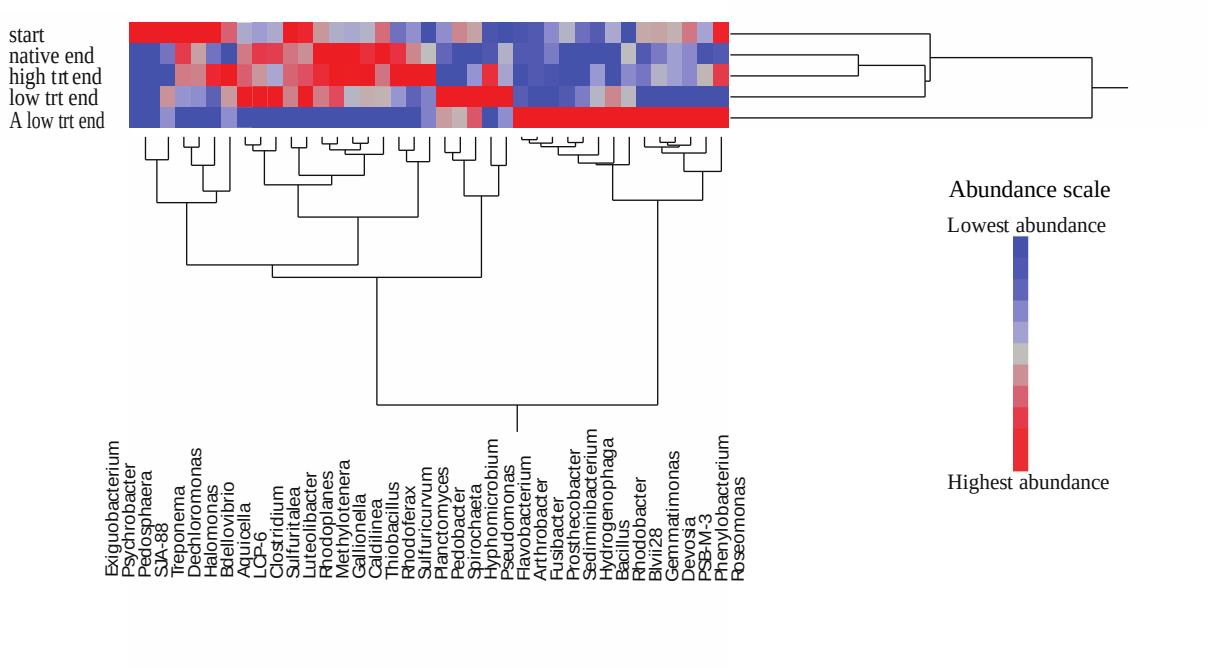


Figure 3.16: Hierarchical cluster analysis of the bacterial genera detected (by 16S rRNA gene sequences) in the sediments of the treatments of wetland site 1 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15), control treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment, A low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high concentrations.

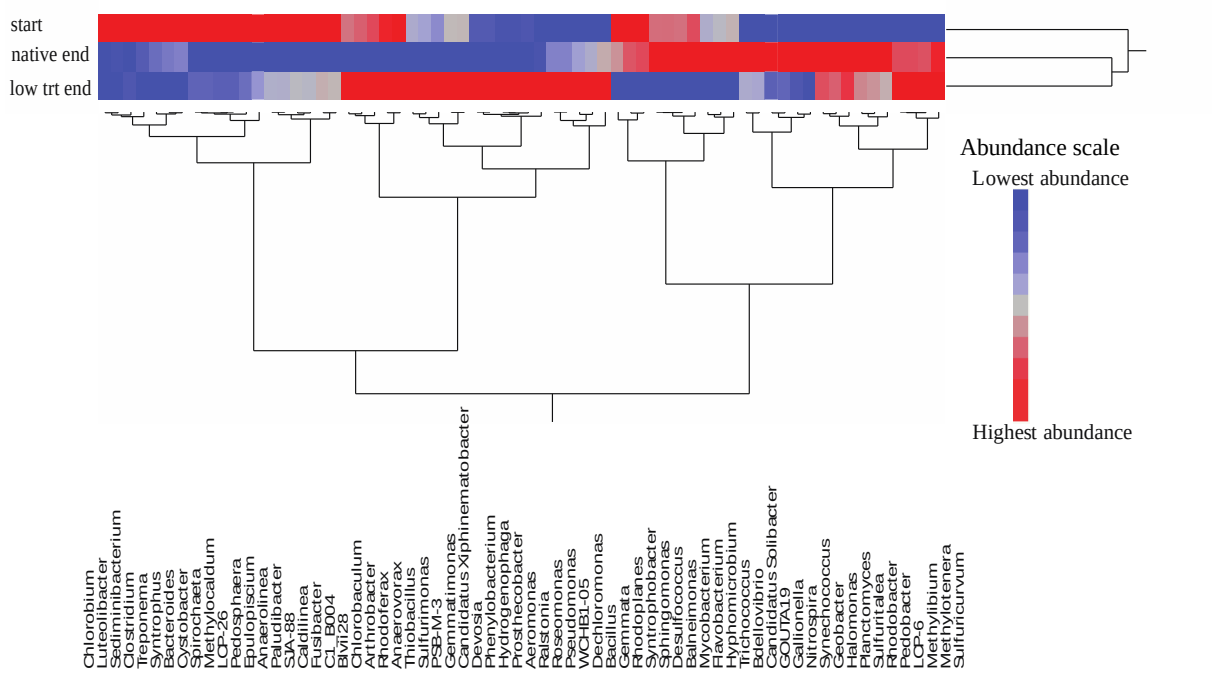


Figure 3.17: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 2 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15), control treatment of the metal microcosm experiment of the native sediment with no metals added, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment. . Adequate DNA was not found in the high treatment (with metals (Pb and Cu) added in lower concentration in the water to the native sediment) in in the water to the native sediment and autoclaved sediment with metals added in high and low concentrations.

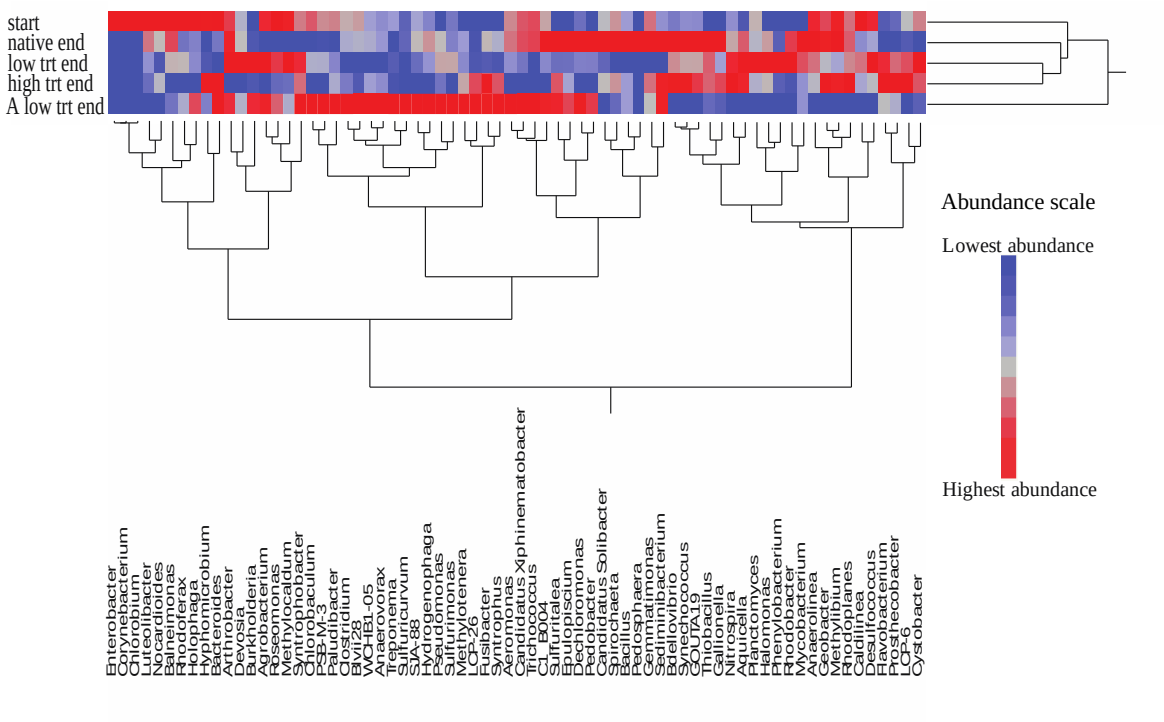


Figure 3.18: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 5 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15) treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment, A low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high concentrations.

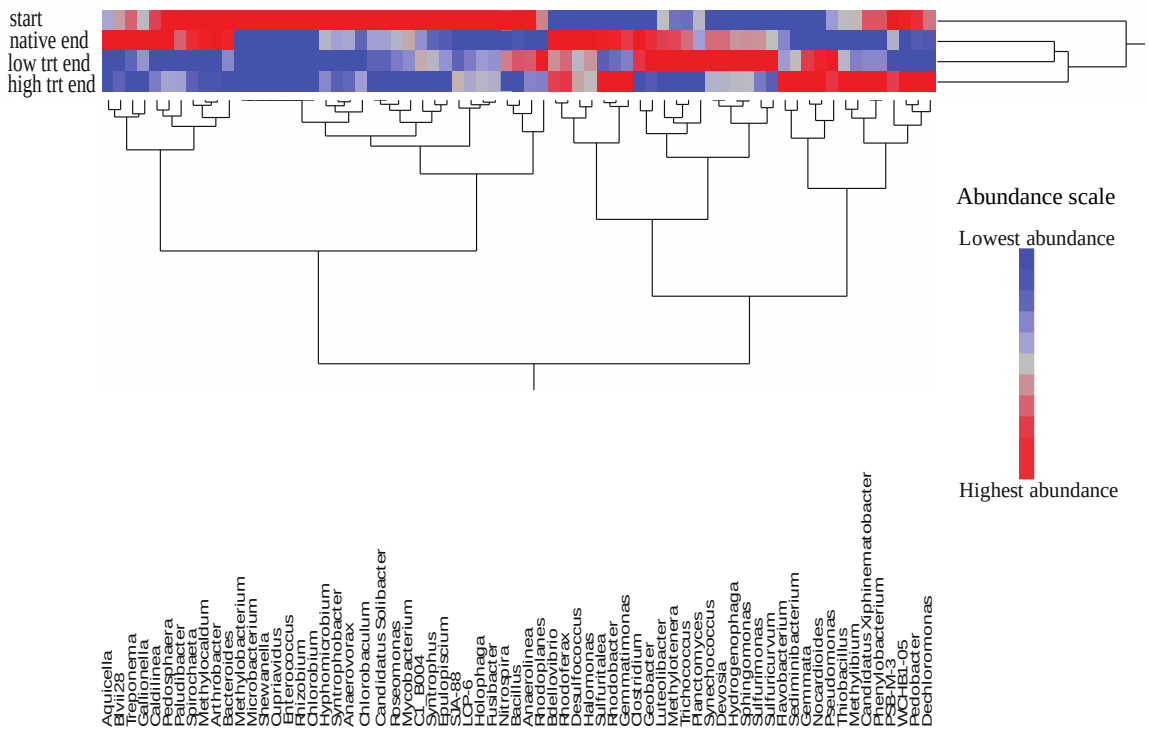


Figure 3.19: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 6 metal microcosm. Where, start (day 0) = beginning treatment of the metal microcosm experiment, native end = end (day 15) treatment of the metal microcosm experiment of the native sediment with no metals added, high trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 15) of the metal microcosm experiment with metals (Pb and Cu) added in lower concentration in the water to the native sediment. Adequate DNA was not found in the autoclaved sediment with metals added in high and low concentrations.

Among all the sites, the bacterial genera *Chlorobium* was an intolerant (to Pb and Cu) bacterial genus identified in the microcosms of wetland sites 2, 5 and 6. *Aquicella* was observed to be less tolerant (to Pb and Cu) in the microcosms of wetland sites 1 and 5, but was intolerant (to Pb and Cu) in the microcosms of wetland sites 5 (Table 3.8). The bacterial genera *Nocardioides, Burkholderia, Holophaga, Syntrophus, Bacteroides, Anaerolinea, Geobacter, Candidatus Solibacter, Cystobacter, Desulfococcus, Syntrophobacter, GOUTA 19, Paludibacter, Methylocaldum, PSB-M-3, Candidatus Xiphinematobacter, Synechococcus, WCHB1-05, C1_B004, Methylibium, Trichococcus, Anaerovorax, Mycobacterium, LCP-6, Sulfurimonas, Epulopiscium, Ralstonia, Bdellovibrio, Flavobacterium, Clostridium, SJA-88, Luteolibacter, Treponema, Caldilinea, Blvii28, Thiobacillus, Rhodoplanes, Sulfuritalea, Rhodoferax, Methylotenera, Planctomyces, Rhodobacter, Gemmatimonas, Spirochaeta, Halomonas, Gallionella, Roseomonas, Fusibacter, Dechloromonas, Bacillus, Devosia, Hydrogenophaga, Prosthecobacter, Sediminibacterium, Phenylobacterium, Sulfuricurvum, Pedobacter, Hyphomicrobium* and *Pseudomonas* were identified as tolerant (to Pb and Cu) (either less or highly) bacterial genera in the microcosms of wetland sites 1, 2, 5 and 6 (Table 3.8). Whereas, *Gemmata* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 2 but was highly tolerant (to Pb and Cu) microcosms of wetland site 6. *Sphingomonas* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 1 but was less tolerant (to Pb and Cu) microcosms of wetland site 6. *Nitrospira* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 6 but was tolerant (to Pb and Cu) in the microcosms of wetland sites 2 and 5. *Balneimonas* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 5 but less tolerant(to Pb and Cu) in the microcosms of wetland site

2. *Chlorobaculum* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 5 but highly tolerant(to Pb and Cu) in the microcosms of wetland site 2 and 6. *Aeromonas* was identified as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 5 but highly tolerant (to Pb and Cu) in the microcosms of wetland site 2. *Pedosphaera* was characterize as sensitive (to Pb and Cu) to high treatments of the metal microcosms of wetland site 1 but tolerant in the microcosms of wetland site 2, 5 and 6. *Arthrobacter* was classified as sensitive(to Pb and Cu) to high treatments of the metal microcosms of wetland site 5 but highly tolerant (to Pb and Cu) in the microcosms of wetland site 1, 2 and 6 (Table 3.8).

Patterns of specific predictive bacterial indicators in the nutrient microcosm experiments

In wetland site 1 nutrient microcosm experiment, the intolerant (to nitrate and phosphate) bacterial genera were: *Chronothrix* and *Psychrobacter*. The sensitive (to nitrate and phosphate) bacterial genera that was observed: *Exiguobacterium*. Among the tolerant bacterial genera, some less tolerant (to nitrate and phosphate) genera were: *Anaerovorax, Aquicella, Bdellovibrio, Clostridium, Flavobacterium, Gemmatimonas, Geobacter, Hydrogenophaga* and more. The highly tolerant (to nitrate and phosphate) genera were: *Arthrobacter, Bacillus, Blvii28, Candidatus Solibacter, Candidatus Xiphinematobacter, Cystobacter, Dechloromonas, Devosia, Fusibacter, Gallionella* and more. The entire list in described in Figure 3.20, Table 3.8.

In wetland site 2 nutrient microcosm experiment, the intolerant (to nitrate and phosphate) bacterial genera were: *Chronothrix* and *Agrobacterium*. No sensitive (to nitrate and phosphate) bacterial genera were observed in the microcosms of wetland site 2. Among the tolerant bacterial genera, some less tolerant (to nitrate and phosphate) genera were: *Chlorobaculum, Anaerovorax, Bdellovibrio, Clostridium, Cystobacter, Flavobacterium, Geobacter, Holophaga* and more. The highly tolerant (to nitrate and phosphate) genera were: *Anaerolinea, Arthrobacter, Bacillus, Blvii28, Candidatus Solibacter, Dechloromonas* and more. The entire list in described in Figure 3.21, Table 3.8.

In wetland site 5 nutrient microcosm experiment, the intolerant (to nitrate and phosphate) bacterial genera were: *Cronothrix, Enterobacter, Brevundimonas, Agrobacterium, Chlorobium, Chlorobaculum* and *Corynebacterium.* The sensitive (to nitrate and phosphate) bacterial genera that were observed: *Burkholderia* and *Aeromonas.* Among the tolerant bacterial genera, some less tolerant (to nitrate and phosphate) genera were: *Aquicella, Blvii28* and more. Some highly tolerant (to nitrate and phosphate) genera were: *Anaerolinea, Anaerovorax, Arthrobacter,*

Bacillus, Bdellovibrio, Caldilinea, Candidatus Solibacter, Cystobacter, Dechloromonas, Devosia, Flavobacterium and more. The entire list in described in Figure 3.22, Table 3.8.

In wetland site 6 nutrient microcosm experiment, the intolerant (to nitrate and phosphate) bacterial genera were: *Cronothrix, Methylobacterium, Microbacterium, Shewanella, Cupriavidus, Enterococcus, Rhizobium, Agrobacterium* and *Stenotrophomonas.* No sensitive (to nitrate and phosphate) bacterial genera were observed in the microcosms of wetland site 6. Among the tolerant bacterial genera, some less tolerant (to nitrate and phosphate) genera were: *Chlorobaculum, Aquicella, Arthrobacter, Balneimonas, Blvii28, Pedosphaera, Pedosphaera* and more. The highly tolerant (to nitrate and phosphate) genera were: *Chlorobium, Bacteroides, Sulfurimonas, Anaerolinea, Anaerovorax, Bdellovibrio, Clostridium, Devosia* and more. The entire list in described in Figure 3.23, Table 3.8.

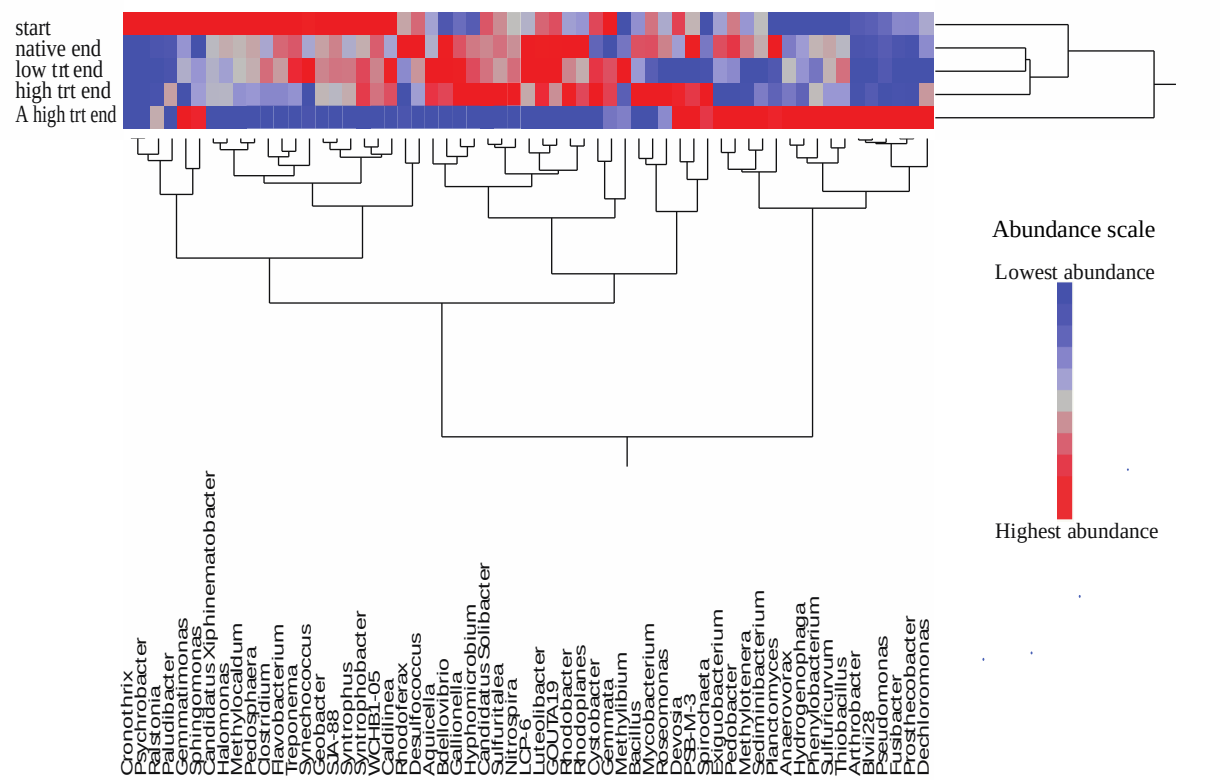


Figure 3.20: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 1 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

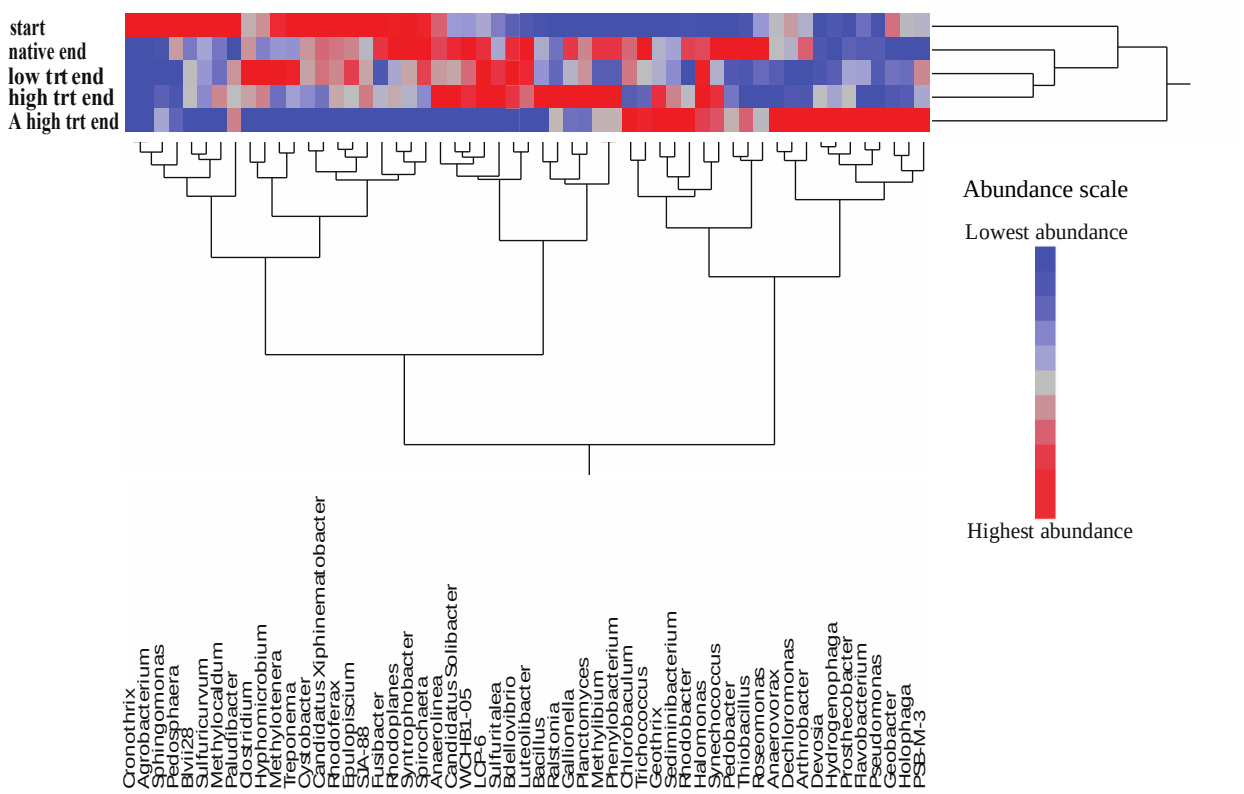


Figure 3.21: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 2 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

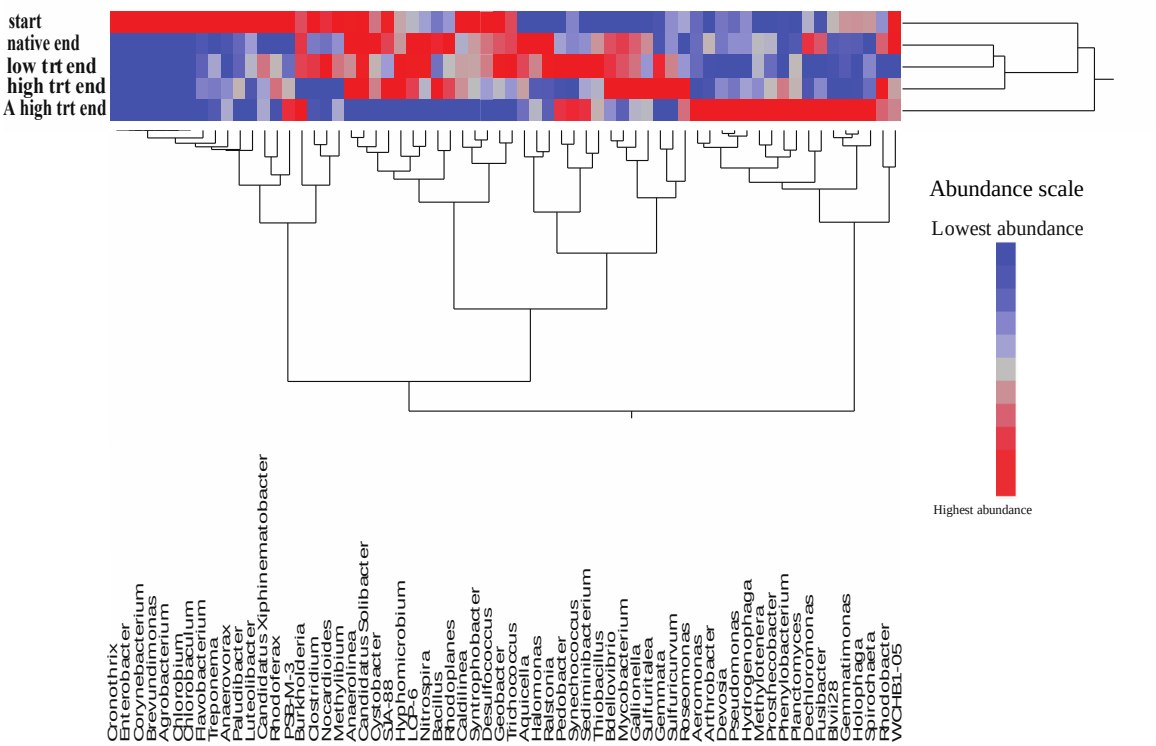


Figure 3.22: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences)detected in the sediments of the treatments of wetland site 5 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment, A high trt end = end treatment (day 15) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the autoclaved sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low concentrations.

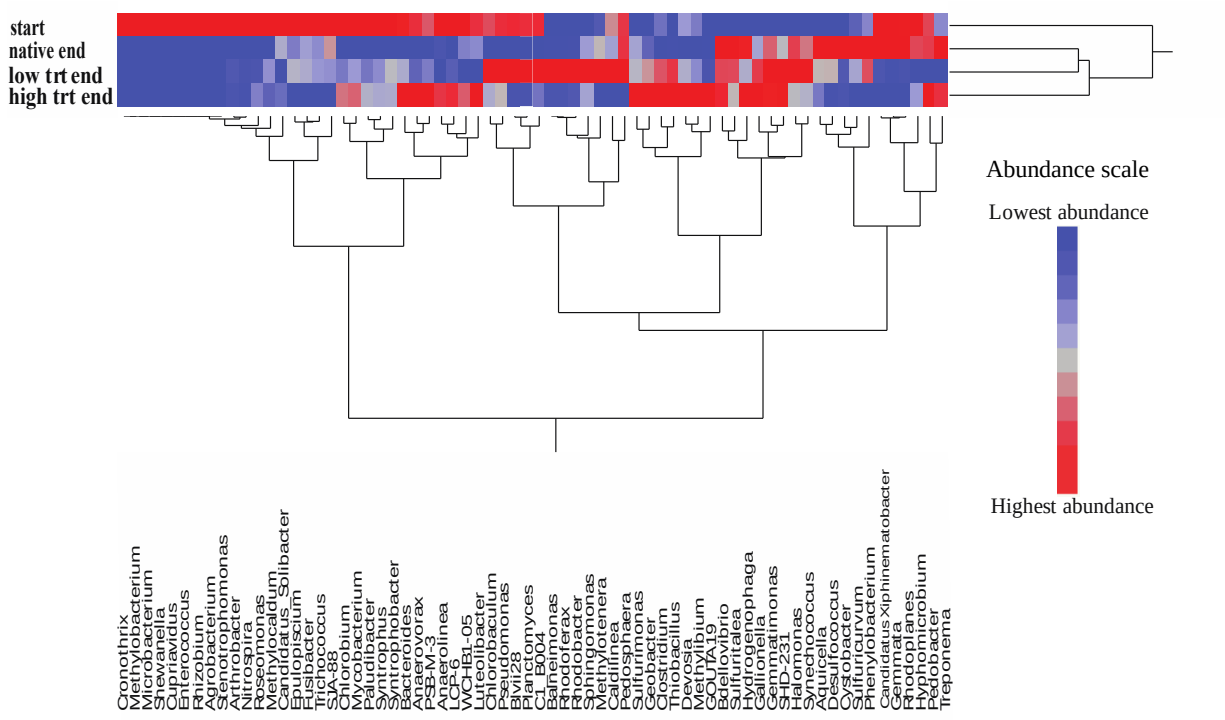


Figure 3.23: Hierarchical cluster analysis of the bacterial genera (by 16S rRNA gene sequences) detected in the sediments of the treatments of wetland site 6 nutrient microcosm. Where, Start (day 0) = beginning treatment of the nutrient microcosm experiment, native end = end (day 30) treatment of the nutrient microcosm experiment of the native sediment with no nutrients added, high trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in higher concentration in the water to the native sediment, low trt end = end treatment (day 30) of the nutrient microcosm experiment with nutrients (nitrate and phosphate) added in lower concentration in the water to the native sediment. Adequate DNA was not found in the autoclaved sediment with metals added in low and high concentrations.

Among all the sites, the bacterial genus *Chronothrix* was an intolerant (to nitrate and phosphate) bacterial genus identified in the microcosms of wetland sites 1, 2, 5 and 6. *Agrobacterium* was an intolerant(to nitrate and phosphate) bacterial genus identified in the microcosms of wetland sites 2, 5 and 6. *Chlorobium* was observed to be highly tolerant(to nitrate and phosphate) in the microcosms of wetland site 6, but was intolerant (to nitrate and phosphate) in the microcosms of wetland sites 5. Similarly, *Chlorobaculum* was observed to be less tolerant (to nitrate and phosphate) in the microcosms of wetland sites 2 and 6, but was intolerant (to nitrate and phosphate) in the microcosms of wetland sites 5 (Table 3.8). Again the bacterial genera of *Bacillus, Dechloromonas, Flavobacterium, Holophaga, Prosthecobacter, Ralstonia, Sediminibacterium, Spirochaeta, Anaerolinea, Anaerovorax, Aquicella, Arthrobacter, Balneimonas, Bdellovibrio, Blvii28, C1_B004, Caldilinea, Candidatus Solibacter, Candidatus Xiphinematobacter, Clostridium, Cystobacter, Desulfococcus, Devosia, Epulopiscium, Fusibacter, Gallionella, Gemmata, Gemmatimonas, Geobacter, GOUTA19, Halomonas, Hydrogenophaga, Hyphomicrobium, LCP-6, Luteolibacter, Methylibium, Methylocaldum, Methylotenera, Mycobacterium, Nitrospira, Paludibacter, Pedobacter, Pedosphaera, Phenylobacterium, Planctomyces, PSB-M-3, Pseudomonas, Rhodobacter, Rhodoferax, Rhodoplanes, Roseomonas, SHD-231, Sphingomonas, Sulfuricurvum, Sulfuritalea, Synechococcus, Syntrophobacter, Syntrophus, Thiobacillus, Treponema, Trichococcus, WCHB1-05, Epulopiscium, Fusibacter* and *SJA-88* were identified as tolerant (to nitrate and phosphate) (either less or highly) bacterial genera in the microcosms of wetland sites 1, 2, 5 and 6 (Table 3.8).

Table 3.8: Category of bacterial Indicator genera (sensitive, tolerant, intolerant) in the metal and nutrient microcosms of wetland site 1,2,5 and 6 in the chapter.

Genera- metal microcosm

Intolarant	**Sensitive**	**Tolerant**	
		Highly tolerant	Less tolerant
Methylobacterium, Microbacterium,Shewanella,Cupriavidus, Enterococcus, Rhizobium, Enterobacter, Corynebacterium, Chlorobium, Exiguobacterium,Psychrobacter, Aquicella	*Agrobacterium,Gemmata,Sphingomonas, Nitrospira,Balneimonas,Chlorobaculum, Aeromonas,Pedosphaera, Arthrobacter*	*Nocardioides, Holophaga, Gemmata,Syntrophus, Bacteroides,Anaerolinea,Geobacter, Candidatus_Solibacter, Cystobacter,Desulfococcus,Syntrophobacter,GOUTA19, Paludibacter,Nitrospira, Clostridium, Blvii28, Methylocaldum,Synechococcus,Candidatus_Xiphinematobacter, WCHB1-05, Aeromonas, Luteolibacter, Caldilinea, C1_B004,Methylibium, Anaerovorax, Treponema, Sulfurimonas,Chlorobaculum, Epulopiscium, Caldilinea Pedosphaera,Bdellovibrio,Flavobacterium, SJA-88,Blvii28 LCP-6, Thiobacillus, Rhodoplanes, Sulfuritalea,Rhodoferax, Methylotenera, Rhodobacter, Gemmatimonas,*	*Candidatus Solibacter,Burkholderia,GOUTA19 Holophaga,Sphingomonas,Syntrophus,Anaerolinea, Geobacter,Candidatus_Solibacter,Cystobacter, Desulfococcus,Syntrophobacter,Paludibacter Nitrospira, Methylocaldum, WCHB1-05, C1_B004, Candidatus_Xiphinematobacter, Synechococcus, Balneimonas, Trichococcus, Mycobacterium, Sulfurimonas Epulopiscium, Ralstonia, Pedosphaera, Bdellovibrio, Flavobacterium, Clostridium, SJA-88, Luteolibacter Treponema, Caldilinea, LCP-6, Thiobacillus, Rhodoplanes Sulfuritalea, Rhodoferax, Methylotenera, Planctomyces Rhodobacter, Spirochaeta, Halomonas,Gallionella Roseomonas, PSB-M-3, Aquicella, Dechloromonas Bacillus, Devosia, Hydrogenophaga, Sediminibacterium Phenylobacterium, Sulfuricurvum, Pedobacter Hyphomicrobium, Pseudomonas*

		Spirochaeta, Halomonas, Gallionella, Roseomonas, PSB-M-3, Fusibacter, Dechloromonas,Bacillus, Devosia, Sediminibacterium, Hydrogenophaga,Prosthecobacter, Phenylobacterium,Sulfuricurvum, Arthrobacter, Pedobacter,Hyphomicrobium,Pseudomonas	

Genera- nutrient microcosm

Intolarant	**Sensitive**	**Tolerant**	
		Highly tolerant	Less tolerant
Psychrobacter, Enterobacte, Corynebacterium, Brevundimonas, Cronothrix,Methylobacterium Microbacterium, Shewanella, Cupriavidus Enterococcus, Rhizobium,Agrobacterium Stenotrophomonas, Chlorobium, Chlorobaculum	*Exiguobacterium, Burkholderia Aeromonas*	*Geothrix,Bacillus, Dechloromonas, Flavobacterium, Holophaga, Prosthecobacter,Ralstonia, Sediminibacterium, Spirochaeta, Chlorobium,Bacteroides, Sulfurimonas, Anaerolinea, Anaerovorax,Arthrobacter,Bdellovibrio, Blvii28,Caldilinea, Candidatus Solibacter, Candidatus Xiphinematobacter, Clostridium Cystobacter, Devosia, Gallionella,Gemmata,Geobacter,GOUTA19 Halomonas,*	*Flavobacterium, Holophaga, Nocardioides, Ralstonia, Sediminibacterium, Spirochaeta, Chlorobaculum, C1_B004, Caldilinea, Anaerovorax,Aquicella, Arthrobacter, Bdellovibrio, Blvii28, Candidatus Xiphinematobacter, Clostridium, Gemmata Cystobacter,Desulfococcus,Epulopiscium,Fusibacter,Gemmatimonas,Geobacter,GOUTA19, Nitrospira, Paludibacter,Pedobacter, Pedosphaera, Phenylobacterium, Halomonas, Hydrogenophaga, Hyphomicrobium, LCP-6, Luteolibacter, Methylibium, Methylocaldum, Methylotenera, PSB-M-3, Pseudomonas, Rhodobacter, Rhodoferax, Rhodoplanes, Roseomonas,SHD-*

		Hydrogenophaga, Hyphomicrobium, LCP-6, Mycobacterium, Luteolibacter, Methylibium, Methylocaldum, Methylotenera, Nitrospira, Paludibacter,Pedobacter, Pedosphaera Phenylobacterium,Planctomyces,PSB-M-3, Pseudomonas, Rhodobacter, Rhodoferax, Rhodoplanes, Roseomonas,Sphingomonas Sulfuricurvum, Sulfuritalea, Synechococcus, Syntrophobacter Syntrophus, Thiobacillus, Treponema, WCHB1-05, Fusibacter,SJA-88	*231,Sphingomonas, Trichococcus Sulfuricurvum, Sulfuritalea, Synechococcus, Syntrophobacter Syntrophus, Thiobacillus, Treponema, Epulopiscium, Fusibacter,Trichococcus, SJA-88*

Discussion:

The goal of this present chapter was to use controlled microcosms constructed from sediments collected from wetlands across a gradient of land use types in an urbanizing watershed to characterize how bacterial and ecotoxicological bioindicators respond to continuous pollution (nutrient and metal) stress in wetland ecosystems.

The concentrations of Pb decreased over time the experiments of the metal microcosm experiment, both in high and low level of treatment (Figure 3.3). The removal mechanisms of metals in wetlands are complex (Xiao et al. 2013). Metals may be removed from water and get retained within the sediments. This removal can happen through abiotic or biotic process (Knox et al. 2006). The abiotic process may involve settling and sedimentation, redox and precipitation. The biotic process involves microorganisms related or mediated sorption and plant uptake (Xiao et al. 2013). Ability of a wetland to retain pollutants like metals depends on the hydrology of the system. If the system is well-drained then the rate of oxidation is high, forming oxide and oxyhydroxides, resulting in metal/oxide complexes (Sinicorpe et al. 1992). These metal microcosm experiments of this present chapter were drained at the interval of 7 days. Figure 3.24 shows that the dissolved oxygen level in mg/L was between 6.5 – 8 mg/L and the oxygen level increased at the end of the experiment with drop in temperature (^{0}C). According to (EPA 1986), The Federal water quality criteria for dissolved oxygen in freshwater bodies should be between 6 mg/L (in warm water) and 6.5 mg/L (in cold water) as a lowest 7 day mean for aquatic life to live. Therefore, the water in the microcosms was not anoxic/hypoxic for life. The oxygen level was not low, so the metals (Pb and Cu) added in the water of the microcosms formed oxide and oxyhydroxides. This resulted in metal/oxide complexes (Sinicorpe et al. 1992) and thus the metals (Pb and Cu) were retained in the sediments of the microcosms. Experiments on

assessment of contaminant such as Pb, Cu and Zn retention in constructed wetland sediments showed that elements of these pollutants (Pb, Cu, Zn) once strongly bound to sediment and form complexes that is unlikely to be broken (Knox et al. 2006). Reduction of Pb and Cu from day 0 or start of the experiment to day 15 or end of this microcosm experiment is consistent with the hypothesis that these microcosm sediments formed complexes such as oxide and oxyhydroxides, particularly for Pb, thus were retained in the sediments .

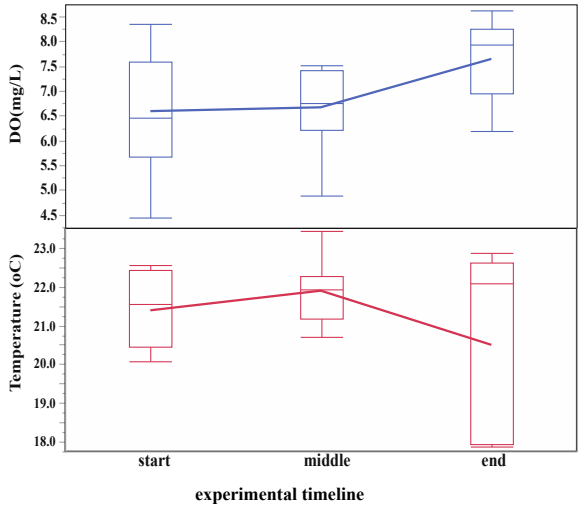


Figure 3.24: Box plot showing the variation of temperature and DO (mg/L) in the metal microcosm from start (day 0) to end of the experiment (Day 15).

In metal microcosm, the root inhibitions of ecotoxicological indicators (*Lepidium sativum* ,*Sinapis alba* and *Sorghum saccharatum*) from microcosms of wetland sediments were lower both in low and high treatments (of Pb and Cu) the microcosms at the end of the experiment (Figure 3.5). But only the effect was significant for *Sorghum saccharatum* (P=0.0153) (Figure 3.7). This again implies a clear reduction in toxicity in the sediments from the start to the end of the experiment, but still toxicity was higher in the treatment microcosm by adding Pb and Cu,

hence there was no facilitation of growth as for the case of the native (control) sediment (Figure 3.5). The effect of experimental timeline (day effect) and treatment level (high and low) on the stem growth inhibition of ecotoxicological indicators on the was not statistically significant as discussed in the results of this present chapter.

The bacterial phyla and genera Shannon, Simpson diversity indices from the sediments of microcosms of wetland sites was lower in low and high treatments for the metals microcosms over time(Figure 3.6). The effect of experimental timeline was statistically significant of the Simpson diversity indices of phyla (P=0.0045) and genera (P=0.0314) (Figure 3.6). The effect of treatment level was not statistically significant as described in results. This indicates the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) were negatively affected by addition of Pb and Cu in the water of the metal microcosms in mg/L, which did not happen in the native (control) sediments (Figure 3.6).

Previously in chapter 1, with increase in metal component 2 in positive axis (or increase in Pb concentration in ppm), the root growth inhibition of the *Lepidium sativum, Sinapis alba, Sorghum saccharatum* decreases or growth was facilitated (Figure 1.10). Unlike this present chapter, it was observed that with increase in Pb concentration, root inhibition of *Sorghum* was increased (Figure 3.7 and Table 3.3). These trends were not very clear or significant in the other root of plant indicator species (*Lepidium sativum* and *Sinapis alba*) even with the stem inhibition of any of the plant indicator species. Pb and Cu was added as a source of pollutant in this experiment. Pb is not an essential micronutrient for plant but species like *Sorghum bicolor* can accumulate Pb in their tissues up to a threshold level for which these plants are used for phytoremediation of Pb (Vamerali et al. 2010). Similarly, *Sorghum saccharatum* of this present chapter experiment grew, when exposed to element like Pb but up to a certain threshold point

which was likely crossed in this microcosm experiment as Pb was added in the water over a period (15 days) but that was not the case in Chapter 1. So, with the increase in Pb concentration, the root inhibition of *Sorghum saccharatum* increased in this present chapter microcosm experiment (Figure 3.7 and Table 3.3).

This was confirmed by the experimental timeline effect, where the concentration of Pb decreased over time, in both in high and low level of treatment significantly (Figure 3.5). At the same time, the root inhibitions of *Sorghum saccharatum* were lower both in low and high treatments for the metal microcosms. This implies that *Sorghum saccharatum* was negatively affected by the Pb addition. With increase in Cu concentration root growth inhibition of *Lepidium sativum* and *Sinapis alba* or stem growth inhibition of *Lepidium sativum, Sorghum saccharatum* and *Sinapis alba* did not have any significant trend as described in results.

For the bacterial indicators, the effect of increasing Cu concentration added in the water of metal microcosm significantly decreased the Shannon, Simpson diversity indices of phyla and genera (Figure 3.8 and Table 3.3). With increasing Pb concentration there were significant increase in the Simpson diversity indices of phyla and Shannon, Simpson diversity indices of genera (Figure 3.8 and Table 3.3).

Hence, it is evident from these results that the impact of Cu, decreased the bacterial diversity and number directly. It appears that *in situ* (Roane and Kellogg 1996) and laboratory studies (Knight et al. 1997) bacterial diversity in contaminated soils can be highly correlated to metals concentrations (Ahmed et al. 2018). Studies suggest that long-term heavy metal such as Pb, Cu, Cd and Zn can affect and can also decrease the microbial biomass, activity and diversity (Chen et al. 2014). Specifically, metals like Cu (alone or in combination) can affect the genetic structure and function of the exposed community (Ahmed et al. 2018). Because of which,

increasing Cu concentration(mg/L) added in the water of metal microcosm significantly decreased the Shannon, Simpson diversity indices of phyla and genera.

Metal contaminated ecosystems were shown to exert a highly selective pressure towards transfer of metal-resistance genes and finally developing resistance to metal concentration (Ryan et al. 2005, Cai et al. 2009, Azarbad et al. 2013, 2016). Several field-related studies have demonstrated a change in the microbial community structure and function with higher levels of metal-resistance genes being stably present, even after long-term exposure of the metal pollutant (Olson and Thornton 1982, Hemme et al. 2010, Kang et al. 2013). In a similar study showed Pb-resistant bacteria tend to grow in contaminated soils after a certain timeline (Margesin and Schinner 1996, Sabry et al. 1997, Konopka et al. 1999). In this present chapter, the metal microcosm experiment with increasing Pb concentration there were significant increase in the Simpson diversity indices of phyla and Shannon, Simpson diversity indices of genera (Figure 3.10, Table 3.3), confirming the growing resistance of the bacterial communities with the added Pb. In Chapter 2 also, a significant amount of Pb-resistant, remediating, precipitating, biomethylating bacterial communities were identified from the wetland site sediments (Table 3.7). Additionally, with increase in of metal component 2 (effect of Pb and Hg concentration in ppm), the Shannon diversity index of phyla, the Simpson diversity index of phyla, genera increased significantly (Figure 2.10 and 2.11). The metal microcosm experiment of this present chapter confirmed this trend of growing resistance of the sediment bacterial communities with the added Pb. Presence of these specific Pb-resistant bacteria have been discussed layer in the discussion.

In the sediment of the metal microcosm, the concentration of metals in the sediments did not significantly change over the time course of the experiments in the metal microcosm

experiment (Figure 3.4), with the exception other than the concentration of As that decreased from start (day 0) after adding to the end (day 15) of the experiment of the metal microcosm experiment (Figure 3.4).

In terms of the relationship, with increase in Ni concentration in the sediments of the metal microcosm, a decrease in root growth inhibition of *Sorghum saccharatum* was observed (Figure 3.9) – Ni is a widely-distributed element, also creates toxic physiological effects on plants when accumulated above a certain body weight especially in the vegetative and reproductive parts (Soon et al. 1980, S. Sengar et al. 2008). Experiments on *Sorghum bicolor* show that sometimes Ni can bioaccumulate in this species and can efficiently take up Ni very efficiently but also can be toxic after a certain level (Al Chami et al. 2015) . In this experiment of the present chapter, *Sorghum saccharatum* also accumulated Ni efficiently and the effect was not toxic until the body weight after accumulation is achieved, hence decrease in root growth inhibition was observed.

With increase in Cd concentration in the sediments of the metal microcosm, an increase in root growth inhibition of *Sorghum saccharatum* and *Sinapis alba* was observed (Figure 3.9). Other research studies have proven that Cd could get accumulated in the roots of *Sinapis* and can cause in the inhibition of root growth (Fargašová 2001). Similarly, root growth in *Sorghum* can be reduced with an increase in Cd supply (Zancheta et al. 2015). With increase in As concentration(ppm) in the sediments of the metal microcosm, an increase in root growth inhibition of *Lepidium sativum* was observed in the results (Figure 3.9). Other researches have also shown that As at a certain concentration, can negatively affect plant development in *Lepidium sativum*(Umar et al. 2013).

With increase in Ag and Ni concentration in the sediments of the metal microcosm, an increase in stem growth inhibition of *Sinapis alba* was observed (Figure 3.9). Studies have shown that growth in the root of *Sinapis alba* can get affected by Ag in ionic as well as in nanoparticle form (Kaduková et al. 2015), similarly Ni also can cause unfavorable changes in shoots and roots of *Sinapis alba* (Matraszek et al. 2017). With increase in Ag concentration in the sediments of the metal microcosm, an increase in stem growth inhibition of *Sorghum saccharatum* was observed. Ag after a certain concentration can affect the growth of *Sorghum saccharatum* negatively(Lee et al. 2012). With increase in Rb concentration in the sediments of the metal microcosm, a decrease in stem growth inhibition of *Sinapis alba* was observed. Implying Rb was not creating any toxic effects of *Sinapis alba* stem growth (Figure 3.9).

With increasing Cd, Zn level, an increase in the Shannon diversity indices of phyla was observed (Figure 3.10). With increasing As and Zn level, an increase in the Simpson diversity indices of phyla was observed (Figure 3.10). With increasing Zn level, an increase in the Simpson diversity indices of genera was observed (Figure 3.10). To survive in toxic environment, some microbes evolved defense mechanisms to metabolize and transform heavy metal into a less toxic forms and consequently become heavy metal resistant microbes are formed. As a result, toxic metals like Cd, As, even micronutrients such as Zn, Ni can exert toxicity to living cells like microbes by growing resistance and thus can less impact the diversity and number of the sediment bacterial community (Gurave et al. 2015, Prabhakaran et al. 2015, Yazdankhah et al. 2018). With increasing Hg level, a decrease in the Shannon diversity indices of phyla was observed (Figure 3.10). Hg is a toxic element that can alter the bacterial community (Gurave et al. 2015, Prabhakaran et al. 2015, Vasileiadis et al. 2015, Yazdankhah et al. 2018).

Nutrient microcosm:

The concentration of nitrate and phosphate decreased over time in the nutrient microcosm experiment, both in high and low level of treatment (Figure 3.3) and both the reductions of nitrate and phosphate concentration were significant (Table 3.6). Treatment wetland systems are used as water remediation processes, such as secondary and tertiary treatment of wastewater, and surface water runoffs. Previous studies have also shown that wetlands can remove nitrate and phosphate from nutrient-rich waters (Gersberg et al. 1986, Bachand and Horne 2000, Reilly et al. 2000, Shannon et al. 2000, Walker and Shannon 2006).

The effect of experimental timeline (day effect) and treatment level (high and low) on the stem and root growth inhibition of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* was not statistically significant (Figure 3.5). The effect of experimental timeline (day effect) was statistically significant on the Shannon, Simpson diversity indices of phyla and genera. The diversity was high at the start of the experiment but decreased at the end (Figure 3.5).

A wetland system can remove nitrogen because of the proximity of aerobic zones that can support autotrophic nitrifying bacterial population and anaerobic zones supporting heterotrophic denitrifying bacterial population. On the other hand, phosphate removal can be done through its adsorption onto soil or sediment, incorporation into microbial mass, uptake in plant biomass and precipitation (Thomas et al. 1995). Studies have shown that nitrate and phosphate can be removed from wetland ecosystems as well as from wetland microcosms very efficiently (Busnardo et al. 1992, Jasper et al. 2014, Kadlec 2016). Similarly, in the present chapter in these nutrient microcosms also, nitrate and phosphate added in water were reduced during the experiment and the residing bacterial population may have played a very important role in this remediation. There was a decrease of Shannon and Simpson diversity indices of phyla and

genera over time in the experiment (Figure 3.6). This suggests that, the reduction (or deficiency) of nitrate and phosphate may have contributed to the decrease of Shannon and Simpson diversity indices of phyla and genera. These results are consistent with the finding that the wetland microbial community was lower in diversity and number associated with phosphate deficiency as in chapter 2. Other studies have also shown that phosphorus deficiency can also negatively affect the growth and development of microorganisms, thus reducing the number and diversity (Kulakovskaya 2014)

Certain trends in the growth inhibition was clear. For example, in *Sinapis alba* (lower treatment level – in root) and *Sorghum saccharatum* (lower and higher treatment level – in root), growth inhibition decreased over time (Figure 3.5). This implies that the reduction (or deficiency) of nitrate and phosphate from start (day 0) to the end (day 30) of the experiment, created a decrease on the growth inhibition or facilitated the growth. In chapter 1, the increasing phosphate resulted in increasing root inhibition significantly (Figure 1.8). The phosphorus cycle is sedimentary but gaseous, forming complexes within organic matter in wetland soil (Kadlec and Wallace 2009). This implies that in these wetland sites the available phosphorus is not adequate for plants to grow properly resulting in growth inhibition. But when phosphorus was supplied outside in the microcosm experiments (as in this present chapter) there was facilitation of growth.

With increasing addition of nitrate in the microcosm water the root growth inhibition of *Sorghum saccharatum* was increased but at the same time the stem growth inhibition decreased significantly with negative estimate values (Figure 3.11 and Table 3.6). Clearly in this case nitrate was affecting root and stem of the same plant differently. Previously in the experimental timeline effect, it was seen that, *Sorghum saccharatum* (lower and higher treatment level – in

root), growth inhibition decreased over time (Figure 3.5) at the same time the concentration of nitrate in the nutrient microcosm experiment, both in high and low level of treatment (Figure 3.3). Hence, the negative effect on root was more pronounced than the stem growth inhibition. Although nutrients like nitrate or a nitrogen form are required for plant, but excess can cause ill-effects (Mitsch and Gosselink 2000, USGS 2017) as here nitrate is causing increase in root growth inhibition.

Pb and Cu concentration along with nitrate and phosphate concentration were observed to be high in the autoclaved sediments (Figure 3.12). This is consistent with the hypothesis that the bacterial community present in the wetland sediments may serve to alleviate the pollutant (Pb, Cu, nitrate and phosphate) concentration as bacterial community were less in number and diversity in the autoclaved sediment compared to non-autoclaved sediment (Figure 3.15). This however, does not rule out the possibility that autoclaving may have changed the physical structure of the sediments that could have affected the XRF methodology in detecting Pb. This remains to be investigated. However, in metal microcosm, the level of all the measured metals, Ag, As, Cd, Fe, Hg, Ni, Pb, Rb, Zn were not observed to be different between autoclaved and non-autoclaved sediments and was also not statistically significant (Figure 3.15). Hence in case of sediment metals the effect of bacteria was not very profound. Metals in sediment once strongly bound to sediment and form complexes that is unlikely to be broken (Knox et al. 2006) hence the metals in the sediments were not likely in an available form within the microcosms.

The effect of autoclaving did not have a specific trend or significant effect of autoclaving on the stem and root growth inhibition of *Lepidium*, *Sinapis* and *Sorghum* in nutrient and metal microcosm (Figure 3.14). Shannon and Simpson diversity indices of phyla and genera observed to higher in the non-autoclaved sediments of metal and nutrient microcosms for both in low and

high level of treatment with few exceptions (Figure 3.15). The autoclave process was performed in this experiment as a negative control to observe the effect of bacteria on the pollutants, when bacterial community is present in low numbers in the sediments. Hence, it is very common to see the effect of autoclaving creating a significant effect on the bacterial community (Figure 3.15). Although certain bacteria did survive the autoclaving process as well and their abundance in the sediments increased after autoclave (Figure 3.16 – 3.23). This might have happened due to two reason, firstly possibly some genera were simply resistant to the autoclave heating process and secondly as the autoclaved sediments were used in the microcosms, the water was changed at 7 day interval and nutrients were also renewed every 7 days. More importantly the metal microcosm experiment ran for 15 days and nutrient microcosm experiment ran for 30 days after the autoclave so it was also possible that some bacterial genera actually grew back during this time.

Patterns of predictive bacterial indicators detected in microcosm experiments:

A bioindicator is a single or group of species whose status, functional abilities or population can depict a picture about the quality of the environment and the cumulative effects of several pollutants present in the environment. Hence, it is important for biological indicators to be sensitive to wide range of biological stresses. The indicators should be able to discriminate human caused changes from all the background "noise" of natural variation (Karr and Chu 1999). Soil microbial communities play a very important role in the ecosystem and provides a multitude of ecosystem services thus impacting the overall functioning of the soil environment. Hence, soil microbial indicators can offer significant advantages over traditional biological and chemical methods, especially at the genus taxonomic level, that might not be available in the higher taxonomical ranks (Hermans et al. 2016).

In this present chapter, the first two major types of microbes that were detected were firstly intolerant (to Pb and Cu) bacterial genera that were present at the start of the experiment (day 0) but disappeared later in all the metal treatments (Table 3.8). The second type was sensitive (to Pb and Cu) bacterial genera, were absent or could not sustain in the high treatments of the metal microcosm at the end of the experiment (day 15) compared to start of the experiment (day 0) (Table 3.8). In the high treatment of the metal microcosm the metals were applied with higher levels of concentration as listed in Table 3.1.

Among all these identified these bacterial genera *Chlorobium* was an intolerant (to Pb and Cu) genus identified among the wetland site microcosms of 2, 5 and 6 but was not detected in the microcosms of wetland site 1. This implies that this genus totally disappeared in all the treatments by the end or day 15 of the experiment of the microcosms of wetland site 2, 5 and 6. Hence, based on these results it could be concluded that *Chlorobium* very sensitive genus when exposed to heavy metal pollution for an extended period especially Pb and Cu. Similarly, the genus *Aquicella* was not detected in the microcosms of wetland site 2, but was observed to be an intolerant (to Pb and Cu) genus in the microcosms of wetland site 6 although was less tolerant in the microcosms of wetland sites 1 and 5. Implying that *Aquicella* also was not a very tolerant genus when exposed to heavy metal pollution for a long period especially Pb and Cu. Now the bacterial genera *Gemmata, Sphingomona, Nitrospira, Balneimonas, Chlorobaculum, Aeromonas, Pedosphaera* and *Arthrobacter* were observed to be resistant to Pb and Cu in most of the microcosms of wetland sites of 1, 2, 5 and 6 but were sensitive or they disappeared in the high concentration in certain microcosms also. For example, *Pedosphaera,* was a tolerant (to Pb and Cu) bacterial genus in the microcosms of wetland sites 2,5 and 6 but was sensitive (to Pb and Cu) in the microcosms of wetland site 1 as it became zero abundance in the high level of

treatment where metals Pb and Cu was added in higher concentration in microcosm water. This implies that pollutant applied to the water of wetland site 1 created more stress compared to other in these sediments residing genera causing them to decrease in abundance, likely because of the sediment and metal interaction in wetland site 1 microcosm. Previous studies have also indicated that exposure to heavy metal pollution can lead to decrease in soil bacterial abundance diversity (Chen et al. 2014, Xie et al. 2016).

Some of the bacterial genera that were observed to tolerant to Pb and Cu (either highly or less) among the microcosms of almost all wetland sites were: *Nocardioides, Burkholderia, Holophaga, Syntrophus, Bacteroides, Anaerolinea, Geobacter, Candidatus Solibacter, Cystobacter, Desulfococcus, Syntrophobacter* and more. The complete list is in Table 3.8. All the tolerant species could withstand the stress factor of Pb and Cu well, but some were less tolerant and some were more or highly tolerant. Although we found the same genus to be less tolerant within the microcosms of a wetland site were highly tolerant into the others, this could be a factor of the wetland site sediment chemistry. The less tolerant bacterial genera were lower in abundance in the high- concentration treatments of the microcosms and the highly tolerant bacterial genera were higher in abundance in the high and low concentration treatments. However, all these tolerant genera also survived the autoclaved treatments of high temperature as well, which could be because of two reasons firstly, these genera were simply resistant to the autoclave heating process and secondly they likely grew back as the experiment went along as discussed before (Table 3.8). But this property of surviving the high heat of these tolerant genera needs to be investigated in future studies.

Among these tolerant genera identified *Flavobacterium, Bacillus, Pseudomonas, Clostridium, Burkholderia, Aeromonas, Ralstonia* and *Arthrobacter* have already been identified

as Pb-resistant, remediating, precipitating, biomethylating and/or Hg resistant and/or Hg bioremediating bacterial genera in chapter 2 from the field study (Gummersheimer and Giblin 2003, Chen et al. 2011, Kafilzadeh et al. 2012, Zhang et al. 2012, 2017, Figueiredo et al. 2014, Tipayno et al. 2018, Ayangbenro et al. 2019). *Clostridium*, *Pseudomonas*, *Bacillus* and *Arthrobacter* have also been identified as Cu- resistant bacterial genera in previous studies such as in (Kunito et al. 1997, He et al. 2010, Santo et al. 2010, Andreazza et al. 2011, Altimira et al. 2012, Berg et al. 2012). *Thiobacillus* also has been reported as resistant to various metals (Tipayno et al. 2018) and specifically to Cd (Zhang et al. 2017, Feng et al. 2018). *Gemmatimonas*, *Candidatus Solibacter* and *Nitrospira* has also been reported as resistant to Cd (Feng et al. 2018). *SJA-88*, *Fusibacter* and *Dechloromonas* have also been reported as resistant to Cd (Zhang et al. 2017). *Rhodoferax* has also been identified as Iron-reducing bacteria (Risso et al. 2009). *Flavobacterium* and *PSB-M-3* have also been reported as resistant/remediating to Cr (Pei et al. 2018). *Sediminibacterium* was also reported as resistant to metal treatments (Kou et al. 2018). *Rhodoplanes* have been detected in environments with high As concentrations (Lakshmi et al. 2011, Wang et al. 2016). Strains of *Halomonas* also been reported as resistant to heavy metals like Pb, Zn and Cd (Manasi et al. 2016). *Desulfococcus* has been reported having metal resistance genes especially to Cd (Naz et al. 2005). *Anaerolinea* and *Geobacter* have also been detected with possible resistant properties for heavy metals like As and Pb (Tipayno et al. 2018). (Kou et al. 2018) has observed *Methylibium* as resistant to many heavy metals like Pb, Cu and Zn. (Ding et al. 2017) have identified *Sphingomonas* as metal (Cr and Cd) resistant bacterial genera. The taxonomical phyla that these specific indicators belong to were: Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria.

As discussed before, it is important for biological indicators to be sensitive to wide range of biological stresses. The indicators should be able to discriminate human caused changes from all the background "noise" of natural variation (Karr and Chu 1999). In the present chapter in the metal microcosm experiment, we could identify a wide range of soil bacterial indicators (specifically in genus taxonomic level) either sensitive to high concentration of Pb and Cu or tolerant to metal treatments.

For nutrient microcosm, nitrate and phosphate were added in high and low concentration to the water of nutrient microcosm experiment. Nutrients such as nitrate and phosphate are essential for the growth of plants and animals but excess presence can cause adverse effects. Nitrate is abundantly present in the environment but phosphorus gets into water by soil erosion. Nitrate and phosphate are also artificially introduced in water through chemical fertilizers to grow crops. Excess runoffs from agricultural fields when gets into water bodies, level of nitrate also becomes excess causing water pollution and eutrophication (USGS 2017).

Nitrate and phosphate were applied as a stress in the water of this microcosm experiment in high and low concentration (Table 3.1) over 30 days. The tolerant (nitrate and phosphate) genera of this stress present in the microcosms of all the wetland sites (1, 2, 5 and 6) were: *Bacillus, Dechloromonas, Flavobacterium, Holophaga, Prosthecobacter, Ralstonia, Sediminibacterium, Spirochaeta, Anaerolinea* and more. The complete list is in Table 3.8. All the tolerant species could withstand the stress factor of nitrate and phosphate well, but some were less tolerant and some were more or highly tolerant. Although we found the same genus to be less tolerant (to nitrate and phosphate) within the microcosms of a wetland site were highly tolerant (to nitrate and phosphate) into the others, this could be a factor of the wetland site sediment chemistry. The less tolerant bacterial genera were lower in abundance in the high-

concentration treatments of the microcosms and the highly tolerant (to nitrate and phosphate) bacterial genera were higher in abundance in the high and low concentration treatments. But most of these tolerant genera also survived the autoclaved treatments as well as did not disappear (Table 3.8). As discussed in the metal microcosm section, this property of bacterial genera surviving the high heat of autoclaving process needs to be investigated further in future studies.

Other than the tolerant bacterial genera, *Cronothrix* was also identified as the most intolerant (to nitrate and phosphate) bacterial genera present among microcosms of all the wetland sites (1, 2, 5 and 6).

In this nutrient microcosm experiment where nitrate was applied in high and low concentration as one the stress factors for 30 days. Nitrate is also formed in water bodies naturally through oxidation of other forms of nitrogen like nitrite, ammonia, and organic nitrogen compounds such as amino acids. Wetlands located especially near farmland also receive a high level of nitrate from the farming activities. Excess nitrate can be removed from the ecosystem by denitrification, which is reduction of nitrate (NO_{3}^{-}) into gaseous forms nitrous oxide (N_2O) and dinitrogen (N_2). However, the release of N_2O is detrimental to the environment as it is a potential greenhouse gas as well (Kadlec and Wallace 2009).Microbes play a very important role in this denitrifying process. Microbes can convert nitrate into dinitrogen or N_2 gas, these microbes are called as denitrifiers (Kadlec and Wallace 2009).

Among the bacterial genera identified in this experiment, *Bacillus, Enterobacter, Pseudomonas, Hyphomicrobium, Arthrobacter, Burkholderia, Rhizobium, Thiobacillus, Flavobacterium, Corynbacterium* and *Agrobacterium* has also been identified as denitrifiers in (Smith and Zimmerman 1981, Gerardi 2006, Castellano-Hinojosa et al. 2017). Among which *Bacillus, Pseudomonas, Hyphomicrobium, Arthrobacter, Thiobacillus* and *Flavobacterium* have

been identified as tolerant (less or highly) and highly abundant bacterial genera to the continuous added nitrate stress in the nutrient microcosm experiments in this present chapter as well as in the field studies from chapter 2 (Table 3.7).

The main function of denitrifying bacteria is to break down nitrate. These findings give us a clear direction to what kind of specific bacterial indicators to look for when there is a nitrate stress, and the direction might be confirmed with the presence of specific functional bacterial genera classified as denitrifiers. The taxonomical phyla of these genera were: Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes.

A major part of the organic phosphorus treatment wetland soils and flocs is microbial (Kadlec and Wallace 2009). In a natural environment, numerous microorganisms in soil and rhizosphere can release total soil phosphorus through solubilizing and mineralization (Bhattacharyya and Jha 2012). These groups of microorganisms are called as phosphorus solubilizing microorganisms (PSMs). These PSMs increase the bioavailability of soil insoluble phosphorus for plants in ecosystems (Zhu et al. 2011). In literatures, the identified phosphorus-solubilizing microorganisms (PSMs) genera are: *Pseudomonas, Agrobacterium* (Babalola and Glick 2012), *Bacillus, Burkholderia* (Mamta et al. 2010, Zhao et al. 2014, Istina et al. 2015, Alori et al. 2017), *Ralstonia, Rhizobium* (Tajini et al. 2012), *Serratia, Rhodococcus, Salmonella* and *Thiobacillus* (Postma et al. 2010, David et al. 2014, Alori et al. 2017), *Flavobacterium, Enterobacter, Streptomyces* (Zhu et al. 2011). In Chapter 2 field studies, the phosphorus solubilizing microorganisms (PSMs) genera that were observed during summer 2017 in wetland site 1-6 were: *Pseudomonas, Agrobacterium, Thiobacillus, Ralstonia, Bacillus, Flavobacterium, Enterobacter, Arthrobacter, Streptomyces* and *Rhizobium* (Table 3.7). But in this microcosm experiment phosphorus was added from was applied in high and low concentration in mg/L as

one the stress factors for 30 days (Table 3.1). Hence there were less abundance of phosphorus-solubilizing microorganisms (PSMs) that were observed in this experiment. For example, genus like *Streptomyces* was not observed at all. Genera like *Enterobacter, Rhizobium, Agrobacterium* were present but were highly intolerant and were not present in any of the treatment microcosms other than the day 0 treatments, where no phosphorus was applied.

Although genera like *Pseudomonas, Thiobacillus* and *Flavobacterium* were present and were tolerant in the microcosms of the wetland sites. But *Pseudomonas* was observed to be less tolerant in the microcosms of the wetland sites 1, 2 and 6. *Thiobacillus* was observed to be less tolerant in the microcosms of the wetland sites 1, 2 and 5. *Flavobacterium* was observed to be less tolerant to nitrate and phosphate in the microcosms of the wetland sites 1, 2 and was not observed in the microcosm of the wetland site 6. Implying that these genera were low in abundance in the higher treatment levels of nutrients (where phosphorus was also applied in higher concentration –Table 3.1) in most of the wetland site microcosms, compared to the lower treatment levels. Genus like *Burkholderia,* although was not detected in summer 2017 in wetland site 1-6 as in chapter 2, but in this nutrient microcosm experiment this genus was not present in the in the higher treatment levels of nutrients and was classified as a sensitive indicator (Table 3.7). Only two genera *Bacillus* and *Ralstonia* were tolerant to the phosphorus stress and were abundantly present in the nutrient microcosm experiment (Table 3.8). Hence these two genera did not respond to the applied phosphate stress. The taxonomical phyla of these genera were Firmicutes and Proteobacteria.

Hence, in this nutrient microcosm experiment where phosphate was applied in high and low concentration as one the stress factors for 30 days other than the sensitive and tolerant and intolerant indicators of stress as phosphate, we also could identify certain genera such as:

Pseudomonas, Thiobacillus, Flavobacterium, Enterobacter, Burkholderia and *Rhizobium* who were mostly less tolerant and were specifically low in abundance in the higher treatment levels of nutrients (where phosphorus was also applied in higher concentration) (Table 3.8). These genera have been identified as phosphorus solubilizing microorganisms (PSMs) among other literatures as discussed before. These findings give us a clear direction to what kind of specific bacterial indicators to look for when there is a phosphate stress, and the direction might be confirmed with the decrease of specific functional bacterial genera classified as phosphorus solubilizing microorganisms (PSMs).

Future Research:

So far we were able to identify specific bacterial genera in relation to metal and nutrient stress. But a limitation to this study is that we were only able to identify taxi up to the genus level. To understand this correlation of bacterial genera and pollutants more specific analysis needs to be made. After long term exposure stress, microbial communities develop resistance systems (Nies 1999, Hemme et al. 2010, Chen et al. 2018). For example, bacterial communities often develop metal resistance genes (MRGs) in response to metal pollution. Widespread metal pollution facilitates co-selection of antibiotic resistance genes (ARGs) and MRGs together(Li et al. 2017). Literatures have shown significant co-occurrence or co-selection of MRGs or ARGs in soil contaminated with metals such as As, Cr, Cd, Cu, Ni, Pb and Zn (Pal et al. 2015, Li et al. 2017, Chen et al. 2019, Liu et al. 2019). Studies have reported horizontal gene transfer of ARGs and MRGs as often these two genes are carried by the same plasmid (Pal et al. 2015, Li et al. 2017, Chen et al. 2019). MRGs and ARGs can respond to the influence of heavy metals (Chen et al. 2019). These studies have also reported genera such as *Escherichia, Staphylococcus, Bacillus,*

Pseudomonas, Burkholderia, Rhizobium, Corynebacterium, Streptococcus, Enterobacter, Cupriavidus, Ralstonia and *Streptococcus* with metal resistance genes in plasmids with ARGs or without ARGs (Boyd and Barkay 2012, Pal et al. 2015). These genera were detected in the present study, in-fact genera such as *Bacillus, Pseudomonas, Burkholderia, Rhizobium, Ralstonia, Streptococcus, Enterobacter* and *Corynebacterium* were abundantly found in the present study and also were identified as metal-resistant genera in the present study. Hence, for future studies identification of specific metal resistance genes such as merA – for Hg resistance (Boyd and Barkay 2012), cop A, cop B, pco A, pco C, pco D – for Cu resistance and pbr T – for Pb resistance (Chen et al. 2019) with or without ARGs, could be investigated to establish the functional capabilities of metal resistance genera such as *Bacillus, Pseudomonas, Burkholderia, Rhizobium, Ralstonia, Streptococcus, Enterobacter* and *Corynebacterium* as detected in present study at the genetic level. This will establish the candidacy of these bacterial genera to be selected as metrics for a multi-metric index more strongly.

Conclusion:

The first two predictions in this chapter were that there would be reduction in the pollutant (Pb, Cu, nitrate and phosphate) added to the water of metal and nutrient microcosms from beginning to the end of the experiments of microcosms and there will be reduction in the growth inhibition responses of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* (the ecotoxicological indicators) grown in the sediments of the of metal and nutrient microcosms from beginning to the end of the experiment in metal and nutrient microcosms. Based on the predictions we clearly see there is reduction of Pb, Cu, nitrate and phosphate added to the water of metal and nutrient microcosms from beginning to the end of the experiments of microcosms. In terms of the bio-indicators, in metal microcosms it was observed that the root inhibition of

Sorghum saccharatum was lower at the end of the experiment compared to the beginning, indicating that reduction of the metals Pb and Cu also reduced the growth inhibition. Some similar trends were also observed in the nutrient microcosms with the indicators *Sinapis alba* and *Sorghum saccharatum* (Figure 3.5, 3.6). The regression model showed that with increase in Pb concentration , root inhibition of *Sorghum saccharatum* was increased. Again, confirming the fact that only reduction of toxic metals like can reduce toxicity in sediments indicated by reduction of root inhibition of *Sorghum* (Figure 3.7). In the nutrient microcosms, it was also observed that with increasing addition of nitrate in the microcosm water the root growth inhibition of *Sorghum saccharatum* was increased. Indicating that although nutrients like nitrate or a nitrogen form are required for plant, but excess can cause ill-effects (Figure 3.11).

The third prediction was that there would be increases in the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) from beginning to the end of the experiment metal and nutrient microcosms as the pollutants (Pb, Cu, nitrate and phosphate) will be reduced. In contrast to the prediction it was observed that the bacterial phyla and genera Shannon, Simpson diversity indices microcosms of wetland sites 1, 2, 5 and 6 were lower in low and high treatments (of Pb and Cu applied in water) of the microcosms at the end of the experiment (Figure 3.8). This indicates the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) were negatively affected by addition of Pb and Cu in the water of the metal microcosms even at reduced condition at the end of the experiment (Figure 3.6). Similar trends were seen in the nutrient microcosm as well. The Shannon, Simpson diversity indices of phyla and genera was high at the start (day 0) of the experiment but decreased at the end (day 30). This implies that, the reduction (or deficiency) of nitrate and phosphate from start (day 0) to the end (day 30) of the experiment caused in the decrease of Shannon and Simpson

diversity indices (Figure 3.6). With increasing Cu concentration added in the water significantly decreased the Simpson diversity indices of phyla and genera with increasing Pb concentration there were significant increase in the Simpson diversity indices of phyla and Shannon, Simpson diversity indices of genera (Figure 3.8), suggesting how metals like Pb and Cu (alone or in combination) can affect the genetic structure and function of the exposed community (Ahmed et al. 2018).

For the nutrient microcosm, a trend of increase in the Shannon, Simpson diversity indices of phyla and genera was observed with increase in the phosphate added in the microcosm water indicating that phosphorus deficiency can also negatively affect the growth and development of microorganisms, thus reducing the number and diversity (Kulakovskaya 2014).

The next prediction was with the increase in the detected sediment metal concentration from beginning to the end the growth inhibition responses of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* (the ecotoxicological indicators) will increase and bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) will decrease in the metal microcosm experiment. The concentration of As detected in the metal microcosm sediments did significantly change from start (day 0) after adding to the end (day 15) of the experiment of the metal microcosm experiment (Figure 3.4). Also, sediment metals such as Ni, Cd, As, Ag, Zn, Fe and Hg detected in the sediment microcosms created significant relationships with the growth inhibition responses of *Lepidium sativum, Sinapis alba and Sorghum saccharatum* (the ecotoxicological indicators) and bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) (Figure 3.9).

After this it was predicted that the bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) and the pollutant concentration in mg/L (Pb, Cu, nitrate and

phosphate) will be lower in the autoclaved sediments of the metal and nutrient microcosms but the growth inhibition responses of the ecotoxicological indicators (*Lepidium sativum, Sinapis alba and Sorghum saccharatum)* will be higher. The results showed that the Shannon and Simpson diversity indices of phyla and genera observed to higher in the non-autoclaved sediments of metal and nutrient microcosms for both in low and high level of treatment with few exceptions. The autoclave process was performed in this experiment as a control to observe the effect of bacteria on the pollutants, when bacterial community is present in low numbers in the sediments. Hence, it was very common to see the effect of autoclaving creating a significant effect on the bacterial community (Figure 3.15). Pb and Cu concentration along with nitrate and phosphate concentration was observed to be high in the autoclaved sediments (Figure 3.13). This confirms that the bacterial community present in the wetland sediments could reduce the pollutant (Pb, Cu, nitrate and phosphate) concentration in the non-autoclaved sediments as bacterial community were less in number and diversity in the autoclaved sediment compared to non-autoclaved sediment (Figure 3.15). Although no significant trends were seen among the ecotoxicological indicators when exposed to autoclaved/non-autoclaved sediments (Figure 3.14).

The last prediction was that there will be some specific bacterial genera present in the sediments of the metal and nutrient microcosms which will be predictive indicators to the added pollutants (Pb, Cu, Nitrate and Phosphate) added to the water of metal and nutrient microcosms. In the present chapter from metal microcosm experiments, a wide range of soil bacterial indicators (specifically in genus taxonomic level) either sensitive to high concentration of Pb and Cu or tolerant genera to metal treatments (such as: *Flavobacterium, Bacillus, Pseudomonas, Clostridium, Burkholderia, Aeromonas, Ralstonia, Arthrobacter, Thiobacillus, Halomonas, Anaerolinea, Methylibium* and *Geobacter)* were identified.

The nutrient microcosm experiment findings give us a clear direction to what kind of specific bacterial indicators to look for when there is a nitrate stress, and the direction might be confirmed with the presence of specific functional bacterial genera classified as denitrifying genera (such as *Bacillus*, *Pseudomonas, Hyphomicrobium, Arthrobacter*, *Thiobacillus* and *Flavobacterium)*. As denitrifiers break nitrate into N_2O and N_2. Also, these findings give us a clear direction to what kind of specific bacterial indicators to look for when there is a phosphate addition, and the direction might be confirmed with the decrease of specific functional bacterial genera (and species) classified as phosphorus solubilizing microorganisms (PSMs) genera (such as: *Pseudomonas*, *Thiobacillus*, *Flavobacterium*, *Enterobacter*, *Burkholderia* and *Rhizobium).*

These results answer the research questions that the ecotoxicological indicators (*Lepidium sativum, Sinapis alba and Sorghum saccharatum)* and sediment bacterial indicators (Shannon and Simpson diversity indices of phyla and genera) in sediments correlate with manipulated changes in the concentration of nutrients (nitrate, phosphate) and metals (Cu, Pb) added to the water of experimental microcosms.

Also, we did observe specific bacterial taxa or bacterial specific assemblages of identified bacterial taxa that can potentially serve as predictive indicators of the induced pollution to the water of experimental microcosms (i.e. *ex ante* impact indicators for ecological risk assessment). Although we detected some predictive genera but only more detailed functional analysis of these taxonomical species can confirm these trends of their response to pollutant loads such as nitrate, phosphate, Cu and Pb.

Bibliography

Ahmed, A. M., E. Lyautey, C. Bonnineau, A. Dabrin, and S. Pesce. 2018. Environmental concentrations of copper, alone or in mixture with arsenic, can impact river sediment microbial community structure and functions. Frontiers in Microbiology 9:1–13.

Ahn, C., P. Gillevet, and M. Sikaroodi. 2007. Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading. Ecological Indicators 7:852=863.

Alori, E. T., B. R. Glick, and O. O. Babalola. 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Frontiers in Microbiology 8:1–8.

Altimira, F., C. Yẽez, G. Bravo, M. González, L. A. Rojas, and M. Seeger. 2012. Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. BMC Microbiology 12.

Andreazza, R., S. Pieniz, B. C. Okeke, and F. A. O. Camargo. 2011. Evaluation of copper resistant bacteria from vineyard soils and mining waste for copper biosorption. Brazilian Journal of Microbiology 42:66–74.

Ayangbenro, A. S., O. O. Bababola, and O. S. Aremu. 2019. Bioflocculant production and heavy metal sorption by metal resistant bacterial isolates from gold mining soil. Chemosphere 231:113–120.

Aylagas, E., Á. Borja, M. Tangherlini, A. Dell'Anno, C. Corinaldesi, C. T. Michell, X. Irigoien, R. Danovaro, and N. Rodríguez-Ezpeleta. 2017. A bacterial community-based index to assess the ecological status of estuarine and coastal environments. Marine Pollution Bulletin 114:679–688.

Azarbad, H., C. A. M. Van Gestel, M. Niklińska, R. Laskowski, W. F. M. Röling, and N. M. Van Straalen. 2016. Resilience of soil microbial communities to metals and additional stressors: DNA-based approaches for assessing "stress-on-stress" responses. International Journal of Molecular Sciences 17:1–20.

Azarbad, H., M. Niklińska, C. A. M. Van Gestel, N. M. Van Straalen, W. F. M. Röling, and R. Laskowski. 2013. Microbial community structure and functioning along metal pollution gradients. Environmental Toxicology and Chemistry 32:1992–2002.

Babalola, O. O., and B. R. Glick. 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. Journal of Food, Agriculture and Environment 10:540–549.

Bachand, P., and A. Horne. 2000. Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature. Ecol. Eng. 14(1–2):17–32.

Baranowski, R., A. Rybak, and I. Baranowska. 2002. Speciation Analysis of Elements in Soil Samples by XRF. Polish Journal of Environmental Studies 11:473–482.

Behrends, L. L. 2007. Use of aquatic plants for removal of nitrate-nitrogen in subsurface flow constructed wetlands.

Berg, J., K. K. Brandt, W. A. Al-Soud, P. E. Holm, L. H. Hansen, S. J. Sørensen, and O. Nybroe. 2012. Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term cu exposure. Applied and Environmental Microbiology 78:7438–7446.

Bhattacharyya, P. N., and D. K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327–1350.

Boyd, E. S., and T. Barkay. 2012. The mercury resistance operon: From an origin in a geothermal environment to an efficient detoxification machine. Frontiers in Microbiology 3:1–13.
Bruker. 2017. No Title.
Burbach, K., J. Seifert, D. H. Pieper, and A. Camarinha-Silva. 2016. Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. MicrobiologyOpen 5:70–82.
Busnardo, M., R. Gersberg, R. Langis, T. Sinicrope, and J. Zedler. 1992. Nitrogen and phosphorus removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:287–307.
Cai, L., G. Liu, C. Rensing, and G. Wang. 2009. Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. BMC Microbiology 9:1–11.
Carey, R., and G. J. Hochmuth. 2012. Regulatory and Resource Management Practices for Urban Watersheds: The Florida Experience. Hort Technology 22:418–429.
Castellano-Hinojosa, A., D. Correa-Galeote, P. Carrillo, E. J. Bedmar, and J. M. Medina-Sánchez. 2017. Denitrification and biodiversity of denitrifiers in a High-Mountain Mediterranean Lake. Frontiers in Microbiology 8:1911.
Al Chami, Z., N. Amer, L. Al Bitar, and I. Cavoski. 2015. Potential use of Sorghum bicolor and Carthamus tinctorius in phytoremediation of nickel, lead and zinc. International Journal of Environmental Science and Technology 12:3957–3970.
Chapman, D. P. M. 1995. Do sediment toxicity tests require field validation? Environmental Toxicological and Chemistry 14:1451-1453.
Chen, B., J. Liu, Z. Wang, L. Dong, J. Fan, and J. Qu. 2011. Remediation of Pb-Resistant Bacteria to Pb Polluted Soil. Journal of Environmental Protection 02:130–141.
Chen, J., F. He, X. Zhang, X. Sun, J. Zheng, and J. Zheng. 2014. Heavy metal pollution decreases microbial abundance, diversity and activity within particle-size fractions of a paddy soil. FEMS Microbiology Ecology 87:164–181.
Chen, J., J. Li, H. Zhang, W. Shi, and Y. Liu. 2019. Bacterial heavy-metal and antibiotic resistance genes in a copper tailing dam area in northern China. Frontiers in Microbiology 10:1–12.
Chen, Y., Y. Jiang, H. Huang, L. Mou, J. Ru, J. Zhao, and S. Xiao. 2018. Long-term and high-concentration heavy-metal contamination strongly influences the microbiome and functional genes in Yellow River sediments. Science of the Total Environment 637–638:1400–1412.
Clapcott, J. E., K. J. Collier, R. G. Death, E. O. Goodwin, J. S. Harding, D. Kelly, J. R. Leathwick, and R. G. Young. 2012. Quantifying relationships between land-use gradients and structural and functional indicators of stream ecological integrity. Freshwater Biology 57:74–90.
Clapcott, J. E., E. O. Goodwin, R. G. Young, and D. J. Kelly. 2014. Une approche multimétrique pour prédire l'intégrité écologique des cours d'eau en Nouvelle-Zélande. Knowledge and Management of Aquatic Ecosystems.
Czerniawska-Kusza, I., & Kusza, G. 2010. The potential of the Phytotoxkit microbiotest for hazard evaluation of sediments in eutrophic freshwater ecosystems. Environmental Monitoring And Assessment. 179:113–121.
Czerniawska-Kusza, I., T. Ciesielczuk, G. Kusza, and A. Cichoń. 2006. Comparison of the

Phytotoxkit microbiotest and chemical variables for toxicity evaluation of sediments. ENVIRONMENTAL TOXICOLOGY 21:367–372.
Dagnino, A., S. Sforzini, F. Dondero, S. Fenoglio, E. Bona, J. Jensen, and A. Viarengo. 2008. A "Weight-of-Evidence" approach for the integration of environmental "Triad" data to assess ecological risk and biological vulnerability. Integrated Environmental Assessment and Management 4:314–326.
David, P., R. S. Raj, R. Linda, and S. B. Rhema. 2014. NoMolecular characterization of phosphate solubilizing bacteria (PSB) and plant growth promoting rhizobacteria (PGPR) from pristine soils. Int. J. Innov. Sci. Eng. Technol. 1:317–324.
Decker, K. L., A. Pocewicz, S. Harju, M. Holloran, M. M. Fink, T. P. Toombs, and D. B. Johnston. 2017. Landscape disturbance models consistently explain variation in ecological integrity across large landscapes. Ecosphere 8.
Dellinger, M., M. Carvan, R. Klingler, J. McGraw, and T. Ehlinger. 2014. An Exploratory Analysis of Stream Teratogenicity and Human Health Using Zebrafish Whole-Sediment Toxicity Test. Challenges 5:75–97.
Díaz Rizo, O., A. Gelen Rudnikas, R. D. Lavin Pérez, G. Arencibia Caraballo, and K. D´Alessandro Rodríguez. 2014. XRF analysis of sediments from Nuevitas Bay (Cuba): assessment of current heavy metal contamination. Nucleus:11–14.
Ding, G.-C., Y. M. Piceno, H. Heuer, N. Weinert, A. B. Dohrmann, A. Carrillo, G. L. Andersen, T. Castellanos, C. C. Tebbe, and K. Smalla. 2013. Changes of Soil Bacterial Diversity as a Consequence of Agricultural Land Use in a Semi-Arid Ecosystem. PLoS ONE 8:e59497.
Ding, Z., J. Wu, A. You, B. Huang, and C. Cao. 2017. Effects of heavy metals on soil microbial community structure and diversity in the rice (Oryza sativa L. subsp. Japonica, Food Crops Institute of Jiangsu Academy of Agricultural Sciences) rhizosphere. Soil Science and Plant Nutrition 63:75–83.
DiScenza, D. J., and A. R. Keimowitz. 2014. Calibration and Evaluation of an X-Ray Fluorescence Method for the Determination of Lead and Arsenic in Soils. Journal of Environmental Analytical Chemistry 01:1–3.
Elder, G. J. 2016. OUP accepted manuscript. Journal Of Tropical Pediatrics:5–8.
EPA. 1985. U.S. Environmental Protection Agency. Freshwater Wetlands for Wastewater Management Handbook. EPA Region IV Atlanta, GA EPA. 904:9-85–135.
EPA. 1986. Ambient Water Quality Criteria for Dissolved Oxygen.
EPA. 2003. Protecting Water Quality from Urban Runoff. United States Environmental Protection Agency:EPA-841-F-03-003.
EPA. 2007. FIELD PORTABLE X-RAY FLUORESCENCE SPECTROMETRY FOR THE DETERMINATION OF ELEMENTAL CONCENTRATIONS IN SOIL AND SEDIMENT.
EPA. 2012. Summary of the Clean Water Act.
Fargašová, A. 2001. Phytotoxic effects of Cd, Zn, Pb, Cu and Fe on Sinapis alba L. seedlings and their accumulation in roots and shoots.
Feng, G., T. Xie, X. Wang, J. Bai, L. Tang, H. Zhao, W. Wei, M. Wang, and Y. Zhao. 2018. Metagenomic analysis of microbial community and function involved in cd-contaminated soil. BMC Microbiology 18:1–13.
Figueiredo, N. L. L., J. Canário, A. Duarte, M. L. Serralheiro, and C. Carvalho. 2014. Isolation and characterization of mercury-resistant bacteria from sediments of tagus estuary (Portugal): Implications for environmental and human health risk assessment. Journal of

Toxicology and Environmental Health - Part A: Current Issues 77:155–168.
Foley, J. A., R. DeFries, G. P. Asner, C. Barford, G. Bonan, S. R. Carpenter, F. S. Chapin, M. T. Coe, G. C. Daily, H. K. Gibbs, J. H. Helkowski, T. Holloway, E. A. Howard, C. J. Kucharik, C. Monfreda, J. A. Patz, I. C. Prentice, N. Ramankutty, and P. K. Snyder. 2005. Global consequences of land use. Science 309:570.
Gerardi, M. H. 2006. Chapter 1 Wastewater Microorgansims. Page (M. H. Gererdi, Ed.).
Gersberg, R. M., B. V. Elkins, S. R. Lyon, and C. R. Goldman. 1986. Role of aquatic plants in wastewater treatment by artificial wetlands. Water Res. 20:363–367.
Ghosh Roy, S. 2020. Biological indicators of toxic stress in wetland sediments. University of Wisconsin Milwaukee.
Gikas, P., E. Ranieri, and G. Tchobanoglous. 2013. Removal of iron, chromium and lead from waste water by horizontal subsurface flow constructed wetlands. Journal of Chemical Technology and Biotechnology 88:1906–1912.
Gummersheimer, B. S., and T. Giblin. 2003. Identification of Lead Resistant Bacteria from a Heavily Contaminated Site Author (s): Beth S . Gummersheimer and Tara Giblin Published by : Beta Beta Beta Biological Society Stable URL : https://www.jstor.org/stable/4608669 Identification of lead resis. jstor 74:48–54.
Gurave, N. A., V. V Korde, S. S. Dhas, and M. Disale. 2015. Isolation and identification of heavy metal resistant bacteria from petroleum soil of Loni, Ahmednagar. European Journal of Experimental Biology 5:6–11.
He, L. Y., Y. F. Zhang, H. Y. Ma, L. N. Su, Z. J. Chen, Q. Y. Wang, M. Qian, and X. F. Sheng. 2010. Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. Applied Soil Ecology 44:49–55.
Hemme, C. L., Y. Deng, T. J. Gentry, M. W. Fields, L. Wu, S. Barua, K. Barry, S. G. Tringe, D. B. Watson, Z. He, T. C. Hazen, J. M. Tiedje, E. M. Rubin, and J. Zhou. 2010. Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. ISME Journal 4:660–672.
Hermans, S. M., H. L. Buckley, B. S. Case, F. Curran-Cournane, M. Taylor, and G. Lear. 2016. Bacteria as emerging indicators of soil condition. Applied and Environmental Microbiology:AEM.02826–16.
Istina, I. N., H. Widiastuti, B. Joy, and M. Antralina. 2015. Phosphate- solubilizing microbe from Saprists peat soil and their potency to enhance oil palm growth and P uptake. Proc. Food Sci. 3:426–435.
Jasper, J. T., Z. L. Jones, J. O. Sharp, and D. L. Sedlak. 2014. Nitrate removal in shallow, Open-water treatment wetlands. Environmental Science and Technology 48:11512–11520.
Jensen, J. 2011. Sediment toxicity and the recoveryof biological integrity in a restored stream channel. Milwaukee. University of Wisconsin Milwaukee.
Kadlec, R. H. 2016. Large constructed wetlands for phosphorus control: A review. Water (Switzerland) 8.
Kadlec, R., and S. Wallace. 2009. Treatment Wetlands. CRC Press Taylor & Francis Group, LLC.
Kaduková, J., O. Velgosová, A. Mrazíková, R. Marcinčáková, and E. Tkáčová. 2015. Assessment of Biologically Synthesized Ag Nanoparticles Toxicity Against E. coli, Staphylococcus aureus, Parachlorella kessleri and Sinapis alba. Nova Biotechnologica et Chimica 14:69–77.
Kafilzadeh, F., R. Afrough, H. Johari, and Y. Tahery. 2012. Range determination for resistance /

tolerance and growth kinetic of indigenous bacteria isolated from lead contaminated soils near gas stations (Iran). European Journal of Experimental Biology 2:62–69.
Kang, S., J. D. Van Nostrand, H. L. Gough, Z. He, T. C. Hazen, D. A. Stahl, and J. Zhou. 2013. Functional gene array-based analysis of microbial communities in heavy metals-contaminated lake sediments. FEMS Microbiology Ecology 86:200–214.
Karr, J. R., and E. W. Chu. 1999. No Title. Page Restoring Life in Running Waters. Island Press, Washington D.C., Covelo California.
Ke, X., C. Wang, D. Jing, Y. Zhang, and H. Zhang. 2015. Assessing water quality by ratio of the number of dominant bacterium species between surface/subsurface sediments in Haihe River Basin. Marine Pollution Bulletin 98:267-273.
Kenna, T. C., F. O. Nitsche, M. M. Herron, B. J. Mailloux, D. Peteet, S. Sritrairat, E. Sands, and J. Baumgarten. 2011. Evaluation and calibration of a Field Portable X-Ray Fluorescence spectrometer for quantitative analysis of siliciclastic soils and sediments. Journal of Analytical Atomic Spectrometry 26:395.
Knight, B., S. P. McGrath, and A. M. Chaudri. 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol 63:39–43.
Knox, A. S., D. L. Dunn, M. H. Paller, E. A. Nelson, W. L. Specht, and J. C. Seaman. 2006. Assessment of contaminant retention in constructed wetland sediments. Engineering in Life Sciences 6:31–36.
Konopka, A., T. Zakharova, M. Bischoff, L. Oliver, C. Nakatsu, and R. F. Turco. 1999. Microbial biomass and activity in lead-contaminated soil. Applied and Environmental Microbiology 65:2256–2259.
Kou, S., G. Vincent, E. Gonzalez, F. E. Pitre, M. Labrecque, and N. J. B. Brereton. 2018. The response of a 16S ribosomal RNA gene fragment amplified community to lead, zinc, and copper pollution in a Shanghai field trial. Frontiers in Microbiology 9:1–23.
Krachler, M., R. Alvarez-Sarandes, and G. Rasmussen. 2016. High-Resolution Inductively Coupled Plasma Optical Emission Spectrometry for 234U/238Pu Age Dating of Plutonium Materials and Comparison to Sector Field Inductively Coupled Plasma Mass Spectrometry. Analytical Chemistry 88:8862–8869.
Kulakovskaya, T. 2014. Phosphorus storage in Microorganisms: Diversity and Evolutionary Insight. Biochemistry & Physiology: Open Access 04:1–4.
Kunito, T., K. Nagaoka, N. Tada, K. Senoo, H. Oyaizu, S. Matsumoto, and K. Saeki. 1997. Characterization of cu-resistant bacterial communities in cu-contaminated soils. Soil Science and Plant Nutrition 43:709–717.
Lakshmi, K. V. N. S., C. Sasikala, V. V. Ramana, E. V. V. Ramaprasad, and C. V. Ramana. 2011. Rhodovulum phaeolacus sp. nov. a phototrophic alphaproteobacterium isolated from a brown pond. Journal of General and Applied Microbiology 57:145–151.
Lee, W. M., J. Il Kwak, and Y. J. An. 2012. Effect of silver nanoparticles in crop plants Phaseolus radiatus and Sorghum bicolor: Media effect on phytotoxicity. Chemosphere 86:491–499.
Li, J., B. Li, Y. Zhou, J. Xu, and J. Zhao. 2011. A rapid DNA extraction method for PCR amplification from wetland soils. Letters in Applied Microbiology 52:626–633.
Li, L. G., Y. Xia, and T. Zhang. 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME Journal 11:651–662.
Liu, K., M. Sun, M. Ye, H. Chao, Y. Zhao, B. Xia, W. Jiao, Y. Feng, X. Zheng, M. Liu, J. Jiao,

and F. Hu. 2019. Coexistence and association between heavy metals, tetracycline and corresponding resistance genes in vermicomposts originating from different substrates. Environmental Pollution 244:28–37.
Mamta, R. P., V. Pathania, A. Gulati, B. Singh, R. K. Bhanwra, and R. Tewari. 2010. timulatory effect of phosphate-solubilizing bacteria on plant growth, stevioside and rebaudioside-A contents of Stevia rebaudiana Bertoni. Appl. Soil Ecol. 46:222–229.
Manasi, N. Rajesh, and V. Rajesh. 2016. Evaluation of the genetic basis of heavy metal resistance in an isolate from electronic industry effluent. Journal of Genetic Engineering and Biotechnology 14:177–180.
Margesin, R., and F. Schinner. 1996. Bacterial heavy metal-tolerance - Extreme resistance to nickel in Arthrobacter spp. strains. Journal of Basic Microbiology 36:269–282.
Matraszek, R., B. Hawrylak-Nowak, M. Chwil, S. Chwil, and M. Rudaś. 2017. Effect of the Interaction of Nickel Stress and Sulphur Supplementation on the Content and Accumulation of Macronutrients in White Mustard (Sinapis Alba L.). Electronic Journal of Polish Agricultural Universities 20.
Messer, T., M. Burchell, F. Birgand, S. Broome, and G. Chescheir. 2017. Nitrate removal potential of restored wetlands loaded with agricultural drainage water: A mesocosm scale experimental approach. Ecological Engineering 106:541–554.
Microbiotest Inc. 2015. Standard Operational Procedure, Phytotoxkit. Seed germination and early growth microbiotest with higher plants.
Mitsch, W. J., and J. G. Gosselink. 2000. Wetlands. Third. John Wiley & Sons., NY.
MP Biomedicals. 2017. Fast DNA TM spin kit.
Munns, W. R., A. W. Rea, G. W. Suter, L. Martin, L. Blake-Hedges, T. Crk, C. Davis, G. Ferreira, S. Jordan, M. Mahoney, and M. G. Barron. 2016. Ecosystem services as assessment endpoints for ecological risk assessment. Integrated Environmental Assessment and Management 12:522–528.
Naz, N., H. K. Young, N. Ahmed, and G. M. Gadd. 2005. Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. Applied and Environmental Microbiology 71:4610–4618.
Nies, D. H. 1999. Microbial heavy-metal resistance. Applied Microbiology and Biotechnology 51:730–750.
Nikinmaa, M. 2014. An Introduction to Aquatic Toxicology. Academic Press; 1 edition (July 24, 2014).
Novotny, V., T. Ehlinger, A. Bartošová, E. Manolakos, and N. O' Reilly. 2005. Developing Risk Propagation Model for Estimating Ecological Responses of Streams to Anthropogenic Watershed Stresses and Stream Modifications. Center for Urban Environmental Studies Publications.
Olson, B. H., and I. Thornton. 1982. The resistance patterns to metals of bacterial population in contaminated land. J. Soil Sci. 33:271–277.
Owens, P. L., R. C. HInkle, and R. G. Best. 1989. Low-Energy Wastewater Recycling Through Wetland Ecosystem: Copper and Zinc in Wetland Microcosm. Page Freshwater wetlands and wildlife. Oak Ridge, Tenesse.
Pal, C., J. Bengtsson-Palme, E. Kristiansson, and D. G. J. Larsson. 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16:1–14.
Pei, Y., Z. Yu, J. Ji, A. Khan, and X. Li. 2018. Microbial community structure and function

indicate the severity of chromium contamination of the Yellow River. Frontiers in Microbiology 9:1–12.
Postma, J., E. H. Nijhuis, and E. Someus. 2010. Selection of phosphorus solubilizing bacteria with biocontrol potential for growth in phosphorus rich animal bone charcoal. Appl. Soil Ecol. 46:464–469.
Prabhakaran, P., Mu. A. Ashraf, and W. S. Aqma. 2015. Microbial Stress Response to Heavy Metal in the Environment. RSC Advances 5:54667.
Ramond, J.-B., P. J. Welz, D. A. Cowan, and S. G. Burton. 2012. Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up. Research in Microbiology 163:28-35.
Reilly, J. F., A. J. Horne, and C. D. Miller. 2000. Nitrate removal from a drinking water supply with large free-surface constructed wetlands prior to groundwater recharge. Ecol. Eng. 14(1–2):33-47.
Risso, C., J. Sun, K. Zhuang, R. Mahadevan, R. DeBoy, W. Ismail, S. Shrivastava, H. Huot, S. Kothari, S. Daugherty, O. Bui, C. H. Schilling, D. R. Lovley, and B. A. Methé. 2009. Genome-scale comparison and constraint-based metabolic reconstruction of the facultative anaerobic Fe(III)-reducer Rhodoferax ferrireducens. BMC Genomics 10:447.
Roane, T. M., and S. T. Kellogg. 1996. Characterization of bacterial communities in heavy metal contaminated soils. Canadian Journal of Microbiology 42:593–603.
Rüegg, S. R., L. R. Nielsen, S. C. Buttigieg, M. Santa, M. Aragrande, M. Canali, T. Ehlinger, I. Chantziaras, E. Boriani, M. Radeski, M. Bruce, K. Queenan, and B. Häsler. 2018. A systems approach to evaluate One Health initiatives. Frontiers in Veterinary Science 5:1–18.
Ryan, R. P., D. J. Ryan, and D. N. Dowling. 2005. Multiple metal resistant transferable phenotypes in bacteria as indicators of soil contamination with heavy metals. Journal of Soils and Sediments 5:95–100.
S. Sengar, R., S. Gupta, M. Gautam, A. Sharma, and K. Sengar. 2008. Occurrence, Uptake, Accumulation and Physiological Responses of Nickel in Plants and its Effects on Environment. Research Journal of Phytochemistry 2:44–60.
Sabry, S. A., H. A. Ghozlan, and D. M. Abou-Zeid. 1997. Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. Journal of Applied Microbiology 82:245–252.
Santo, C. E., P. V. Morais, and G. Grass. 2010. Isolation and characterization of bacteria resistant to metallic copper surfaces. Applied and Environmental Microbiology 76:1341–1348.
Shannon, R. D., O. P. Flite, and M. S. Hunter. 2000. Subsurface flow constructed wetland performance at a Pennsylvania campground and conference center. J. Environ. Qual. 29:2029-2036.
Sinicorpe, T. L., R. Langis, R. M. Gersberg, M. J. Busnardo, and J. B. Zedler. 1992. Metal removal by wetland mesocosms subjected to different hydroperiods. Ecological Engineering 1:309–322.
Smith, M. S., and K. Zimmerman. 1981. Nitrous Oxide Production by Nondenitrifying Soil Nitrate Reducers1. Soil Science Society of America Journal 45:865.
Sokal, R., and J. Rohlf. 1981. Biometry. Second. W.H. Freeman and Company, San Fransisco.
Soon, Y. K., B. T.E., and J. R. Moyer. 1980. Land application of chemically treated sewage sludge III. Effects on soil and plant heavy metal content. J. Environ. Qual. 9:497–504.

Suter, G. I. 2006. Ecological Risk Assessment. CRC Press.
Szoboszlay, M., A. Dohrmann, C. Poeplau, A. Don, and C. Tebbe. 2017. Impact of land use change and soil organic carbon quality on microbial diversity in soils across Europe. FEMS Microbiol Ecol.
Tajini, F., M. Trabelsi, and J. J. Drevon. 2012. Combined inoculation with Glomus intraradices and Rhizobium tropici CIAT899 increases phosphorus use efficiency for symbiotic nitrogen fixation in common bean (Phaseolus vulgaris L.). Saudi J. Biol. Sci. 19:157–163.
Tallis, H., P. Kareiva, M. Marvier, A. Chang, and A. H. Mwinyi. 2008. Practical Conservation and Economic Development. Proceedings of the National Academy of Sciences 105:9457–9464.
Thomas, P. R., P. Glover, and T. Kalaroopan. 1995. An evaluation of pollutant removal from secondary treated sewage effluent using a constructed wetland system. Water Sci. Tech. 32:87-93.
Tipayno, S. C., J. Truu, S. Samaddar, M. Truu, J. K. Preem, K. Oopkaup, M. Espenberg, P. Chatterjee, Y. Kang, K. Kim, and T. Sa. 2018. The bacterial community structure and functional profile in the heavy metal contaminated paddy soils, surrounding a nonferrous smelter in South Korea. Ecology and Evolution 8:6157–6168.
Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol 5:240–245.
Umar, S., N. Gauba, N. A. Anjum, and T. O. Siddiqi. 2013. Arsenic toxicity in garden cress (Lepidium sativum Linn.): Significance of potassium nutrition. Environmental Science and Pollution Research 20:6039–6049.
USGS, U. S. G. S. 2017. Nitrogen and Water. U.S. Department of the Interior.
Vamerali, T., M. Bandiera, and G. Mosca. 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environmental Chemistry Letters 8:1–17.
Vasileiadis, S., E. Puglisi, M. Trevisan, K. G. Scheckel, K. A. Langdon, M. J. McLaughlin, E. Lombi, and E. Donner. 2015. Changes in soil bacterial communities and diversity in response to long-term silver exposure. FEMS Microbiology Ecology 91:1–11.
Vymazal, J. 2007. Removal of nutrients in various types of constructed wetlands. Science of the Total Environment 380:48–65.
Walker, C. W., and R. D. Shannon. 2006. Nitrate and phosphate removal effects of compost amendments in wetland mesocosms. American Society of Agricultural and Biological Engineers 49:1773–1778.
Wang, H., C. W. Marshall, M. Cheng, H. Xu, L. Hu, X. Yang, and T. Zheng. 2017. Changes in land use driven by urbanization impact nitrogen cycling and the microbial community composition in soils. Scientific Reports 7.
Wang, L., and J. Lyons. 2003. Wang2003.pdf. Page 227 Biological Response Signatures: Indiactor Patterns Using Aquatic Communities. CRC Press.
Wang, N. F., T. Zhang, X. Yang, S. Wang, Y. Yu, L. L. Dong, Y. D. Guo, Y. X. Ma, and J. Ye Zang. 2016. Diversity and composition of bacterial community in soils and lake sediments from an arctic lake area. Frontiers in Microbiology 7:1–9.
Webb, J. M.-S. 1998. Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. Journal of Applied Microbiology 84:240-248.
Wilson, M. A., and S. R. Carpenter. 1999. Economic valuation of freshwater ecosystem services in the United States: 1971-1997. Ecological Applications 9:772–783.
Wohl, D. L., S. Arora, and R. Gladstone. 2004. Functional redundancy supports biodiversity and

ecosystem function in a closed and constant environment. Ecology 85:1534–1540.
Xiao, H., S. Zhang, J. Zhai, Q. He, A. Mels, K. Ning, and J. Liu. 2013. Retention and distribution of Cu, Pb, Cr, and Zn in a full-scale hybrid constructed wetland receiving municipal sewage. Water Science and Technology 67:2257–2264.
Xie, Y., J. Fan, W. Zhu, E. Amombo, Y. Lou, L. Chen, and J. Fu. 2016. Effect of heavy metals pollution on soil microbial diversity and Bermudagrass genetic variation. Frontiers in Plant Science 7.
Yazdankhah, S., E. Skjerve, and Y. Wasteson. 2018. Antimicrobial resistance due to the content of potentially toxic metals in soil and fertilizing products. Microbial Ecology in Health and Disease 29:1548248.
Yoder, C., and E. Rankin. 1995. Biological response signatures and the area degradation value:new tools for interpreting multi-metric data, in W.S. Davis and T.P. Simons(Eds). Pages 263–286 Biological Assessment and Criteria: Tools for Water Resource Planning and Decision Making. Lewis Publishers, Boca Raton, FL.
YSI. 2020. YSI a xylem brand.
Yu, Y., H. Wang, J. Liu, Q. Wang, T. Shen, W. Guo, and R. Wang. 2012. Shifts in microbial community function and structure along the successional gradient of coastal wetlands in Yellow River Estuary. European Journal of Soil Biology 49:12–21.
Zancheta, A. C. F., C. A. De Abreu, F. C. B. Zambrosi, N. de M. Erismann, and A. M. M. A. Lagôa. 2015. Cadmium Accumulation by Jack-Bean and Sorghum in Hydroponic Culture. International Journal of Phytoremediation 17:298–303.
Zhang, D., J. Luo, Z. Lee, Y. Maspolim, R. Gersberg, and Y. Liu. 2016. Characterization of bacterial communities in wetland mesocosms receiving pharmaceutical-enriched wastewater. Ecological Engineering 90:215–224.
Zhang, M. J., F. K. Huang, G. Y. Wang, X. Y. Liu, J. K. Wen, X. S. Zhang, Y. S. Huang, and Y. Xia. 2017. Geographic distribution of cadmium and its interaction with the microbial community in the Longjiang River: Risk evaluation after a shocking pollution accident. Scientific Reports 7:1–12.
Zhang, W. hui, Z. Huang, L. yan He, and X. fang Sheng. 2012. Assessment of bacterial communities and characterization of lead-resistant bacteria in the rhizosphere soils of metal-tolerant Chenopodium ambrosioides grown on lead-zinc mine tailings. Chemosphere 87:1171–1178.
Zhao, K., P. Penttinen, X. Zhang, X. Ao, M. Liu, X. Yu, and Q. Chen. 2014. Maize rhizosphere in Sichuan, China, hosts plant growth promoting Burkholderia cepacia with phosphate solubilizing and antifungal abilities. Microbiological Research 169:76–82.
Zhu, F., L. Qu, X. Hong, and X. Sun. 2011. Isolation and characterization of a phosphate-solubilizing halophilic bacterium Kushneria sp. YCWA18 from Daqiao saltern on the coast of yellow sea of China. Evidence-based Complementary and Alternative Medicine 2011.
Zięba-Palus, J., Kunicki, M. 2006. Application of the micro-fTIR spectroscopy, Raman spectroscopy and XRF method examination of inks. Forensic Science International 158:164–172.

Appendices

Appendix 1.1: Analysis of Variance for the effects of predicted yearly nutrient loading on (A) Stem Inhibition and (B) Root Inhibition among wetland sites 1-6.
Factors included seed species (*Lepidium sativum*, *Sinapis alba*, *Sorghum saccharatum*), nitrate-nitrite load (Kg/yr) and phosphate load (Ky/yr).

Source	DF	SS	F Ratio	Prob > F
(A) Stem Inhibition				
Seed species	2	0.87377	16.1445	<.0001*
Nitrate-Nitrite	1	0.24738	9.1415	0.0041*
Phosphate	1	0.08137	3.0068	0.0898
Nutrient Interaction	1	0.18764	6.9338	0.0116*
(B) Root Inhibition				
Seed species	2	0.0820	0.6990	0.5024
Nitrate-Nitrite	1	0.0282	0.4809	0.4916
Phosphate	1	0.0014	0.0246	0.8761
Nutrient Interaction	1	0.0007	0.0112	0.9162

Appendix1.2: Analysis of Variance for the effects of measured nutrient concentrations on (A) Stem Inhibition and (B) Root Inhibition among wetland sites 1-6. Factors included seed species (*Lepidium sativum, Sinapis alba, Sorghum saccharatum),* nitrate concentration (mg/L) and phosphate concentration (mg/L).

Source	DF	SS	F Ratio	Prob > F
(A) Stem Inhibition				
Seed species	2	0.1752	3.7271	0.0551
Nitrate	1	0.0174	0.7416	0.4060
Phosphate	1	0.0375	1.5963	0.2304
Nutrient Interaction	1	0.0293	1.2475	0.2859
(B) Root Inhibition				
Seed species	2	0.0478	0.7520	0.4924
Nitrate	1	0.1215	3.8218	0.0743
Phosphate	1	0.2253	7.0879	0.0207*
Nutrient Interaction	1	0.2333	7.3402	0.0190*

Appendix1.3: Analysis of Variance for the effects of predicted metal loading (Kg/yr) on (A) Stem Inhibition and (B) Root Inhibition among wetland sites 1-6. Factors included sampling time, predicted total loadings (Kg/yr) for Cd, Cu , Pb, Zn, and seed species (*Lepidium sativum, Sinapis alba, Sorghum saccharatum*)

Source	DF	SS	F Ratio	Prob > F
(A) Stem Inhibition				
Seed species	2	0.7259	12.0298	<.0001*
Cd	1	0.0298	0.9863	0.3167
Cu	1	0.0062	0.2047	0.6489
Pb	1	0.0682	2.2601	0.1512
Zn	1	0.0003	0.0104	0.9076
Metal Interaction terms	1	0.0182	0.6045	0.4629
(B) Root Inhibition				
Seed species	2	0.0861	0.8102	0.4359
Cd	1	0.0547	1.0304	0.3064
Cu	1	0.0001	0.0001	0.9990
Pb	1	0.3144	5.9165	0.0168*
Zn	1	0.0132	0.2486	0.6119
Metal Interaction terms	1	0.0280	0.5268	0.4625

Appendix1.4: Analysis of Variance for the effects of measured metal concentration Principal Component on (A) Stem Inhibition and (B) Root Inhibition among wetland sites 1-6. Factors included sampling time, metal PC1 and PC2, and seed species (*Lepidium sativum, Sinapis alba, Sorghum saccharatum*)

Source	DF	SS	F Ratio	Prob > F
(A) Stem Inhibition				
Seed species	2	0.7259	10.8653	0.0002*
Metal PC 1	1	0.0297	0.8887	0.3511
Metal PC 2	1	0.0007	0.0197	0.8892
Metal Interaction	1	0.0110	0.3282	0.5697
(B) Root Inhibition				
Seed species	2	0.0861	0.8389	0.4391
Metal PC 1	1	0.1478	2.8808	0.0969
Metal PC 2	1	0.5251	10.2325	0.0026*
Metal Interaction	1	0.2072	4.0374	0.0508

*Appendix 2.1:*Analyis of Variance for the effects of predicted yearly nutrient loading on Shannon diversity index, Simpson diversity index and total number of identified phyla and genera among wetland sites 1-6. Factors included nitrate-nitrite load (Kg/yr)) and phosphate load (Ky/yr).

Source	DF	SS	F Ratio	Prob > F
(A) Phyla Shannon diversity index				
Nitrate-Nitrite	1	0.8489	2.1758	0.1522
Phosphate	1	0.9057	2.3214	0.1397
Nutrient Interaction	1	0.6422	1.6461	0.2108
(B) Phyla Simpson diversity index				
Nitrate-Nitrite	1	0.1515	5.8671	0.0219*
Phosphate	1	0.1725	6.6813	0.0150*
Nutrient Interaction	1	0.1054	4.0831	0.0526
(C) Genera Shannon diversity index				
Nitrate-Nitrite	1	1.7510	1.3311	0.2580
Phosphate	1	1.6565	1.2592	0.2710
Nutrient Interaction	1	0.9611	0.7307	0.3997
(D) Genera Simpson diversity index				
Nitrate-Nitrite	1	0.0860	10.6436	0.0028*
Phosphate	1	0.0776	9.6040	0.0043*
Nutrient Interaction	1	0.0862	10.6695	0.0028*

Appendix 2.2: Analyis of Variance for the effects of measured nutrient concentrations on Shannon diversity index, Simpson diversity index and total number of identified phyla and genera among wetland sites 1-6. Factors included nitrate concentration (mg/L) and phosphate concentration (mg/L).

Source	DF	SS	F Ratio	Prob > F
(A) Phyla Shannon diversity index				
Nitrate	1	0.0205	0.8933	0.3649
Phosphate	1	1.1500	50.1017	<.0001*
Nutrient Interaction	1	0.1048	4.5654	0.0559
(B) Phyla Simpson diversity index				
Nitrate	1	0.0665	77.2115	<.0001*
Phosphate	1	0.0197	22.8275	0.0006*
Nutrient Interaction	1	0.0861	99.9433	<.0001*
(C) Genera Shannon diversity index				
Nitrate	1	0.0613	0.1528	0.7033
Phosphate	1	2.6256	6.5481	0.0266*
Nutrient Interaction	1	0.3652	0.9108	0.3604
(D) Genera Simpson diversity index				
Nitrate	1	0.0035	7.1689	0.0215*
Phosphate	1	0.0011	2.1868	0.1673
Nutrient Interaction	1	0.0049	10.1546	0.0087*

Appendix 2.3: Analyis of Variance for the effects of predicted metal loading (Kg/yr) on Shannon diversity index, Simpson diversity index and total number of identified phyla and genera among wetland sites 1-6. Factors included sampling time, predicted total loadings (Kg/yr) for Cd, Cu , Pb, Zn.

Source	DF	SS	F Ratio	Prob > F
(A) Phyla Shannon diversity index				
Cu	1	4.5531	18.9091	0.0002*
Pb	1	2.3646	9.8202	0.0045*
Zn	1	4.3734	18.1629	0.0003*
Cd	1	2.3709	9.8461	0.0045*
Metal Interaction terms	1	1.5567	6.4649	0.0179*
(B) Phyla Simpson diversity index				
Cu	1	0.5241	38.8365	<.0001*
Pb	1	0.2829	20.9650	<.0001*
Zn	1	0.5171	38.3168	<.0001*
Cd	1	0.1420	10.5207	0.0031*
Metal Interaction terms	1	0.0812	6.0138	0.0209*
(C) Genera Shannon diversity index				
Cu	1	0.2161	0.1954	0.662
Pb	1	0.0177	0.0160	0.9003
Zn	1	0.3419	0.3091	0.5828
Cd	1	1.2712	1.1493	0.2932
Metal Interaction terms	1	2.7329	2.4708	0.1276
(D) Genera Simpson diversity index				
Cu	1	0.0568	7.7270	0.0098*
Pb	1	0.0885	12.0378	0.0018*
Zn	1	0.0707	9.6255	0.0045*
Cd	1	0.0304	4.1309	0.0520
Metal Interaction terms	1	0.0293	3.9897	0.0559

Appendix 2.4: Analysis of Variance for the effects of measured metal concentration Principal Component on Shannon diversity index, Simpson diversity index and total number of identified phyla and genera among wetland sites 1-6. Factors included sampling time, metal PC1 and PC2.

Source	DF	SS	F Ratio	Prob > F
(A) Phyla Shannon diversity index				
Metal PC 1	1	0.1404	1.7176	0.2024
Metal PC 2	1	5.5359	67.7363	<.0001*
Metal Interaction	1	1.4883	18.2105	0.0003*
(B) Phyla Simpson diversity index				
Metal PC 1	1	0.0232	1.8714	0.1826
Metal PC 2	1	0.3487	28.1249	<.0001*
Metal Interaction	1	0.0062	0.5001	0.4855
(C) Genera Shannon diversity index				
Metal PC 1	1	5.8019	4.5623	0.0413*
Metal PC 2	1	0.3671	0.2887	0.5952
Metal Interaction	1	4.4353	3.4876	0.0720
(D) Genera Simpson diversity index				
Metal PC 1	1	0.0055	3.0043	0.0945
Metal PC 2	1	0.2227	121.2653	<.0001*
Metal Interaction	1	0.0634	34.5527	<.0001*

Appendix 3.1: Effect test from ANOVA showing the significance of experimental duration and treatment level (high and low) on metal (Pb and Cu) added in mg/L in the water and metal detected in ppm in the sediments of metal microcosm and nutrients (nitrate and phosphate) added in mg/L in the water of nutrient microcosm.

	Metal microcosm - metals added in water					
	Pb (mg/L)			**Cu (mg/L)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.000001	24.763	<.0001*	0.0003	1.1749	0.319
Treatment level	0.000067	0.5031	0.61	0.0005	0.6749	0.515
	Nutrient microcosm - nutrients added in water					
	Nitrate (mg/L)			**Phosphate (mg/L)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	9067	6.4182	0.0036*	26	11.9591	<.0001*
Treatment level	79	0.0561	NS	6	2.5600	NS

	Metal microcosm - metal detected in sediments								
	Ag (ppm)			**As (ppm)**			**Cd (ppm)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.0012	0.3312	NS	0.0767	4.2484	0.0223*	0.0054	0.4785	NS
Treatment level	0.0017	0.4522	NS	0.0307	1.7017	NS	0.0194	1.7162	NS
	Fe (ppm)			**Hg (ppm)**			**Ni (ppm)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.0002	0.0096	NS	0.0540	2.7600	NS	0.000131	0.252300	NS
Treatment level	0.0001	0.0032	NS	0.0025	0.1261	NS	0.000078	0.149100	NS
Effect Source	**Pb (ppm)**			**Rb (ppm)**			**SS**	**F ratio**	**P value**

Experimental duration	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	0.0473	0.3754	NS
Treatment level	0.000011	1.9716	NS	0.0057	0.2966	NS	0.0008	0.0067	NS
	0.000011	2.1328	NS	0.0083	0.4319	NS			

Appendix 3.2: Effect test from ANOVA showing the significance of experimental duration and treatment level (high and low) on the root and stem growth inhibition of *Lepidium sativum, Sinapis alba, Sorghum saccharatum* in nutrient and metal microcosm experiments.

Metal microcosm									
	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.0351	0.3183	NS	0.0222	0.225	NS	0.4030	4.6128	0.0153*
Treatment level	0.0714	0.6479	NS	0.3206	3.245	0.0487*	0.0375	0.4296	NS
	root inhibition *Lepidium*			**stem inhibition *Sinapis***			**stem inhibition *Sorghum***		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.0291	0.5276	NS	0.0462	0.4937	NS	0.1199	0.4036	NS
Treatment level	0.0572	1.0377	NS	0.0040	0.0424	NS	0.0338	0.1137	NS
Nutrient microcosm									
	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Experimental duration	0.0393	0.1591	NS	0.0231	0.0955	NS	0.0145	0.1907	NS
Treatment level	0.0038	0.0153	NS	0.2499	1.0313	NS	0.0351	0.4619	NS
	root inhibition *Lepidium*			**stem inhibition *Sinapis***			**stem inhibition *Sorghum***		

Effect Source	SS	F ratio	P value	SS	F ratio	P value	SS	F ratio	P value
Experimental duration	0.0628	1.0958	NS	0.0441	0.6668	NS	0.0526	0.1553	NS
Treatment level	0.0083	0.1440	NS	0.0414	0.6269	NS	0.2100	0.6195	NS

Appendix 3.3: Effect test from ANOVA showing the significance of experimental duration and treatment level (high and low) on the Shannon and Simpson diversity indices of phyla and genera in nutrient and metal microcosm experiments.

	Shannon diversity index of phyla			Simpson diversity index of phyla		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Experimental duration	0.0014	0.0039	NS	21.7560	10.2461	0.0045*
Treatment level	1.1319	1.5537	NS	2.2168	0.5220	NS
	Shannon diversity index of genera			**Simpson diversity index of genera**		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Experimental duration	0.2765	3.3789	NS	1688.4	5.3548	0.0314*
Treatment level	0.0553	0.3376	NS	410.21	0.6505	NS
	Shannon diversity index of phyla			**Simpson diversity index of phyla**		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Experimental duration	0.4023	8.0020	0.0104*	30.8410	18.1680	0.0004*
Treatment level	0.1136	1.1295	NS	2.4055	0.7085	NS
	Shannon diversity index of genera			**Simpson diversity index of genera**		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Experimental duration	0.0804	14.0738	0.0013*	512	4.7310	0.0418*
Treatment level	0.0143	1.2515	NS	93	0.4282	NS

Appendix 3.4: Effect test from ANOVA showing the significance of autoclave and treatment level (high and low) on metal (Pb and Cu) added in mg/L in the water and metal detected in ppm in the sediments of metal microcosm and nutrients (nitrate and phosphate) added in mg/L in the water of nutrient microcosm.

Metal microcosm - metals added in water						
	Pb (mg/L)			**Cu (mg/L)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.000003	5.4463	0.0270*	0.0005	1.4882	NS
Treatment Level	0.000000	0.129	NS	0.000027	0.0813	NS
Nutrient microcosm - nutrients added in water						
	Nitrate (mg/L)			**Phosphate (mg/L)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	568.266	2.1855	NS	5.7843	8.1497	0.0080*
Treatment Level	0.7657	0.0029	NS	3.1910	4.4959	0.0430*
Metal microcosm - metal detected in sediments						
	Ag (ppm)			**As (ppm)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.0014	0.9676	NS	0.0179	1.4974	NS
Treatment Level	0.00004	0.0283	NS	0.00004	0.0035	NS
	Cd (ppm)			**Fe (ppm)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.0226	2.8822	NS	0.00003	0.0028	NS
Treatment Level	0.0027	0.3370	NS	0.0098	0.8337	NS
	Hg (ppm)			**Ni (ppm)**		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**

Autoclave	0.0050	0.4840	NS	0.00005	0.2657	NS
Treatment Level	0.0179	1.7298	NS	0.0002	0.7770	NS
		Pb (ppm)			**Rb (ppm)**	
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	1.4E-05	2.9337	NS	0.000084	0.0091	NS
Treatment Level	9.02E-10	0.0002	NS	0.0110	1.1761	NS
		Zn (ppm)				
Effect Source	**SS**	**F ratio**	**P value**			
Autoclave	0.00002	0.0003	NS			
Treatment Level	0.0011	0.0169	NS			

Appendix 3.5 : Effect test from ANOVA showing the significance of autoclave and treatment level (high and low) on the root and stem growth inhibition of Lepidium sativum, Sinapis alba and Sorghum saccharatum in nutrient and metal microcosm experiments.

Metal microcosm									
	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.1947	3.8788	NS	0.0000004	0.0001	NS	0.0096	0.2644	NS
Treatment Level	0.1349	2.6879	NS	0.1556	2.6968	NS	0.0749	2.0614	NS
	root inhibition *Lepidium*			**root inhibition *Sinapis***			**root inhibition *Sorghum***		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.0284	0.7453	NS	0.0730	1.6546	NS	0.0246	0.2347	NS

Treatment Level	0.0404	1.0592	NS	0.0060	0.1354	NS	0.0039	0.0368	NS

Nutrient microcosm

	root inhibition *Lepidium*			root inhibition *Sinapis*			root inhibition *Sorghum*		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.0280	0.1574	NS	0.0048	0.0368	NS	0.0692	1.9933	NS
Treatment Level	0.0038	0.0212	NS	0.1631	1.2496	NS	0.0521	1.5004	NS

	root inhibition *Lepidium*			root inhibition *Sinapis*			root inhibition *Sorghum*		
Effect Source	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**	**SS**	**F ratio**	**P value**
Autoclave	0.0413	1.0854	NS	0.0250	0.6798	NS	0.0206	0.1847	NS
Treatment Level	0.0012	0.0325	NS	0.0032	0.0879	NS	0.2930	2.6264	NS

Appendix 3.6: Effect test from ANOVA showing the significance of autoclave and treatment level (high and low) on the Shannon and Simpson diversity indices of phyla and genera in nutrient and metal microcosm experiments.

Metal microcosm

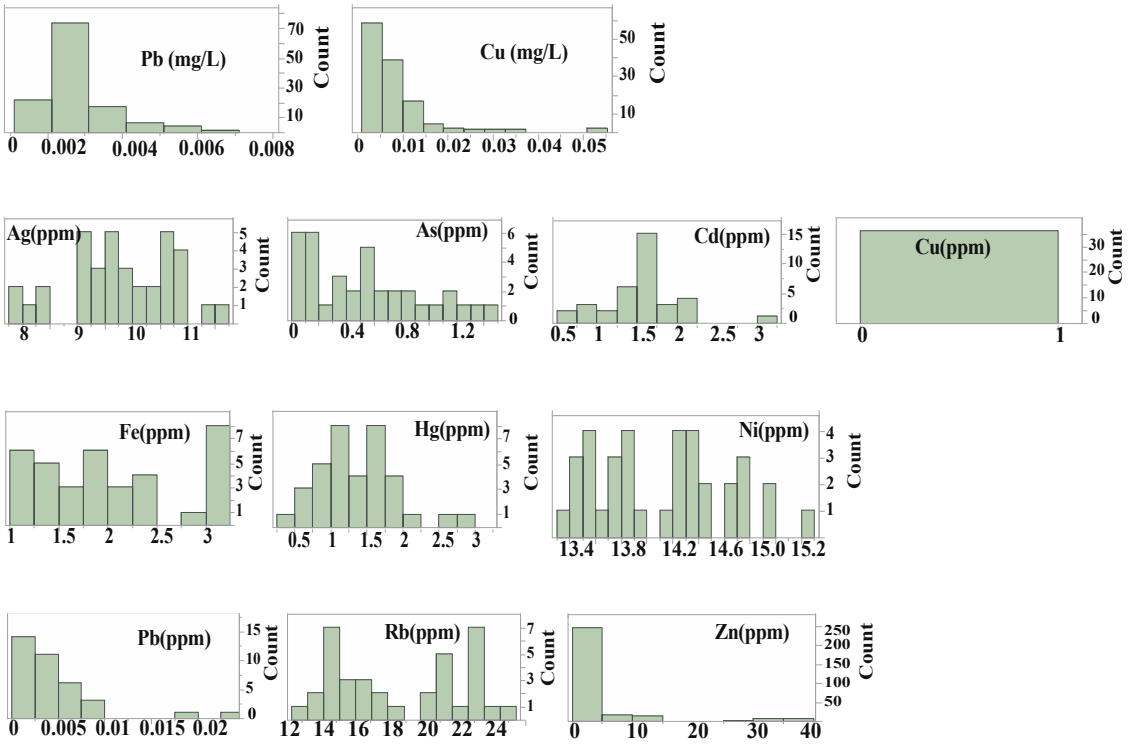
	Shannon diversity index of phyla			Simpson diversity index of phyla		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Autoclave	1.0944	10.5168	0.0176*	19.5739	6.5414	0.0430*
Treatment Level	0.3149	3.0262	NS	6.6395	2.2188	NS

	Shannon diversity index of genera			Simpson diversity index of genera		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Autoclave	1.0162	10.4778	0.0178*	2739	6.6515	0.0418*
Treatment Level	0.1587	1.6364	NS	684	1.6610	NS

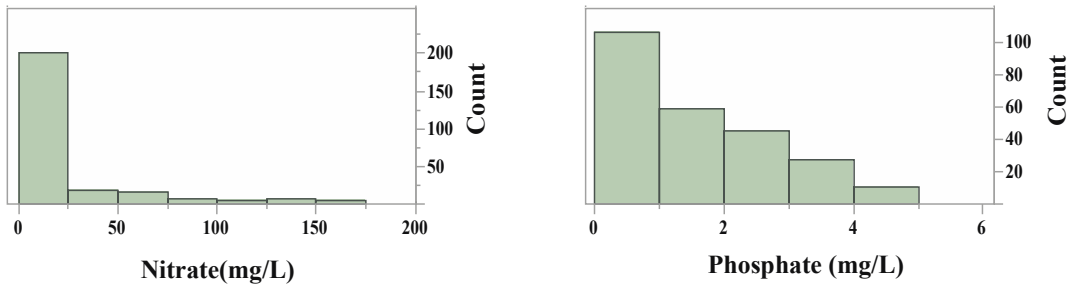
Nutrient microcosm

	Shannon diversity index of phyla			Simpson diversity index of phyla		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Autoclave	0.7580	174.8159	<.0001*	23.2464	52.4199	0.0004*

Treatment Level	0.0502	11.5729	0.0145*	0.2258	0.5091	NS
	Shannon diversity index of genera			**Simpson diversity index of genera**		
Effect Source	**SS**	**F Ratio**	**P value**	**SS**	**F Ratio**	**P value**
Autoclave	0.0232	19.4954	0.0045*	7	0.0805	NS
Treatment Level	0.0192	16.1119	0.0070*	183	2.1770	NS



Appendix 3.7: Frequency distributions with histograms (n or readings taken = 124) showing metal concentration (mg/L) of the added metals Pb and Cu in the water of the metal microcosm and frequency distributions with histograms (n or readings taken = 36) showing metal concentration (ppm) measured from the sediments of metal microcosm.



Appendix 3.8: Frequency distributions with histograms (n or readings taken =124) showing nutrient concentrations (mg/L) of the added nutrients nitrate and phosphate in the water of the nutrient microcosm.

Appendix A:

Journal of Environmental Protection and Ecology 20, No 2, 608–619 (2019)

Industrial pollution

EFFECT OF METALS ON GROWTH INHIBITONS OF SINAPIS, SORGHUM AND LEPIDIUM IN TEXTILE DYE WASTE CONTAMINATED SOIL, INDIA

SUBHOMITA GHOSH ROY[a], TIMOTHY J. EHLINGER[b], MARISSA R. JABLONSKI[b]*

[a]*Department of Biological Sciences, University of Wisconsin Milwaukee, 53 201 Milwaukee, WI, USA*
[b]*Masters of Sustainability Peacebuilding (MSP), University of Wisconsin Milwaukee, 53 201 Milwaukee, WI, USA*
E-mail: *marissajablonski@gmail.com*

Abstract. A large portion of India textile dye industry exists in the informal economy, dumping their wastewater without treatment into nearby land and water. This wastewater has the potential for toxic impacts as dyes are contaminated with heavy metals like copper (Cu), zinc (Zn), lead (Pb), etc. The goal of this study is to use ecotoxicological methods to characterise the extent of toxic stress in the adjacent ecosystems to informal dyers in two different locations in India. 23 soil samples were col-lected around the city of Bangalore, India during July 2016. Using the standard operational procedure of the Phytotoxkit, Microbiotest™, growth of the indicator plants (*Sinapis*, *Lepidium*, and *Sorghum)* was measured in dye contaminated and control soils. Percentage of growth inhibition was calculated with respect to the control soil for each plant. The contaminated soil and commercial dyes were also tested with XRF (X-ray fluorescence) for presence and concentration of metals (ppm). Our study showed that despite the low level of metals present in the soil, growth inhibition was observed in the indicator plants. Suggesting that in combination toxic metals even at low levels can cause an impact on the ecosystem.

Keywords: heavy metals, eco-toxicological tests, growth inhibition, dye.

AIMS AND BACKGROUND

Industrial pollution continues to be a major cause of environmental degradation in air, soil and water[1–3]. Soil samples are excellent media to monitor human caused metal pollution, since metals are normally deposited on topsoil and serves as metal sinks[4]. Therefore, analyses of metals in soils offer an ideal means to monitor pol-lution of soils and environment quality.

Metals today have a great ecological significance due to their toxicity and accu-mulation by leaching into water, taken up by plants or by getting semi-permanently bonded by soil components like clay or organic matter5. Anthropogenic sources of

* For correspondence

metals exist in various industrial point sources[6]. Contaminated sites often support some plant species accumulating high concentrations of metals like lead (Pb), copper (Cu), zinc (Zn) and many more[7].

In India, a major source of metal contamination in topsoil is from textile dye industrial waste. A significant portion of the dying industry occurs within an informal economy. Mainly due to economic stress, dyers lack means to treat their wastewater and mix their wash and dye batches in extremely high concentrations of dye (measured as high as 12 g/l) in each bath, which gets dumped onto soil after use. This dumping is fatal as these colours are concentrated with metals such as Pb, Cu, Zn, etc. with possibility of seeping into water and routing them artificially into the environment and human systems[8].

Dye-waste contamination is currently getting a lot of attention because of its possible toxic effects. But there is still a gap that can show how the biota is getting affected by the metal contamination of dye-waste, from the lowest to the highest trophic level by bio-accumulation or magnification. In this study, a standard eco-toxicological test was chosen to assess the possible threats that are affecting the biota from metal pollution of dye industries in India. Eco-toxicological approaches are of paramount importance for testing the potential effect of contaminants on biota[9], as they are based on standardised protocols, the results are reproducible and therefore provide the advantage of allowing easy comparisons and interpretations[10].

The aim of this project was to determine if growth inhibition of the indicator plants (*Lepidium*, *Sinapis* and *Sorghum*) can detect toxic contamination from dye waste.

EXPERIMENTAL

Two dye-dumping locations: Locations A (current dumping site) and B are about 16 and 80 km from Bangalore city, India, respectively (Fig. 1) were chosen. Total of 23 samples were collected using a sediment core sampler (depth 15–20 cm from surface) from both locations. Location B is considered an historic dumping site but has new infrastructure to carry wastewater to a community dumping site, built between 2013–2015.

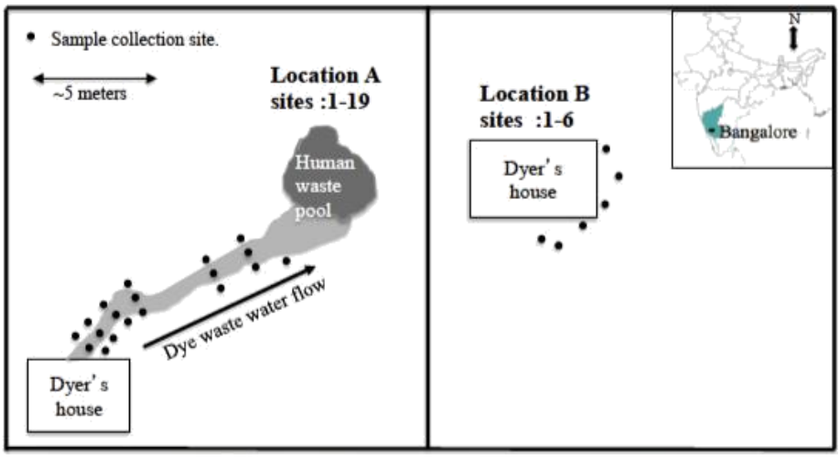


Fig. 1. Sediment sampling map from dye-waste dumping areas in location A and location B near Bangalore, Karnataka, India with the sampling collection sites

In location A, sediment samples were collected from a dye unit waste stream that connects with a human waste pond. The sediment samples were collected in a linear transect: in the mid-stream, south and north borders of the stream or pool respectively (Fig. 1). In location B, sediment samples were collected from 6 sites (approximately 1 m apart) around a dyer house (Fig. 1). The sediments were stored on ice and were brought back to the laboratory. In lab, every sample were divided and stored into two parts, one stored for metals and the second for eco-toxicological tests.

Analysis of metals and minerals was completed using X-ray fluorescence (XRF) using handheld INNOV-XSYSTEMS (Model α-4000, Serial 11392). The instrument was calibrated with Standard Reference Materials according to EPA method 6200 for soil[11]. Three trial readings (in ppm) for every metal were taken for each soil sample collected in field. Large rocks, organic debris, twigs, leaves roots were removed before taking the readings[12]. For eco-toxicological tests, root and stem growth inhibitions (PHYTOTOXKIT™) of three plant indicators (*Sorghum saccharatum, Lepidium sativum* and *Sinapis alba*) were measured. This test method PHYTOTOXKIT™ has been developed by MICROBIOTESTS Inc., Belgium and these indicator species are widely used to assess sediment contamination[10,13]. Seeds from each species were grown in control and test sediments saturated with distilled water, for 72 h at 25 ºC in dark in polyvinylchloride plates (21 × 15.5 × 0.8 cm). In each plate, 10 seeds of the same plant were placed on a filter paper (placed on the sediments) in one row at equal distance. Digital images were taken of all plates. Root and stem lengths were measured using Image J software[14]. The proportion of root to shoot length inhibition of the test sample plants were calculated with

respect to the control plant growth. All lab work was performed at Environmental Engineering Laboratory, Civil Engineering Department, Bangalore Institute of Technology. Statistical Analysis was conducted using JMP® Version 13 (Ref. 15). The metal concentration (ppm) data were log transformed and proportional data were arc-sine transformed prior to analysis to correct for heteroscedastic variance.

RESULTS AND DISCUSSION

The main goal of this project was to determine if these dye-contaminated soils high in metals cause growth inhibition among indicator plants *Sinapis*, *Sorghum* and *Lepidium*. The levels of metals were tested in both of our testing locations and in commercially available dyes. The elements detected in commercial dyes in ppm levels were: Fe, Cu, rubidium (Rb), strontium (Sr), zirconium (Zr), Mo, Pb and gold (Au) (Fig. 2).

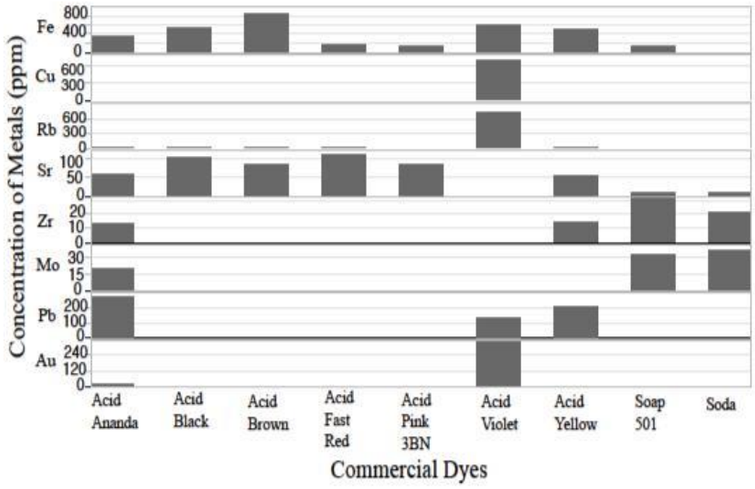


Fig. 2. Concentration of detected metals (ppm) in all the commercial dyes tested (each value of the metal concentration for every dye is the median of 3 recorded values)

The metals detected in ppm levels in location A were: molybdenum (Mo), Fe, Pb, Zr, Sr, Rb, Zn, Co and titanium (Ti) (Fig. 3). The relationships between the metals were analysed using principal component analysis. In location A, principal component 1 was associated with the levels of Ti, Mo, Rb, Fe, Zr, Pb and Co and principal component 2 was associated with the levels of Zn and Sr (Fig. 4 and Table 1). For all the plant (*Lepidium*, *Sinapis*, and *Sorghum*) species, as principal component 1 (Ti, Mo, Rb, Fe, Zr, Pb and Co) increased, the proportion inhibition of root and stem growth decreased, showing a negative estimate values in the linear regression model but as principal component 2 (Sr and Zn) increased the

proportion growth inhibition increased with positive values (Fig. 5 and Table 2). Also, in location A, the metals that had most significant principal components (PC) scores were Sr (0.81 in PC2), Zn (0.81 in PC2), Zr (0.94 in PC1), Rb (0.92 in PC1), Fe (0.94 in PC1) (Table 1).

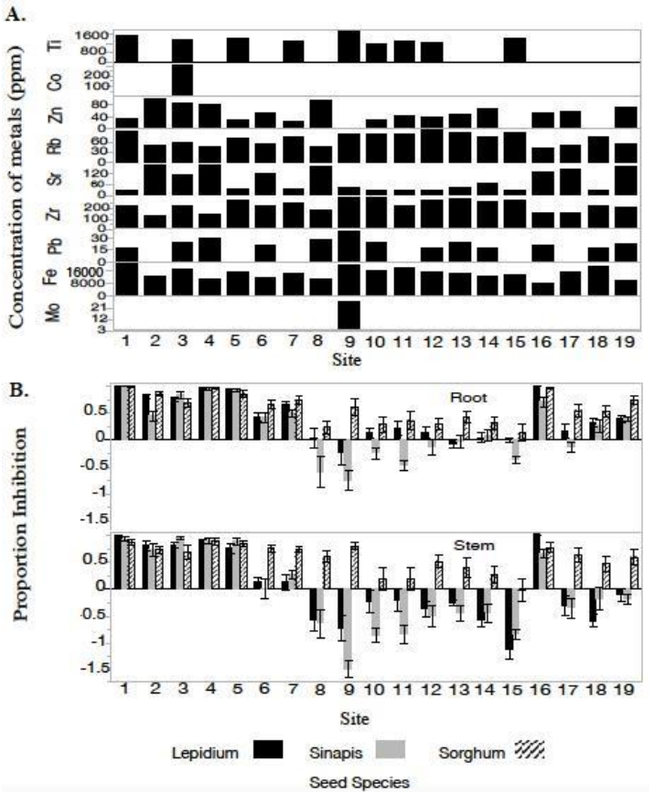


Fig. 3. Concentration of detected metals in ppm (*Y*-axis) at location A sampling collection sites (*X*-axis) (each value of metal concentration in the graph is the median of 3 recorded values at each sampling site) – A; proportion inhibition of root and stem of *Lepidium*, *Sinapis* and *Sorghum* (*Y*-axis) at location A sampling collection sites (*X*-axis) (each value of proportion inhibition in the graph is the mean of 10 inhibition values of each seed species) – B

612

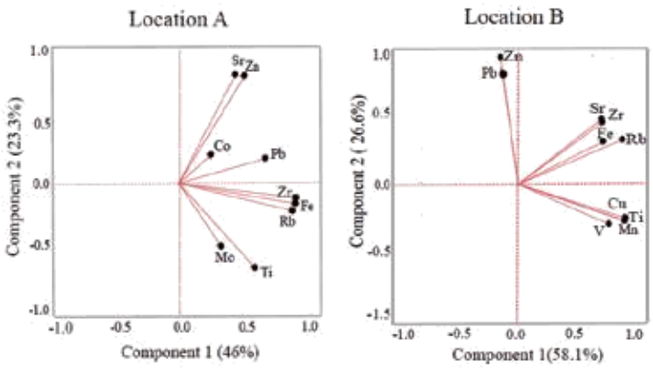


Fig. 4. Scatter plots of the scores for pairs of principal components overlaid with a matrix of 2D representations of metals present in ppm level from locations A and B (labelled biplot rays show eigenvectors of endpoints relative to each component in multivariate space; *X*-axis represents com-ponent 1 and *Y*-axis represents component 2)

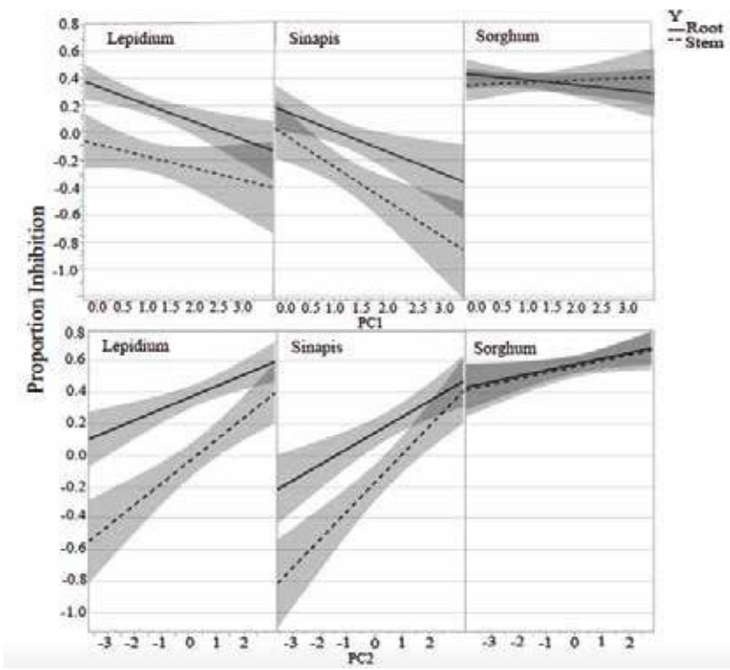


Fig. 5. Line of fit showing linear regression for proportion growth inhibition of *Lepidium*, *Sinapis* and *Sorghum* root (solid) and stem (dotted) and principal components PC1 (top row) and PC2 (bot-tom) of detected metals for study location A

613

The metals detected in ppm levels in location B were: Fe, Pb, Zr, Sr, Rb, Zn, Cu, manganese (Mn), vanadium (V) and Ti (Fig. 6). Principal component 1 was associated with the levels of Ti, V, Mn, Cu, Rb, Sr, Zr, and Fe and component 2 was associated with the levels of Pb and Zn (Table 1 and Fig. 4). In all plants (*Lepidium*, *Sinapis*, and *Sorghum*) as component 1 increased, the proportion inhi-bition increased with positive estimate values in the linear regression model and as the elements associated with component 2 increased, growth inhibition of all plants decreased with a negative value (Fig. 7 and Table 2). Also, in location B, the metals that had most significant principal components (PC) scores were Pb (0.86 in PC2), Zn (0.96 in PC 2), Rb (0.92 in PC1), Mn (0.94 in PC1), Cu (0.94 in PC1), Ti (0.93 in PC1) and V (0.80 in PC1) (Table 1).

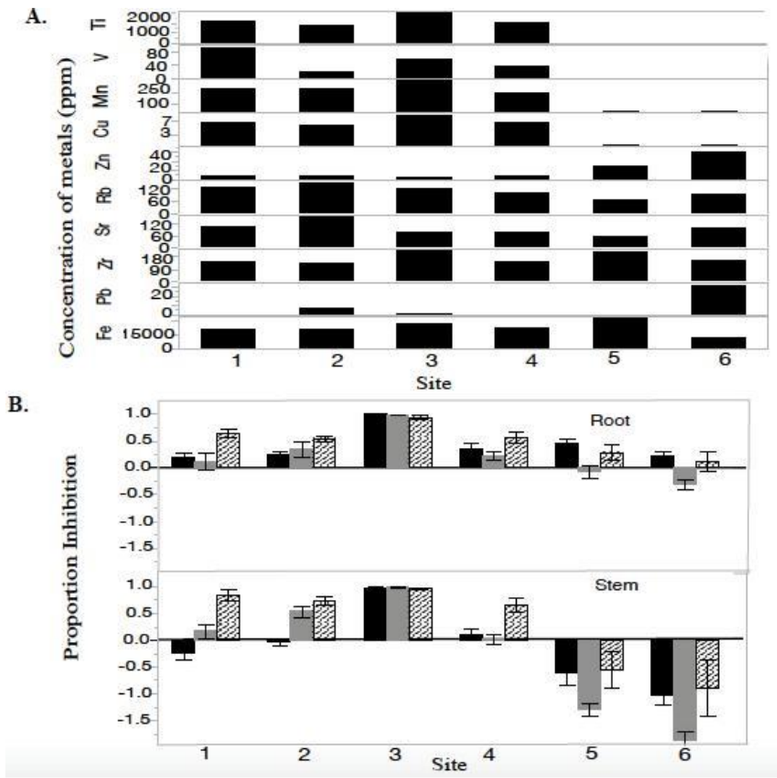


Fig. 6. Concentration of detected metals in ppm (*Y*-axis) at location B sampling collection sites (*X*-axis) (each value of metal concentration in the graph is the median of 3 recorded values at each sampling site) – A; proportion inhibition of root and stem of *Lepidium*, *Sinapis* and *Sorghum* (*Y*-axis) at location B sampling collection sites (*X*-axis) (each value of proportion inhibition in the graph is the mean of 10 inhibition values of each seed species) – B

614

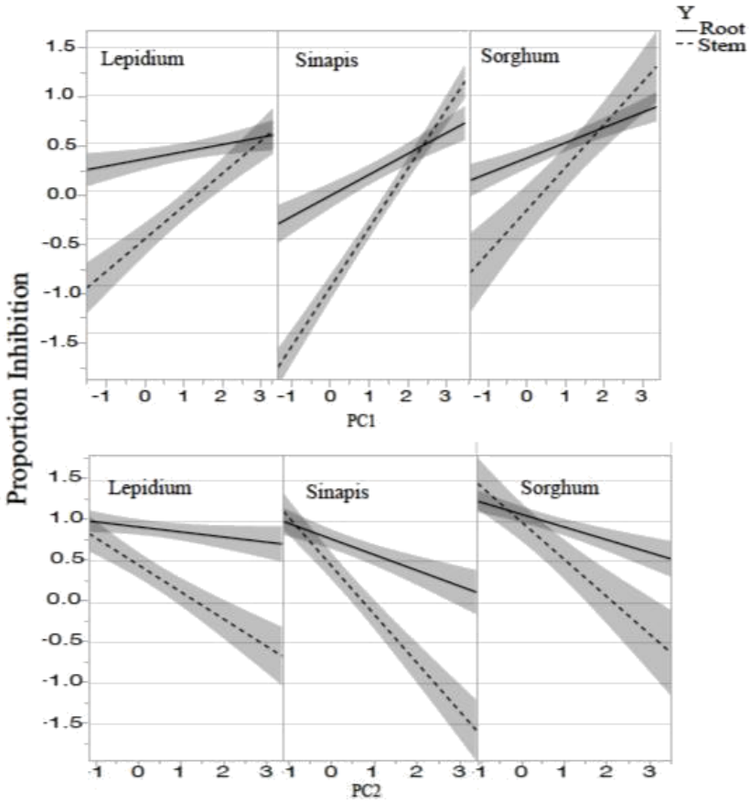


Fig. 7. Line of fit showing linear regression for proportion growth inhibition of *Lepidium, Sinapis* and *Sorghum* root (solid) and stem (dotted) and principal components PC1 (top row) and PC2 (bot-tom) of detected metals for study location B

Table 1. Loading score for principal components in locations A and B, India

Location A			Location B		
metals	PC1	PC2	metals	PC 1	PC2
Sr	0.45	0.81	Pb	–0.14	0.86
Zr	0.94	–0.15	Zn	–0.15	0.96
Ti	0.61	–0.63	Sr	0.74	0.47
Co	0.26	0.21	Zr	0.73	0.50
Zn	0.53	0.81	Rb	0.92	0.34
Rb	0.92	–0.21	Fe	0.75	0.32
Mo	0.34	–0.47	Mn	0.94	–0.26
Pb	0.70	0.19	Cu	0.94	–0.28
Fe	0.94	–0.11	Ti	0.93	–0.28
			V	0.80	–0.30

615

The purpose of this study was to characterise how the dye waste can have a toxic impact on indicator plants (*Lepidium*, *Sinapis* and *Sorghum*) of ecosystem. In general, in location A the sites 1–5, had higher level of root and stem growth inhibition and metal concentration (Fig. 3). Where, principal component 1 was associated with the levels of Ti, Mo, Rb, Fe, Zr, Pb and Co and principal component 2 was associated of Zn and Sr (Fig. 4 and Table 1). Similarly, in location B the sites 1–4, had higher level of root and stem growth inhibition and metal concentration (Fig. 6). Where, principal component 1 was associated with the levels of Ti, V, Mn, Cu, Rb, Sr, Zr, and Fe and principal component 2 was associ-ated with Pb and Zn (Fig. 4 and Table 1). *Lepidium* root, *Sinapis* root and stem showed a significant relationship with principal component 1 both in locations A and B (Table 2). For principal component 2, *Sinapis*, *Sorghum* root and *Lepidium*, *Sinapis* stem showed a significant relationship with principal component 2 both in locations A and B (Table 2).

Table 2. Linear regression estimate values along with probability levels from line of fit for propor-tion growth (root and stem) inhibition of *Lepidium*, *Sinapis* and *Sorghum* across locations A and B sites, India

Test parameters	Principal components	Location A		Location B	
		estimate	probability	estimate	probability
Lepidium – root	PC1	–0.1369	0.0015*	0.0699	0.0108*
Sinapis – root	PC1	–0.1483	0.0073*	0.2034	<0.0001*
Sorghum – root	PC1	–0.0388	0.2762	0.1487	<0.0001*
Lepidium – stem	PC1	–0.0923	0.1691	0.3180	<0.0001*
Sinapis – stem	PC1	–0.2425	0.0008*	0.5764	<0.0001*
Sorghum – stem	PC1	0.0172	0.6772	0.4166	<0.0001*
Lepidium – root	PC2	0.0763	0.0004*	–0.0581	0.0565
Sinapis – root	PC2	0.1067	<0.0001*	–0.1803	<0.0001*
Sorghum – root	PC2	0.0386	0.0288*	–0.1466	<0.0001*
Lepidium – stem	PC2	0.1460	<0.0001*	–0.3120	<0.0001*
Sinapis – stem	PC2	0.1897	<0.0001*	–0.5579	<0.0001*
Sorghum – stem	PC2	0.0392	0.0551	–0.4306	<0.0001*

Fe, Cu, Rb, Sr, Zr and Pb were detected in the sample soil as well as in the commercially available dye sample (Figs 2, 3 and 6).

Cu is an essential element for all living organisms including humans at low levels of intake. But, at higher levels, toxic effects can occur. There are several instances of bioaccumulation in plants and even animals[16,17]. The maximum con-centration in one of the commercial dyes (with Cu as a component) was as high as 703 ppm (Fig. 2). This could raise a serious reason of concern, as these dyes are the sources of toxic contamination of Cu of the ecosystem at the studied loca-tion B (Fig. 6).

The second detected metal of concern was Fe. This is the second most abundant metal on the earth crust[7,8] and one of the vital components of organisms[18]. But there is evidence of Fe toxic accumulation in plants[18] and the maximum level of Fe in both study locations A and B were as high as 19,506 ppm and B: 32,851 ppm, respectively (Figs 2, 3, 6 and Table 1) and in the commercial dyes.

One of the main metals of concern in this study is Pb which is a naturally oc-curring metal and is commonly found as a toxic element in paints and dyes[19]. Pb was detected in commercial dyes in significant levels (268 ppm: Fig. 2) as well as in the study locations A and B (Figs 3 and 6). Pb is bio accumulated by terrestrial and aquatic plants and animals[20]. Therefore, chances of adverse biological effects on the ecosystem of biota of both these study locations, by the presence of high Pb from the commercial dyes is strongly possible as well.

The other metals of concern were Rb, Sr and Zr. Both Sr and Zr are naturally occurring elements and can reportedly bio accumulate in plants and then in higher trophic level organisms[21–23]. Rb is not an essential component of living matter, but is a toxic agent that may partly substitute for potassium[24]. These metals were observed in the commercial dyes as well as in the soil ecosystem of the study locations A and B, proving the dyes to be the possible source of contamination (Figs 2, 3, 6 and Table 1).

In terms of the effect of metals on the growth inhibitions of *Sinapis*, *Sorghum* and *Lepidium* (root and stem) in textile dye waste contaminated soil within loca-tion A, as principal component 2 (level of Sr and Zn) increased the proportion growth inhibitions of *Sinapis*, *Sorghum* and *Lepidium* (root and stem) increased with positive values (Fig. 5 and Table 2). Implying the negative effects of these elements on the indicator plants. Zn is a toxic metal known to be bioaccumulated in living organisms[17,20]. Also, as discussed before, Sr is a metal that was detected in the sample soil as well as in the commercial dyes with known toxic effects of bio-accumulation[21]. In location B, for *Lepidium*, *Sinapis*, and *Sorghum* (root and stem) as component 1 (level of Ti, V, Mn, Cu, Rb, Sr, Zr, and Fe) increased, the proportion inhibition increased with positive estimate values in the linear regres-sion model (Fig. 7 and Table 2). As previously discussed, Fe, Cu, Rb, Sr and Zr, all these elements either have the property of bio-accumulation at higher trophic level or is toxic to living beings[16–18,21–24].

Eco-toxicological tests are used for testing the potential effect of contaminants on biota[25]. Our pilot study showed that these standard plant indicators *Sinapis*, *Sorghum* and *Lepidium* with the root and stem growth inhibition results are highly capable of displaying toxic effects from textile dye waste and hence can be easily applied to these field studies.

CONCLUSIONS

This was a pilot study attempting to characterise and evaluate the degree of growth inhibition on indicator plants due to metal contamination dumped from dye-waste. Some trends were seen that answered the question of that combinations of metals found in dye-dump sites, can have toxic effects on the biota[9] and simple eco-toxicological tools can display these results easily to understand these effects. More data is required to study these effects in more details, especially on higher trophic levels where the risk becomes proportionally higher.

REFERENCES

1. A. de BARTOLOMEO, L. POLETTI, G. SANCHINI, B. SEBASTIANI, G. MOROZZI: Relationship among Parameters of Lake Polluted Sediments Evaluated by Multivariate Statistical
   Analysis. Chemosphere, **55** (10), 1323 (2004).
2. A. LANDJAO, G. ARANA, A. de DIEGO, N. ETXEBARRIA, O. ZULOAGA, D. AMOUR-
   OUX: Analysis of Heavy Metal Distribution in Superficial Estuarine Sediments (Estuary of Bilbao, Basque Country) by Open-focused Microwave-assisted Extraction and ICP-OES. Che-mosphere, **56** (11), 1033 (2004).
3. M. MIRO, J. M. ESTELA, V. CERDA: Application of Flowing Stream Techniques to Water
   Analysis: Part III. Metal Ions: Alkaline and Alkaline-earth Metals, Elemental and Harmful Transition Metals, and Multi Elemental Analysis. Talanta, **63** (2), 201 (2004).
4. A. K. KRISHNA, P. K. GOVIL: Heavy Metal Distribution and Contamination in Soils of Thane-Belapur Industrial Development Area, Western Indian. Environ Geol, **47**, 1054 (2005).
5. P. ACERO, J. M. A. MANDADO, J. GOMEZ, M. GIMENO, L. AUQUE, F. TORRIJO: Envi-ronmental Impact of Heavy Metal Dispersion in the Huerva River (Iberian Range, NE Spain). Environ Geology, **43**, 950 (2003).
6. G. NILGUN, A. OMAR, T. GURDAL: Investigation of Soil Multi-element Composition in Antalya, Turkry. Environ Int, **29**, 631 (2004).
7. S. MASU, V. RUS: Studies Regarding the Establishment of Perennial Plant Communities Stimu-lated with Biosolids on Fly Ash Dumps. J Environ Prot Ecol, **14 (**1), 204 (2013).
8. S. MASU, V. RUS, M. ALBELESCU: Studies on Plant Growth and Metal Bioaccumulation in
   Crops on Fly Ash Disposal Site. J Environ Prot Ecol, **14** (3), 986 (2013).
9. M. A. HASSAAN, A. E. NEMR: Health and Environmental Impacts of Dyes: Mini Review. American Journal of Environmental Science and Engineering, **1** (3), 64 (2017).
10. P. CALOW, V. E. FORBES: Does Ecotoxicology Inform Ecological Risk Assessment? Environ.
    Sci Technol, 147 (2003).
11. USEPA: SW-846 Test Method 6200, Field Portable X-ray Fluorescence Spectrometry for the
    Determination of Elemental Concentrations in Soil and Sediment. Retrieved from https://www. epa.gov/sites/production/files/2015-12/documents/6200.pdf, 2007.
12. J. ZIEBA-PALUS, M. KUNICKI: Application of the Micro-FTIR Spectroscopy, Raman Spec-troscopy and XRF Method Examination of Inks. Forensic Sci Int, **158** (2–3), 164 (2006).
13. I. CZERNIAWSKA-KUSZA, T. CIESIELCZUK, G. KUSZA, A. CICHON: Comparison of the
    Phytotoxkit Microbiotest and Chemical Variables for Toxicity Evaluation of Sediments. Environ
    Toxicol, **21** (4), 367 (2006).
14. C. A. SCHEIDER, W. S. RASBAND, K. W. ELICIERI: NIH Image to ImageJ: 25 Years of Im-age Analysis. Nat methods, **9** (7), 671 (2012).
15. JMP® Version 13. SAS Institute Inc., Cary, NC, 1989–2019.

16. E. MALINOWSKA, K. JANKOWSKI, B. WISNIEWSKA-KADZAJAN, J. SOSNOWSKI, R. KOLCZAREK et al.: Content of Zinc and Copper in Selected Plants Growing along a Mo-torway. Bull Environ Contam Toxicol, **95** (5), 638 (2015).
17. J. YOON, X. CAO, Q. ZHOU, L. MA: Accumulation of Pb, Cu, and Zn in Native Plants Growing on a Contaminated Florida Site. Sci Total Environ, **368** (2–3), 456 (2006).
18. E. L. CONOLLY, M. L. GUERINOT: Iron Stress in Plants. Genome Biol, **3** (8), 1024 (2002).
19. ATSDR: Toxicological Profile for Lead. Retrieved from http://www.atsdr.cdc.gov/, 2007.
20. R. CARDWELL: Do Cd, Cu, Ni, Pb, and Zn Biomagnify in Aquatic Ecosystems? Rev Environ Contam Toxicol, **226**, 101 (2013).
21. ATSDR: Toxicological Profile for Strontium. Retrieved from http://www.atsdr.cdc.gov/, 2004.
22. I. C. SMITH, B. L. CARSON: Trace Metals in the Environment: Zirconium. Ann Arbour Sci Pub Inc, Ann Arbour, **3**, 405 (1978).
23. National Council on Radiation Protection and Measurements (U.S.): Radiological Assessment: Predicting the Transport, Bioaccumulation, and Uptake by Man of Radionuclides Released to the Environment. Recommendations of the National Council on Radiation Protection and Mea-surements. NCRP, Bethesda, MD, 1984.
24. H. B. STEINBACH: Comparative Biochemistry of the Alkali Metals. Ch. 15. In: Comparative Biochemistry a Comprehensive Treatise (Eds F. Marcel, H .S. Mason). Academic Press, New York and London, 1962.
25. D. P. M. CHAPMAN: Do Sediment Toxicity Tests Require Field Validation? Environ Toxicol Chem, **14** (9), 1451(1995).

Received 26 April 2019
Revised 18 May 2019

Subhomita Ghosh Roy

Place of Birth: Kolkata, India

A. EDUCATION AND PROFESSIONAL PREPARATION:

2014- present PhD, Biological Sciences, UW- Milwaukee.

2010-2012 MS, Biological Sciences, UW- Milwaukee.

B. DISSERTATION TITLE:

Biological Indicators of toxic stress in wetland sediments

C. APPOINTMENTS:

2014 – present Teaching Assistant, Department of Biological Sciences. UW- Milwaukee.

2020 – present Visiting Instructor - Department of Biological Sciences, Marquette University

2019 Adjunct Faculty, Bryant and Stratton college, Milwaukee

2013-2014 Water sustainability consultant, Biome Environmental Solutions Pvt. Ltd

2010-2012 Teaching Assistant, Department of Biological Sciences. UW- Milwaukee.

D. PUBLICATIONS:

Ghosh Roy, Subhomita. (2012). An Evaluation of the effect of Stormwater Treatment ponds on wetland and stream quality indicators. MS Thesis. University of Wisconsin-Milwaukee.

Ehlinger, T. J., Jensen, J., Dellinger, M., Ghosh Roy, S., Mcguire, A., Ortenblad, A. (2012). Monitoring of Stream Habitat & Aquatic Biotic Integrity. Pike River North Branch, Racine county, Wisconsin. Interim Report summary submited to Mount Pleasant Stormwater Utility District, Department of Biological Sciences, University of Wisconsin-Milwaukee.

Ghosh Roy, S., Ehlinger, T. J., Jablonski, M.R. (2019). Effect of Metals on growth inhibitions of *Sinapis, Sorghum* and *Lepidium* in textile dye waste contaminated soil, India. Journal of Environmental Protection and Ecology 20, No 2, 608-619.

Ghosh Roy, S., Ehlinger, T. J. (2019). Biological indicators of toxic stress in wetland sediments. Manuscript in preparation.

E. AWARDS:

Richard P. Howmiller Graduate Award, May 2015, Department of Biological Sciences. UW-Milwaukee.

Clifford H Mortimer Award, April 2012, Department of Biological Sciences. UW-Milwaukee.

Chancellor's Graduate Student Award (2010 – 2012), 2014, 2015, Department of Biological Sciences. University of Wisconsin Milwaukee (UWM).

Graduate School Travel Award, UWM, August 2018.

F. RECENT PRESENTATIONS:

An Evaluation of the effect of Stormwater Treatment ponds on wetland and stream quality indicators, MS Thesis Defense, UWM, December 2012.

Biological Science Symposium, UWM, Poster Presentation: Seasonality Variation in Pore Water and Sediment Toxicity in Residential Stormwater Treatment Wetland System, April 2012.

Society for Freshwater Science (SFS) Annual Meeting, Poster presentation: Evaluating wetland function with respect to water quality indicators, May2015.

PRRSUM (Partnership for River Restoration and Science in the Upper Midwest): (along with Dr. Neil O' Reilly and Dr. Stephen Andrew Mcguire), 2016.

Ecological Society of America, Poster presentation: Biological indicators of pollution stress in wetland sediments, August 2018.

Biological Science Symposium, UWM, Presentation: Biological indicators of toxic stress in wetland sediments, April 2019.