UNDERSTANDING THE ROLE THAT BIOFILMS PLAY IN THE SPECIATION AND REMOVAL OF ARSENIC FROM WATER IN NON-AERATED AND AERATED CONSTRUCTED WETLAND MESOCOSMS

COMPRÉHENSION DU RÔLE DES BIOFILMS DANS LA SPÉCIATION ET L'ÉLIMINATION DE L'ARSENIC DE L'EAU DANS LES MÉSOSCOSMES DE ZONES HUMIDES ARTIFICIELLES, AÉRÉS ET NON AÉRÉS

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Abstract

A potentially cheap and effective way to remove the toxic metalloid, arsenic, from water may be to use constructed wetland systems. There are many interacting factors that influence arsenic removal in constructed wetlands. Factors previously assessed include pH, redox, the availability of nutrients, the types of substrates used and the type of microbial activity present in the wetlands. This thesis, while including and assessing previously studied aspects affecting arsenic removal, expanded the understanding of how novel *Equisetum* sp. (horsetails), biofilms and aeration contribute and drive arsenic removal in constructed wetland systems.

In the first study, the ability of three species of horsetail plants (*Equisetum hyemale, Equisetum fluviatile* and *Equisetum scirpoides*) to take up arsenate under hydroponic conditions at 1 and 10 mg/L was assessed. This was done to to test how tolerant the plants were to arsenic and to select the species that would be best suited for planting in constructed wetlands treating arsenic. The speciation of arsenic in *E. hyemale* and *E. fluviatile* tissues using X-ray absorption spectroscopy (XAS) was used to understand the kinds of arsenic species found in the plants after exposure and gain a better understanding of how the plants physiologically responded to high concentrations of arsenic. All *Equisetum* sp. removed less arsenic from the hydroponic solution at the higher As(V) dose than at the lower As(V) dose (78%, 71% and 53% at 1 mg/L As(V) compared to 67%, 55% and 43% at 10 mg/L As(V) for *E. fluviatile*, *E. hyemale* and *E. scirpoides* respectively). Plant tissue arsenic concentrations increased with increasing arsenic dose, where arsenic concentrations were higher in the roots than the shoots for all *Equisetum* sp. The plant species that showed the highest arsenic root and shoot arsenic concentrations during growth at the highest As(V) dose was *E. hyemale* (Shoots: 162 mg/kg dw As, roots: 510 mg/kg dw As). There was a higher proportion of As(III) and As(III)-S species in the plant tissues of *E. fluviatile*, at the highest dose of As(V). As(III)-S, found in the roots of *E. fluviatile*, was hypothesized to be a thiolated arsenic species that restricted the movement of arsenic to the shoots. Most of the arsenic in *E. hyemale* remained as As(V) at both doses and may be the reason why *E. hyemale* was able to take up more arsenic in the shoots.

To underpin the remaining studies described in this thesis, a total of 12 mesocosms (3 non-aerated planted, 3 non-aerated unplanted, 3 aerated planted and 3 aerated unplanted) were set up to test their ability to remove 1 mg/L As(V) of arsenic from water. The mesocosms were planted with *E. hyemale*. The experiment lasted 33 weeks. The mesocosms were fed a nutrient solution that supported the growth of plants and microbes on a weekly basis. Arsenic was added 1 day after fresh nutrients were added to the mesocosms. The mesocosms were extensively monitored by collecting data on water quality, nutrient cycling, microbial activity and plant growth. Porewater samples were also collected to determine arsenic concentrations and identify the species of arsenic present. Water samples were also filtered to collect biofilm samples to identify the prokaryotes in the porewater through next generation 16s rRNA sequencing. At the end of the 33 week experiment, the mesocosms were dismantled and biofilm and plant samples were collected. Biofilms were detached from gravels to determine the concentration of arsenic in the biofilms as well as the mineral composition through X-ray diffraction analysis (XRD) as well as the types of arsenic species found in the biofilms through X-ray absorption spectroscopy (XAS). Detached gravel and root biofilms were also collected for next generation 16s rRNA sequencing. The arsenic remaining in the biofilm detachment solutions were used to attempt to determine the amount of arsenic that was adsorbed onto the biofilms and substrates. Plant tissues were collected to determine the concentration of arsenic as well as the type of species found in the plant tissues through XAS (roots and shoots).

In the second study, we examined the fate and removal of arsenic in the mesocosms. It was discovered that non-aerated mesocosms removed more arsenic (% removal of 70%) than aerated mesocosms (% removal of 40%) because the non-aerated biofilms (115 mg/kg dw As) contained more arsenic than the aerated biofilms (30 mg/kg dw As). Based on mass balance calculations, non-aerated mesocosms retained more arsenic in biofilms (19 - 37%) and through adsorption on to biofilms and substrates (18 - 24%) than aerated mesocosms (biofilms 4 - 6%, substrate/biofilm adsorbed (8 - 9%). Plants accounted for <0.4% of the total arsenic retained in the mesocosms in all planted mesocosms. Further examination of the mineral composition of the biofilms from the mesocosms revealed that non-aerated

mesocosms may have retained arsenic through the biogenic formation of pyrite (2 - 15 wt%) that can adsorb/co-precipitate arsenic with iron oxyhydroxides. This may have occurred due to the activity of sulfate reducing and fermentative bacteria. The sulfate reducing bacteria likely produced hydrogen sulfide and the fermentative bacteria lowered the pH through the release of organic acids and carbon dioxide that caused the dissolution of ankerite and release of iron(II). The reaction of iron(II) and hydrogen sulfide may have led to the formation of pyrite. The abiotic formation of As-S species such as orpiment (As_2S_3) in lower quantities was not ruled out.

In the third study, arsenic speciation was examined in the porewater, detached gravel biofilms and plants in order to further explain removal in both types of mesocosms. Although we know that non-aerated mesocosms removed more arsenic than aerated mesocosms from the previous investigation, porewater samples taken 20 min, 1 day and 6 days after addition on weeks 1, 6 and 14 revealed that non-aerated mesocosms rapidly removed the maximum amount of arsenic 1 day after addition whereas aerated mesocosms required more than 1 day to hit maximal removal. Most of the arsenic in the porewater from oxidizing aerated mesocosms remained as As(V) by day 6 for weeks 1, 6 and 14. There was a higher proportion of DMA(V) and MMA(V) in the reducing non-aerated porewaters. As(III) found in the non-aerated gravel biofilms was likely released and converted into the methylated arsenic by porewater bacteria. Non-aerated gravel biofilms also contained a high proportion of As-S species, indicating the potential formation of abiotic orpiment. Biofilms from aerated mesocosms showed no arsenic transformations. There was no discernable speciation pattern between plant tissues from non-aerated and aerated mesocosms, as most of the arsenic was found as As(V) and As(III) with some samples showing the formation of As-S species hypothesized to be arsenic bound to thiols/phytochelatins, mostly in roots, similar to our hydroponic As(V) uptake study in Chapter 3.

Finally, in the last study of the thesis we examined the identity, microbial activity and ecophysiological role of the prokaryotes found in non-aerated and aerated porewaters and detached gravel and root biofilms. It was found that although the microbial activity of porewater prokaryotes from non-aerated mesocosms remained higher than that of prokaryotes from aerated mesocosms throughout the exposure period, the microbial activity of prokaryotes in non-aerated mesocosms decreased over the exposure period while the activity in aerated mesocosms remained the same. This may have been due to the production of more toxic arsenic species in the non-aerated mesocosms. We identified the most common and abundant prokaryotic genera in porewaters, gravel and root biofilms from our mesocosms through 16s rRNA next generation sequencing. There was a higher richness and diversity of prokaryotes in biofilms than in porewaters for all mesocosms. There was a higher diversity of prokaryotes in porewaters from aerated mesocosms compared to those from non-aerated porewaters. Root biofilms in non-aerated planted mesocosms had a higher prokaryote diversity than in non-aerated porewater. Both non-aerated porewaters and biofilms are characterised by having prokaryotes that are capable of fermentation, sulfide reduction and sulfide oxidation irrespective of planting status. Prokaryotes found in porewater and biofilms from non-aerated mesocosms were largely dominated by highly versatile facultative and strict anaerobic prokaryotes capable of fermentation (*Candidatus* Omnitrophus, *Endomicrobium*, *Candidatus* Competibacter) , nitrate reduction/denitrification (*Candidatus* Omnitrophus, *Candidatus* Competibacter, *Thauera*), sulfate reduction (*Desulfomonile*) and sulfide oxidation (*Thiovirga*, *Sulfuricurvum*, *Thauera*) with the capability of switching metabolisms depending on the availability of carbon, nitrogen and sulfur. These organisms led to the removal of nitrate and carbon within 24 hrs of nutrient addition. The presence of fermentative and sulfate reducing prokaryote may have also led to the formation of pyrite prior to addition of arsenic. In contrast, prokaryotes found in porewater and biofilms from aerated mesocosms were dominated by aerobic prokaryotes capable of aerobic respiration and assimilating nitrogen and sulfate for cell growth but not removal of these nutrients (TM7a, *Nakamurella*, Pir4 lineage, *Chryseobacterium*, *Mycobacterium*, *Micropruina*, *Nocardioides*, *Chryseobacterium*, *Comamonas*, Candidatus *Xiphinematobacter* as well as others).

Résumé

L'utilisation de terres humides artificielles représente une méthode potentiellement économique et efficace pour éliminer l'arsenic, un métalloïde toxique, de l'eau. Plusieurs facteurs interagissent et influencent l'élimination de l'arsenic dans ces milieux humides. Parmi les facteurs déjà évalués figurent le pH, le potentiel redox, la disponibilité des nutriments, les types de substrats utilisés et l'activité microbienne présente. Cette thèse, tout en intégrant et en évaluant les aspects précédemment étudiés concernant l'élimination de l'arsenic, approfondit la compréhension du rôle de nouvelles espèces d'Equisetum (prêles), des biofilms et de l'aération dans ce processus au sein des zones humides artificielles.

Dans un premier temps, la capacité de trois espèces de prêles (*Equisetum hyemale*, *Equisetum fluviatile* et *Equisetum scirpoides*) à absorber l'arséniate en culture hydroponique à des concentrations de 1 et 10 mg/L a été évaluée. L'objectif était de tester la tolérance des plantes à l'arsenic et de sélectionner les espèces les plus adaptées à la plantation dans des milieux humides artificiels pour le traitement de l'arsenic. La spéciation de l'arsenic dans les tissus *d'Equisetum hyemale* et *d'Equisetum fluviatile*, analysée par spectroscopie d'absorption des rayons X (XAS), a permis d'identifier les différentes espèces d'arsenic présentes dans les plantes après exposition et de mieux comprendre leur réponse physiologique à de fortes concentrations d'arsenic. Toutes les espèces *d'Equisetum* ont éliminé moins d'arsenic de la solution hydroponique à forte dose d'As(V) qu'à faible dose (78 %, 71 % et 53 % à 1 mg/L d'As(V), contre 67 %, 55 % et 43 % à 10 mg/L d'As(V) pour *E. fluviatile*, *E. hyemale* et *E. scirpoides*, respectivement). Les concentrations d'arsenic dans les tissus végétaux ont augmenté avec la dose d'arsenic, et étaient plus élevées dans les racines que dans les parties aériennes pour toutes les espèces d'Equisetum. L'espèce végétale présentant les concentrations les plus élevées d'arsenic dans les racines et les parties aériennes lors de sa croissance à la dose la plus élevée d'As(V) était *E. hyemale* (parties aériennes : 162 mg/kg de matière sèche As, racines : 510 mg/kg de matière sèche As). On a observé une proportion plus élevée d'espèces As(III) et As(III)-S dans les tissus végétaux d'*E. fluviatile*, à la dose la plus élevée d'As(V). L'As(III)-S, présent dans les racines *d'E. fluviatile*, serait une espèce d'arsenic thiolée limitant la diffusion de l'arsenic vers les parties aériennes. La majeure partie de l'arsenic présent chez *E. hyemale* est demeurée sous forme d'As(V) aux deux doses, ce qui pourrait expliquer sa capacité d'absorption plus importante dans les parties aériennes.

Douze mésocosmes (3 non aérés et plantés, 3 non aérés et non plantés, 3 aérés et plantés, et 3 aérés et non plantés) ont été mis en place pour tester leur capacité à éliminer 1 mg/L d'arsenic As(V) de l'eau. Les mésocosmes plantés contenaient de l'Eucalyptus hyemale. L'expérience a duré 33 semaines. Une solution nutritive favorisant la croissance des plantes et des micro-organismes a été ajoutée chaque semaine aux mésocosmes. L'arsenic a été ajouté un jour après chaque apport de solution nutritive. Les mésocosmes ont fait l'objet d'un suivi rigoureux, avec la collecte de données sur la qualité de l'eau, le cycle des nutriments, l'activité microbienne et la croissance des plantes. Des échantillons d'eau interstitielle ont également été prélevés pour déterminer les concentrations d'arsenic et identifier les espèces présentes. Des échantillons d'eau ont été filtrés afin de recueillir des échantillons de biofilm pour identifier les procaryotes présents dans l'eau interstitielle par séquençage de nouvelle génération de l'ARNr 16S. À la fin de l'expérience de 33 semaines, les mésocosmes ont été démantelés et des échantillons de biofilm et de plantes ont été prélevés. Les biofilms ont été détachés des graviers pour déterminer la concentration d'arsenic et la composition minérale par diffraction des rayons X (DRX), ainsi que les espèces d'arsenic présentes par spectroscopie d'absorption des rayons X (XAS). Des biofilms détachés des graviers et des racines ont également été prélevés pour un séquençage de nouvelle génération de l'ARNr 16S. L'arsenic restant dans les solutions de détachement des biofilms a été utilisé pour tenter de déterminer la quantité d'arsenic adsorbée sur les biofilms et les substrats. Des tissus végétaux ont été prélevés afin de déterminer la concentration d'arsenic ainsi que les espèces présentes par spectroscopie d'absorption des rayons X (XAS) (racines et parties aériennes).

Dans une deuxième étude, nous avons examiné le devenir et l'élimination de l'arsenic dans les mésocosmes. Nous avons constaté que les mésocosmes non aérés éliminaient plus d'arsenic (élimination de 70 %) que les mésocosmes aérés (élimination de 40 %), car les biofilms non aérés (115 mg/kg de matière sèche As) contenaient plus d'arsenic que les biofilms aérés (30 mg/kg de matière sèche As). Selon

les calculs de bilan massique, les mésocosmes non aérés ont retenu davantage d'arsenic dans les biofilms (19 à 37 %) et par adsorption sur les biofilms et les substrats (18 à 24 %) que les mésocosmes aérés (biofilms : 4 à 6 %, adsorption sur substrat/biofilm : 8 à 9 %). Les plantes représentaient moins de 0,4 % de l'arsenic total retenu dans les mésocosmes plantés. L'analyse de la composition minérale des biofilms a révélé que les mésocosmes non aérés pourraient avoir retenu l'arsenic par la formation biogénique de pyrite (2 à 15 % en poids), capable d'adsorber/coprécipiter l'arsenic.

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List of Acronyms and Abbreviations

16s rRNA	16S ribosomal ribonucleic acid
AMD	Acid mine drainage
Anammox	Anaerobic ammonia oxidation
APS	Advanced Photon Source
As(V)	Arsenate
As(III)	Arsenite
BCF	Bioconcentration factor
BOD	Biological oxygen demand
CAO	Chemolithotrophic As(III) oxidizers
CBE	Citrate-bicarbonate-EDTA
CLPP	Community Level Physiological Profiling
CLS	Canadian Light Source
COD	Chemical oxygen demand
CW	Constructed wetland
DMA(III)	Dimethylarsinous acid
DMA(V)	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonium
DO	Dissolved oxygen
E0	Absorption edge energy
EPS	Extracellular polymeric substances
FDA	Fluorescein-diacetate
FWS	Free water surface
GSH	Glutathione
HF	Horizontal subsurface flow
HN-AD	Heterotrophic nitrification and aerobic denitrification
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
ICP-MS	Inductively coupled plasma – mass spectrometry
IIRPs	Immobile iron-rich particles
LCF	Linear combination fitting
LOD	Limit of detection
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
MSD	Microbial sulfur disproportionation
ORP	Redox potential
OT	Operation time
OTU	Operational taxonomic unit
PC	phytochelatin
PB	Phosphate buffer
PHA	Polyhydroxyalkanoate
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
SOB	Sulfur oxidizing bacteria
SRB	Sulfate reducing bacteria
SRM	Standard reference material
TC	Total carbon
TIC	Total inorganic carbon
TETRA	Tetramethylarsonium

TF	Translocation factor
TMA(III)	Trimethylarsine
TMAO	Trimethylarsine oxide
TN	Total nitrogen
TOC	Total organic carbon
TSS	Total suspended solids
VF	Vertical subsurface flow
WHO	World Health Organization
XRF	X-ray fluorescence spectroscopy
XAS	X-ray absorption spectroscopy
XANES	X-ray absorption near-edge structure
XRD	X-ray diffraction
VF	Vertical subsurface flow
VFA	Volatile fatty acids
ZVI	Zero valent iron

Chapter 1 – Introduction

1.1 Background

The use of passive constructed wetlands to treat various domestic and industrial wastewaters has become a popular choice because they are relatively cheap and easy to maintain in comparison to other treatment technologies. Depending on their design and the type of wastewater being treated (organic or inorganic), constructed wetlands are able to treat various wastewaters effectively due to chemical, biological and physical processes that occur within them, where the goal is either to retain the chemicals of concern in the wetlands or to help degrade them into less toxic chemicals for safe release into the environment. In particular, the use of constructed wetlands for the treatment of mining wastewaters that contain toxic heavy metals and metalloids has been well established. Arsenic contamination of water is of particular concern during mining operations of iron and gold ores, as well as other ores. Although there has been some effort in utilizing constructed wetlands for the removal of arsenic from water, there has been considerable difficulty in establishing effective and consistent removal of high concentrations of arsenic using constructed wetland systems in field conditions because arsenic is very sensitive to changes in conditions within a wetland system which affects its speciation and ultimately how much arsenic is removed from the water. Previous studies have examined the effects of environmental conditions in constructed wetlands on the removal of arsenic from water, however these studies were commonly observational, did not investigate the role of biofilm, and did not directly investigate the role of aeration. A detailed assessment of how arsenic is removed in constructed wetlands under both reducing and oxidizing conditions is therefore required, along with an understanding of how bacterial biofilms and plants interact with arsenic and each other under these conditions.

1.2 Research Objectives

1. Screen the potential abilities for *E. fluviatile*, *E. hyemale* and *E. scirpoides* to treat arsenic contaminated water.

2. Evaluate the effects of aeration and plant presence on the fate and removal of arsenic in treatment wetland mesocosms.

3. Evaluate the effects of aeration and plant presence on the transformation and speciation of arsenic in treatment wetland mesocosms.

4. Evaluate the interplay and effects of aeration and plant presence on the microbial community in treatment wetland mesocosms treating arsenic contaminated water.

1.3 Thesis Organization

The thesis consists of seven chapters, presented in manuscript style with four appendices, as listed below:

Chapter 1: Brief background on the research topic, research objectives and the organization structure of the thesis

Chapter 2: Literature review on arsenic and the current state of knowledge about arsenic in constructed wetlands

Chapter 3: An assessment of the uptake abilities and speciation of As(V) in *Equisetum* sp. plants under hydroponic conditions

Chapter 4: Assessment of the ability of non-aerated and aerated planted and unplanted lab scale constructed mesocosms to remove arsenic from water and determine its fate, through mass balance calculations

Chapter 5: Determination of the speciation of arsenic in porewater, plant and biofilm samples from the non-aerated and aerated planted and unplanted lab scale constructed mesocosms, to help explain arsenic removal

Chapter 6: Assessment of the identity and ecophysiological role of prokaryotes through 16s rRNA sequencing to try and understand the cycling of S, N and C and how it may influence arsenic removal

Chapter 7: Summary of the main outcomes for the objectives set out by the thesis and recommendations for future work

Appendix A contains supplemental information for Chapter 3.
Appendix B contains supplemental information for Chapter 4.
Appendix C contains supplemental information for Chapter 5.

1.4 Co-authorship statement

Chapters 3 through 6 were prepared or submitted for publication in peer-reviewed journals. I am the primary author of the published and submitted chapters, with guidance and assistance of the co-authors indicated below. All contribution statements are given at the end of each chapter.

Chapter 3 was prepared for publication in Sustainability and co-authored by Dr. Michelle Nearing, Dr. Iris Koch, and Dr. Kela Weber.

Chapter 4 was prepared for publication in Environments and co-authored by Dr. Iris Koch and Dr. Kela Weber.

Chapter 5 has not been submitted for publication and is co-authored by Dr. Debora Meira, Dr. Iris Koch and Dr. Kela Weber.

Chapter 6 has not been submitted for publication and is co-authored by Dr. Sarah Wallace, Dr. Iris Koch and Dr. Kela Weber.

Chapter 2 – Literature Review

2.1 Arsenic contamination of water around the world

Arsenic is a toxic metalloid that can occur naturally in the environment. It is a component of 245 naturally occurring minerals found in the earth's crust (Mandal & Suzuki, 2002). It is most commonly found in association with arsenopyrite (FeAsS), sulfide minerals that include realgar (As_4S_4) and orpiment (As_2S_3), arsenolite (As_2O_3) and other oxide, phosphate, carbonate and silicate minerals (Shankar et al., 2014). Arsenic may be released from these minerals as the result of weathering and geothermal activity, which in turn may contaminate natural water sources (Bundschuh & Maity, 2015; Mandal & Suzuki, 2002). The release of arsenic into ground water sources is usually mediated by adsorption-desorption, precipitation-dissolution, oxidation-reduction, and ion-exchange processes, and can be affected by grain size and organic content of soils and sediments through which ground water runs through as well as biological activity and other aquifer characteristics (Jain & Ali, 2000). Reductive dissolution of iron oxides is a common cause of arsenic contamination of ground water if arsenic is part of the host minerals or if the arsenic is adsorbed to iron oxides minerals (Shankar et al., 2014).

In many regions around the world, the concentration of arsenic in water may be 10 to 100 times higher than the maximum concentration limit of approximately 10 µg/L set by the World Health Organization's (WHO) guidelines (World Health Organization, 2003). Chronic ingestion of inorganic arsenic through food and water at concentrations >10 µg/L can be harmful to the body and potentially cause health problems including cancer, while acute concentrations of 60 mg/L can be fatal (S. Wang & Mulligan, 2006b). Some regions of the world where arsenic may be found in natural aquifers at very high concentrations include the Western USA, (Utah, Nevada, California, and Arizona), Northern Mexico, Chile, Peru, Argentina, India, Bangladesh (West Bengal, and Calcutta), Mongolia, China, Taiwan, Thailand, Vietnam, Cambodia, Nepal, Hungary, Romania and parts of Finland (Jain & Ali, 2000; Mandal & Suzuki, 2002; Shankar et al., 2014; Sharma & Sohn, 2009; R. Singh et al., 2015; Smedley & Kinniburgh, 2002).

2.2 Arsenic contamination of water in Canada

In Canada, arsenic is mainly associated with sulphide minerals (Moncur et al., 2009). Compared to other parts of the world, the problem of naturally high arsenic concentrations in ground and surface waters in Canada is not as widespread. In most cases natural surface and ground water concentrations of arsenic in Canada range between 1.0 – 5.0 µg/L (S. Wang & Mulligan, 2006b), there are some geologic "hotspots" where arsenic contamination of groundwater can occur. These mineralogical hotspots include the Northwest Territories, Baffin Island, Nova Scotia, Newfoundland, and British Columbia (Boyle et al., 1998; Grosz et al., 2004). In these regions of Canada, arsenic may be a problem when installing water wells but the biggest threat to water sources comes from the gold mining and smelting industries (Ashton & Riese, 1989; Azcue et al., 1995, 1994a, 1994b; Azcue & Nriagu, 1995; Barringer et al., 2013; Bright et al., 1996; Brooks, 1982; Wagemann et al., 1978). Parts of Northern Ontario have also seen arsenic contamination of water that is attributable to the mining of gold, silver, cobalt, zinc, lead, iron and nickel (Azcue & Nriagu, 1995; Paliouris & Hutchinson, 1991; S. Wang & Mulligan, 2006b). In the Canadian prairies, mine tailings and waste piles from gold mining activity in Northern Manitoba and uranium tailings in Northern Saskatchewan have been contaminating local lakes (Donahue & Hendry, 2003; Salzsauler et al., 2005; Simpson et al., 2011). Other minor sources of arsenic in ground water in Canada may come from extraction, processing and combustion of coal, the manufacture and use of herbicides for non-agricultural purposes as well as the use of chromated copper arsenate (CCA) as a wood preservative for wood that is mainly used for industrial/infrastructural purposes (Atiang' et al., 2025; Garelick et al., 2009; Mandal & Suzuki, 2002; Moghaddam & Mulligan, 2008; Morais et al., 2021; S. Wang & Mulligan, 2006b).

2.3 Arsenic speciation and chemistry

Arsenic may exist in one of four oxidation states (-3, 0, +3 and +5). The -3 oxidation state in the form of arsine gases (AsH_3) and the zero oxidation state (ie. elemental As or As0) are rarely found in nature and

only exist in highly reducing environments (Cullen & Reimer, 1989; Garelick et al., 2009; Sharma & Sohn, 2009; S. Wang & Mulligan, 2006b). The main soluble arsenic species found in natural waters include inorganic As(V) and As(III). Both As(V) and As(III) species may coexist in natural waters but the pH and redox conditions may determine which species is proportionally higher in concentration (Shankar et al., 2014). A higher proportion of the arsenic is found as As(V) in aerobic (oxidizing) waters as arsenate in various anionic forms (pH<2: H_2AsO_4, pH 2-11: $HAsO_4^{2-}$ and AsO_4^{3-}) while a higher proportion of the arsenic may be found as As(III) in anaerobic (reducing) waters as uncharged arsenite at pH <9, H_3AsO_3 (Kadlec & Wallace, 2008; Sharma & Sohn, 2009). Although less common in water, organic species of arsenic may also be present in aquatic environments, where the inorganic arsenic species are converted to methylated arsenic species by microbial activity. Inorganic arsenic can be methylated to form the trivalent species monomethylarsonous acid [MMA(III)], dimethylarsinous acid [DMA(III)] and trimethylarsine [TMA(III)], as well as the more commonly occurring, and more stable pentavalent species monomethylarsonic acid [MMA(V)], dimethylarsinic acid [DMA(V)] and trimethylarsine oxide (TMAO) (Wang & Mulligan, 2006b).

2.4 Human toxicity and health effects

Arsenic contamination of water from anthropogenic activities poses a serious and significant risk to local environments, ecosystems, and to human health. The mobility of the inorganic arsenic species is affected by the nature and availability of absorbent surfaces and the process of adsorption itself is affected by many factors such as pH, redox and the presence of organic matter and competing ions (Sharma & Sohn, 2009).

The toxicity of arsenic depends on the chemical form (inorganic or organic) and the amount that is absorbed by the organism. The inorganic species (As(III) and As(V)) are more toxic than the pentavalent organic species (DMA, MMA, TMA, TMAO). Only one form of arsenic, arsenobetaine, has not been proven to be toxic (and is thus considered non-toxic), and is commonly found in seafood; all other arsenic species should be considered to have some degree of toxicity (Caumette et al., 2011).

As(III) may be transported into cells via membrane proteins (aquaglyceroporins) that transport compounds such as glycerol and urea (Liu et al., 2002). Once As(III) enters cells, it may disrupt enzyme activities by binding to thiol and hydroxide groups found in their active sites which in effect will disrupt many important metabolic activities (Abernathy & Ohanian, 1992; Ganie et al., 2024; Ratnaike, 2003). Such enzymes may include glutathione reductases, glutathione peroxidases, thioredoxin reductases, and thioredoxin peroxidases that are important for redox balance within cells (Ganie et al., 2024; Sharma & Sohn, 2009). Arsenate, on the other hand, is an analog of phosphate and may be transported through phosphate transporters (Ganie et al., 2024; Shankar et al., 2014). Arsenate does not react directly with enzymes until it is converted to As(III) in vivo. In humans and rats the acute toxicity of arsenic is as follows: MMA(III) ≈ DMA(III) > As(III) > As(V) > DMA(V) > MMA(V) (Petrick et al., 2000; Shankar et al., 2014; R. Singh et al., 2015; Styblo et al., 2000). DMA(III) and MMA(III) are more toxic than arsenite because of a higher affinity for thiol ligands and sulfur rich proteins (Petrick et al., 2000; Styblo et al., 2000).

Exposure to arsenic in humans is usually through ingestion of arsenic contaminated water and food. Acute arsenic poisoning in humans may cause nausea, vomiting, abdominal pains, hypotension, EKG abnormalities, respiratory distress/failure, renal damage/failure as well as neurological effects such as headaches, delirium, encephalopathy, and seizures (Campbell & Alvarez, 1989; Dueñas-Laita et al., 2005; Health Canada, 2006; Kuivenhoven & Mason, 2023; Ratnaike, 2003). Although acute exposure to highly toxic arsine gas (AsH_3) is less common, it can occur in industrial workplace settings and acute toxicity is characterised by hemolysis which may lead to hypoxia and renal failure if left untreated (Blackwell & Robbins, 1979; Danielson et al., 2006; Pullen-James & Woods, 2006).

Chronic exposure to arsenic in humans may cause noncancerous diseases that include cardiovascular, dermal, renal, neurological and endocrine diseases (Abernathy & Ohanian, 1992; Kuivenhoven & Mason, 2023; Shankar et al., 2014; Yoshida et al., 2004). Arsenic, particularly the inorganic species, are known

Group 1 carcinogens in humans and may cause lung, kidney, skin, bladder liver and prostate cancer (Ganie et al., 2024; Kuivenhoven & Mason, 2023; Shankar et al., 2014; Yoshida et al., 2004).

2.5 Water treatment technologies for Arsenic

Technologies used for the removal of arsenic from water include air or chemical oxidation/ precipitation; coagulation/coprecipitation with alum, iron or lime; sorption using iron coated sand or activated alumina and ion-exchange resins; and membrane filtration techniques, which include nanofiltration, reverse osmosis and electrodialysis (Health Canada, 2006; Mohan & Pittman, 2007). Although air and chemical oxidation of arsenic are simple processes, using air oxidation is a slow process while chemical oxidation requires stringent control over pH conditions of the water.

The chemicals used for coagulation/coprecipitation technologies may be relatively cheap but may require pre-oxidation of arsenite (if present) before addition of the chemicals, which also requires proper pH control over the process. The sedimentation and filtration steps at the end of the process may produce large amounts of toxic sludge which require proper disposal (Sullivan et al., 2010).

Sorption and ion exchange technologies have become more widely available but suffer mainly from the fact that the adsorbent material must be regenerated or replaced, which may also produce large amounts of toxic sludge.

Common membrane filtration technologies such as reverse osmosis and nanofiltration can effectively remove many different contaminants from water and do not produce toxic sludges or wastewater but have very high capital, running and maintenance costs. Electrodialysis is the only membrane technology that produces toxic wastewater.

In the mining industry, the generation of highly polluted acid mine drainage (AMD) waters with concentrations of arsenic and other metals that range in the hundreds to thousands of parts per million are usually treated using oxidation/precipitation and coagulation and coprecipitation methods (Lizama-Allende et al., 2011). The toxic sludges produced must go into settling or sedimentation ponds. Again, as stated before, the generation of toxic sludges adds costs to the process, especially if the sludges are not dewatered (Johnson & Hallberg, 2005). Other methods that are used to treat AMD waters include anoxic limestone drains, but these limestone drains may fail due to a process called “armouring”. In this process, high ferrous iron-containing wastewater can lead to the oxidation of ferrous iron to form a layer of iron hydroxide on the limestone, which prevents the neutralizing activity of the limestone on the wastewater (Johnson & Hallberg, 2005).

2.6 Natural wetlands

An alternative method to arsenic treatment is a constructed wetland system. Wetlands can be defined as land that is saturated with water for long enough to allow for aquatic processes to dominate. They are characterized by poorly drained soils, with vegetation and microbial communities that are adapted to saturated soils (Kadlec & Wallace, 2008; Kennedy & Mayer, 2002). Wetlands are transitional between terrestrial and aquatic systems. They are generally classified as bogs, fens, swamps, marshes, peat lands, muskegs, sloughs and, in the Canadian prairies, potholes (Kadlec & Wallace, 2008; Kennedy & Mayer, 2002). The type of wetland that develops may be determined by the climate, hydrological processes and the hydroperiod of a particular area (Kennedy & Mayer, 2002).

Wetlands provided a number of direct and indirect anthropogenic benefits and can be thought of as “Nature's kidneys”, where they act as physical, chemical and biological filters. They include physical benefits such as flood mitigation, coastal protection, aquifer recharge and sediment trapping (Kennedy & Mayer, 2002). Wetlands are also important in improving water quality because they aide in the degradation, assimilation, precipitation and adsorption of nutrients and anthropogenic contaminants. Due to their high primary productivity and low decomposition rates, wetlands may act as biogeochemical sinks for important nutrients such as carbon, nitrogen, phosphorus and sulfur (Kennedy & Mayer, 2002). Wetlands are also places of great biological diversity which support a wide variety of birds, mammals, fish and other aquatic life.

2.7 Constructed treatment wetlands

There is evidence that the United Kingdom has been using natural wetlands to treat wastewaters for more than a century and as early as the 1900s in North America natural wetlands have been used for water treatment where some of the oldest treatment wetlands include such as the Great Meadows natural wetland in Lexington Massachusetts (1912), the Cootes Paradise natural wetland close to Hamilton, Ontario (1919), the Brillion Marsh in Wisconsin (1923) and the natural cypress swamp close to Waldo, Florida (1939) (Vymazal, 2011, 2022). There was a shift away from using natural wetlands for treatment of wastewaters in the 1950s because humans began to realize their important indirect and direct anthropogenic functions (as outlined in the previous section) as well as more appreciation for their intrinsic value. To exploit the physical, chemical and biological functions of natural wetlands to treat wastewaters, pioneering research on the use of man-made constructed wetlands began in the 1950s with Dr. Kathe Seidel in Germany at the Max Planck Institute (Kadlec & Wallace, 2008; Kennedy & Mayer, 2002; Vymazal, 2011, 2022). Researchers realized that engineered wetlands are preferred over natural wetlands mainly because natural wetlands may not perform as well as man-made constructed wetlands because they may not be designed around treating a particular contaminant(s). Since the 1950s the number of constructed wetlands used in Europe and the United Kingdom grew to over 6000 while North America lagged behind at over 1000 constructed wetlands, mainly found in the United States and very few in Canada (Kennedy & Mayer, 2002). Constructed wetlands have been used to treat point sources of pollution which include primary and secondary sewage, and industrial and agricultural wastewaters, as well as non-point sources of pollution such as municipal storm waters and acid mine drainage waters (Kennedy & Mayer, 2002; S. Wu et al., 2015).

2.8 Types of constructed wetlands

2.8.1 Free water surface constructed wetlands

Free water surface (FWS) constructed wetlands bear the most resemblance to natural wetlands such as ponds and marshes. FWS wetlands are usually composed of shallow basins that contain 20-30 cm of soil and they are flooded with 20-40 cm of water (Vymazal, 2010, 2022). Water flow and infiltration may be controlled by berms, dikes, and liners (Kadlec and Wallace 2008b). FWS wetlands are usually planted with emergent macrophytes which typically cover more than 50% of the surface of the wetland (Vymazal, 2010, 2022). FWS wetlands are efficient at removing organic contaminants via microbial degradation and TSS (Total Suspended Solids) via sedimentation as well as reducing BOD (Vymazal, 2010, 2022). FWS wetlands are also good at nitrogen removal from water through volatilization of ammonia in open water areas, nitrification (oxidation of ammonia to nitrite and nitrate) in the aerobic water column and denitrification (reduction of nitrate, ultimately to N_2) in anaerobic carbon rich littler layers. FWS wetlands are generally poor at removing phosphorus because it is not adsorbed or precipitated and input of phosphorus occurs due to the the decay of dead plant matter. FWS wetlands have become popular in North America and Australia and more recently in European countries (Vymazal, 2010, 2022). FWS wetlands have been traditionally used to treat effluent from secondary and tertiary sewage treatment processes (i.e., lagoons, trickling filters and activated sludge systems) but have been used to treat other wastewater types such as storm water runoff, and agricultural and food processing wastewaters (Kadlec & Wallace, 2008; Vymazal, 2010, 2022). FWS wetlands provide ancillary benefits by creating wetland habitats that support various fauna and flora.

2.8.2 Horizontal subsurface flow constructed wetlands

Horizontal subsurface flow (HF) constructed wetlands consist of planted gravel or rock beds with an impermeable bottom liner where water flows below the surface of the rock bed in a horizontal flow direction. HF wetlands act like filtration beds where contaminants are removed by microbial degradation through aerobic planted zones associated with plant roots as well as deeper anoxic and anaerobic zones. They are effective at removing TSS via sedimentation and organic matter through microbial degradation (Vymazal, 2010, 2022). HF wetlands are poor at removing ammonia due to low dissolved oxygen (DO) concentrations that do not allow for ammonia oxidation to nitrite and nitrate but denitrification may be high

for wastewaters with high nitrate concentrations. They are also poor at removing phosphorus unless the substrates used are rich in aluminum and iron oxyhydroxides, which can remove phosphorus via adsorption or ligand exchange. The use of HF constructed wetlands were the first type of constructed wetland that became popular and spread throughout Europe since the 1960s and eventually gained acceptance in the 1990s in North America, Australia, Asia and Africa (Vymazal, 2010, 2022). HF wetlands have traditionally been used to treat domestic and municipal wastewaters but more recently have been used to treat industrial and agricultural wastewaters as well as landfill leachates and runoff waters.

2.8.3 Vertical subsurface flow constructed wetlands

Most vertical subsurface flow (VF) constructed wetlands are filled with substrates such as sand or gravel materials, which may be used alone or in combination, as various layers to control the flow rate of the water through the bed media. VF wetlands may or may not be planted with vegetation. VF wetlands may be configured in a few different operational modes with respect to how 1) wastewater is introduced and 2) treated in the wetland:

Wastewater may be introduced to the VF wetland from the top of the wetland a few centimeters below the top of the substrate and allowed to flow by gravity to the bottom of the wetland (Kadlec & Wallace, 2008; Vymazal, 2010, 2022). Wastewater may also be loaded from the bottom of the VF wetland and move towards the top, again just below the surface of the substrate (Kadlec & Wallace, 2008). In some cases, the surface of the VF wetland is flooded where the wastewater is allowed to move above the level of the substrate.

There are a number of ways in which the wastewater may be treated and this may revolve around whether oxidizing, reducing or both conditions need to be promoted for proper treatment, as well as the amount of time the wastewater is in contact with components of the VF wetland. If oxidizing conditions need to be promoted, single pass VF wetlands in series or recirculating gravity flow systems can be used to create oxidizing conditions. For example, nitrification may be promoted to treat high ammonia waste streams (Kadlec & Wallace, 2008). In VF wetlands that are flooded and allowed to stand for a number of days, reducing conditions may be promoted. These conditions could allow, for example, denitrification of high nitrate wastewaters to occur. In cases where both nitrification and denitrification are desired, the use of tidal flow systems (also called fill and drain systems) are used where the wastewater is pulse loaded and subjected to both oxidizing and reducing conditions (Kadlec & Wallace, 2008).

As we can see, VF wetlands can be very effective in removing high nitrogen containing wastes and biosolids that come from domestic and municipal wastewaters. They can also be used to treat effluent from refinery and food processing industries as well as compost leachates and airport runoff (Kadlec & Wallace, 2008; Vymazal, 2010, 2022).

2.9 Current knowledge on the removal of arsenic using lab scale constructed wetlands

This review will address the current knowledge on the treatment of arsenic contaminated water sources in lab scale constructed wetland mesocosms (CWs). The relevant scientific literature was obtained by using science and engineering primary literature databases and using the search terms "arsenic" and "constructed wetlands" "treatment wetlands" and/or “pilot wetlands”. Research Rabbit, a free online citation-based literature mapping tool was used to ensure that all the relevant literature relating to arsenic treatment in constructed wetlands was captured. This review excludes studies that used unplanted systems alone, such as column experiments or bioreactors with no plants, to remove arsenic from water. A total of forty-three studies were identified and selected for review. To better understand the literature, we must first attempt to describe how the experiments were designed and the range of conditions that were used to try and treat arsenic from contaminated water. Then, based on this understanding, this review will attempt to describe the most important arsenic removal mechanisms in constructed wetlands. This review will also attempt to identify knowledge and data gaps that may need to be filled to improve the design of our study.

2.10 Types of CWs studied for removing arsenic from water

As we can see from Table 2-1, the types of CWs used to treat arsenic from water include vertical flow CWs (VF), horizontal flow CWs and free water surface CWs (FWS). In all cases where studies used VF and HF CWs, all were setup as single standalone systems as opposed to a series of CWs connected in series, as was seen in more studies that used FWS CWs. The substrate volume and depth information were calculated from the fill height of the substrate from each of the studies. This is because treatment of the arsenic in the CWs is primarily mediated by the biotic and abiotic conditions and activities occurring within the substrate. Substrate depth among the three types of CWs appear to be roughly the same and do not exceed 0.51 m. Although there was a wide variation in substrate volume for all the types of CWs, on average FWS CWs had a larger substrate volume because the substrate volume in studies that used FWS connected in series were multiplied by the number of CWs in that series. FWS CWs were also not as deep as VF and HF CWs, as FWS CWs are theoretically supposed to resemble shallower wastewater treatment ponds that generally are wider than they are deep.

Table 2-1: Summary of the type, configuration, volume and depth of constructed wetlands studied for treating arsenic from water from the literature

CW Type	Study	Configuration[a]	Volume (L)	Depth (m)
VF	**Al-Abbas and Ismail 2024**	Single	149.3	0.25
	Baquir et al. 2024	Single	864	0.9
	Rabbi et al. 2024	Single	9.5	0.25
	Xu et al. 2024	Single	3.1	0.3
	Zhao et al. 2024	Single	883.6	0.45
	Liu et al. 2022	Single	6.8	0.6
	Fan et al. 2021	Single	937.5	0.75
	Nguyen et al. 2021	Single	228.2	0.58
	Li et al. 2014	Single	48	0.4
	Rahman et al. 2014	Single	18.5	0.3
	Wu et al. 2014	Single	6.8	0.6
	Arroyo et al. 2013[b]	Single	10.1	0.5
	Lizama-Allende et al. 2012	Single	5.5	0.7
	Zurita et al. 2012	Single	20.4	0.16
	Lizama-Allende et al. 2011b	Single	5.5	0.7
	Rahman et al. 2008b	Single	18.5	0.3
	Average		118.7	0.51
	Stdev		279.2	0.19
HF	**Palacios Robles et al. 2024**	Single	302.4	0.24
	Lizama-Allende et al. 2021	Single	225	0.3
	Corroto et al. 2019	Single	144	0.3
	Hua et al. 2019	Single	17.9	0.15
	Lizama-Allende et al. 2018	Single	225	0.3
	Türker et al. 2016	Single	1100	0.55

Table 2-1: Continued

CW Type	Study	Configuration[a]	Volume (L)	Depth (m)
HF	**Lizama-Allende et al. 2014**	Single	78	0.65
	Valles-Aragón et al. 2014	Single	262.5	0.35
	Olmos-Márquez et al. 2012	Single	225	0.3
	Rahman et al. 2011	Single	26.6	0.3
	Singhakant et al. 2009a	Single	2000	1
	Singhakant et al. 2009b	Single	2000	1
	Rahman et al. 2008a	Single	37.5	0.25
	Buddhawong et al. 2005[b]	Single	30	0.2
	Average		490.1	0.43
	Stdev		726.8	0.28
FWS	**Park et al. 2022**	Single	20	0.15
	Thathong et al. 2019	Single	360	0.4
	Hendriksea et al. 2018	Series (5)	177.5	0.2
	Zhang et al. 2017a	Single	3.3	0.16
	Zhang et al. 2017b	Single	1.2	0.14
	Schwindaman et al. 2014a	Series (4)	817	0.3
	Schwindaman et al. 2014b	Series (4)	817	0.3
	Arroyo et al. 2013[b]	Single	7	0.35
	Nakwanit et al. 2011	Single	11200	0.8
	Spacil et al. 2011	Series (4)	931.7	0.25
	Dorman et al. 2009	Series (4)	5740.1	0.62
	Eggert et al. 2008	Series (4)	1891	0.3
	Sundberg and Hassan 2007	Series (4)	1134.6	0.3
	Buddhawong et al. 2005[b]	Single	22.5	0.15
	Ye et al. 2003	Single	75.1	0.13
	Average		1546.5	0.3
	Stdev		3044.3	0.19

[a] Series refers to CWs connected in series with the number per series shown in brackets

[b] Arroyo et al. 2013 and Buddhawong et al. 2005 appear twice in the table because they compare two different types of CWs in their studies.

2.11 Operating conditions

The hydraulic retention time (HRT), sometimes also referred to as the residence time, of the wastewater in the CWs also varied widely from one study to the next. For consistency, the HRT for CWs connected in series was calculated as the total time the wastewater was in each CW in the series. The average HRT from all studies where a hydraulic retention time was provided was 5 ± 4 days with a range of 0.1 – 16 days (Al-Abbas & Ismail, 2024; Arroyo et al., 2013; Baquir et al., 2024; Corroto et al., 2019; Dorman et al., 2009; Eggert et al., 2008; Fan et al., 2021; Hendrikse et al., 2018; Hua et al., 2019; J. Li et al., 2014; H. Liu et al., 2022; Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021; Nakwanit et al., 2011; Nguyen et al., 2021; Olmos-Márquez et al., 2012; Palacios Robles et al., 2024; Rabbi et al., 2024; K. Z. Rahman et al., 2011, 2014, 2008ba, 2008aa; Schwindaman et al., 2014a, 2014b; Singhakant et al., 2009a, 2009b; Spacil et al., 2011; Sundberg, 2006; Sundberg-Jones & Hassan, 2007; Thathong et al., 2019; Türker,

2016; Valles-Aragón et al., 2014; Wu et al., 2014; Xu et al., 2024; Ye et al., 2003; J. Zhao et al., 2024; Zurita et al., 2012). The average total operating time (OT) for these CWs was 205 ± 129 days with a range of 28 – 517 days. We can think of these studies as studies that treated multiple batches or loadings of arsenic contaminated wastewater. For the remaining studies that did not report an HRT, the HRT was equal to the total operating time of the CWs which was 120 ± 49 days with a range of 50 – 231 days (Buddhawong et al., 2005; Olmos-Márquez et al., 2012; J.-H. Park et al., 2022; Z. Zhang et al., 2017a, 2017b). We can think of these studies as treating the wastewater in a single batch. The important thing to conclude from the information above is that increasing total operating time or HRT did not necessarily mean increased removal of arsenic from the CWs because conditions that included substrate type, wastewater characteristics/composition, environmental conditions (pH, redox etc.) and the type of biological activity occurring in the CWs influenced how quickly and efficiently arsenic was removed, as will be discussed in the subsequent sections below.

2.12 Substrate types and composition

The types of substrates used in CWs removing arsenic from water vary widely in the literature. Many studies use single substrates that include generic gravels (Buddhawong et al., 2005; Fan et al., 2021; Hua et al., 2019; Lizama-Allende et al., 2011, 2012; K. Z. Rahman et al., 2011, 2014, 2008aa, 2008ab), polypropylene granules (Al-Abbas & Ismail, 2024), limestone (Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021), zeolites (Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021), bauxite (Hua et al., 2019), alum (Hua et al., 2019), ceramsite (Wu et al., 2014; Xu et al., 2024), clay materials (Rabbi et al., 2024), egg shells (Rabbi et al., 2024), date palm fiber (Rabbi et al., 2024) minerals with high iron oxide content (Thathong et al., 2019; Valles-Aragón et al., 2014; J. Zhao et al., 2024; Zurita et al., 2012), pyrite (J. Zhao et al., 2024) and high manganese oxide content (Wu et al., 2014). Other studies have chosen to mix inorganic substrates together, such as mixing generic sands with gravels (Al-Abbas & Ismail, 2024; Baquir et al., 2024; Olmos-Márquez et al., 2012; Singhakant et al., 2009a, 2009b) or mixing gravel or soils with coal cinder (J. Li et al., 2014), clay (Arroyo et al., 2013) and iron oxide containing minerals (Corroto et al., 2019; H. Liu et al., 2022).A few studies have used cocopeat as their main organic substrate (Lizama-Allende et al., 2011, 2012) while other studies have mixed inorganic gravels, limestone and sands with organic substrates that included soils with high organic carbon content (loamy, black or potting soils) (Nakwanit et al., 2011; Nguyen et al., 2021; Palacios Robles et al., 2024; Türker, 2016; Ye et al., 2003), organic peat (Türker, 2016) and cocopeat/coconut shell (Lizama-Allende et al., 2014; Nakwanit et al., 2011). There are a few studies that used unique substrates that included hydrosoils and sediments from field scale CWs and local natural rivers. Park et al., 2022 used sediments from a field scale CW used for water purification. Other researchers used CWs connected in series that contained hydrosoils that came from local sources such as creeks or rivers and amended some of their CWs with inorganic and organic components including sand, gravel, peat moss, shredded hardwood, hay, gypsum and zero valent iron (Dorman et al., 2009; Eggert et al., 2008; Schwindaman et al., 2014a, 2014b; Spacil et al., 2011; Sundberg-Jones & Hassan, 2007; Z. Zhang et al., 2017a, 2017b).

Another important feature of the substrates is whether or not they are able to support plant and/or microbial life. None of the studies adequately describe the nutrient composition of their substrates if such nutrients were even present. The most basic nutrients for plants would be a source of nitrogen and phosphate while a readily available carbon source such as simple sugars or carbohydrates would support microbial life. It is important to note here, that in some studies, the lack of nutrients in the substrate is sometimes compensated by the presence of nutrients for plants and/or microbes in the wastewater (See Section 2.14).

CWs filled with substrates with potentially little to no nutrient content include CWs filled mostly with inorganic substrates that include gravels/stones, limestone, zeolites, iron/manganese oxide containing minerals, clays and various sands (Arroyo et al., 2013; Buddhawong et al., 2005; Corroto et al., 2019; Fan et al., 2021; Hua et al., 2019; H. Liu et al., 2022; Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021; Olmos-Márquez et al., 2012; K. Z. Rahman et al., 2011, 2014, 2008aa, 2008bb; Singhakant et al., 2009a, 2009b; Valles-Aragón et al., 2014; Wu et al., 2014; Xu et al., 2024; Zurita et al., 2012).

CWs that were filled with or mixed with organic materials (wood mulches, hay, and peat moss) or soils that are presumed to contain organics (especially black, potting, loamy soils) likely supported plant and microbial growth (Palacios Robles et al., 2024). The use of cocopeat (coconut coir) alone or mixed with inorganic substrates in some studies likely did not support the growth of plants as it is poor in N, P and K but may have supported the growth of microbes as it is rich in mostly carbohydrates and lignin (Lizama-Allende et al. 2014, Lizama-Allende et al. 2012, Lizama-Allende et al. 2011b).

In some studies, hydrosoils/sediments from natural sources or field scale treatment CWs and even drinking water treatment residue were used as CW substrates and it was difficult to assess the nutrient content of these soils (J.-H. Park et al., 2022; Thathong et al., 2019; Xu et al., 2024) while other studies amended soils with plant nutrients (Spacil et al., 2011) or added organic matter such as pine mulch (Eggert et al., 2008) or organic matter of unknown origin and type to promote microbial growth (Dorman et al., 2009; Hendrikse et al., 2018; Sundberg-Jones & Hassan, 2007).

2.13 Wastewater characteristics

There are two main categories of arsenic contaminated waters treated using CWs. They include synthetic waters and waters treated from real-world sources, as shown in Table 2-2 and Table 2-3. The synthetic ground waters were made using tap water and were formulated to mimic the composition of arsenic contaminated ground waters from contaminated water wells in the U.S.A.(H. Liu et al., 2022) and Bangladesh (Schwindaman et al., 2014a, 2014b). In one study, arsenic was added to native ground water of unknown origin or composition (Valles-Aragón et al., 2014). Other CWs treated synthetic mine wastewaters (Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021; Rabbi et al., 2024). Park et al., 2022 used water from an in-situ water purification wetland in Okcheon, Chungcheongbuk-do, South Korea used to remove pollutants from the Daecheong Lake that is used for drinking water and spiked the water with DMA(V). Synthetic domestic wastewaters contaminated with arsenic were also treated in CWs. Although there was no obvious reason given for using arsenic contaminated domestic wastewater in some CW studies (Fan et al., 2021; J. Li et al., 2014; J. Zhao et al., 2024), other studies were particularly interested in how arsenic removal in CWs changed when changing the redox and the type of microbial activity in their CWs by varying the concentration of arsenic, carbon and sulfate in their wastewater (K. Z. Rahman et al., 2011, 2014, 2008aa, 2008bb). Synthetic industrial wastewaters are used in CW studies for two reasons, it is more practical to formulate the mixtures and it allows researchers to focus on the most toxic components of the wastewaters that pose the highest risk to human health and the environment. The synthetic industrial wastewaters were designed to simulate wastewaters generated from mining and processing of minerals (Arroyo et al., 2013; Buddhawong et al., 2005; Hua et al., 2019; Thathong et al., 2019) and those generated from fossil fuel extraction and combustion (Dorman et al., 2009;; Spacil et al., 2011; Sundberg-Jones & Hassan, 2007). The remaining studies used synthetic waters designed to simulate drinking water naturally contaminated by arsenic in places like China, Mexico and Thailand (Olmos-Márquez et al., 2012; Singhakant et al., 2009b, 2009a; Wu et al., 2014; Xu et al., 2024).

Significantly fewer studies were focused on treating arsenic from real-world arsenic contaminated waters, as shown in Table 2-3. There was only one study that examined the removal of arsenic from ground water in Mexico (Zurita et al., 2012) and another for drinking water in Thailand (Nakwanit et al., 2011). The remaining studies examined removal of arsenic from industrial wastewaters that included mining (Nguyen et al., 2021; Palacios Robles et al., 2024; Türker, 2016), reverse osmosis rejection water (Corroto et al., 2019) wastewaters associated with fossil fuel extraction and combustion (Hendrikse et al., 2018; Ye et al., 2003), leather tannery wastewater (Al-Abbas & Ismail, 2024) and upflow anaerobic sludge blanket reactor effluent water containing metals/metalloids (Baquir et al., 2024).

Eggert et al., 2008 is the only study that compared the removal of arsenic from both synthetic and real-world industrial wastewater (flue gas desulfurization waters) in their CWs.

2.14 Wastewater composition

As shown in Table 2-2 and Table 2-3, most of the CWs in the literature treated As(V) and As(III) contaminated waters but many studies did not report what species of arsenic were present in their

wastewaters, particularly studies that treated real-world arsenic contaminated waters. Only a single study by Park et al. 2022 attempted to treat DMA(V) contaminated water.

Although most studies attempted to remove only arsenic from water using CWs, many studies had multiple contaminants that were being treated along with arsenic. The other contaminants included Fe, Zn, Pb, B, Cu, Mn, Li, Co, Ni, Sr, Ba, F, Cl, Al, Ga, V, Mo, Se, Cr, Zn, Se, Hg, Cd, cyanide solids and other organics. Studies that treated arsenic along with other contaminants usually came from studies that treated wastewaters that came from real-world industrial sources or synthetic wastewaters that mimicked real-world industrial wastewaters. The presence of other contaminants may have interfered with the removal of arsenic. In other cases, such as with the Lizama-Allende et al. papers, the presence of Fe(II) that formed Fe(III) oxyhydroxides upon neutralization by substrates, particularly limestone gravel, may have enhanced arsenic removal through arsenic coprecipitation with the iron oxides.

The arsenic contaminated water being treated in CWs may also be divided up into those that contained no nutrients for plants and/or microbes and those that contained nutrients for plants and/or microbes as shown in Table 2-2 and Table 2-3. As with the substrates in Section 2.12, nutrients for plants is defined as the presence of nitrogen or phosphate that is present or added to the wastewater and nutrients for microbes is defined as readily oxidizable carbon sources such as sugars or carbohydrates that are present in or added to the wastewater. A large proportion of studies did not provide nutrients in their wastewaters to support plant or microbial growth. In a study by Li et al., 2014, they stated they used synthetic sewage solution but do not state the composition of their solution. Other studies only provided nutrients for plant or microbial growth and fewer studies provided nutrients for both. For those studies that do not provide nutrients to either plants or microbes, the implicit conclusion that that can be made is that these studies were largely relying on abiotic removal of arsenic and not the direct and indirect mechanisms of removal that may be provided by microbes and plants (covered in Section 2.16).

Table 2-2: The types of arsenic contaminated synthetic waters treated in CWs from the literature

Water type	Study	[As species] (ppm)	Other target contaminants	Nutrients
Ground	**Liu et al. 2022**	As(V) 0.1 ppm	None	Plants
	Schwindaman et al. 2014a	As(V) 0.12 ppm, As(III) 0.18 ppm	None	None
	Schwindaman et al. 2014b	As(V) 0.12 ppm, As(III) 0.18 ppm	None	None
	Valles-Aragón et al. 2014	As(III) 0.09066 ppm	None	None
Surface	**Park et al. 2022**	DMA(V) 7.5 ppm	None	Plants
	Lizama-Allende et al. 2021	Unknown 2.93 ppm	Fe, Zn	None
	Lizama-Allende et al. 2018	Unknown 1 ppm	Fe, Pb, Zn	None
	Lizama-Allende et al. 2014	As(V) 2.6 ppm	Fe, B	None
	Lizama-Allende et al. 2012	As(V) 3.08 ppm	Fe, B	None
	Lizama-Allende et al. 2011b	As(V) 0.89 ppm	B, Cu, Fe, Mn, Zn	None
Domestic	**Fan et al. 2021**	As(V) 0.0508, 0.0998, 0.4728 ppm	None	Both
	Li et al. 2014	Unknown 1 ppm	F	Unknown
	Rahman et al. 2014	As(V) 0.2 ppm	None	Both
	Rahman et al. 2011	As(V) 0.89 ppm	None	Both
	Rahman et al. 2008a	As(V) 0.2 ppm	None	Both
	Rahman et al. 2008b	As(V) 0.2 ppm	None	Both
Industrial	**Hua et al. 2019**	Unknown 1 ppm	Al, Ga, V, Mo	Plants
	Thathong et al. 2019	Unknown 0.5 ppm	Fe	None
	Arroyo et al. 2013	Unknown 20 ppm	Zn	None
	Spacil et al. 2011	As(III) 0.018 ppm	Se, hydrocarbons	*Both
	Dorman et al. 2009	Unknown 0.23 ppm	Cr, Zn, Se, Hg	None
	Eggert et al. 2008	Unknown 0.171 ppm	Hg, Se	None
	Sundberg and Hassan 2007	As(III) 0.34 ppm	Hg, Se	None
	Buddhawong et al. 2005	As(V) 0.5 ppm	Zn	Plants
Drinking	**Wu et al. 2014**	As(V) 0.5 ppm	None	Plants
	Olmos-Márquez et al. 2012	As(III) 31.3 ppm	None	None
	Singhakant et al. 2009a	As(III) 4.7 ppm	None	Plants
	Singhakant et al. 2009b	As(III) 4.1 ppm	None	Plants

Table 2-3: The types of real-world arsenic contaminated waters treated in CWs from the literature

Water type	Study	[As species] (ppm)	Other target contaminants	Nutrients
Ground	**Zurita et al. 2012**	Unknown 0.034 ppm	F, Cl	Plants
Drinking	**Nakwanit et al. 2011**	Unknown 0.36 ppm	None	None
	Xu et al. 2024	As(V) 1 ppm	None	None
Domestic	**Zhao et al. 2024**	As(V) 0.1 ppm	None	Both
Industrial	**Al-Abbas and Ismail 2024**	Unknown 1.5 ppm	Cr, TDS, COD	Both
	Baquir et al. 2024	As(V) 4.6 ppm	Cr(VI)	Microbes
	Palacios Robles et al. 2024	Unknown 0.36 ppm	Li, Cr, Al, Co, Ni, Cu, Zn, Sr, Cd, Ba, Pb	Both
	Rabbi et al. 2024	As(III) 13.9 ppm	Cd, Cr, Pb, Zn, Cu, Ni	Both
	Nguyen et al. 2021	Unknown 0.36 ppm	Cd, Zn, Cu, Fe, Mn	None
	Corroto et al. 2019	As(V) 0.085 ppm	None	Plants
	Hendrikse et al. 2018	Unknown 0.026 ppm	B, Cu, Pb, Zn, hydrocarbons	Microbes
	Türker et al. 2016	Unknown 0.049 ppm	None	None
	Ye et al. 2003	Unknown 0.5 ppm	Se, B, CN	None

*Series C CWs had no nutrients in water, Series A CWs had a feed additive that likely supported both plants and microbes and Series S CWs had sucrose added to water that likely only supported microbes

2.15 Environmental conditions

The environmental conditions for using CWs to remove arsenic from water were divided between climate-controlled conditions and conditions where no climate control was used. Climate-controlled conditions refer to the use of green houses or growth chambers to mainly control temperature and sometimes humidity as well (Buddhawong et al., 2005; Hua et al., 2019; J.-H. Park et al., 2022; K. Z. Rahman et al., 2011, 2014, 2008ba, 2008aa; Schwindaman et al., 2014a, 2014b; Spacil et al., 2011; Ye et al., 2003; Z. Zhang et al., 2017a). Studies where no climate control was used refers to studies where CWs were exposed to outdoor conditions, outdoor open-air greenhouses or indoor greenhouses with no climate control. Based on the Köppen-Geiger classification for climate (Beck et al., 2018), most of the outdoor studies were done in temperate subtropical (Dorman et al., 2009; Eggert et al., 2008; Fan et al., 2021; Hendrikse et al., 2018; Nguyen et al., 2021; Sundberg-Jones & Hassan, 2007; Wu et al., 2014), dryer Mediterranean climates (Arroyo et al., 2013; Türker, 2016; Zurita et al., 2012), continental humid (J. Li et al., 2014) tropical savanna (Singhakant et al., 2009a, 2009b), tropical temperate (Rabbi et al., 2024), and humid subtropical climates (Baquir et al., 2024). For studies that used open air greenhouses (with at least a roof to prevent rain fall), the CWs were exposed to cold semi-arid climates (Lizama-Allende et al., 2018, 2021), tropical savanna climate (Thathong et al., 2019), humid subtropical climate (Corroto et al., 2019; Z. Zhang et al., 2017b) and marine climates (Lizama-Allende et al., 2011, 2012, 2014). The remaining studies used indoor greenhouses with no climate control (H. Liu et al., 2022; Olmos-Márquez et al., 2012; Valles-Aragón et al., 2014) or the climate control conditions were not stated (Al-Abbas & Ismail, 2024; Xu et al., 2024; J. Zhao et al., 2024).

Many studies did not collect data or track changes in redox potential and/or pH (Al-Abbas & Ismail, 2024; Arroyo et al., 2013; Hua et al., 2019; J. Li et al., 2014; Lizama-Allende et al., 2018; Palacios Robles et al., 2024; Rabbi et al., 2024; K. Z. Rahman et al., 2008ba, 2008aa; Türker, 2016; Wu et al., 2014; Ye et al., 2003; Z. Zhang et al., 2017a; J. Zhao et al., 2024; Zurita et al., 2012) or did not collect this data at all from the pore water in their CWs (Baquir et al., 2024; Eggert et al., 2008; H. Liu et al., 2022; Nakwanit et

al., 2011; Xu et al., 2024; Z. Zhang et al., 2017b). By not tracking this data, the studies made it difficult to assess or deduce the type of microbial activity that may have been occurring in the CWs as well as the speciation and removal of arsenic along with other metals and nutrients in the CWs. This is especially important for CWs that were exposed to outdoor conditions where seasonal changes may have brought about large changes in temperature, pH and redox that may have influenced the abiotic and biotic conditions in the CWs and may have changed the arsenic removal efficacy of the CWs (Arroyo et al., 2013; Baquir et al., 2024; Eggert et al., 2008; J. Li et al., 2014; H. Liu et al., 2022; Lizama-Allende et al., 2018; Nakwanit et al., 2011; Rabbi et al., 2024; Türker, 2016; Wu et al., 2014; Z. Zhang et al., 2017b; Zurita et al., 2012).

2.16 Biological activity

2.16.1 Plants

Twenty-one different plant species have been planted in CWs treating arsenic from water. The top three most common plant species include *Typhya sp.* (Arroyo et al., 2013; Dorman et al., 2009; Eggert et al., 2008; Hendrikse et al., 2018; Nguyen et al., 2021; Schwindaman et al., 2014a, 2014b; Spacil et al., 2011; Sundberg-Jones & Hassan, 2007; Türker, 2016; Ye et al., 2003), *Phragmites australis* (Arroyo et al., 2013; Baquir et al., 2024; Fan et al., 2021; Hua et al., 2019; Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021; Ye et al., 2003) and *Juncus effuses* (Buddhawong et al., 2005; Corroto et al., 2019; Palacios Robles et al., 2024; K. Z. Rahman et al., 2011, 2014, 2008ba, 2008aa; Wu et al., 2014). The less common plant species include *Schoenoplectus* sp. (Dorman et al., 2009; Eggert et al., 2008; Sundberg-Jones & Hassan, 2007; Valles-Aragón et al., 2014), *Iris pseudacorus* (H. Liu et al., 2022; J.-H. Park et al., 2022; Ye et al., 2003)*Scirpus* sp. (Ye et al., 2003; Z. Zhang et al., 2017a, 2017b), *Cyperus* sp.(Corroto et al., 2019; Nakwanit et al., 2011), *Eleocharis macrostachya* (Olmos-Márquez et al., 2012; Valles-Aragón et al., 2014), *Chrysopogon zizanioides* (Singhakant et al., 2009a, 2009b), *Colocasia esculenta* (Nakwanit et al., 2011; Thathong et al., 2019), *Pteris vittata* (Wu et al., 2014; Xu et al., 2024), *Zantedeschia aethiopica* (Zurita et al., 2012), *Anemopsis californica* (Zurita et al., 2012), *Thalia dealbata* (Ye et al., 2003), *Polypogon monspeliensis* (Ye et al., 2003), *Sagittaria latifolia* (Ye et al., 2003), *Canna* sp. (J. Li et al., 2014), *Canna indica* (Al-Abbas & Ismail, 2024; Rabbi et al., 2024) and *Acorus calamus* (J. Li et al., 2014; J. Zhao et al., 2024). Many of these plants are commonly found growing around natural wetlands and have also been used in other CWs treating different kinds of wastewaters (Kadlec & Wallace, 2008). These same plant species have also been found growing in soils and sediments contaminated with high arsenic concentrations (Anh et al., 2011; Bergqvist & Greger, 2012; P. Chang et al., 2005; Dale & Freedman, 1982; Dushenko et al., 1995; Hozhina et al., 2001; Larios et al., 2012; Liu et al., 2006; Michel & Henein, 2007; Usman et al., 2012). The only plant that is not considered a plant that is commonly able to grow in wetlands is *P. vittata* but this plant was likely chosen for its ability to hyperaccumulate arsenic in it's shoots from soil and under hydroponic conditions (Fayiga et al., 2005; L. Q. Ma et al., 2001).

In the majority of studies which have used plants in their constructed wetlands to remove arsenic and other contaminants in their wastewater, it was not possible to tell if the plants grew well because plant growth measurements such as biomass, plant density, plant/shoot count, plant height were a) ignored entirely (Al-Abbas & Ismail, 2024; Baquir et al., 2024; Dorman et al., 2009; Eggert et al., 2008; Lizama-Allende et al., 2011; Palacios Robles et al., 2024; Sundberg-Jones & Hassan, 2007), b) only reported at the beginning of their experiment (Arroyo et al., 2013; Hua et al., 2019; J. Li et al., 2014; Lizama-Allende et al., 2012; Olmos-Márquez et al., 2012; Schwindaman et al., 2014a, 2014b; Singhakant et al., 2009a, 2009b; Spacil et al., 2011; Thathong et al., 2019; Türker, 2016; Valles-Aragón et al., 2014; J. Zhao et al., 2024; Zurita et al., 2012), c) only provided at the end of the experiment (Ye et al., 2003; P. Zhang et al., 2017; Z. Zhang et al., 2017a) or d) the plant growth measurement metrics at the start of the experiment were not the same as the metric measured at the end of the experiment, and therefore no assessment of plant growth could be made (Lizama-Allende et al., 2014; Nakwanit et al., 2011; Wu et al., 2014; Xu et al., 2024). The remaining studies correctly measured initial and final plant growth measurements that either showed plant growth (Buddhawong et al., 2005; Corroto et al., 2019; Nguyen et al., 2021; Rabbi et al., 2024; K. Z.

Rahman et al., 2011, 2008aa) or showed decreased plant growth or plant death by the end of their experiment (Fan et al., 2021; Hendrikse et al., 2018; K. Z. Rahman et al., 2014, 2008b).

2.16.2 Bacteria and biofilms

The metabolic activity, diversity and abundance of bacteria and biofilms within CWs, is usually determined by abiotic factors such as wastewater composition, redox conditions and the ambient temperature of their surrounding environment.

The bacteria in studies that use CWs to treat arsenic from water can largely be assumed to come from the substrates and/or the wastewater used in the CWs. There are only three studies that have used activated sludge to inoculate their CWs with bacteria (Al-Abbas & Ismail, 2024; H. Liu et al., 2022; Xu et al., 2024). Only a few studies have shown the potential presence and activity of microbial communities in their CWs through 16S rRNA sequencing from genetic material removed from CW substrates, typically at the end of the arsenic exposure period (Arroyo et al., 2013; H. Liu et al., 2022; Nguyen et al., 2021; J.-H. Park et al., 2022; Xu et al., 2024; J. Zhao et al., 2024). Xu et al., 2024 found that the identifiable bacterial community in all their CW treatments was largely dominated by Proteobacteria and Bacteroidetes where the genera *Thauera* and *Plasticicumulans* were found in all arsenic and untreated controls, but *Pseudomonas* were found in the highest abundance in treatments with the highest arsenic concentrations. Zhao et al., 2024 found that CWs with pyrite, magnetite and quartz sand all had a high abundance of bacteria from the families Micrococcaceae and Hyphomonadaceae but a high abundance of Opitutaceae was unique to pyrite filled CWs. The bacteria found in CWs with iron containing substrates were found to have a higher occurrence of enzymes involved in iron and sulfur metabolism but all CWs, irrespective of the substrates used, had enzymes involved with arsenic metabolism. Liu et al., 2022, found that CWs with immobile iron-rich particles in the substrate contained a high abundance of anaerobic As(III) oxidizing bacteria from the families Myxococcaceae (15%) and Fimbriimonadaceae (15%) which removed more arsenic than CWs without immobile iron-rich particles. J.-H. Park et al., 2022, found that both planted and unplanted CWs which both removed all of the DMA(V) from the water, primarily through demethylation, had a similar composition of bacteria that included bacteria from the classes Deltaproteobacteria (33-34%), Betaproteobacteria (13-15%) and Bacteroidetes (18-21%). Nguyen et al., 2021 found that soil sampled from planted CWs had a higher diversity of bacteria in planted compared to unplanted CWs but that unplanted CWs removed more arsenic than planted CWs. Arroyo et al., 2013 found that both planted and unplanted CWs had a high abundance of Alpha, Beta, Delta and Gamma proteobacteria (27 - 95%) compared to other bacterial taxa and that CWs planted with *P. australis* or *T. latifolia* had a higher diversity of bacteria than unplanted CWs. CWs planted with *P. australis* had the highest diversity of bacteria (i.e. *P. australis* > *T. latifolia* > unplanted).

Other studies have deduced the presence and activity of bacteria indirectly through changes in the concentration and speciation of nutrients such as carbon, nitrogen, sulfur and through changes in redox potential of the wastewater (Al-Abbas & Ismail, 2024; Baquir et al., 2024; Buddhawong et al., 2005; Dorman et al., 2009; Eggert et al., 2008; Fan et al., 2021; Lizama-Allende et al., 2014; Palacios Robles et al., 2024; K. Z. Rahman et al., 2011, 2014, 2008ba, 2008aa; Singhakant et al., 2009a, 2009b; Spacil et al., 2011; Valles-Aragón et al., 2014; Z. Zhang et al., 2017a, 2017b). For example, a few of the studies have shown denitrification and sulfate reduction activity along with a reduction in total organic carbon (TOC) or chemical oxygen demand (COD) (Fan et al., 2021; K. Z. Rahman et al., 2011, 2014, 2008ba, 2008aa; Z. Zhang et al., 2017a). The remaining studies either largely ignore the activity of bacteria or there was little evidence of bacterial activity (Corroto et al., 2019; Hendrikse et al., 2018; Hua et al., 2019; J. Li et al., 2014; Lizama-Allende et al., 2011, 2012, 2021; Nakwanit et al., 2011; Olmos-Márquez et al., 2012; Schwindaman et al., 2014a, 2014b; Sundberg-Jones & Hassan, 2007; Thathong et al., 2019; Türker, 2016; Wu et al., 2014; Ye et al., 2003; Zurita et al., 2012).

2.17 Fate of arsenic in constructed wetlands

In most of the studies examining arsenic removal in CWs, it was difficult to ascertain what removal mechanism(s) were at play and was largely left to speculation because of data gaps such as missing water

quality measurements that sometimes included important parameters such as pH and redox measurements, along with a lack of information on the type of microbial activity in the CWs. Most studies also lacked any information on arsenic speciation in their substrates and porewaters. As a result, only broad insights into why and how arsenic was removed from the CWs in the current literature could be made. The removal mechanism(s) in these studies are largely left to speculation and only broad insights can be made. Some of these insights include: 1) the type and composition of the substrate may help retain arsenic in the CWs, especially if the substrate are known to adsorb arsenic, 2) the composition and speciation of nutrients and other metals may influence arsenic removal, 3) the presence of plants and microbes as well as their interactions with each other and with substrates depend on the prevailing redox and pH conditions in the CW and 4) CWs setup outdoors and subjected to seasonal changes may experience changes in removal efficacy due to changes in plant and microbial growth.

To better understand how arsenic is removed in CWs, some studies have used a mass balance approach to determine what component or part of their CWs most of the arsenic is found in. For studies that calculate a mass balance, the concentration of arsenic remaining in the effluent water, sediment and plants are determined at the end of the experiment and then multiplied by a volume or mass to determine the mass of arsenic in each compartment and is usually represented as a percentage of the total mass of arsenic added to the CWs (Baquir et al., 2024; Fan et al., 2021; Lizama-Allende et al., 2014; Olmos-Márquez et al., 2012; J.-H. Park et al., 2022; K. Z. Rahman et al., 2011, 2014; Singhakant et al., 2009a, 2009b; Wu et al., 2014; Xu et al., 2024; Ye et al., 2003; Z. Zhang et al., 2017a; J. Zhao et al., 2024). In some cases, the mass of arsenic that was missing from the mass balance was operationally defined as unaccounted/other or was surmised to potentially be the result of microbial volatilization of arsenic (Baquir et al., 2024; Fan et al., 2021; Olmos-Márquez et al., 2012; K. Z. Rahman et al., 2011, 2014; Singhakant et al., 2009a; Wu et al., 2014, 2014; Xu et al., 2024; Ye et al., 2003; J. Zhao et al., 2024). From the studies that calculate a mass balance from their CWs, it was found that arsenic accumulated in the following components in the following descending order: sediments/substrates (64 ± 25 %, range 16 – 100%) > effluent (27 ± 22%, range 0.0 – 72%) > plants (5 ± 7%, range 0 – 34%) > unaccounted (13 ± 9%, range 1 – 25%).

Although the speciation of arsenic was not determined, the removal of arsenic by the substrates in the Lizama-Allende et al., 2014 is largely abiotic in nature as a result of the type of substrate, composition and pH of the wastewater. The acidic wastewater contained arsenic, iron as well as other metals and a pH shift occurred when the wastewater was placed in contact with the limestone or zeolite filled CWs forming iron (III) oxide which adsorbed the arsenic and effectively retained all of the arsenic in the substrates. Lizama-Allende et al., 2011, 2012, 2018, 2021, have also shown this same removal mechanism in their CWs but no mass balance was shown in these studies.

In other CW studies, the speciation and fate of arsenic was less predictable, due to the activity of plants and microbes and their potential interactions. Although microbes may directly influence the speciation of arsenic, they may also simultaneously be responsible for the speciation of iron, manganese and sulfur if present in the wastewater and/or substrates which may be important in determining arsenic removal. Usually under oxidizing conditions, iron(II) and manganese (II) oxidizing bacteria are able to form biogenic iron(III) oxyhydroxides ($Fe(OH)_3$, FeOOH) and manganese oxyhydroxides (MnO_2, Mn_3O_4 $Mn(OH)_2$) respectively, which can remove arsenic from solution through adsorption (Hedrich et al., 2011; Hohmann et al., 2010; Katsoyiannis & Zouboulis, 2004, 2006; Nealson, 2006; Omoregie et al., 2013; Ozaki et al., 2013). Under reducing conditions, sulfate may be transformed into sulfide by sulfate reducing bacteria (SRB) in the presence of As(III) or through ubiquitous bacterial reduction of As(V) and have been shown to form arsenic sulfides such as orpiment (As_2S_3) or realgar (As_4S_4) in both bioreactors and under natural conditions (Battaglia-Brunet et al., 2012; Jackson et al., 2013; Keimowitz et al., 2007; Kirk et al., 2004; Rodriguez-Freire et al., 2014; Teclu et al., 2008). The potential for anaerobic iron(III) and sulfate reducing bacteria to form pyrite which is subsequently able to adsorb arsenic has largely been ignored in the CW literature (Bostick & Fendorf, 2003; Bulut et al., 2014; Couture et al., 2013; Farquhar et al., 2002; Fischer et al., 2021; Han et al., 2013; M.-K. Lee et al., 2018; Pi et al., 2017; Saunders et al., 2018; F. Sun et al.,

2012; Wolthers et al., 2005; X. Zhang et al., 2022). Bacteria may also remobilize adsorbed arsenic from Fe(III) and Mn(III) oxides through reduction or through oxidation of arsenic bearing sulfides (Horvath et al., 2014; J.-H. Huang, 2014; Islam et al., 2004). In most cases, plants take up a very small proportion of the arsenic added to CWs but may have an indirect influence on arsenic removal, either by providing nutrients to microbes through carbon rich root exudates or may provide oxygen rich zones around their roots that may form iron plaques for arsenic to adsorb to (Das et al., 2017; Frémont et al., 2022; H. Li et al., 2011; K. Mei et al., 2021; X. Q. Mei et al., 2009; C. Wu et al., 2012).

Park et al., 2022, Liu et al., 2022 and Zhang et al., 2017a are the only studies that examined arsenic speciation in both pore waters and substrates which also calculated a mass balance. Examination of speciation in Park et al., 2022 showed that DMA(V) was demethylated to form As(III) and As(V) in the substrate but not in the porewater. The substrate in planted CWs also showed a small proportion of arsenic sulfide (As_2S_3). The very high removal by sediments (>98%) in both planted and unplanted CWs in this study was likely due to the microbial demethylation activity that allowed for adsorption of As(III) and As(V) despite not knowing the composition of the substrate. Liu et al., 2022 showed that despite the presence of a higher ratio of As(V):As(III) in both CWs with immobile iron-rich particles (IIRPs) and control CWs without IIRPs, the CWs with IIRP CWs may have removed more arsenic through adsorption of As(V) in the first 10 cm of the wetlands and the presence of anaerobic As(III) oxidizing bacteria in deeper sections of the substrate in these CWs may have helped enhance this adsorption. Zhang et al., 2017a found that CWs treating wastewater with high phosphate concentrations increased the ratio of As(III) to As(V) in the porewater and substrates while the opposite was true for wastewater with low phosphate concentrations. The CWs with low phosphate concentrations removed more arsenic in their sediments (78 – 80%) than the CWs treated with high phosphate (50 – 55%) likely because there was better As(V) adsorption by the substrates due to less interference by phosphate, lower plant growth and lower microbial reduction of iron (III) oxides from the substrate and As(V) in the water which effectively helped reduce the mobility of arsenic.

The remaining studies that calculate a mass balance do not account for arsenic speciation in their CWs. Fan et al., 2021 showed that with increasing arsenic concentrations their gravel likely adsorbed no more than 30% of the arsenic and that plant uptake was reduced with increasing concentration due to arsenic toxicity. Wu et al., 2014 found that CWs with manganese sand removed 76 – 83% of the arsenic from water and removed more arsenic than CWs with ceramsite sand that only removed 34 – 47% of the total arsenic added. Olmos-Márquez et al., 2012 showed that planted CWs removed more arsenic in the first 15 cm of their substrates than unplanted CWs because the plants may have been responsible for the conversion of As(III) to As(V) through root zone oxygenation and As(V) adsorption to their substrates. Singhakant et al., 2009a and 2009b showed that deep CWs removed more arsenic than their shallow planted as well as their unplanted deep and shallow control mesocosms with increasing HRT. They believed that the decomposition of plant roots may have promoted the formation of arsenic sulfides by microbes. Rahman et al., 2011 and 2014 found that shifting the redox conditions by varying the concentration of carbon and sulfate in their CWs led to the accumulation of 42 – 50% of the total mass of arsenic in their plants, mostly in the roots. Ye et al., 2003 used multiple plant species in their CW but only 2% of the total mass of arsenic ended up in the plants and 51% ended up in the sediments, presumably through some amount of adsorption. The results from Schwindaman et al., 2014a show that oxidizing series CWs that received regular amendments of zero valent iron (ZVI) removed more arsenic than oxidizing series CWs without ZVI and reducing CWs with and without ZVI. The issue with the mass balance from this study is that it was not reflective of the overall removal efficiency of the CWs because the mass balance was done only for the first CW in each series and a large proportion of the arsenic was likely unaccounted for and remained in the other CWs in each series.

Chapter 3 – Uptake and speciation of arsenic in hydroponically exposed horsetail plants (*Equisetum* sp.)

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Abstract

Our study examined the arsenic uptake abilities of three horsetail species (*Equisetum fluviatile*, *Equisetum hyemale* and *Equisetum scirpoides*) and the speciation of arsenic in *E. fluviatile* and *E. hyemale* when exposed to 1 and 10 mg/L of arsenate under hydroponic conditions. The concentration of arsenic in the hydroponic solution of both treatments decreased, with more removal for the 1 mg/L treatments. *E. hyemale* removed the most arsenic from the hydroponic solutions, followed by *E. scirpoides* and *E. fluviatile*, respectively. In all plants, root and shoot arsenic concentrations increased with arsenic dose. Root arsenic concentrations were always higher than shoot concentrations. We also demonstrate that *E. hyemale* and *E. scirpoides* are able to grow at both arsenic doses. Through linear combination fittings of XANES spectra for root and shoot tissues from *E. fluviatile* and *E. hyemale*, we discovered that *E. fluviatile* reduces As(V) to As(III) which then reacts with thiols to form As-S complexes in the roots at the highest dose. The As-S complexes in the roots may have restricted arsenic translocation to the shoots. In contrast, the arsenic in *E. hyemale* tissues mostly remained as As(V) at all doses. The ability of *E. hyemale* to grow and remove high concentrations of arsenic may make it a good candidate for use in constructed wetlands treating arsenic from water.

Keywords: arsenic; horsetails; hydroponic uptake; bioavailability; arsenic speciation; constructed wetlands

3.1 Introduction

Some common plant genera that have been used in constructed wetlands (CWs) for treating arsenic from water include *Phragmites, Typha, Juncus, Schoenoplectus* and *Scirpus* (Arroyo et al., 2013; Buddhawong et al., 2005; Corroto et al., 2019; Dorman et al., 2009; Eggert et al., 2008; Fan et al., 2021; Hendrikse et al., 2018; Hua et al., 2019; Lizama-Allende et al., 2011, 2012, 2014, 2018, 2021; Nguyen et al., 2021; K. Z. Rahman et al., 2011, 2014, 2008aa, 2008ba; Schwindaman et al., 2014a, 2014b; Spacil et al., 2011; Sundberg-Jones & Hassan, 2007; Türker, 2016; Wu et al., 2014; Ye et al., 2003). This may be because these plant species have also been found growing in soils and sediments contaminated with high arsenic concentrations (Andrejić et al., 2023; Anh et al., 2011; Bergqvist & Greger, 2012; P. Chang et al., 2005; Dale & Freedman, 1982; Dradrach et al., 2020; Dushenko et al., 1995; Hozhina et al., 2001; Larios et al., 2012; W. Liu et al., 2006; Michel & Henein, 2007; Usman et al., 2012).

Strangely, there have been no studies that have utilized horsetail plants (*Equisetum* sp.) in CWs treating arsenic despite the fact that these plants have been shown growing in the same arsenic contaminated environments as the aforementioned plant species and are able to accumulate relatively high arsenic concentrations (Bergqvist & Greger, 2012; Brooks et al., 1981; Dale & Freedman, 1982; Dradrach et al., 2020; Dushenko et al., 1995; Hozhina et al., 2001; I. Koch et al., 2000; Matanzas et al., 2017; Meharg, 2003; Mir et al., 2007; Wanat et al., 2014). Horsetail plants (*Equisetum* sp.), are primitive fern-like plants that can live in both aquatic and terrestrial habitats and are taxonomically allied with fern genera *Pteris, Pityrogramma* and *Adiantum*, which all have some species that are able to hyperaccumulate arsenic in their tissues (Campos et al., 2015; Kachenko et al., 2007; L. Q. Ma et al., 2001; Raj et al., 2015; N. Singh et al., 2010; Sridokchan et al., 2005; Wanat et al., 2014). *Equisetum* sp. were initially investigated for their ability to potentially accumulate gold and act as indicator species, along with other plants growing in highly mineralized soils (Brussel, 1978; Cannon et al., 1968; Erdman & Olson, 1985; Girling et al., 1978, 1979; Girling & Peterson, 1980; Warren & Delavault, 1950). There was some controversy as to how much gold could be accumulated by *Equisetum* sp. at the time and the general consensus after some more investigation and improved analytical methods was that *Equisetum* sp. accumulated more arsenic than gold (Brooks, 1982; Brooks et al., 1981).

The best way to assess the ability of some plants to grow and remove arsenic from water in CWs is to use hydroponic experiments, which maximize the bioavailability of arsenic to the plants (phytoavailability) by removing the effect of soils and sediments; hydroponic conditions also mimic the water saturated conditions of CWs. Specifically, the bioavailability of arsenic to plants may be affected by

the nature (organic or inorganic) and availability of absorbent surfaces, which in turn is governed by the pH, redox and presence of other competing ions in soils and sediments (Meharg & Hartley-Whitaker, 2002; Mohan & Pittman, 2007; Moreno-Jiménez et al., 2010; Pi et al., 2015; Sharma & Sohn, 2009; S. Wang & Mulligan, 2006a).

In this study we exposed *E. fluviatile*, *E. hyemale* and *E. scirpoides* to 1 and 10 mg/L of As(V) under hydroponic conditions. These concentrations reflect those found in contaminated ground waters and mine effluents, respectively (Wang and Mulligan, 2006b). We assessed 1) the concentration of arsenic in *Equisetum* sp. tissues and 2) arsenic speciation in fresh tissues using X-ray absorption near-edge structure (XANES) spectroscopy. This study will ultimately be used to determine if *Equisetum* sp. is suitable for planting in CWs treating arsenic contaminated water.

3.2 Materials and Methods

3.2.1 Experimental conditions and setup

The uptake experiments were conducted as two separate experiments. In the first experiment, we used stationary phase *E. fluviatile* and *E. hyemale* plants obtained from Ergonic Resources Ltd. Saskatoon, SK. For each horsetail species, the plants were washed with tap water to remove soil, pebbles and other debris and then divided into 9 plastic 600 mL containers containing hydroponic nutrient solution to allow for a 13-day acclimation period at room temperature under white fluorescent lights. The nutrient solution was made in tap water following Hoagland-Arnon No. 2 nutrient solution for macronutrients (Hoagland and Arnon, 1950) with a modified micronutrient solution (5 mg/L NaFeEDTA, 0.5 mg/L H_3BO_3, 0.5 mg/L $MnCl_2*4H_2O$, 0.05 mg/L $ZnCl_2*2H_2O$, 0.02 mg/L $CuCl_2*2H_2O$ and 0.05 mg/L $Na_2MoO_4*2H_2O$). Following the 13-day acclimation period, fresh Hoagland's solution with 0 mg/L, 1 mg/L and 10 mg/L of As(V) in triplicate were added to each of the 9 pots for each horsetail species ($Na_2HAsO_4*7H_2O$). The treatments were kept at room temperature under white fluorescent lights for 16 days. On days 0 and 16, all containers were topped off to the 600 mL mark with tap water and 5 mL samples of the hydroponic solution were taken and frozen for later dilution with 2% nitric acid and analysis of total arsenic using ICP-MS. On the last day of the experiment, all plants were sequentially washed with tap water and ultrapure water (<18 Ω) and blotted dry with paper towel. Three shoot and root tissue samples were taken from each treatment, wrapped in tin foil and flash frozen using liquid nitrogen and stored at -80°C for X-ray absorption near-edge structure (XANES) spectroscopy speciation analysis. The remaining plants were stored at -20°C and later processed for total arsenic analysis.

In the second experiment, we wanted to test the uptake ability and growth of horsetails that were actively growing, for comparison to the stationary phase plants in the first experiment. We did not have healthy *E. fluviatile* plants growing in soil or under hydroponic conditions at the time so we decided to use *E. scirpoides* instead as there has been only one study that documented the uptake of arsenic in *E. scirpoides* plants conducted by Brooks et al., 1981. The *E. hyemale* and *E. scirpoides* plants in this case originated from The Bloomin Bog Water Gardens, Ilderton ON. These plants were grown in Vigoro 3 in 1 garden mix (black humus, Canadian sphagnum peat moss and compost) under green house conditions. Wet weight stem cuttings from the soil grown *E. hyemale* and *E. scirpoides* plants were taken and then the stems were placed in separate glass tubes with Hoagland's solution to allow the cuttings to establish roots which took 2-3 weeks. In a similar way to the first experiment, in triplicate and for each horsetail species, plants were placed in separate glass test tubes with 15 mL of Hoagland's solution containing 0 mg/L, 1 mg/L and 10 mg/L of As(V). All treatment tubes were placed at room temperature under white fluorescent lights for 45 days. To determine the initial and final concentration of total arsenic in the Hoagland's solutions, water samples from all treatment tubes were sampled on day 0 and 45 and stored in a refrigerator at 4°C until analysis for total arsenic by ICP-MS (within a 2-week time period). On the last day of the experiment, all plants were sequentially washed with tap water and ultrapure water (>18 MΩ-cm) and blotted dry with paper towel. The final wet weight biomass of the plants was recorded. Plants were then processed for total arsenic analysis.

3.2.2 Total Arsenic Analysis

To determine the total amount of arsenic in *Equisetum* sp. and its distribution in the plants, the plants were divided up into root and shoot tissues. There was no obvious formation of iron root plaques on the surface of the roots that could adsorb/coprecipitate arsenic. Only the arsenic found in the roots was considered and any arsenic found on the surface of the roots was likely washed away. The wet weights of the tissues were determined and recorded; the tissues were then dried in an oven at 65°C for 24–48 hrs and weighed again to record their dry weights. These measurements are used to determine the % moisture in the tissues and hence allow for the calculation of both dry and wet weight concentrations of arsenic in the tissues after acid digestion. Root and shoot tissues were ground into powder form. A quantity of 0.5 g of powdered plant sample was weighed into a pre-weighed digestion tube containing a boiling chip, although for some samples less powder was available (minimum 0.02 g). Then, 10 ml of 70% nitric acid (Fisher TraceMetal™ Grade, ON, Canada) was added to each digestion tube and placed on a heat block (VWR International, ON, Canada) at 121°C; each sample was gently vortexed (Fisher Vortex Genie 2, ON, Canada) every 30-45 min. The digestion continued until all plant materials were dissolved and most of the acid had evaporated to leave 1–2 mL of digest solution. The digests were then diluted with 10 mL of ultrapure (>18 MΩ-cm) water and the final weight of the solution, digest tube and boiling chip was recorded. The digest solutions were then filtered through 0.45 µm syringe filters using 10 mL syringes into new 15 mL c-tubes (Fisherbrand, ON, Canada). The filtered digests were stored at 4°C. The digests were diluted appropriately (30–100x) using 2% nitric acid prior to total arsenic analysis using ICP-MS (Elan DRC II, Perkin Elmer, MA, USA.).

For the Hoagland's solutions that were sampled from the treatments over the duration of the plant uptake experiments, the unfiltered samples were also diluted appropriately (2–100x) using 2% nitric acid solution and run on the ICP-MS instrument.

Calculations for whole plant arsenic concentrations (Eq. S1), tissue and plant arsenic bioconcentration factors (BCFs) and root to shoot translocation factors (TFs) are shown in the Supplementary data in Eq. S1 to S4, respectively.

3.2.3 X-Ray absorption near-edge spectroscopy (XANES)

Flash frozen *E. fluviatile* and *E. hyemale* fresh shoot and root tissues were analyzed at the Advanced Photon Source (APS) synchrotron facility (Illinois, USA) to obtain XANES spectra, which allowed the identification of the arsenic species and proportions in the plant tissues through linear combination fitting of the data.

Through a partnership of the APS X-ray Science Division and the Canadian Light Source (CLS@APS), the bending magnet beam line at Sector 20 (20-BM) was used. XANES spectra of the arsenic Kα-edge (11868 eV) were recorded in fluorescence mode using a solid-state Ge detector (Canberra model GL0055PS) while monitoring incident and transmitted intensities in N_2-filled transmission ionization chambers. Frozen plant samples were placed in a sample holder with minimum sample handling. This consisted of cutting shoots for a given sample to lengths of approximately 4–5 mm and layering the sections to make a sample approximately 5 mm x 2 mm x 2mm in size; roots were folded and forced with tweezers into the sample holder to make a similarly sized sample, also with minimal handling. Samples were held between two layers of Kapton™ tape and immediately frozen in liquid N_2, where they were kept until placement in a liquid N_2 cryostat maintained at 100K (Model 22 CTI Cryodyne Refrigerator System, Janis) for analysis. A total of 3 to 5 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. The Si(111) double crystal monochromator was calibrated using the first inflection point of the gold LIII absorption edge (11919.7 eV). A reference gold foil was measured simultaneously with all samples. Additional experimental setup information can be found in Smith et al., 2005 and Nearing et al., 2014. XANES spectra of the arsenic Kα-edge (11868 eV) were fit within -20 to +30 eV from the arsenic binding energy using Athena software. Frozen liquid As(III) and As(V) standards (Koch et al., 2011), and liquid DMA(V) and MMA(V) (Smith et al., 2005) previously measured by our group were used for fittings.

3.3 Results

3.3.1 Removal of As(V) from Hoagland's solution

The initial and final arsenic concentrations in the hydroponic solutions are shown for the first experiment and second experiment in Table 3-1. In the first experiment, there was no significant difference in arsenic concentrations in the hydroponic solution between day 0 and day 16 for treatments with *E. fluviatile* and *E. hyemale* at the 1 mg/L As(V) dose (ANOVA post hoc Tukey $p>0.05$). When *E. fluviatile* and *E. hyemale* were exposed to the 10 mg/L As(V) dose, the concentration of arsenic in the hydroponic solution by day 16 was significantly less than the amount initially added on day 0 (ANOVA post hoc Tukey $p<0.05$).

In the second experiment, there was no significant difference in arsenic concentrations in the hydroponic solution between day 0 and day 45 for treatments with *E. hyemale* and *E. scirpoides* at the 1 mg/L As(V) dose (ANOVA post hoc Tukey $p>0.05$). When *E. hyemale* and *E. scirpoides* were exposed to the 10 mg/L As(V) dose, the remaining concentration of arsenic in the hydroponic solution by day 45 was significantly less than the concentration initially added on day 0 for *E. hyemale* only (ANOVA post hoc Tukey $p<0.05$).

E. hyemale at the 1 and 10 mg/L As(V) dose in both experiments showed no significant difference in the concentrations of arsenic remaining in the hydroponic solution when comparing day 16 and day 45 concentrations (ANOVA post hoc Tukey $p>0.05$).

There were no significant differences in the % arsenic removals for *Equisetum* sp. exposed to the same arsenic dose within each experiment ($p>0.05$ ANOVA post hoc Tukey test). There were also no significant differences in the % arsenic removals for *E. hyemale* exposed to the same arsenic dose when comparing the two different experiments ($p>0.05$ ANOVA post hoc Tukey test).

Table 3-1: The initial and final concentration of arsenic in the Hoagland's solution at the start and end of each hydroponic experiment and the percent removal by the *Equisetum* sp. plants

Species	As(V) Treatment (mg/L)	Total As in Hoagland's (mg/L)		As removal (%)
		Initial total [As] (mg/L)[a]	Final total [As] (mg/L)[b]	
E. fluviatile	**1**	0.84 ± 0.04	0.18 ± 0.02	78 ± 1
	10	8.2 ± 0.4	2.7 ± 0.3■	67 ± 4
E. hyemale	**1**	0.74 ± 0.05	0.23 ± 0.09	69 ± 13
	10	7.3 ± 0.7	3.9 ± 0.92■	46 ± 15
E. hyemale	**1**	1.2 ± 0.3	0.34 ± 0.18	71 ± 15
	10	11 ± 3	5.0 ± 3.5■ Δ	55 ± 32
E. scirpoides	**1**	1.2± 0.3	0.55 ± 0.29	53 ± 24
	10	11 ± 3	6.3 ± 1.7	43 ± 15

All values are average ± SD for n=3 replicates. All control treatments with no arsenic had a total arsenic concentration of ≤ 0.01 mg/L for n=20 samples.

■ = Indicates for which arsenic dose and what *Equisetum* sp. showed significantly less arsenic remaining in the hydroponic solution for each separate experiment ($p<0.05$ ANOVA post hoc Tukey test).

Δ = Indicates for which experiment and for what arsenic dose there was significantly less arsenic remaining in the hydroponic solution when comparing *E. hyemale* in both experiments ($p<0.05$ ANOVA post hoc Tukey test).

[a]In the first experiment, initial total [As] were sampled after adding the solutions to the pots while in the second experiment, initial total [As] were sampled from the original stock solutions on day 10, 23 and 45 and averaged.

[b]The final total [As] for the first hydroponic experiment with *E. fluviatile* and *E. hyemale* occurred on Day 16 and for the second experiment with *E. hyemale* and *E. scirpoides* occurred on Day 45.

3.3.2 Hydroponic uptake of As(V) by *E. hyemale*, *E. fluviatile* and *E. scirpoides*

As shown in Table 3-2, both tissue and plant arsenic concentrations increased with increasing As(V) concentration. All tissue and plant arsenic concentrations were significantly greater for plants exposed to the 10 mg/L As(V) treatment in comparison to the control ($p<0.05$) and 1 mg/L As(V) treatments ($p<0.05$). Only *E. hyemale* in the first experiment at the 10 mg/L As(V) treatment showed significantly greater arsenic concentrations in the root tissues as compared to the shoot tissues ($p<0.05$). Root arsenic concentrations for *E. hyemale* in the first experiment at the 10 mg/L As(V) dose was also significantly greater than *E. fluviatile* root arsenic concentrations at the same As(V) dose ($p<0.05$). *E. hyemale* plants had a higher arsenic concentration than the other *Equisetum* sp. at all arsenic treatment concentrations but this was not significant, likely due to the very high variability seen in the root arsenic concentrations. The equivalent average wet weight tissue arsenic concentrations are given in Appendix A Table S1.

Table 3-2: Average dry weight (mg/kg dw) tissue arsenic concentrations in *Equisetum* sp. at the end of each experiment

		[As] (mg/kg dw)		
Species	**Treatment (mg/L)**	**Shoot**	**Root**	**Plant[a]**
E. fluviatile	**0**	0.37 ± 0.04	1.5 ± 0.6	1.4 ± 0.5
	1	3.6 ± 0.3	9.2 ± 0.7	8.8 ± 0.6
	10	37 ± 3†§	62 ± 6†§	60 ± 6†§
E. hyemale	**0**	0.39 ± 0.07	7.2 ± 7.1	6.1 ± 6.2
	1	7.4 ± 1.0	21 ± 15	16 ± 9
	10	39 ± 13†§	210 ± 100†§Δ□	170 ± 70†§
E. hyemale	**0**	0.68 ± 0.43	0.58 ± 0.94	0.56 ± 0.24
	1	17 ± 7	33 ± 10	16 ± 6
	10	162 ± 12†§	510 ± 420§	170 ± 40†§
E. scirpoides	**0**	0.44 ± 0.37	3.0 ± 4.3	0.59 ± 0.63
	1	14 ± 4	24 ± 4.3	14 ± 4
	10	83 ± 22†§	570 ± 170†§	120 ± 40†§

All values are average ± SD for n=3 plants per treatment.
a= Calculated using weighted averages of the shoot and root tissue.
†= Indicates the treatment dose for which there was a significantly higher tissue/plant arsenic concentration compared to the control treatment for each *Equisetum sp.* in each experiment ($p<0.05$ ANOVA post hoc Dunnett test).
§= Indicates significantly higher tissue/plant arsenic concentration at the 10 mg/L As(V) dose compared to the 1 mg/L As(V) dose for each *Equisetum sp.* in each experiment ($p<0.05$ ANOVA post hoc Tukey test).
Δ= Indicates root arsenic concentrations significantly higher than shoot arsenic concentrations for the same *Equisetum* sp. within the same experiment ($p<0.05$ ANOVA post hoc Tukey test).
□= Indicates what *Equisetum* sp. had a significantly greater arsenic concentration for the same tissue type and treatment dose in each experiment ($p<0.05$ ANOVA post hoc Tukey test).

3.3.3 Bioconcentration and translocation of As(V) in *Equisetum* sp.

As shown in Table 3-3 for both the first and second experiment, root BCFs were greater than shoot BCFs for all As(V) treatments and *Equisetum* species tested but this was only significant for *E. fluviatile* in the first experiment and *E. scirpoides* in the second experiment ($p<0.05$) likely because there was very high variability in root concentrations for *E. hyemale* in both experiments. With respect to whole plant BCFs, only *E. fluviatile* showed a significantly greater removal of arsenic at the 1 mg/L As(V) dose compared to

the 10 mg/L As(V) dose. Whole plant BCFs for the remaining *Equisetum* sp. were greater than for *E. fluviatile* but this was not significant. The relatively high BCF values for the remaining *Equisetum* sp. also showed little difference, indicating that the As(V) dose had not influenced their capacity to remove arsenic from solution.

E. hyemale in the first experiment also a significantly greater shoot BCF at the 1 mg/L As(V) dose compared to the 10 mg/L As(V) dose ($p<0.05$). *E. hyemale* also had a significantly greater shoot BCF than *E. fluviatile* at the 1 mg/L As(V) dose in the first experiment ($p<0.05$). *E. hyemale* in the second experiment had a significantly higher shoot BCF than *E. scirpoides* at the 10 mg/L As(V) ($p<0.05$). *E. hyemale* in the second experiment had a significantly higher shoot BCF than *E. hyemale* in the second experiment at the 10 mg/L As(V) dose.

TFs for all treatment doses remained below 1 as a result of high root to shoot arsenic concentrations in all *Equisetum* sp. In the first experiment, TFs for *E. fluviatile* exposed to 10 mg/L As(V) were significantly greater than for *E. fluviatile* exposed to the 1 mg/L As(V) dose as well as *E. hyemale* exposed to the 10 mg/L As(V) dose ($p<0.05$). In the second experiment, TFs for *E. scirpoides* exposed to 1 mg/L As(V) was significantly greater than *E. scirpoides* exposed to 10 mg/L As(V). Calculated average wet weight BCFs and TFs are given in Appendix A Table S2.

Table 3-3: Calculated average dry weight tissue bioconcentration factors (BCFs) and translocation factors (TFs) in *E. fluviatile*, *E. hyemale* and *E. scirpoides*

Species	Treatment (mg/L)	Shoot BCF	Root BCF	Plant BCF	TF
E. fluviatile	**1**	4.3 ± 0.3	$10.9 \pm 0.9^{\dagger\#}$	$10.4 \pm 0.8^{\dagger}$	0.40 ± 0.03
	10	4.6 ± 0.5	$7.7 \pm 0.9^{\#}$	7.3 ± 0.8	$0.60 \pm 0.05^{\dagger\S}$
E. hyemale	**1**	$9.9 \pm 0.8^{\dagger\S}$	27 ± 18	21 ± 10	0.46 ± 0.23
	10	5.5 ± 1.6	30 ± 16	24 ± 12	0.22 ± 0.15
E. hyemale	**1**	14 ± 6	28 ± 9	14 ± 5	0.55 ± 0.24
	10	$15 \pm 1^{\S\Delta}$	46 ± 38	15 ± 4	0.44 ± 0.23
E. scirpoides	**1**	12 ± 3	$20 \pm 4^{\#}$	12 ± 3	$0.59 \pm 0.08^{\dagger}$
	10	8 ± 2	$51 \pm 15^{\#}$	10 ± 4	0.15 ± 0.04

All values are average ± SD for n=3 plants per treatment.
† = Indicates which treatment concentration yielded a significantly greater BCFs or TFs for each *Equisetum* sp. ($p<0.05$ Welch's t-test).
§ = Indicates which *Equisetum* sp. yielded a significantly greater BCFs or TFs for the same treatment concentration within each experiment ($p<0.05$ Welch's t-test).
Δ = Indicates in which experiment *E. hyemale* yielded a significantly greater BCFs or TFs for the same treatment concentration ($p<0.05$ Welch's t-test).
= Indicates that root BCFs were significantly greater than shoot BCFs for the same treatment for each *Equisetum* sp. ($p<0.05$ Welch's t-test).

Table 3-4: Arsenic speciation in *E. hyemale* and *E. fluviatile* tissues

The speciation of arsenic in flash frozen *E. hyemale* and *E. fluviatile* root and shoot tissues exposed to 1 and 10 mg/L As(V) from the first experiment was examined using XANES. At the time of XANES analysis, only these two *Equisetum* sp. were available for speciation analysis.

As shown in Figure 3-1, the three main arsenic species that were identified in the tissues of both species are inorganic species: arsenate [As(V)], arsenite [As(III)], and an As(III) compound matched to arsenic(III) triglutathione [$As(SG)_3$] with corresponding white line K-edge energies of 11875, 11872 and 11870 eV respectively. The As(III)-S compound matched to $As(SG)_3$ is referred to as $As(SG)_3$ but the structure of the compound in the plant tissues was not further elucidated (XANES analysis only gives information on the oxidation state and what the arsenic is immediately bound to, i.e. nearest neighbor).

From Figure 3-2 it can be seen that for *E. fluviatile* exposed to 1 mg/L As(V) for 16 days, roots contained 45% As(V), 41% As(III) and 15% As(III)-S while shoots contained 100% As(V). When *E. fluviatile* plants were exposed to 10 mg/L As(V) for 16 days, roots contained 15% As(V), 24% As(III) and 62% As(III)-S while shoots contained 46% As(V) and 54% As(III).

From Figure 3-2, it can also be seen that for *E. hyemale* exposed to 1 mg/L As(V) for 16 days, roots contained 100% As(V) while arsenic in the shoots was not detectable through XAS. This may have occurred because of the overall low arsenic concentration in this sample (2.5 mg/kg ww, Appendix A Table S1), and because of the heterogeneity of the sample (to preserve arsenic species, samples were not homogenized prior to XAS analysis). When *E. hyemale* plants were exposed to 10 mg/L As(V) for 16 days, roots contained 91% As(V) and 9% As(III)-S while shoots contained 54% As(V) and 46% As(III).

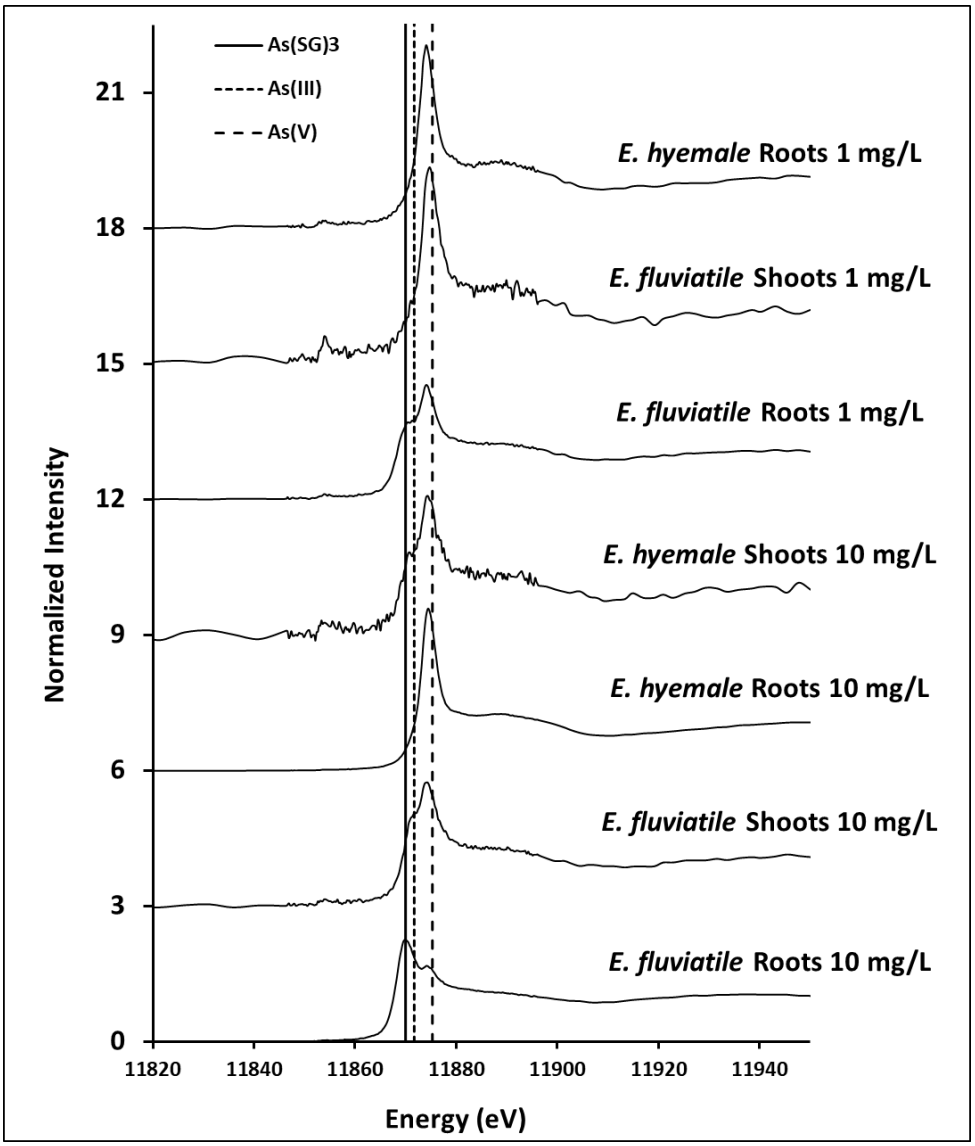

Figure 3-1: X-ray absorption near-edge structure (XANES) spectra of arsenic in shoot and root tissues of *E. fluviatile* and *E. hyemale* exposed to 1 and 10 mg/L of As(V) in hydroponic culture for 16 days. Arsenic in shoots of *E. hyemale* exposed to 1 mg/L As(V) was not detectable and hence the XANES spectrum could not be included in the figure.

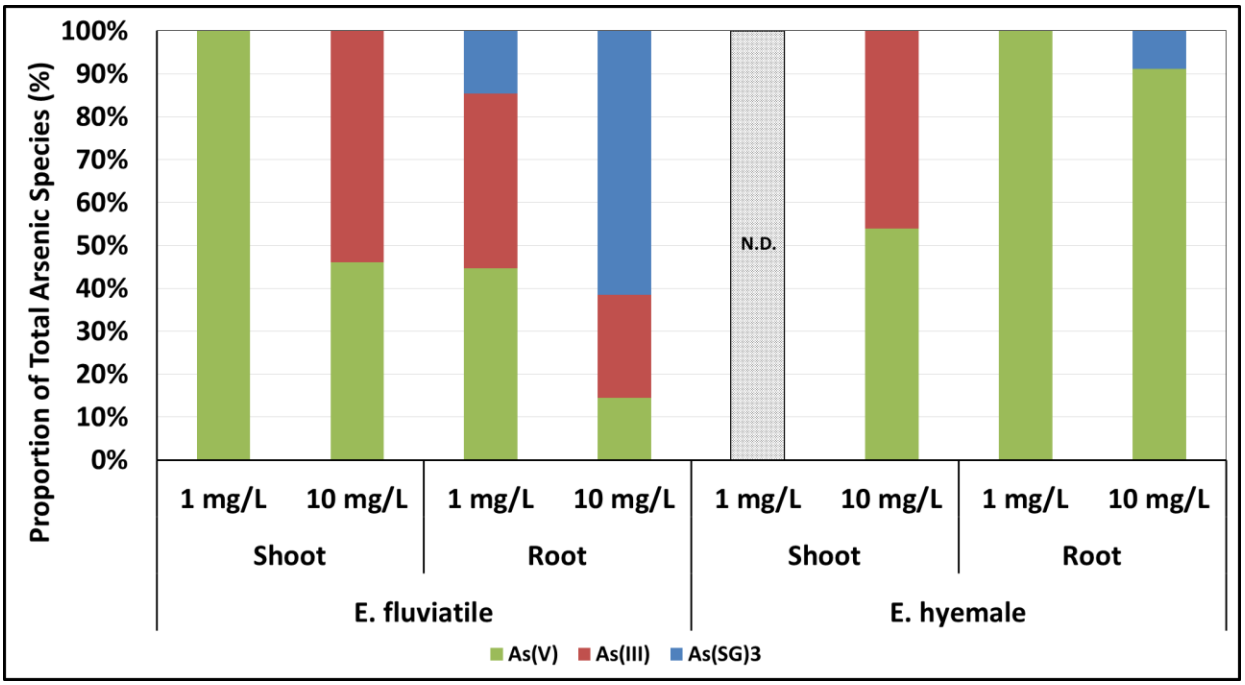

Figure 3-2: Proportion of arsenic compounds determined by linear combination fitting of XANES data for shoot and root tissues of *E. fluviatile* and *E. hyemale* exposed to 1 and 10 mg/L of As(V) in hydroponic culture for 16 days. Arsenic (III) triglutathione [As(SG)3] was used for fitting As(III)-S species but the structure of this arsenic compound was not further elucidated. N.D. = Not Detectable for ww tissue concentrations of arsenic less than 10 mg/kg .

3.4 Discussion

Under most conditions, As(V) and As(III) are generally considered to be the most phytoavailable and hence most common species of arsenic found in plants (N. Li et al., 2016). In plant roots, As(V) is an analog of phosphate and may be transported through phosphate transporters while As(III) may be transported into cells via membrane proteins (aquaglyceroporins) that transport compounds such as glycerol, urea and silicic acid (Abbas et al., 2018; Kumar et al., 2022; N. Li et al., 2016; Z. Liu et al., 2002; Panda et al., 2010; Shankar et al., 2014; F. J. Zhao et al., 2009).

As shown in Table 3-1, the total arsenic concentration remaining in the Hoagland's solutions in both hydroponic experiments at both arsenic doses decreased over time but the decrease in arsenic concentration was only significant for *E. fluviatile* and *E. hyemale* in the first experiment and *E. hyemale* in the second experiment at the 10 mg/L dose. Although there was a higher % arsenic removal from the hydroponic solution for the 1 mg/L As(V) dose compared to the 10 mg/L As(V) dose for all *Equisetum* sp. in each experiment, the increased removal was not significant. There was also no difference between the % arsenic removal from the hydroponic solution at either arsenic dose when comparing *E. hyemale* in both experiments.

In concordance with arsenic removal from the Hoagland's solution, we see that in Table 3-2, all *Equisetum* sp., arsenic concentrations in the shoots, roots and whole plants at the 10 mg/L dose were significantly greater than for the 1 mg/L dose likely because there was more arsenic available for uptake. Root arsenic concentrations at the 10 mg/L dose for *E. hyemale* were significantly greater than the root arsenic concentrations for *E. fluviatile* at the same dose, only in the first experiment. Root arsenic concentrations were higher than shoot arsenic concentrations for all *Equisetum* sp., but this was only significant for *E. fluviatile* and *E. scirpoides* at both exposure doses because there was very high variability in the root arsenic concentrations for *E. hyemale* at both doses and in both experiments. The high variability

in root arsenic concentrations in plants may be due to competitive inhibition by phosphate in the Hoagland's solution which may have reduced the amount of arsenic taken up by *E. hyemale* in our experiments (Garbinski et al., 2019; Kumar et al., 2022; F. J. Zhao et al., 2009). This high variability in root arsenic concentrations also influenced the concentration of arsenic in shoot tissues for *E. hyemale* in both experiments. Although the difference was not significant, shoot arsenic concentrations for *E. hyemale* in the second experiment were greater than shoot arsenic concentrations in the first experiment at both arsenic doses. This was perhaps because of a longer arsenic exposure period in the second experiment and/or the plants in the second experiment were younger and actively growing which may have allowed for more phosphate and arsenic uptake, particularly since arsenic mimics phosphate (Marsh et al., 2000; Sandil et al., 2023).

Unlike *Equisetum* sp. collected from arsenic contaminated soils that had tissue BCFs < 1 (dry weight concentrations), the BCFs from our study, shown in Table 3-3 (also dry weight concentrations), have tissue BCF values much greater than 1, which would indicate greater bioavailability of arsenic to our plants under hydroponic conditions (Andrejić et al., 2023; Anh et al., 2011; Bergqvist & Greger, 2012; Dradrach et al., 2020; Dushenko et al., 1995; Matanzas et al., 2017; Usman et al., 2012; Wanat et al., 2014; Xue et al., 2014). Only a fraction of the total amount of arsenic found in some contaminated soils and sediments (<2-50%) is available for plant uptake (P. Chang et al., 2005; Larios et al., 2012; Wanat et al., 2014). The bioavailability of arsenic in soil is governed by variables such as arsenic speciation, soil composition, pH and redox conditions as well as the biological activities of microbes and plants (Abbas et al., 2018; Badri & Vivanco, 2009; Frémont et al., 2022; Jia et al., 2014). It was clear that under our hydroponic conditions, where arsenic bioavailability was maximized, *E. hyemale* and *E. scirpoides* tolerated higher arsenic concentrations in their tissues than *E. fluviatile*.

E. fluviatile may have restricted both the transport of arsenic into the roots as well as translocation to the shoots. As shown in Figure 3-1, the primary arsenic species found in *E. fluviatile* and *E. hyemale* are As(V) and As(III) with more As(III)-S appearing in *E. fluviatile*. From Figure 3-2 and Appendix A Table S3, we show that the proportion of As(III) increases with increasing arsenic dose for *E. fluviatile* and *E. hyemale* shoots. The greatest differences in arsenic speciation between the two *Equisetum* sp. were seen in the proportion of arsenic species in the roots. *E. hyemale* formed very little to no reduced arsenic species in the roots. It may be that *E. hyemale* was avoiding the toxic effects of arsenic by enzymatically reducing As(V) to As(III) and expelling As(III) from the roots through aquaglyceroporin transporters, out of the plant (Kumar et al., 2022).

For *E. fluviatile*, the proportion of As(III)-S increased with increasing arsenic dose in the roots. Although we did not identify the exact identity and concentration of the As(III)-S molecule in the plant tissues, we hypothesize that these molecules may be $As(GS)_3$, PC precursor molecules or that PCs may have been directly formed (Hartley-Whitaker et al., 2001; Meharg et al., 2008; Schat et al., 2002; Sneller et al., 1999).To reduce oxidative stress and cellular damage, plants may directly store $As(GS)_3$ or phytochelatins in root vacuoles (S. Pandey et al., 2015; Raab et al., 2005; Song Won-Yong et al., 2010). We propose that *E. fluviatile*, like other non-hyperaccumulating plants, restricted the translocation of arsenic from the roots to the shoots by forming and storing As-complexes primarily in their roots (Batista et al., 2014; Briat, 2010; Duan et al., 2011; W.-J. Liu et al., 2010; Meharg et al., 2008; Mishra et al., 2017; Raab et al., 2005; F.-J. Zhao et al., 2010). This function of As-S complexes contrasts with their hypothesized role in hyperaccumulating ferns, namely that they may act as temporary ligands for transport of As(III) into vacuoles in the shoots (Briat, 2010; Indriolo et al., 2010; Pickering et al., 2006; Watanabe et al., 2014; X. Yang et al., 2009). However, As-S complexes are found in only low amounts in shoots of hyperaccumulating ferns, with arsenic found as As(III) coordinated to oxygen (Kachenko et al., 2010; X.-W. Li et al., 2009b; Pickering et al., 2006).

Most of the studies that have examined arsenic speciation in *Equisetum* sp. have come from studies that collected plants from arsenic contaminated soils/sediments, where most studies examined speciation in the shoots (I. Koch et al., 2000; Kuehnelt et al., 2000; Mir et al., 2007) and only a single study by Bergqvist and Greger, 2012 examined arsenic speciation in both roots and shoots. These studies used conventional

speciation methods that used wet extraction and chromatographic separation coupled with ICP-MS detection that were designed to detect inorganic and organic arsenic species, but not the less stable As-S species that would require different extraction methods and chromatographic conditions (Bluemlein et al., 2008, 2009; Montes-Bayón et al., 2004; Nearing et al., 2014; Raab et al., 2005, 2004a, 2004b). Similar to our study, most of the arsenic in *Equisetum* sp. in these studies was detected as inorganic As(V) and As(III) in both tissues. Unlike our study, these studies also detected much smaller proportions of MMA, DMA and TMAO in the tissues, likely derived from microbial or fungal activity in the soil/sediments (Cullen, 2014).

Although we did not measure wet biomass growth in the first experiment, there were no obvious or visible signs of plant toxicity. To improve upon the first experiment, we obtained growth data in the second experiment by measuring the wet weight biomass at the beginning and the end of the exposure period for *E. hyemale* and *E. scirpoides*, as shown in Appendix A Figure S1. First, we show that, on average, there was an increase in wet biomass by the end of the second experiment but this increase was only significant for *E. scirpoides* from the 1 and 10 mg/L As(V) treatments. Secondly, based on the final wet biomass, we were able to show that the *Equisetum* sp. exposed to 1 and 10 mg/L As(V) in the second experiment were able to grow as well as the controls ($p>0.05$ ANOVA post hoc Dunnettt test). Lastly, we show that actively growing young *E. hyemale* plants exposed for a longer period of time in the second experiment are able to take up more arsenic than stationary phase *E. hyemale* plants in the first experiment exposed to arsenic for a shorter period of time.

Based on both the plant growth and arsenic concentrations found in the present study, *Equisetum* sp. are likely arsenic tolerant non-hyperaccumulators, reminiscent of the tolerance and arsenic accumulation shown in other fern species *Pteridium aquilinum* and *Athyrium yokoscense* (Van et al., 2006; Visoottiviseth et al., 2002; Wanat et al., 2014). Although it is counter-intuitive to believe that arsenic may enhance the growth of plants, work with hyperaccumulating ferns (plants that accumulate arsenic in the 1000s of mg/kg dw) has shown that exposure to arsenic may enhance nutrient uptake to enhance growth (Campos et al., 2015, 2018; Cao et al., 2004; X. Liu et al., 2016; Peng et al., 2023; N. Singh et al., 2010; Sridokchan et al., 2005; Tu & Ma, 2005). Based on the results of our hydroponic study, *E. hyemale* may be well suited for arsenic removal in CWs treating arsenic from water.

3.5 Conclusion

In this study we have demonstrated the ability of *E. fluviatile*, *E. hyemale* and *E. scirpoides* to take up As(V) and remove it from the hydroponic nutrient solution. From our study and from previous studies in soil, it is clear that *E. fluviatile*, *E. hyemale* and *E. scirpoides* are arsenic tolerant plants which are able to withstand higher arsenic concentrations in their environment and in their tissues. A higher concentration of arsenic was found in the roots than in the shoots in all *Equisetum* sp. at all doses. With increasing As(V) dose, *E. hyemale* plants had the highest plant arsenic concentrations in both experiments (up to 170 mg/kg dw As) despite the high variability in tissue arsenic concentrations in both experiments.

XANES analysis on *E. fluviatile* and *E. hyemale* root and shoot tissues revealed that arsenic is primarily found as As(V) and As(III) at the lower arsenic doses but at the high arsenic dose, there are higher proportions of reduced arsenic species in the tissues which include As(III) and As(III)-S. At the high arsenic dose, *E. fluviatile* may be restricting the movement of arsenic by forming As(III)-S complexes which are sequestered and remain stable in the acidic environment of root vacuoles. On the other hand it is less clear what role As(III)-S plays in *E. hyemale* which did not show a higher proportion of As(III)-S with increasing arsenic exposure but instead As(V) was the predominant arsenic species in the roots, potentially indicating an as of yet unknown mechanism for preventing the reduction of As(V) to As(III) and restricting the translocation of arsenic to the shoots. This allowed for *E. hyemale* to accumulate more arsenic in the shoots than the other *Equisetum* species at both arsenic doses, in both experiments. In the second experiment, the initial and final biomass of *E. hyemale* and *E. scirpoides* were measured to measure growth, and both plant species were able to grow at both arsenic doses.

Due to the ability of *E. hyemale* plants to accumulate the highest concentrations of arsenic in their roots, translocate more of the arsenic to the shoots and grow in the presence of arsenic, this plant species may be well suited for use in constructed wetlands treating arsenic from water.

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Author Contributions

Antoine Hnain: Conceptualization, methodology, investigation, formal analysis, data curation, writing - original draft.; **Michelle Nearing:** Data acquisition and processing.; **Iris Koch:** Writing – review & editing, supervision, funding acquisition **Kela Weber:** Writing – review & editing, resources, supervision, funding acquisition.

Chapter 4 – Arsenic removal is mediated by biofilms in non-aerated and aerated wetland mesocosms

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Abstract

Planted and unplanted recirculating constructed wetland mesocosms maintained under non-aerated (reducing) and aerated (oxidizing) conditions were compared in their ability to remove 1 mg/L As(V) from simulated domestic wastewater over a 33-week period. Each week, one day prior to the addition of arsenic, non-aerated mesocosms removed 70% of the nitrate and 95% of the organic carbon likely through denitrification while aerated mesocosms removed organic carbon only (97%). Upon arsenic addition and treatment for the remainder of the week, non-aerated mesocosms removed more arsenic (72%) than aerated mesocosms (43%). For the first time in the literature, we estimated the proportion of arsenic found in biofilms detached from gravel and incorporated this aspect into mass balance calculations. We discovered that biofilms from non-aerated mesocosms contained a higher proportion of arsenic (28%) than biofilms from aerated mesocosms (5%). Plants from both types of mesocosms removed a negligible amount of arsenic (<0.41%). Through X-ray fluorescence (XRF) analysis, it was discovered that biofilms from non-aerated mesocosms had the highest iron concentrations (35,000 mg/kg) and this iron was identified as pyrite (2–15 wt%) through X-ray diffraction (XRD) analysis. The iron concentration of aerated biofilms was no different than that found in clean gravels (17,500 mg/kg) and no iron minerals were detectable in these samples using XRD analysis. Although some of the arsenic removal in both types of wetlands was thought to be attributable to adsorption onto substrates and biofilms, as well as uptake into biofilms, removal of arsenic in the non-aerated mesocosms was likely enhanced by the presence of pyrite because pyrite can adsorb arsenic directly, or indirectly through the formation of iron oxides that can adsorb or coprecipitate with the iron oxides.

Keywords: constructed wetlands; arsenic; water treatment; biofilms; pyrite; mass balance; aeration

4.1 Introduction

Arsenic is a toxic metalloid that can occur naturally in the environment. It is a component of 245 naturally occurring minerals found in the earth's crust most commonly found in association with iron sulfide and oxide minerals (Mandal & Suzuki, 2002). Arsenic can be released from these minerals through weathering, geothermal and biological processes into natural waters and aquifers primarily as arsenate (As(V)) and arsenite (As(III)) (Bundschuh & Maity, 2015). Some regions of the world where arsenic may naturally be found at very high concentrations in aquifers include parts of the Western USA, South America, India, Bangladesh and other East and Southeastern Asian countries (R. Singh et al., 2015). In Canada, natural surface and ground water concentrations of arsenic commonly range between <1.0 to 5.0 µg/L, which is well below the maximum concentration limit of 10 µg/L set by the World Health Organization's (WHO) guidelines (WHO, 2022). However, there are some regions of Canada where drinking and surface waters are contaminated by arsenic from both natural and anthropogenic sources (McGuigan et al., 2010).

Technologies used for the removal of arsenic from water include: 1) air or chemical pre-oxidation and precipitation; 2) chemical coagulation and coprecipitation with alum, iron or lime; 3) sorption using iron coated sand or activated alumina and ion-exchange resins; and 4) membrane filtration techniques, which include nanofiltration, reverse osmosis and electrodialysis (Mohan & Pittman, 2007; R. Singh et al., 2015). These removal technologies may be costly because they may require a high degree of process control as well as the input of energy, chemicals and the replacement of adsorbent materials. Additional costs may be incurred due to the frequent removal of toxic sludges (weeks to months), that require dewatering and disposal. An advantage of using CWs for treating arsenic is that they can last for years before the wetlands need to be decommissioned. The iron oxide sludges generated from the aforementioned conventional treatment technologies are bulkier, difficult to dewater and are more difficult to stabilize to prevent arsenic leaching when compared to sulfidic sludges that can potentially be generated in CWs (Georgaki et al., 2004; Q. Hu et al., 2024; Ouyang et al., 2023; Tanapon et al., 2008; Yong Gan et al., 2005).

Low cost, passive treatment systems such as engineered constructed wetlands (CWs) (also commonly referred to as treatment wetlands) can be employed to remove arsenic from water. CWs have been shown to be effective in removing toxic heavy metals and metalloids that include Cd, Cr, Cu, Fe, Hg,

Mn, Ni, Pb, Se and Zn from acid mine drainage, industrial wastewater sources, storm water, groundwater and sewage sludge (Yu et al., 2021; Z. Zhang et al., 2024).

The studies that have used CWs for the removal of arsenic from water, and have also tracked the fate of the arsenic in the wetlands through mass balance calculations, have largely ignored the amount of arsenic found in biofilms, despite acknowledging that abiotic conditions (substrate type, pH, redox and dissolved oxygen) and biotic conditions (species and activity of plants and bacteria) may influence arsenic removal in wetlands. Most of these studies were set up outdoors or in greenhouses with no climate control, and the prevailing redox conditions supporting either exclusively oxidizing (Lizama-Allende et al., 2014; Olmos-Márquez et al., 2012; Singhakant et al., 2009b) or reducing conditions (Fan et al., 2021; H. Liu et al., 2022; Singhakant et al., 2009a; Wu et al., 2014). Fan et al. (2021) showed that the removal efficiency of arsenic in their outdoor CWs was better in the hot summer months than in the cold winter months. The studies of Liu et al. (2022), Lizama-Allende et al. (2014), and Wu et al. (2014) showed that abiotic co-precipitation with and/or adsorption of arsenic to iron and manganese oxide rich substrates were largely unchanged through different seasons. Other studies did not account for changes in environmental conditions during their experiments (Olmos-Márquez et al., 2012; Singhakant et al., 2009a, 2009b). This made it difficult to assess whether the variables they tested influenced arsenic removal to any significant degree. The remaining studies used climate-controlled growth chambers where reducing conditions prevailed in the wetlands or redox conditions were manipulated by changing the concentration of arsenic, carbon and sulfate in the wastewater (J.-H. Park et al., 2022; K. Z. Rahman et al., 2011, 2014; Z. Zhang et al., 2017a).

To address some of the knowledge gaps found in the literature and improve our understanding of arsenic removal in constructed wetlands, our study 1) directly compares the ability of planted and unplanted mesocosms to remove arsenic, added as arsenate (As(V), from water under either non-aerated (reducing) or actively aerated (oxidizing) conditions; 2) estimates the proportion of the arsenic mass removed from the water that is attributable to biofilms; 3) examines the metal and mineral composition of biofilms; and 4) proposes removal mechanisms for arsenic from reducing and oxidizing treatment systems.

4.2 Materials and Methods

4.2.1 Construction and operation of the mesocosms

Twelve mesocosm systems were set up indoors and constructed of PVC columns (90 cm high, 25 cm diameter). As shown in Figure 4-1, each mesocosm had a small centrifugal pump (March Series 1, 1A-MD1/2, 11.3 L/min, Glenview, Illinois) which allowed the water to move up to a perforated PVC inflow pipe located ~12 cm below the surface of the gravel bed and to percolate through the gravel substrate and collect at the bottom of the mesocosm for recirculation through a perforated PVC outflow pipe. Black PVC piping through which water circulated served as an injection and sampling port. A water overflow port was located ~5 cm below the surface of the gravel to maintain a water height of approximately 75 cm in each mesocosm. The perforated PVC outflow pipe at the bottom of each mesocosm was connected to a drainage port with a valve that allowed the mesocosms to be drained when needed.

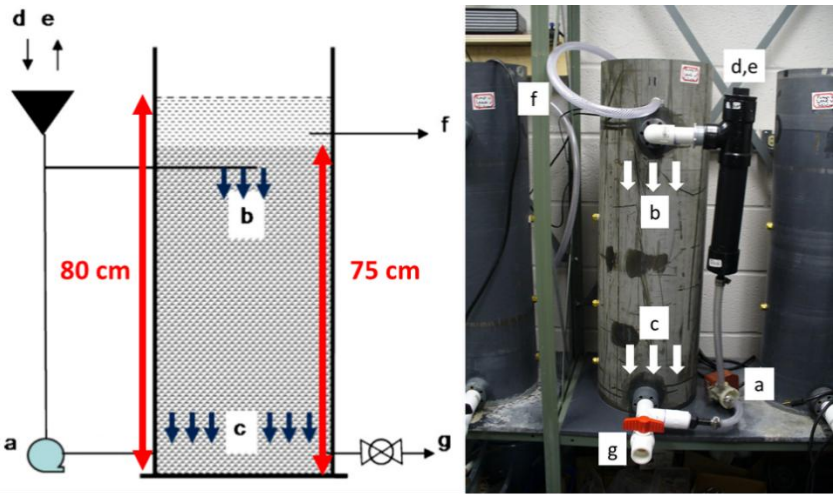

Figure 4-1: Schematic diagram of wetland mesocosms modified from Weber & Legge, 2011 showing a = water pump, b = outflow pipe, c = inflow pipe, d/e = injection/sampling port, f = overflow port and g = drainage port

The first six systems (M1–M6) were designed to be non-aerated and to produce reducing conditions, while the remaining six columns (M7–M12) were designed to produce oxidizing conditions through aeration using 6" aeration stone discs (Figure S2) and an air pump (Q2 air pump, Fluval Mansfield, MA, U.S.A.) with a 6-way air manifold for air distribution (Atlantic Pond Supply, Moncton NB, Canada). To seed the mesocosms with a microbial consortia, an activated sludge sample (~6 L) was collected from a local wastewater treatment plant (Cataraqui Bay Waste Water Treatment Plant, Kingston ON, Canada) and ~160 mL of activated sludge was layered with the pea gravel at 60, 40 and 20 cm depths from the bottom of the mesocosms, with a final pea gravel height of 80 cm from the bottom. Each mesocosm had approximately 72 kg of gravel (Section 3.0.4 will describe mineralogy) with an initial porosity of approximately 0.30. An analysis of the minerology of the substrate is described in Section 4.2.10.

Twelve liters of a simulated domestic wastewater solution as described in Weber & Legge (2011) to support the growth of microbes and plants was freshly prepared and fed to each mesocosm every 7 days. The recipe for this solution is shown in Appendix B Table S4. To monitor any changes in the free pore volume in each of the mesocosms, the volume of simulated domestic wastewater solution required to fill the mesocosms was measured at the beginning of each week. Changes in free pore volume in the mesocosms may be affected by changes in biofilm growth, root growth, or the dissolution/precipitation of mineral phases (See Appendix B Figure S3).

4.2.2 Arsenic exposure experiments

After 3.9 years of operating and maintaining the mesocosms the six non-aerated and six aerated mesocosms were further divided into three planted and three unplanted treatments, where planted mesocosms were planted with rough horsetail (*Equisetum hyemale*) as shown in Figure S4. Ten *E. hyemale* stem cuttings from plants growing in greenhouse conditions were planted in M1–M3 and M7–M9. The average initial wet biomass of plants in each planted mesocosm was 10.6 ± 1.9 g with an initial plant density of 0.13 shoots/in^2. Incandescent grow lights were placed above the planted mesocosms (Otte-Lite Grow, 20W, 950 lumens, OttLite Technologies, Inc., FL, U.S.A.). The plants were allowed to establish themselves for 16 weeks. At the end of the 16-week plant establishment period, the average plant density for non-aerated and aerated mesocosms was 0.11 ± 0.07 shoots/in^2 and 0.12 ± 0.03 shoots/in^2. The unintended growth of common liverworts (*Marchantia polymorpha*) grew only in the non-aerated planted systems potentially because their spores/gemmae were transferred to the mesocosms along with *E. hyemale* plants and the conditions in these mesocosms favored their growth. Arsenic exposure experiments began after the plant establishment period. On a weekly basis, arsenic was added to

the wastewater of all mesocosms 24 hrs after addition of fresh nutrients to a final concentration of 1 mg/L of As(V) (Added from a stock solution of 10000 mg/L As(V) prepared using $Na_2HAsO_4 \cdot 7H_2O$, 98% Alfa Aesar). The arsenic exposure experiment lasted 33 weeks to reflect a typical period of time with no snow cover in southern Ontario, Canada. The total mass of arsenic added to each set of mesocosms over the experimental period is shown in Appendix B

Table S5.

4.2.3 Water quality and nutrient cycling measurements

Water quality parameters were measured on a daily basis (5 days a week) using YSI Professional Plus probes (YSI Inc., Yellow Springs, OH) for the first 16 weeks from the sampling port (Figure 4-1e). To ensure the comparability of all water measurements, mesocosms were topped up with tap water each day to account for water lost through evaporation/evapotranspiration. The parameters measured include water temperature (ºC), conductivity (µS/cm), pH, ORP (oxidation reduction potential mV), DO (dissolved oxygen mg/L), NH_4^+–N (ammonium nitrogen mg/L) and NO_3^-–N (nitrate nitrogen mg/L). All probes were calibrated on a monthly basis according to the manufacturer's instructions.

Water samples for total organic carbon (TOC) analysis were collected from freshly prepared wastewater samples prior to addition to the mesocosms and from the mesocosm after 7 days of treatment for weeks 1, 3, 5, 7, 9, 11, 13 and 15. Water samples for TOC analysis were stored at -20ºC until analysis on a TOC-TN analyzer (Analytik Jena TOC/TN_b multi NC 2100S, Jena, Germany).

4.2.4 Determination of final plant and biofilm masses

Final plant biomass: At the end of the 33-week arsenic exposure experiment, *E. hyemale* plants were recovered from the planted mesocosms, divided into root and shoot tissues and were weighed to obtain their final wet biomasses. *M. polymorpha* plants that only grew in the non-aerated planted mesocosms (M1–3) alongside *E. hyemale* were also recovered. As *M. polymorpha* plants are non-vascular plants with no distinction between root or shoot tissues, whole plants were weighed to obtain their final wet biomass.

Final biofilm biomass: To estimate the total mass of dry biofilm in each of the mesocosms approximately 38 g of wet gravel with attached biofilms was excavated from each layer (0–20 cm, 20–50 cm and 50–80 cm) and weighed in pre-weighed and clean porcelain crucibles and dried at ~105°C for 24 hrs. The dried gravel with biofilms were then cooled in a desiccator and weighed. The weighed samples were then ignited at 550°C in a muffle furnace for 1 hr, allowed to cool overnight inside the furnace, and weighed. The amount of dry biofilm on the gravel was calculated as the difference between the dry mass after drying at 105°C and the mass after ignition at 550°C. This method was adapted from a standard method 2540 G for total, fixed and volatile solids in solids and semisolid samples (American Public Health Association et al., 1998). To estimate the mass of biofilm in each layer for each mesocosm the mass of biofilm in kg lost on ignition was divided by the kg of gravel that was remaining after ignition (kg biofilm lost/kg gravel ignited) and was then multiplied by the total mass of gravel estimated for that layer in kg (gravel layer volume in L x gravel density in kg/L). To estimate the total final dry biofilm mass in each mesocosm, the biofilm biomasses from all three gravel layers were added together.

4.2.5 Determination of arsenic in plants

To determine the total concentration of arsenic in *E. hyemale* shoots and roots as well as whole *M. polymorpha* plants, all plant materials were dried in an oven at 65ºC for 24–48 hrs and weighed. A quantity of 0.5 g of homogenized plant sample was weighed in a pre-weighed digestion tube containing a boiling chip, although for some samples less powder was available (minimum 0.02 g). Three digestion tubes with 0.5 g of dry Bush Twigs and Leaves GBW 07603, a standard reference material (SRM), were digested alongside plant tissues in an effort to determine how effective the digestion procedure was by measuring the % recovery of arsenic from the SRM. Ten millilitres of 70% nitric acid (Fisher TraceMetal™ Grade, ON, Canada) were added to each digestion tube and placed on a heat block (VWR International, ON,

Canada) at 121°C; each sample was gently vortexed (Fisher Vortex Genie 2, ON, Canada) every 30–45 min. The digestion continued until all plant materials were dissolved and most of the acid had evaporated to leave 1–2 mL of digest solution. The digests were then diluted with 10 mL of ultrapure (>18 MΩ-cm) water and the final weight of the solution, digest tube and boiling chip was recorded. To remove particulates, the digest solutions were then filtered through 0.45 µm syringe filters using 10 mL syringes into new 15 mL c-tubes (Fisherbrand, ON, Canada). Whole plant dry weight arsenic concentrations for *E. hyemale* were calculated using a weighted average ((Root dry mass/ Total dry plant mass) x Dry root [As]) + ((Shoot dry mass/ Total dry plant mass) x Dry shoot [As]). No weighted average was required to obtain dry weight arsenic concentrations for *M. polymorpha* as they are non-vascular plants with no roots or shoots and therefore were digested as whole plants.

4.2.6 Simultaneous detachment of biofilms and recovery of desorbed arsenic from biofilms and substrates

A phosphate buffer (PB) solution was used because it has been shown in previous studies to detach biofilm and desorb arsenic from biofilm EPS, calcareous and/or iron oxyhydroxide rich substrates (Marzi et al., 2022; Neupane et al., 2014; Saba et al., 2019; Weber & Legge, 2010). Specifically, 25 g of gravel from three depths (top 0–20 cm, middle 20–50 cm and bottom 50–80 cm) from each mesocosm were placed in Erlenmeyer flasks with 100 mL of PB solution (10 mM pH 7.4) and shaken for 3 hrs at 100 rpm (Innova 2100 Platform shaker, New Brunswick Scientific Inc., CT, U.S.A.). To collect the detached biofilms, the PB solutions were decanted into new 50 mL tubes for each sample and the solutions were filtered using pre-weighed 47 mm, 0.45 µm mixed cellulose ester filter paper (MilliporeSigma, MA, U.S.A.) and a vacuum pump (Buchi V-500, BUCHI Corporation, DE, U.S.A). PB filtrates were analyzed for total arsenic, and the filter paper was prepared and digested in the same way as plant tissues.

4.2.7 Recovery of amorphous iron oxide associated arsenic

The gravels that remained in the Erlenmeyer flasks from the PB solution detachment procedure received 100 mL of citrate-bicarbonate-EDTA (CBE) solution and were shaken for 30 min at 100 rpm. CBE solution was used to dissolve amorphous iron oxides that may be bound to arsenic (M. Rahman et al., 2014). Solutions were filtered, with filtrates and filter papers analyzed as described for the PB detachment procedure.

4.2.8 Determination of arsenic in mesocosm water samples, digests and biofilm filtrates

Mesocosm water samples were collected after 6 days of treatment for weeks 1, 6, 14, 16, 25 and 33 to determine total arsenic concentrations and calculate a percent removal efficiency ((Initial Total [As] - Final Day 6 Total [As]) / Initial Total [As]) x 100). All digests, water samples and biofilm filtrates were stored at 4°C. All digests, wastewater samples and biofilm filtrates were diluted using 2% nitric acid and then analyzed by ICP-MS to obtain total arsenic concentrations. The diluted plant digests were analysed on a PerkinElmer Elan DRC II ICP-MS and all other samples were analysed on a PerkinElmer NexION 310D ICP-MS. ICP-MS instrument conditions are shown in Appendix B Table S6 along with all QA/QC procedures.

4.2.9 Mass balance calculations for wetland compartments

Water: As stated earlier, the free pore water volume in each mesocosm was measured on a weekly basis during wastewater renewal. This allowed for accurate calculation of the total mass of arsenic (in mg) treated in each mesocosm on a weekly basis and by extension helped determine the total mass of arsenic treated in each mesocosm during the whole 33-week experiment.

Plants: To obtain the mass of arsenic in the *E. hyemale* plants, the total dry weight arsenic concentration in whole plants, obtained through a weighted average of the dried root and shoot tissues, was multiplied by the total mass of the plants for each planted mesocosm. Likewise, for the non-aerated mesocosms (M1–3) with *M. polymorpha* plants, the total dry weight arsenic concentrations were multiplied by the total dry mass of *M. polymorpha* in each of these mesocosms. For the non-aerated mesocosms, the

mass of arsenic found in whole *E. hyemale* plants was summed with the mass of arsenic found in whole *M. polymorpha* plants.

Biofilms: The solids remaining on the filters after filtration of the PB-detached and CBE-detached suspensions were assigned as the biofilm component, made up of microbial mass, extracellular polymeric substances (EPS), and possibly precipitated minerals. To estimate the mass of arsenic found in biofilms for each mesocosm, the total dry weight arsenic concentrations from the filter from each layer of each mesocosm were multiplied by the total dry mass of solids retained by the filters from their corresponding layer and summed.

Adsorbed As: The mass of arsenic found in PB filtrates was assigned as the arsenic that was adsorbed to the gravel and to biofilm EPS that was easily desorbed and solubilized. For each mesocosm, the mass of arsenic found in PB filtrates was obtained by multiplying the concentration of arsenic found in each filtrate by the volume of the filtrate for each gravel layer. To obtain the total mass of arsenic removed by PB filtrates for an entire mesocosm, the masses of arsenic for each gravel layer were summed for each mesocosm and multiplied by the ratio of the total mass of gravel in the mesocosms (~72 kg) to the total mass of gravel used for detachment (3 layers x 0.025 kg = ~0.075 kg).

Iron associated As: The mass of arsenic found in CBE filtrates was assigned as the arsenic that was associated with iron oxides, which were assumed to have been dissolved by the CBE. The calculations to obtain the mass of arsenic in CBE filtrates and ultimately each entire mesocosm was the same as for PB filtrates.

Unaccounted: The unaccounted mass of arsenic or missing arsenic was calculated by subtracting the total mass of arsenic added to the mesocosms from the mass of arsenic found in water, plants, biofilms, adsorbed and iron associated arsenic.

4.2.10 Metal content of dried biofilms using X-ray fluorescence spectroscopy

To detach biofilms and easily detached particulates from the surface of gravel substrates (herein referred to as surface solids), approximately 38 g of wet gravel from three gravel layers (0–20 cm, 20–50 cm and 50–80 cm) from each mesocosm was weighed in 50 mL centrifuge tubes and 15 mL of double deionized water was added to the tubes. Each sample was sonicated for 1–2 minutes (Branson Ultrasonics™ Sonic Dismembrator 150T). A similar mass of cleaned gravel samples with no biofilms attached were treated in the same way and used as negative controls. The resulting liquid slurry mixtures were then decanted into new 50 mL tubes and centrifuged at 4000 rpm for 5 min. Most of the supernatant was removed and the resulting pellet was transferred to XRF cups (SCP Science) and the cups were placed in an oven at 60°C until they were dry. Mylar X-ray film (Chemplex Industries Inc.) was used to seal the XRF cups and a handheld XRF analyzer was used to measure the concentration of metals in the dried pellet (Olympus Vanta L Series used with a Vanta work station). XRF QA/QC procedures are described in Appendix B.

4.2.11 X-ray diffraction analysis of substrate attached biofilms and clean substrates

Dried pellets from layers 0–20 cm, 20–50 cm and 50–80 cm from mesocosms M2, M4, M8 and M12 along with three control gravels with no biofilms were analyzed further using X-ray diffraction analysis (XRD). All samples were ground using a pestle and mortar and placed in a zero-background silicon sample holder (ZBH) and the samples were analyzed on a Malvern-Panalytical Empyrean X-ray Diffractometer (Malvern Panalytical Ltd, UK). Each sample was run for 2 hrs at a two-theta angle range of 5–90 and step size 0.0001, equipped with a PIXcel3D Medipix3 detector, a Reflection-transmission spinner sample stage (minimum step size Phi: 0.1), a Cu X-ray generator with K-Alpha1 [Å] =1.54060, K-Alpha2 [Å] =1.54443 and operated at a current of 40 mA and 45 kV volts. For phase identification, Rietveld refinement and phase quantification, an opensource software package called Profex was used to process the data from the instrument (Doebelin & Kleeberg, 2015; Rietveld, 1969).

4.3 Results

4.3.1 Measured wastewater characteristics

In Table 4-1, the temperature and conductivity of the water inside the mesocosms remained relatively constant throughout the 33-week arsenic exposure experiment for all mesocosms ($p>0.05$ ANOVA). All mesocosms also significantly reduced the concentration of TOC initially present in the wastewater ($p<0.05$ ANOVA with a post hoc Tukey test – See Figure S5) but there was a significantly higher concentration of IC remaining in non-aerated mesocosm compared to the aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test). In all aerated mesocosms, the DO, ORP, and pH were all significantly higher than those found in the non-aerated mesocosms ($p<0.05$ ANOVA with a post hoc Tukey test). The NH_4^+–N concentration for non-aerated mesocosms stayed relatively the same as the concentration initially added to the wastewater but was significantly lower in aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test). The concentration of NO_3^-–N in non-aerated mesocosms was significantly lower than the concentrations found in aerated mesocosms and in the initial wastewater ($p<0.05$ ANOVA post hoc Tukey test). Although all mesocosms significantly reduced the concentration of arsenic initially present in the wastewater ($p<0.05$ ANOVA post hoc Tukey test), non-aerated mesocosms reduced the concentration of arsenic significantly more than aerated mesocosms ($p<0.05$ ANOVA with a post hoc Tukey test).

Table 4-1: Characteristics of influent wastewater and pore water in the mesocosms (Average ± S.D.)

Parameter	Initial Wastewater Characteristics	Overall Porewater Characteristics			
		NA-P	NA-UP	A-P	A-UP
Temperature (ºC)	18 ± 3^{b}	20 ± 2^{ab}	20 ± 2^{ab}	20 ± 2^{a}	20 ± 2^{a}
Conductivity (µS/cm)	670 ± 50^{a}	670 ± 40^{a}	670 ± 40^{a}	670 ± 40^{a}	680 ± 40^{a}
DO (mg/L)	6.4 ± 1.6^{b}	0.05 ± 0.07^{c}	0.13 ± 0.16^{c}	7.0 ± 0.9^{a}	7.2 ± 0.9^{a}
ORP (mV)	146 ± 81^{a}	-230 ± 20^{d}	-240 ± 23^{d}	52 ± 20^{c}	67 ± 20^{b}
pH	7.11 ± 0.07^{b}	6.8 ± 0.2^{c}	6.8 ± 0.1^{c}	7.47 ± 0.09^{a}	7.49 ± 0.07^{a}
Total As (mg/L)	1.018 ± 0.01^{a}	0.311 ± 0.141^{c}	0.253 ± 0.118^{c}	0.590 ± 0.142^{b}	0.575 ± 0.161^{b}
NH_4^+–N (mg/L)	19 ± 2^{b}	20 ± 2^{a}	20 ± 2^{ab}	10 ± 1^{d}	11 ± 1^{c}
NO_3^-–N (mg/L)	37 ± 10^{b}	10 ± 13^{c}	12 ± 15^{c}	47 ± 20^{a}	40 ± 11^{ab}
TOC (mg/L)	276 ± 49^{a}	13 ± 1^{b}	13 ± 1^{b}	7.2 ± 0.7^{b}	6.7 ± 0.8^{b}
IC (mg/L)	21 ± 17^{b}	64 ± 4^{a}	60 ± 2^{a}	8.3 ± 1.4^{c}	7.2 ± 1.2^{c}

Note: All fresh wastewater measurements were taken on a weekly basis (n= 33) from the nutrient tank before loading into the mesocosms while pore water measurements were taken on a daily basis from the sampling port from Mon-to Fri of each week of the experiment (n = 165). TOC and IC measurements are the average of day 7 measurements that were taken on odd weeks 1, 3, 5, 7, 9, 11 and 15 (n=8) for all mesocosms. The total arsenic concentrations for each of the mesocosm types are the average of n = 18 samples where day 6 samples were taken on weeks 1, 6, 14, 16, 25 and 33 (n = 3 mesocosms per treatment x 6 sampling points). An ANOVA post hoc Tukey test was used for each measurement in the table to compare values between the mesocosm treatment types. Letters were assigned in alphabetical order (across mesocosm types for any specific measurement) beginning with the highest value for each measurement and measurements with different letters denoted values that were significantly different from each other ($p<0.05$).

4.3.2 Plant and biofilm growth

The wet biomass growth of *E. hyemale* and *M. polymorpha* plants are shown in Figure 4-2. *E. hyemale* struggled to grow in the non-aerated mesocosms in comparison to the aerated mesocosms as only one of the non-aerated mesocosms, M2, showed an increase in final wet biomass whereas in the aerated mesocosm, both M7 and M8 showed an increase in final wet biomass by the end of the experiment. *M. polymorpha* on the other hand grew very well on the surface of non-aerated mesocosms that were also planted with *E. hyemale*. No initial wet biomass for *M. polymorpha* is shown because it is likely that *M. polymorpha* grew from spores/gemmae that originated in the soil that *E. hyemale* was originally cultivated

in. The *M. polymorpha* spores/gemmae were likely transferred into the mesocosms along with *E. hyemale* upon planting the mesocosms. Although we do not know exactly why *M. polymorpha* did not also grow in the aerated planted mesocosms, we suspect that the bubbles created by aeration prevented the growth of *M. polymorpha* by preventing the spores/asexual gemma from attaching to the gravel.

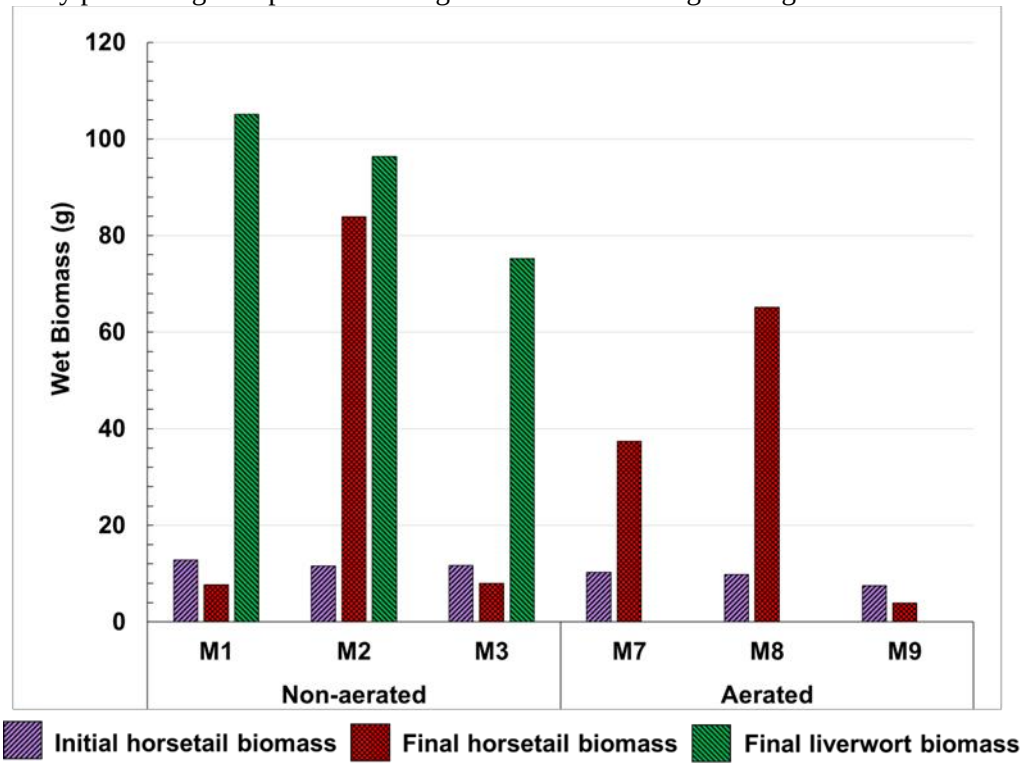

Figure 4-2: Wet biomass measurements used to assess plant growth for *E. hyemale* (horesetail) and *M. polymorpha* (liverwort) growing in the mesocosms

The average dry weight of biofilms determined through loss on ignition of attached gravel biofilms from the three gravel layers from each type of mesocosm is shown in Figure 4-3 A. The biofilm biomass in non-aerated unplanted as well as aerated planted and unplanted mesocosms were significantly greater than the biofilm biomass found in non-aerated planted mesocosms ($p<0.05$ ANOVA post hoc Tukey test). There was a greater amount of dry biofilm biomass within the mesocosms than dry plant biomass by the end of the 33-week arsenic exposure experiment (two orders of magnitude difference) for all planted mesocosms.

The average final dry weight biomasses for *M. polymorpha* and *E. hyemale* plants from non-aerated and aerated planted mesocosms are shown in Figure 4-3 B. Although there was a higher average dry biomass for *E. hyemale* that grew in the aerated planted mesocosms, the average dry mass was not significantly different from *E. hyemale* that grew in non-aerated planted mesocosms ($p>0.05$ ANOVA post hoc Tukey test). Similarly, *M. polymorpha* had a greater average dry biomass than *E. hyemale* in non-aerated planted mesocosms, but this difference was not significantly different ($p>0.05$ ANOVA post hoc Tukey test).

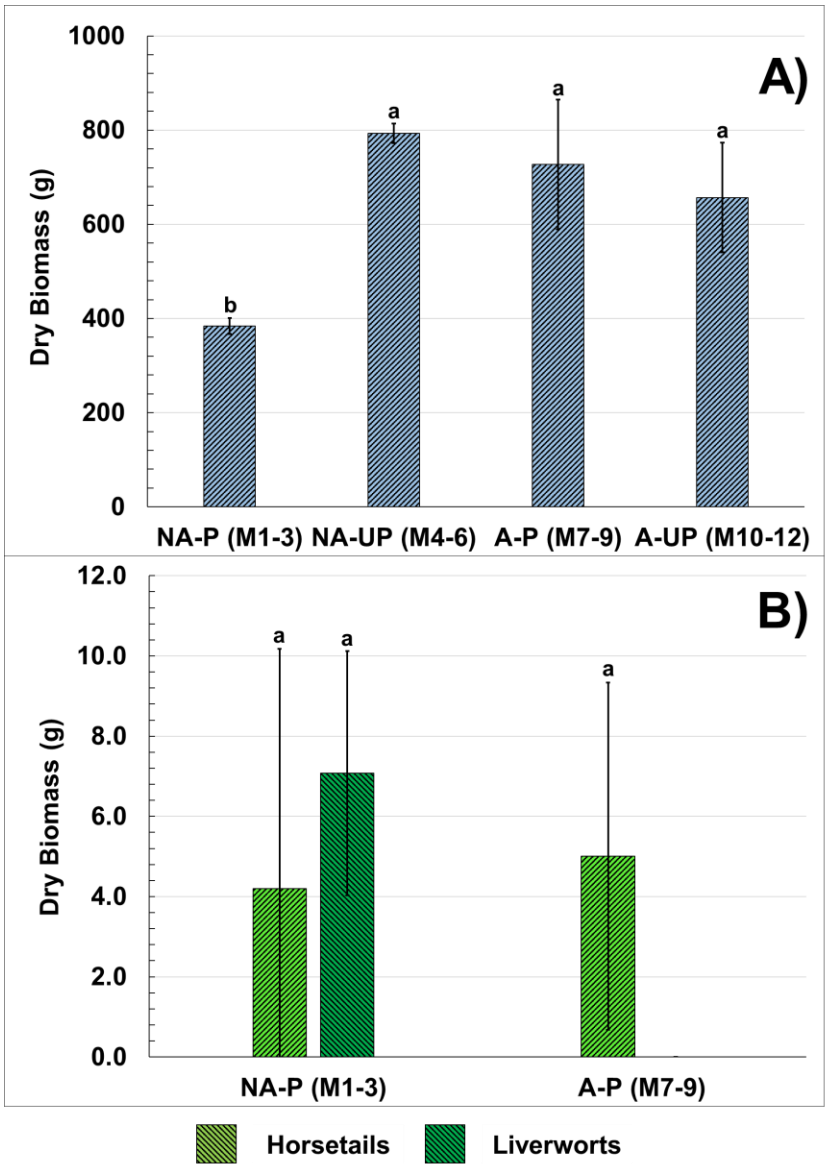

Figure 4-3: Final dry mass of A) biofilms in the mesocosms (Average ± S.D., n=9) and B) the final average dry plant biomasses for *E. hyemale* and *M. polymorpha* (Average ± SD, n=3).

As can be seen from Figure 4-4 A, the dry weight arsenic concentrations for the non-aerated mesocosms were significantly greater than the concentrations found in biofilms from aerated planted and unplanted mesocosms ($p<0.05$ ANOVA post hoc Tukey test).There was no significant difference in the average dry weight arsenic concentrations for planted and unplanted mesocosms of the same type ($p>0.05$ ANOVA post hoc Tukey test).

The average dry weight arsenic concentrations for *M. polymorpha* (liverwort) and *E. hyemale* (horsetail) for planted mesocosms are shown in Figure 4-4 B. *M. polymorpha* plants had a higher arsenic concentration than *E. hyemale* plants in the non-aerated systems. Although *E. hyemale* plants in non-aerated systems had a higher concentration of arsenic than for *E. hyemale* plants in aerated systems, the difference was not significant ($p>0.05$ ANOVA post hoc Tukey test).

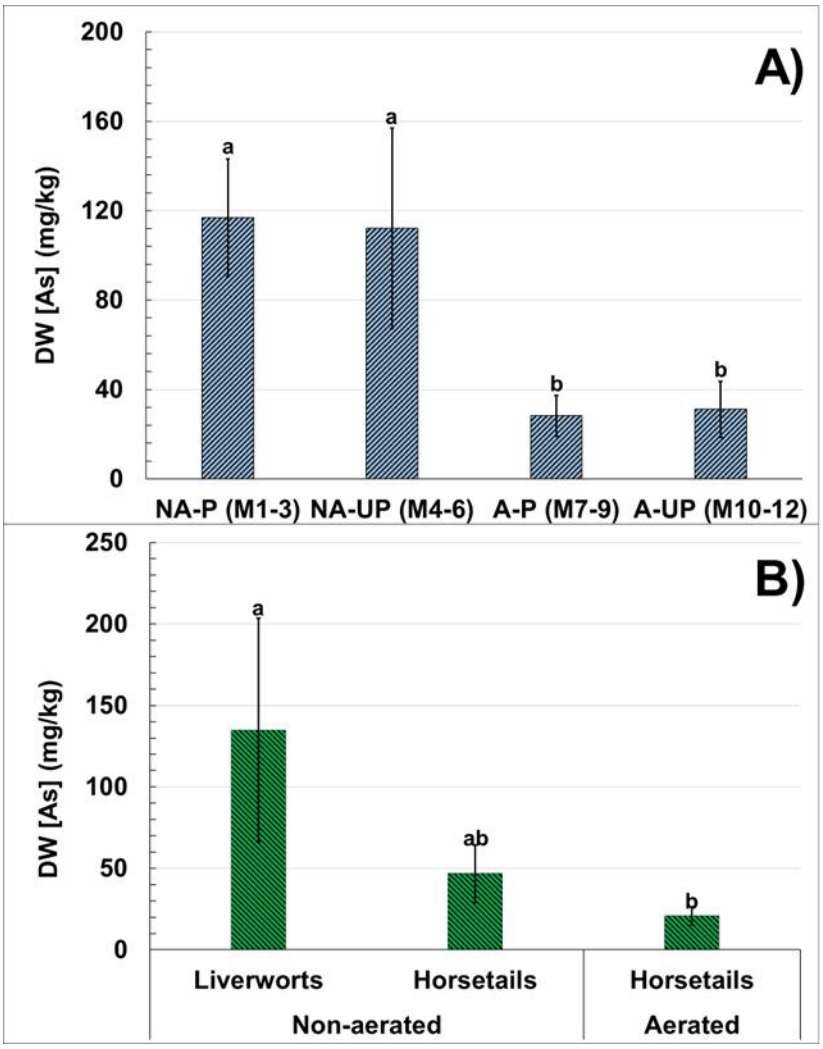

Figure 4-4: Final dry weight arsenic concentrations for A) detached biofilms from three gravel layers for each mesocosm type (Average ± S.D., n=9) and B) for liverworts and horsetails in non-aerated and aerated planted mesocosms (Average ± S.D., n=3).

4.3.3 Mass balance of arsenic in the different components of the mesocosms

The mass proportions of arsenic for water, biofilms (detached using PB and CBE solutions), plants, adsorbed (PB filtrates), iron associated (CBE filtrates) and unaccounted arsenic are shown in Figure 4-5. The largest difference between non-aerated and aerated mesocosms is that the non-aerated mesocosms had a larger proportion of arsenic in biofilms, adsorbed arsenic and iron associated arsenic than aerated mesocosms. This led to less arsenic remaining in the wastewater for non-aerated mesocosms than for aerated mesocosms. In both planted non-aerated and aerated mesocosms, the proportion of arsenic in plants was negligible (<0.4%).

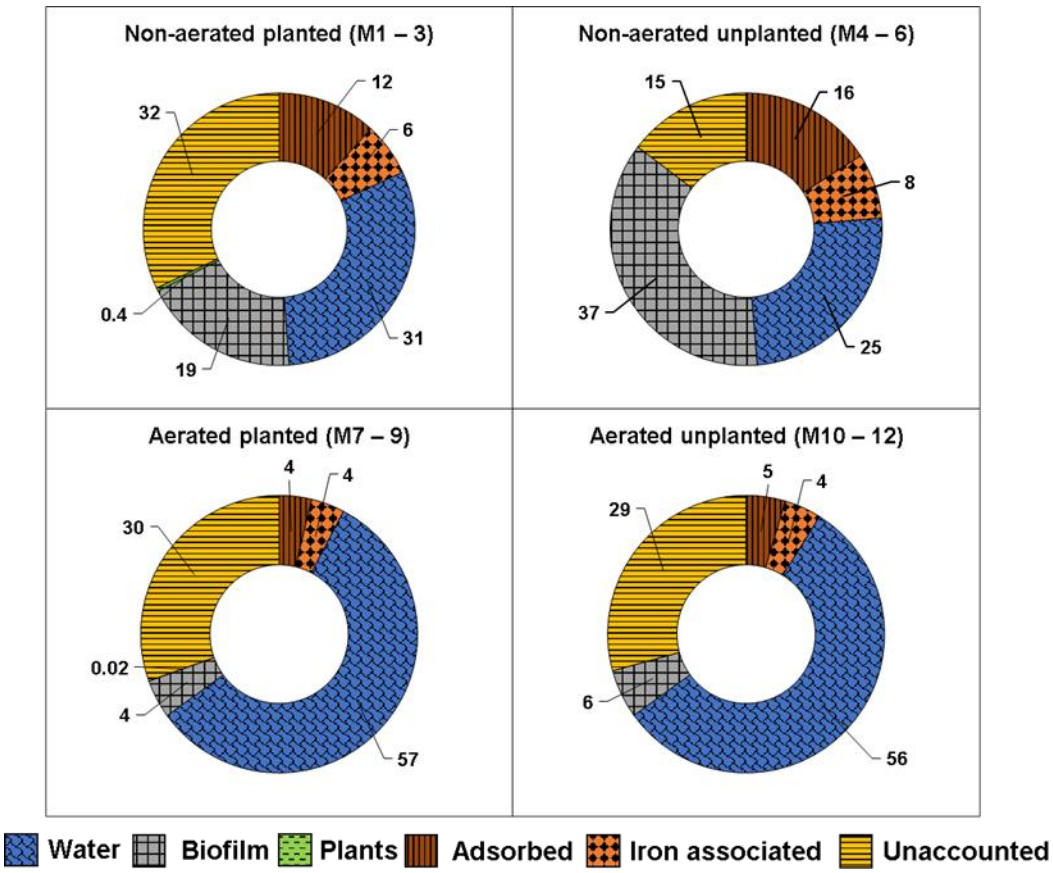

Figure 4-5: The mass balance of arsenic in different compartments of the mesocosms (Average ± S.D., n=3).

4.3.4 Metal composition of biofilms

The concentrations of iron and arsenic obtained through XRF analysis of the gravel-detached surface solids and clean gravels are shown in Figure 4-6 A and B. There were no significant differences in metal concentrations between planted and unplanted mesocosms for either type of system ($p>0.05$ ANOVA post hoc Tukey test).

As shown in Figure 4-6 A, surface solids from non-aerated mesocosms had a significantly higher arsenic concentration than surface solids from aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test) and in comparison, to clean control gravels ($p<0.05$ ANOVA post hoc Dunnett test). Arsenic concentrations for surface solids from aerated mesocosms were not significantly greater than the concentrations found in clean control gravels ($p>0.05$ ANOVA post hoc Dunnett test).

In Figure 4-6 B, biofilms from non-aerated mesocosms showed a significantly greater iron concentration than the clean control gravels ($p<0.05$ ANOVA post hoc Dunnett test) but only surface solids from non-aerated planted mesocosms showed a significantly greater iron concentration than surface solids found in aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test). Iron concentrations for surface solids from aerated mesocosms were not significantly greater than the concentrations found in clean control gravels ($p>0.05$ ANOVA post hoc Dunnett test).

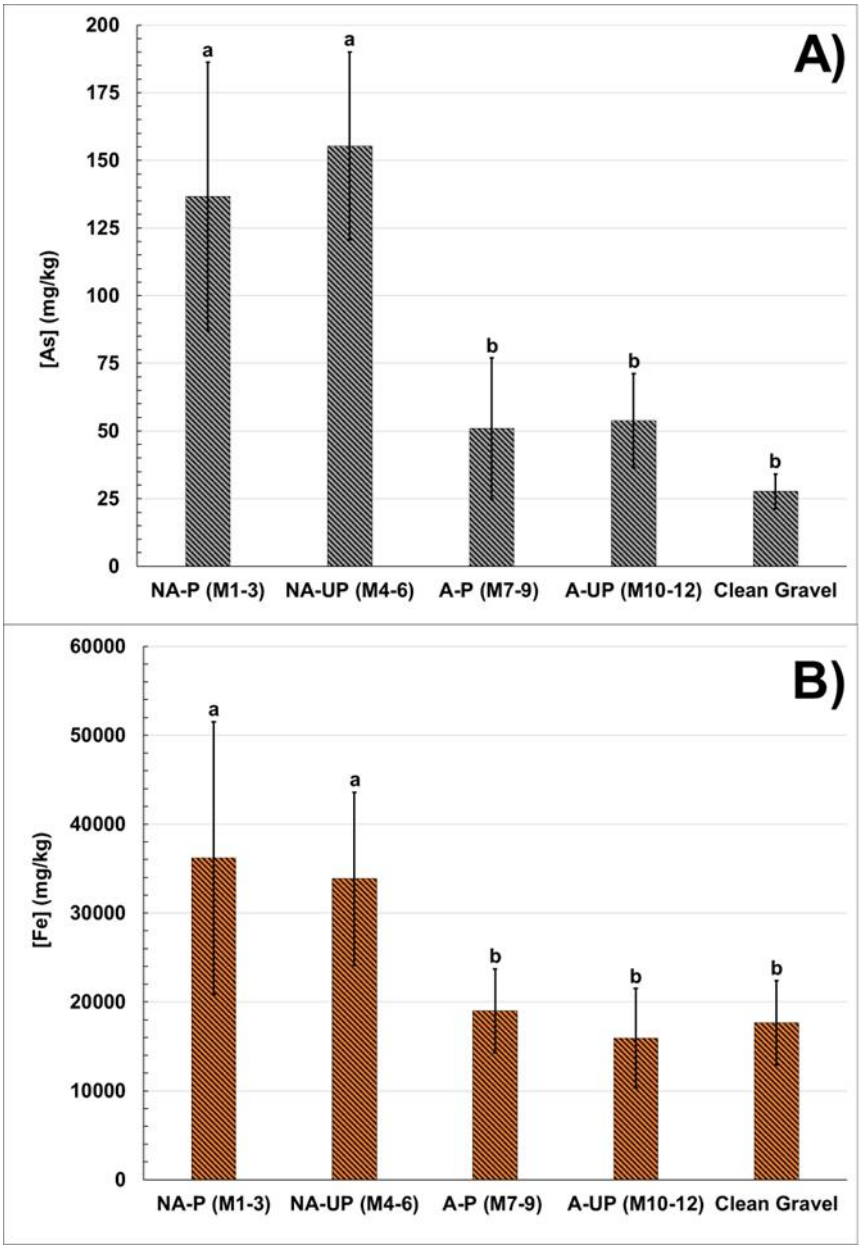

Figure 4-6: The average concentration of A) arsenic and B) iron obtained from XRF analysis of dried surface solids from three gravel layers (0–20, 20–50 and 50–80 cm) for each mesocosm type (Average ± S.D., n=9) compared to clean gravel (Average ± S.D., n=3).

4.3.5 Identification and quantification of minerals in biofilms and substrates

From Table 4-2, we can see that all samples, including controls, largely contained 36.3 ± 13.6 % dolomite ($CaMg(CO_3)_2$), 21.3 ± 12.7 % iron containing ankerite ($CaFeMg(CO_3)_2$), 21.4 ± 8.5 % calcite ($CaCO_3$) and 17.1 ± 5.7 % quartz (SiO_2), consistent with the use of pea gravel. Pyrite (FeS_2), was detected and quantified only in the non-aerated mesocosms and the quantity of this mineral increased with depth. In the non-aerated planted system (M2), there was no pyrite detected in the surface solids from gravel located in the top gravel layer (0–20 cm) of the mesocosm but the phase quantity of pyrite in the biofilms increased from 5.4% in the middle (20–50 cm) gravel layer to 14.6% in the surface solids located in the bottom (50–80 cm) gravel layer. For the non-aerated planted system (M4), there was a similar increase in the quantity

of pyrite in surface solids with increasing depth, where surface solids from the top (0–20 cm), middle (20–50 cm) and bottom (50–80 cm) contained 1.7%, 3.6% and 6.3% pyrite.

With increasing gravel layer depth and pyrite quantity, there was also an increase in the concentration of arsenic measured through XRF analysis for the non-aerated planted and unplanted mesocosms only.

Table 4-2: The relative mineral phase quantities determined from XRD analysis and the arsenic concentrations determined by XRF analysis for clean gravels and gravels from non-aerated and aerated planted and unplanted mesocosms

		Relative mineral phase quantity (wt %)					XRF
Sample ID	**Layer**	**Dolomite**	**Ankerite**	**Calcite**	**Quartz**	**Pyrite**	**[As] (ppm)**
Clean Gravel Control 1	**NA**	33	30	22	12	0	24
Clean Gravel Control 2		36	29	26	7	0	24
Clean Gravel Control 3		20	45	25	8	0	35
M2 (NA-P)	**0–20 cm**	61	10	12	17	0	77
	20–50 cm	42	16	17	19	5	141
	50–80 cm	32	18	15	20	15	210
M4 (NA-UP)	**0–20 cm**	56	0	20	19	2	111
	20–50 cm	37	24	23	23	4	176
	50–80 cm	27	11	33	18	6	194
M8 (A-P)	**0–20 cm**	36	17	18	25	0	65
	20–50 cm	48	27	10	15	0.0	32
	50–80 cm	14	29	20	21	0.0	34
M12 (A-UP)	**0–20 cm**	52	12	12	23	0.0	61
	20–50 cm	26	9	43	20	0.0	24
	50–80 cm	24	43	24	9	0.0	<LOD

4.4 Discussion

Under the indoor laboratory conditions in our study, the lack of aeration in the non-aerated mesocosms produced low DO concentrations and negative ORP measurements which allowed for reducing conditions to prevail. The reducing conditions provided the environment for the growth and activity of bacteria that can survive in anoxic to anaerobic conditions. This was supported by the high TOC consumption in these mesocosms that likely led to the growth and activity of denitrifying bacteria. This resulted in low concentrations of NO_3^-–N but little to no removal of ammonium due to the lack of nitrification activity. A study by Fan et al., (2021) showed that the addition of up to 0.5 mg/L of As(V) to their wetlands under reducing conditions did not disrupt nitrogen removal in relation to their control wetlands with no arsenic and showed relatively high denitrification activity (94% NO_3^-–N removal) and low nitrification (34% NH_4^+–N removal).

The wastewater from our non-aerated mesocosms also smelled of rotten eggs, suggestive of hydrogen sulfide gas formation, most likely by sulfate reducing bacteria. Many studies have indirectly shown the potential presence of sulfate reducing bacteria in their constructed wetlands treating arsenic by demonstrating a simultaneous drop in the concentration of TOC and sulfate (K. Z. Rahman et al., 2011, 2014, 2008aa, 2008bb). Other studies have directly shown the presence of sulfate reducing bacteria in their wetlands through 16s rRNA sequencing when a carbon source and sulfate is provided/present in some form (Nguyen et al., 2021; Z. Zhang et al., 2017a).

The high IC concentrations in the wastewater may be the result of high bicarbonate concentrations in the wastewater. The dissolution of our carbonate-based substrates (Table 4-2) used in the mesocosms was likely driven by a few mechanisms, which may have occurred simultaneously. The fermentative

metabolism of the bacteria in the non-aerated mesocosms likely generated organic acids that can directly react with our substrates to form bicarbonate. Carbon dioxide, also formed through fermentation, may have formed carbonic acid in the wastewater that can directly react with substrates to form bicarbonate. Alternatively, as a result of the pH conditions in the mesocosms, carbonic acid may have dissociated to directly form bicarbonate.

The present study is the first to maintain oxidizing conditions (high DO concentrations and positive ORP) through active aeration and allow the determination of this effect/factor on arsenic removal in wetland mesocosms. In the aerated systems, there was very high nitrification, resulting in lower ammonium concentrations, but very little denitrification activity. The amount of TOC removal activity was approximately the same as in non-aerated systems except that it is likely that aerobic heterotrophic bacteria consumed the carbon provided in the aerated systems. The aeration likely forced the carbon dioxide produced by aerobic bacteria out of the wastewater into the atmosphere as it was being formed, preventing the formation of carbonic acid and producing a higher pH in these systems. The release of carbon dioxide is supported by the much lower IC concentrations measured in the aerated systems. The conditions discussed here and reported in Table 4-1 were similar to those obtained prior to adding arsenic to the mesocosms, when establishing them for this investigation (

Table S7), suggesting that arsenic addition did not appear to disrupt the general functioning of the mesocosms.

From Table 4-1 we can see that both non-aerated and aerated mesocosms were able to remove arsenic from the wastewater and that non-aerated mesocosms removed more arsenic than aerated mesocosms. The presence of plants in either type of mesocosm did not have any obvious direct effects on arsenic removal. This was also reflected in the mass balance, shown in Figure 4-5, where non-aerated mesocosms had a lower average mass of arsenic remaining in the wastewater than aerated mesocosms did. We believe that the two different redox conditions created and maintained in both types of mesocosms had the largest influence on arsenic removal from the wastewater because of the proposed microbial activity and biogeochemical cycling occurring in the two types of mesocosms. Although we did not examine arsenic speciation in water of mesocosms in this study, based on the pH and redox conditions shown in Table 4-1, we hypothesize that most of the added arsenic remained as As(V) despite bacterial activity in the aerated oxidizing mesocosms, while a higher proportion of the As(V) in the non-aerated mesocosms may have been transformed into As(III) and methylated arsenic species by bacteria. K. Z. Rahman et al., (2008a) has shown that promoting reducing conditions by adding TOC and sulfate in their mesocosms improved removal and promoted the reduction of As(V) to As(III) as well as other arsenic species that they could not identify. Other studies have shown that iron oxide rich substrates are able to effectively remove As(V) and As(III) from wastewater but not in the presence of high phosphate concentrations (H. Liu et al., 2022; Z. Zhang et al., 2017a). J.-H. Park et al., (2022) has shown that under reducing conditions, DMA(V) was demethylated into As(V) and As(III) only in the substrate and not the porewater, and was removed through adsorption. The measurement of arsenic speciation in all components of the mesocoms is the topic of a separate study.

The present study is the first to estimate the amount of biofilm in lab-scale constructed wetlands treating arsenic and estimate the amount of arsenic retained in the biofilms relative to the starting amount added to the system. While the biofilm biomass in both non-aerated and aerated systems appear to be relatively similar to each other in Figure 4-3 A, with the exception of the biofilms from non-aerated planted mesocosms, the concentration of arsenic in Figure 4-4 A. for the non-aerated systems were significantly greater than those found in the aerated mesocosms. The biofilms from Figure 4-5 in non-aerated planted and unplanted systems removed 19 and 37% of the total arsenic added to the mesocosms respectively compared to only 4 and 6% in the planted and unplanted aerated systems respectively.

There may be multiple mechanisms of arsenic removal simultaneously operating at the same time in both non-aerated and aerated mesocosms. In both types of systems, arsenic was likely directly taken up by biofilms and adsorbed to biofilm EPS. Differences in pH, redox conditions and potential differences in the microbial composition, physical properties and chemical composition of the EPS may have influenced the capacity for arsenic adsorption (Guo et al., 2016; N. Li et al., 2017; Sheng & Yu, 2006). Biofilm EPS are composed of lipids, proteins and carbohydrates that have phosphate, amino, carboxyl, hydroxyl and

sulfhydryl groups that are capable of binding arsenic through surface complexation, electrostatic or covalent interactions (L. Huang et al., 2022; W.-J. Ma et al., 2021; Saba et al., 2019; Tian et al., 2019; Waqar et al., 2025). Another possible mechanism in both mesocosm types was adsorption of arsenic to the carbonate-based substrates and possibly iron oxide inclusions (Aredes et al., 2012; Jones & Loeppert, 2013; Marzi et al., 2022). The presence of phosphate in the water may have interfered with arsenic adsorption to the carbonate based substrates (Sø et al., 2012). Although we could not differentiate between the proportion of arsenic adsorbed to substrates and those adsorbed to biofilm EPS in each of the mesocosms in Figure 4-5, a higher proportion of arsenic was found in the PB solution from non-aerated mesocosms than aerated mesocosms, indicating more arsenic was adsorbed in the non-aerated mesocosms. There was also a higher proportion of iron associated arsenic in the non-aerated mesocosms than in the aerated mesocosms, as indicated by the higher concentration of arsenic in the CBE solution. This would indicate that more arsenic may have coprecipitated with iron in the non-aerated mesocosms. In the aerated mesocosms, aerobic conditions would not have favoured the presence of Fe(II) through the dissolution of iron in the substrate, thereby not producing a secondary source of Fe(III) hydroxides or other iron minerals. It was hypothesized that any iron minerals in the aerated mesocosms were native to the gravel, present prior to the start of the study and is supported by Figure 6B. Thus, the arsenic associated with these iron minerals was hypothesized to have been adsorbed to pre-existing forms, rather than a result of co-precipitation with iron.

To determine why more arsenic was found in non-aerated surface solids as well as why there was more arsenic associated with their adsorbed and iron associated fractions, further examination of the metal content of the surface solids was done using XRF analysis. Figure 4-6 A and B, revealed that surface solids from non-aerated systems contained a significantly greater iron and arsenic concentration than surface solids from aerated systems or from clean gravels. To identify and quantify the minerals in the surface solids, XRD analysis revealed that non-aerated systems contained large quantities of iron sulfides (2–15 wt%) and that the quantity of iron sulfide in the surface solids increased with sampling depth, as shown in Table 4-2. The increasing pyrite quantity with gravel depth also corresponded with an increase in arsenic concentrations in the surface solids for the non-aerated planted and unplanted mesocosms, as determined through XRF analysis. This may be because the biofilms at the deeper gravel layers (20 cm and below) had a more consistent reducing environment that promoted more pyrite formation and concomitant arsenic removal than the biofilms found in the surface gravel layers (0–20 cm). The iron sulfide minerals were likely formed and accumulated in the non-aerated systems during the 3.9 years prior to treatment with arsenic and likely continued forming during treatment as well. Some of the sulfate added to the non-aerated mesocosms (approximately 48 mg/L sulfate per week) was converted to hydrogen sulfide (identified by smell) likely by sulfate reducing bacteria (Castro et al., 2000; Park & Faivre, 2022; Thauer et al., 2007). The likely activity of fermentative bacteria that produced organic acids and carbon dioxide may have led to the dissolution of carbonate minerals, particularly iron containing ankerite, which released iron (II) into the wastewaters (Ortiz-Castillo et al., 2021; Samuels et al., 2020; Y. Wu et al., 2017). The presence of iron (II) and hydrogen sulfide then likely led to the formation of pyrite (FeS_2) which we hypothesize were trapped in the EPS and inside the bacteria from non-aerated biofilms (Duverger et al., 2020; Y. Park & Faivre, 2022; Picard et al., 2016; Remoundaki et al., 2008; Teclu et al., 2008; Thiel et al., 2019). Biogenic, laboratory synthesized and natural pyrite has been shown to effectively remove As(V) and As(III) from water through direct adsorption onto pyrite, formation of arsenian pyrite or co-precipitation with iron oxyhydroxides formed on the surface of the pyrite as a result of pyrite oxidation due to abiotic oxidative reactions involving Fe(II) and As(III) (Bostick & Fendorf, 2003; Bulut et al., 2014; Farquhar et al., 2002; Fischer et al., 2021; T. Li & Guo, 2024; Luo, Wang, et al., 2025; Saunders et al., 2018; X. Zhang et al., 2022). The main interfering ions for arsenic adsorption to the iron oxides and carbonate containing gravel in the non-aerated mesocosms may have been bicarbonate and phosphate (H. Liu et al., 2025; Stachowicz et al., 2007; Sudhakar et al., 2021). Despite potential interferences, the presence of pyrite in the non-aerated mesocosms, formation of iron oxyhydroxides, and possibly the coprecipitation of arsenic with iron may explain why there was a higher proportion of adsorbed arsenic and iron associated arsenic in these mesocosms. Although there was no arsenian pyrite or iron oxyhydroxide minerals detected using XRD

analysis, their presence cannot entirely be ruled out as they may not have been detectible using XRD due to low concentrations and/or because the precipitates were amorphous (non-crystalline) in nature.

The other possible removal mechanism for arsenic in our non-aerated mesocosms is the formation of orpiment (As_2S_3). Bacteria may form orpiment in the presence of a carbon source and under neutral to mildly acidic conditions (Newman et al., 1997; Rodriguez-Freire et al., 2014, 2016). Most of the literature on arsenic removal in lab-scale constructed wetlands only speculate about the formation of arsenic sulfide precipitates in their treatment systems (K. Z. Rahman et al., 2008aa, 2008bb; Singhakant et al., 2009a; Z. Zhang et al., 2017b). Only one study by J.-H. Park et al., (2022) showed that a very small proportion of what they believe is orpiment was detected in sediments of planted systems through linear combination fitting of XANES data. The formation of orpiment was also not detected by XRD analysis in our biofilms likely due to low concentrations. We believe that the formation of orpiment wasn't favoured because > 90% of the TOC, provided as molasses in the wastewater solution, was consumed by the bacteria 1 day prior to addition of As(V) during each week of treatment, as shown in Figure S5. The mesocosm conditions and timing of arsenic addition in this study was hypothesized to favour the formation of pyrite over orpiment.

There was considerable variability in the growth of *E. hyemale* in both non-aerated and aerated mesocosms as shown in Figure 4-2, likely due to the toxic effects of arsenic and possibly hydrogen sulfide in the non-aerated mesocosms (Fan et al., 2021; Lamers et al., 2013; K. Z. Rahman et al., 2014, 2008bb; P. Zhang et al., 2017). The removal of nutrients such as nitrate (due to denitrification) and iron (due to pyrite formation) specifically in the non-aerated mesocosms may have further contributed to the variability in plant growth. On the other hand, *M. polymorpha* that unintentionally grew and propagated through spores/asexual gemma (Kato et al., 2020) along side *E. hyemale* on the surface of non-aerated mesocosms (M1–3) were less affected by arsenic or sulfide toxicity and had a significantly higher biomass and arsenic concentration than *E. hyemale* in both types of mesocosms. *M. polymorpha* has been demonstrated to be able to accumulate high concentrations of arsenic and has a robust antioxidant response to deal with arsenic toxicity (Dutta et al., 2024; M. Li et al., 2024). The dry biomass of plants in both types of mesocosm along with the accumulated arsenic concentrations were significantly less than that found in biofilms as shown in Figure 4-2 A and B as well as Figure 4-3 A and B respectively. From the mass balance in Figure 4-5, it is clear that arsenic uptake by plants under the conditions we used in this study did not significantly contribute to arsenic removal, as they accumulated no more than 0.4% of the total mass of arsenic from the wastewater. Other studies have shown that between 0.02–10.0% of the total mass of arsenic treated in planted constructed wetlands was removed by plants (Baquir et al., 2024; Fan et al., 2021; Olmos-Márquez et al., 2012; J.-H. Park et al., 2022; Rabbi et al., 2024; Schwindaman et al., 2014a; Singhakant et al., 2009a, 2009b; Wu et al., 2014; Ye et al., 2003). The removal of arsenic by plants is difficult to predict in constructed wetlands as there are many interacting abiotic and biotic factors that may directly or indirectly affect the speciation and bioavailability of arsenic to plants (H. Li et al., 2011; Neubauer et al., 2007; Olmos-Márquez et al., 2012; K. Z. Rahman et al., 2011, 2014, 2008aa; Singhakant et al., 2009a; Z. Zhang et al., 2017b, 2017a).

In Figure 4-5, we can see that there was between 14 – 34% of the arsenic that was unaccounted for in the mass balance. In other constructed wetland studies, up to 30% of the arsenic was unaccounted for (Fan et al., 2021; Olmos-Márquez et al., 2012; K. Z. Rahman et al., 2011, 2014; Schwindaman et al., 2014a; Singhakant et al., 2009a; Wu et al., 2014; Ye et al., 2003; Z. Zhang et al., 2017a). There may be a few reasons why there was a loss (unaccounted amount) of arsenic from the mesocosms in our study. The primary loss of arsenic in the mesocosms is likely a consequence of losing biofilms and any solids that can adsorb arsenic when the mesocosms were drained to add fresh wastewater on a weekly basis. Another possibility is that both adsorbed arsenic and arsenic associated with the biofilms remained on the walls and tubing of the mesocosms when they were dismantled at the end of the experiment. The formation and loss of arsenic through volatilization likely occurred in trace quantities (< 1%) as demonstrated in many other studies (Majumder et al., 2013; Mestrot et al., 2011; Meyer et al., 2007; Prieto et al., 2016; K. Z. Rahman et al., 2014; Webster et al., 2016).

4.5 Conclusion

Our investigation into the removal of arsenic from water using non-aerated and aerated, planted and unplanted constructed wetland mesocosms has shown that non-aerated mesocosms are able to remove more arsenic (72%) than aerated mesocosms (43%) and that planting either type of mesocosm had a negligible effect on removal. Weekly exposure to arsenic did not disrupt the denitrification (70%) and organic carbon removal activities (95%) of the non-aerated mesocosms. The non-aerated mesocosms continued to produce hydrogen sulfide through sulfate reduction as indicated by the odour of the porewater. Aerated mesocosms also continued to remove organic carbon (97%) but no denitrification or sulfate reduction occurred in these mesocosms.

Based on the mass balance calculations that incorporated the amount of arsenic present in the biofilms of the mesocosms, it was demonstrated that non-aerated biofilms accounted for 28% of the removal while aerated biofilms only accounted for 5%. Through examination of the metal content using XRF and identification of the mineral phases found in gravel surface solids using XRD, it was discovered that non-aerated surface solids had the highest iron content of 35,000 mg/kg and that this was attributed to the presence of 2–15 wt% of pyrite. The formation and accumulation of pyrite was hypothesized to be attributable to the presence and activity of sulfate reducing bacteria and production of hydrogen sulfide along with the dissolution of iron containing ankerite by fermentative bacteria in the non-aerated mesocosms. The formation of arsenian pyrite or orpiment may also have been possible but were not detected using the analytical methods used in this study. The metal and mineral phase content of aerated mesocosms were not distinguishable from clean gravel, with no iron containing mineral phases identified in these samples.

Although sorption onto the substrates and onto the EPS of biofilms, as well as arsenic uptake into biofilms were likely operating simultaneously in both types of mesocosms, it is likely that the pyrite formed in non-aerated mesocosms enhanced the removal of arsenate from the water either through direct adsorption to the pyrite or through adsorption/co-precipitation with iron oxides on the surface of pyrite.

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Author Contributions

Antoine Hnain: Writing – original draft, conceptualization. **Iris Koch:** Writing – conceptualization, review & editing, supervision, funding acquisition. **Kela Weber:** Writing – conceptualization, review & editing, supervision, funding acquisition.

Chapter 5 – Arsenic speciation in wastewater, plants and biofilms from non-aerated and aerated laboratory scale constructed wetlands

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Abstract

A study was undertaken to examine how arsenic speciation in wastewater, biofilms and plants may influence and explain the removal of arsenic from lab-scale non-aerated and aerated constructed wetlands. The study design consisted of twelve lab-scale wetlands divided into 4 treatments (non-aerated planted and unplanted, aerated planted and unplanted), and the dosing arsenic species and concentration was 1 mg/L of As(V), representative of contaminated water in leachate or mine waste scenarios. Only non-aerated systems, which removed more arsenic than aerated mesocosms, contained a higher proportion of methylated arsenic species (MMA(V) and DMA(V)), hypothesized to be due to microbial activity. Speciation was unchanged (remained as As(V)) for aerated systems by day 6 for weeks 1, 6 and 14, corresponding with less removal of arsenic. Greater arsenic removal in the non-aerated systems corresponded with higher arsenic concentrations in biofilms and the presence of reduced As(III) and As-S species, detected with X-ray absorption spectroscopy (XAS), whereas only As(V) was seen in biofilms from aerated systems. Previous findings of pyrite in the studied non-aerated systems suggested a hypothesis of arsenic removal in non-aerated systems via As(V) adsorption to pyrite but the finding of As-S species by XAS, which may possibly include amorphous orpiment, suggests that the removal mechanism likely involves complexation with sulfides. In aerated systems, where only As(V) species were seen in biofilms, the primary removal mechanism was hypothesized to be adsorption onto carbonates and extracellular polymeric substances (EPS) produced by the aerated gravel biofilms. There was no clear pattern of arsenic speciation in the plants that would help explain arsenic removal, perhaps due to a dose dependent response by the plants.

Keywords: constructed wetlands, arsenic, water treatment, wastewater, biofilms, plants

5.1 Introduction

Arsenic is a toxic metalloid that can occur naturally in the environment. It is a component of 245 naturally occurring minerals found in the earth's crust, most commonly found in association with sulfide and oxide minerals (Mandal & Suzuki, 2002). Arsenic may exist in one of four oxidation states (-3, 0, +3 and +5). The -3 oxidation state in the form of arsine gases (AsH_3) and the zero oxidation state (ie. elemental As or As^0) are rarely found in nature and only exist in highly reducing environments (Cullen & Reimer, 1989; Garelick et al., 2009; S. Wang & Mulligan, 2006b). The most common form of arsenic found in water is As(V) and As(III) where the redox conditions and pH may determine which species is proportionally higher in concentration (Garelick et al., 2009). A higher proportion of the arsenic is found as As(V) in aerobic (oxidizing) waters as arsenate in various anionic forms (pH<2: H_2AsO_4, pH 2-11: $HAsO4^{2-}$ and $AsO4^{3-}$) while a higher proportion of the arsenic may be found as As(III) in anaerobic (reducing) waters as uncharged arsenite at pH <9, H_3AsO_3 (Kadlec & Wallace, 2008; Sharma & Sohn, 2009). Inorganic arsenic can be methylated to form trivalent species monomethylarsonous acid [MMA(III)], dimethylarsinous acid [DMA(III)] and trimethylarsine [TMA(III)], as well as the more commonly occurring, and more stable pentavalent species monomethylarsonic acid [MMA(V)], dimethylarsinic acid [DMA(V)] and trimethylarsine oxide (TMAO) (Wang & Mulligan, 2006b). The toxicity of arsenic largely depends on the oxidation state, chemical form (inorganic or organic) and the quantity of arsenic an organism is exposed to. In humans and rats the toxicity of arsenic is as follows: MMA(III) ≈ DMA(III) < As(III) < As(V) < DMA(V) < MMA(V) (Petrick et al., 2000; Styblo et al., 2000). Chronic ingestion of inorganic arsenic through food and water at concentrations >10 µg/L can be harmful to the body and potentially cause health problems including cancer, while acute concentrations of 60 mg/L can be fatal (S. Wang & Mulligan, 2006b).

Some regions of the world where arsenic may naturally be found at very high concentrations in aquifers include parts of the Western USA, South America, India, Bangladesh and other East and Southeastern Asian countries (R. Singh et al., 2015). In Canada, natural surface and ground water concentrations of arsenic range between <1.0 to 5.0 µg/L, which is well below the maximum concentration limit of 10 µg/L set by the World Health Organization's (WHO) guidelines (WHO, 2022; Health Canada,

2006). However, there are some regions of Canada where drinking and surface waters are contaminated by arsenic from both natural and anthropogenic sources (McGuigan et al., 2010).

Technologies used for the removal of arsenic from water include: 1) air or chemical oxidation and precipitation; 2) chemical coagulation and coprecipitation with alum, iron or lime; 3) sorption using iron coated sand or activated alumina and ion-exchange resins; and 4) membrane filtration techniques, which include nanofiltration, reverse osmosis and electrodialysis (Mohan & Pittman, 2007; R. Singh et al., 2015). These removal technologies may be costly because they may require a high degree of process control as well as the input of energy, chemicals and the replacement of adsorbent materials. Additional costs may be incurred resulting from the generation of toxic sludges that may require dewatering and disposal.

In most of the literature on arsenic removal in constructed wetlands, the speciation of arsenic in constructed wetlands has largely been ignored despite the fact that arsenic speciation may help predict the removal and ultimate fate of the arsenic in these treatment systems. Of the few studies that have examined arsenic speciation in constructed wetlands, most have focused on looking at arsenic speciation in wastewater and/or substrates (Liu et al., 2022; Park et al., 2022; Rahman et al., 2008a; Zhang et al., 2017a) or wastewater alone. None of the studies have directly examined the speciation of arsenic in the biofilms attached to their substrates or in plants, despite acknowledging that plants and microbes and their potential interactions may influence arsenic speciation and removal from the wastewater. Under some circumstances, biofilms may lead to the formation of biogenic minerals that remove arsenic through adsorption, or arsenic may be removed through precipitation/co-precipitation with other elements produced by the biofilms to form arsenic-bearing minerals. The minerals and/or precipitates formed by biofilms are largely governed by the species of bacteria in the biofilms, which is determined by the abiotic conditions in the wetlands, particularly conditions such as redox, dissolved oxygen concentrations, pH and the wastewater composition. The uptake and speciation of arsenic by biofilms may explain why some studies show that a large proportion of the mass of arsenic (39% or greater) is found in wetland substrates, particularly in wetlands that do not deliberately use arsenic adsorbing substrates (Olmos-Márquez et al., 2012; J.-H. Park et al., 2022; Singhakant et al., 2009a, 2009b; Ye et al., 2003).The interaction of biofilms and plants may also be important to understand arsenic retention in constructed wetlands. Although plants can directly take up arsenic from the water in constructed wetlands, most studies show that a small proportion of the mass of arsenic added, usually <1 to 9.5% of the total mass of arsenic added, is found in plants (Fan et al., 2021; Olmos-Márquez et al., 2012; J.-H. Park et al., 2022; Schwindaman et al., 2014a; Singhakant et al., 2009a, 2009b; Wu et al., 2014; Ye et al., 2003). However, other studies have shown that under changing redox conditions or under high phosphate concentrations, 13 to 50% of the arsenic added to the wetlands was found in plants (Rahman et al., 2011, 2014; Zhang et al., 2017a). Depending on the direction of the redox shift, reductive or oxidative dissolution of arsenic adsorbed to minerals in the substrate or associated with biofilm microbes could occur, thereby releasing arsenic back into the wastewater and increasing its bioavailability to the plants (Rahman et al., 2011, 2014). In our previous study in Chapter 4, we determined that non-aerated planted and unplanted mesocosms removed more arsenic than aerated planted and unplanted mesocosms because the biofilms produced large quantities of pyrite and possibly other As-S minerals under the reducing conditions which helped retain arsenic in the non-aerated mesocosms.

To further understand and support our previous results, the current study focuses on the speciation of arsenic in biofilms, wastewater and plants in non-aerated and aerated, planted and unplanted, laboratory scale mesocosms, interrogating how interactions among the three components may influence the removal of arsenic from wastewater and potentially providing further insights on how to improve arsenic removal in constructed wetlands.

5.2 Materials and Methods

A thorough description of the design, construction and operation of our non-aerated and aerated planted and unplanted mesocosms exposed to 1 mg/L As(V) has previously been described in detail in Chapter 4. This chapter also describes the measurements that were made to evaluate wastewater conditions, nutrient dynamics and arsenic removal through measurements of temperature, conductivity, dissolved oxygen (DO), redox potential (ORP), pH, NO_3^--N. NH_4^+-N, total organic carbon (TOC), total inorganic

carbon (TIC) and total arsenic concentrations. The procedures for preparing and digesting plant and biofilm samples prior to determination of total arsenic concentrations via ICP-MS analysis can also be found in Chapter 4.

5.2.1 Determination of total arsenic concentrations in wastewater

Porewater samples were collected to determine how arsenic concentrations in the mesocosms changed over time upon addition of As(V) that occurred 1 day after fresh nutrients were added. Samples were collected 20 min, 1 day and 6 days after addition of As(V) for weeks 1, 6 and 14. All water samples were stored at 4°C. Prior to ICP-MS analysis (NexION 310D PerkinElmer, MA, USA.) to determine arsenic concentrations, all water samples were diluted 10x using 2% nitric acid by mass. Calibration curves with 0, 2.5, 25, 50 and 100 ppb of As(V) ($Na_2HAsO_4 \cdot 7H_2O$, 98% Alfa Aesar) were used to calculate total arsenic concentrations in all samples. ICP-MS instrument conditions for the analysis were the same as the conditions described in Appendix B for Chapter 4.

5.2.2 Determination of arsenic speciation in wastewater

Prior to analysis, the day 6 wastewater samples from week 1 were diluted 10x by mass using ultrapure (>18 MΩ-cm) water and analysed on an in-house HPLC-ICP-MS (Thermo Fisher UltiMate 3000 UHPLC System, Perkin Elmer NexION 310D, MA, USA) with a Hamilton PRP-X100, 4.6 x 150 mm, 5 µm pore size anion exchange column. PeakFit v4.12 (SeaSolve Software Inc. San Jose, CA, USA) was used to manually identify peaks and quantify the area under their curves. As shown in Appendix C, a calibration curve of peak area vs. concentration of each arsenic species is shown in Figure S7 A–D, which was used to determine the concentration of each arsenic species in the mesocosm water samples.

The remaining day 6 wastewater samples for weeks 6 and 14 were shipped with ice packs to ALS Environmental (Vancouver, BC, Canada) for arsenic speciation analysis using ion-pair reverse phase HPLC-ICP-MS with a collision reaction cell to reduce polyatomic atomic interferences for arsenic (Afton et al., 2008; Garbarino et al., 2002). Instrument conditions for the in-house ICP-MS and QA/QC procedures for the analysis of arsenic concentrations in the wastewater samples are as described in Appendix B for Chapter 4. Appendix C shows the QA/QC procedures used for all the mesocosm water speciation data along with the % recoveries of arsenic from the HPLC columns (Table S10).

5.2.3 Arsenic speciation in flash frozen plant tissues and biofilms

To better understand the speciation of arsenic in fresh plant tissues and biofilms from the mesocosms, samples were collected from the mesocosms upon dismantling, and prepared for X-ray absorption spectroscopy (XAS) analysis. XAS analysis was selected as the arsenic speciation analysis method for plants because of the well-known poor (or highly variable) extraction efficiency for plants, when wet extraction HPLC-ICPMS methods are used (I. Koch et al., 2000; Nearing et al., 2014); similar poor extraction efficiency for biofilms was suspected. Whole *M. polymorpha* plants along with *E. hyemale* root and shoot tissues from non-aerated planted mesocosms (M1-M3) were collected and flash frozen using liquid nitrogen. *E. hyemale* root and shoot tissues were also collected and flash frozen from aerated planted mesocosms (M7 and M8). As there was very little M9 root biomass to begin with, most of the root material was used for acid digestions and only M9 shoots were collected and flash frozen, meaning M9 roots were not available for XAS analysis. The tissues were placed between two strips of Kapton™ tape, cut to approximately 1 cm x 0.5 cm (L x W) pieces, and stored at -80ºC.

Using the same but slightly modified biofilm detachment procedure that was used in Chapter 4, thirty millilitres of PBS solution was added to previously flash frozen gravel attached biofilms that came from 0-20 cm, 20-50 cm and 50-80 cm layers from each of the different mesocosm treatments (non-aerated planted mesocosm M2, non-aerated unplanted mesocosm M4, aerated planted mesocosm M8 and aerated unplanted mesocosm M12). The tubes were placed on an end-over-end shaker for 3 hrs at 40 rpm. The detached biofilms were then filtered onto 0.22 µm filter paper (EZFlow® 47 mm nylon membrane, Foxx Life Sciences, NH, U.S.A) and a second layer of filter paper was layered on top of the first filter paper with

the biofilm to help contain the wet biofilm. The filter papers were then placed between two strips of Kapton tape, cut to the same dimensions as the plant material, flash frozen in liquid nitrogen, and stored at -80ºC.

X-ray absorption near edge structure (XANES) analysis was used to perform arsenic speciation analysis of flash frozen plants tissues and detached biofilms. XANES spectra were collected at the Sector 20 insertion device beamline (20ID-C) of the Advanced Photon Source (CLS@APS), within the X-Ray Science Division (XSD), Argonne National Laboratory (Illinois, USA). XANES spectra of the As Kα-edge were recorded in fluorescence mode by using a four-element silicon drift detector (Vortex®-ME4 with Xspress 3 pulse processor) while monitoring incident and transmitted intensities in straight ion chamber detectors filled with nitrogen gas. Samples were packed in an aluminum sample holder, held between two layers of Kapton™ tape. During irradiation and measurements, the samples collected at Sector 20 were kept at 80 K inside a stainless-steel chamber with Kapton™ tape windows (APS construction, with NorCal clamps) kept under a 8 mTorr vacuum (Agilent Technologies Turbo-V 81-AG) with electronic cooling (Lakeshore 331 Temperature Controller with DT-600 Series silicon dioxide cryogenic temperature sensor). XANES spectra were fit within -150 to -30 eV to E0 using Athena from the Demeter 0.9.26 software package (Ravel & Newville, 2005). As(V), As(III), DMA(V) and MMA(V) arsenic standards for linear combination fitting (LCF) analysis were prepared separately by taking 1000 mg/L stock solutions prepared from salts and soaking four evenly cut strips of Kimwipes tissue (As(V): $Na_2HAsO_4 \cdot 7H_2O$ 98% Alfa Aesar, As(III): $NaAsO_2$ Baker Analyzed Reagent, DMA(V) $C_2H_7AsO_2$ >99% Fluka and MMA(V): $CH_3AsO(OH)_2$). The Kimwipe tissues were air dried overnight and then stacked on top of each other and placed between two pieces of Kapton tape and cut to the same dimensions mentioned before for plant and biofilm samples. Arsenic (III) glutathione was synthesized following the protocol described in Raab et al., 2004b and prepared in the same way as the other standards for XANES analysis. Previous scans of orpiment and realgar were also used for LCFs.

5.3 Results

5.3.1 Arsenic concentration and speciation in wastewater

To build on previous measurements of total arsenic concentrations in wastewater that were done as part of mass balance calculations in Chapter 4, Table 5-1 shows changes in arsenic concentrations over time during week 1, 6 and 14 with sampling at different time points during the week (20 min, 1 day and 6 days addition of arsenic). These total arsenic concentration values were used to calculate proportions for the various potential arsenic species present in the wastewater for day 6 for weeks 1, 6 and 14 shown in Figure 5-1. Day 6 samples for weeks 1, 6 and 14 were selected because the speciation data for the 20 min and day 1 timepoints were unreliable due to issues with our own in-house HPLC-ICP-MS (PerkinElmer NexION 310D).

From Table 5-1, for each week and all mesocosm types, day 1 wastewater arsenic concentrations were significantly less than arsenic concentrations 20 min after arsenic addition ($p<0.05$ ANOVA post hoc Tukey test). Although planted non-aerated and aerated mesocosms showed a slightly higher wastewater arsenic concentration than their unplanted counterparts, the difference was not significant for all weeks and all time points ($p>0.05$ ANOVA post hoc Tukey test). For non-aerated mesocosms for all weeks, there was no significant decrease in arsenic concentrations between day 1 and day 6 arsenic concentrations ($p>0.05$ ANOVA post hoc Tukey test). The opposite was true for aerated mesocosms, where day 6 wastewater arsenic concentrations were not always significantly less than day 1 arsenic concentrations, especially after week 1 ($p>0.05$ ANOVA post hoc Tukey test). For all time points and all weeks, non-aerated mesocosms had significantly lower arsenic concentrations than aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test).

Table 5-1: Total arsenic concentrations determined for wastewater collected on weeks 1, 6 and 14 at 20 min, 1 day and 6 days after addition of 1000 ppb As(V). Values are averages ± S.D., n=3.

		Wastewater arsenic concentrations (ppb)			
Week	**Time point**	**NA-P**	**NA-UP**	**A-P**	**A-UP**
1	**20 min**	658 ± 36^{b}	618 ± 60^{b}	871 ± 3^{a}	879 ± 43^{a}
	Day 1	69 ± 34^{e}	57 ± 7^{e}	565 ± 17^{bc}	509 ± 47^{c}
	Day 6	62 ± 43^{e}	44 ± 14^{e}	341 ± 24^{d}	303 ± 21^{d}
6	**20 min**	836 ± 133^{bc}	834 ± 124^{bc}	1229 ± 18^{a}	1227 ± 29^{a}
	Day 1	380 ± 82^{d}	273 ± 24^{d}	924 ± 44^{b}	856 ± 51^{bc}
	Day 6	340 ± 145^{d}	260 ± 34^{d}	692 ± 29^{c}	661 ± 43^{c}
14	**20 min**	1134 ± 230^{ab}	1090 ± 79^{bc}	1370 ± 42^{a}	1369 ± 70^{a}
	Day 1	486 ± 26^{d}	509 ± 48^{d}	1067 ± 18^{bc}	1040 ± 49^{bc}
	Day 6	446 ± 105^{d}	365 ± 43^{d}	856 ± 39^{c}	851 ± 27^{c}

[a–e] An ANOVA post hoc Tukey test was used to determine which arsenic concentrations were significantly different from each other ($p<0.05$). Letters were assigned in alphabetical order beginning with the highest arsenic concentrations.

In Figure 5-1, As(V) and DMA(V) were found in the highest proportions in wastewater from non-aerated plant and unplanted by day 6 for weeks 1 and 6 but not for week 14, where most of the arsenic was found as As(V). The speciation of arsenic from wastewater from aerated planted and unplanted mesocosms by day 6 for all weeks primarily contained As(V).

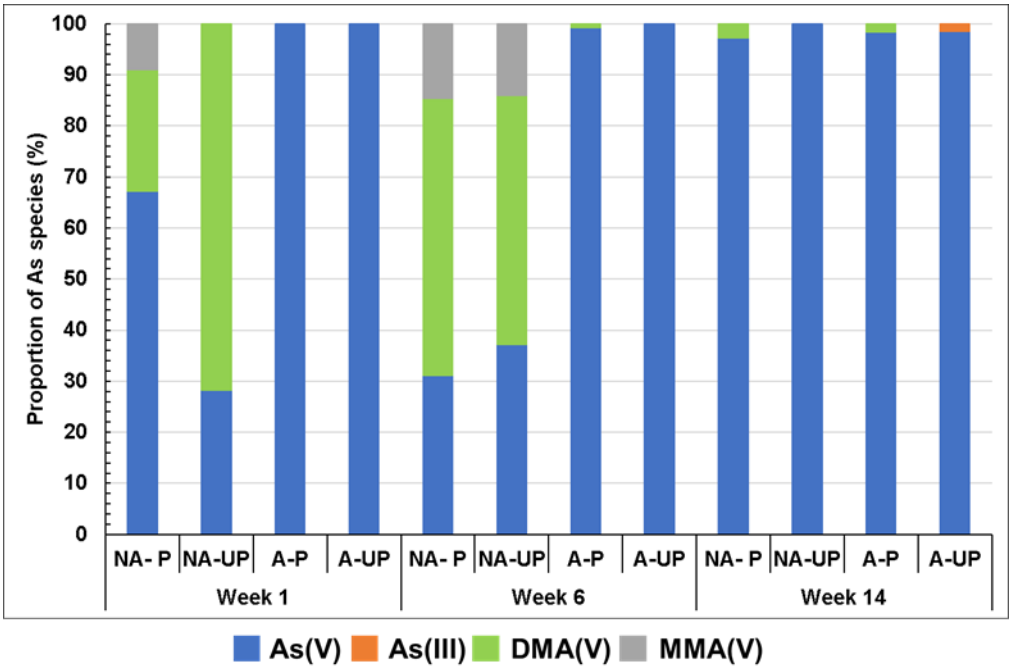

Figure 5-1: The proportion of arsenic species found in wastewater collected on Day 6 for week 1, week 6 and week 14 as determined by HPLC-ICP-MS analysis for non-aerated planted (NA-P), non-aerated unplanted (NA-UP), aerated planted (A-P) and aerated unplanted (A-UP) mesocosms. Values are averages with no S.D. shown, where all samples had an n=3 except for week 1 NA-P, NA-UP and week 14 A-P where the data for M1, M4, M6 and M7 were excluded due to poor column recoveries.

5.3.2 Arsenic speciation in biofilms

The proportions of arsenic species in detached biofilms from the non-aerated planted mesocosm are shown in Figure 5-2 A, demonstrating an increase in the proportion of As-S species (51 – 63%) and As(III) (15 – 29%), but a decrease in the proportion of As(V) (34 – 8%) with depth. The detached biofilms from the non-aerated unplanted mesocosm shown in Figure 5-2 B appear to have a very similar speciation pattern as the non-aerated plant mesocosm, except for the presence of a trace amount of MMA(V) (1%) in the 0–20 cm layer. The relative proportion of arsenic species in the biofilms for the 0–20 cm and the bottom 50–80 cm layer for the non-aerated unplanted mesocosm were very similar but there was an increase in the proportion of As(III) glutathione only for the middle 20–50 cm layer. The biofilms in the aerated planted (Figure 5-2 C) and unplanted mesocosms (Figure 5-2 D) contained 100% As(V) in all gravel layers. All corresponding XANES spectra with identified arsenic species that were found in all biofilm samples are shown in Figure S6 A–D. Linear combination fittings and arsenic species proportions along with a measure of goodness of fit for all biofilm samples are shown in supplementary Table S8.

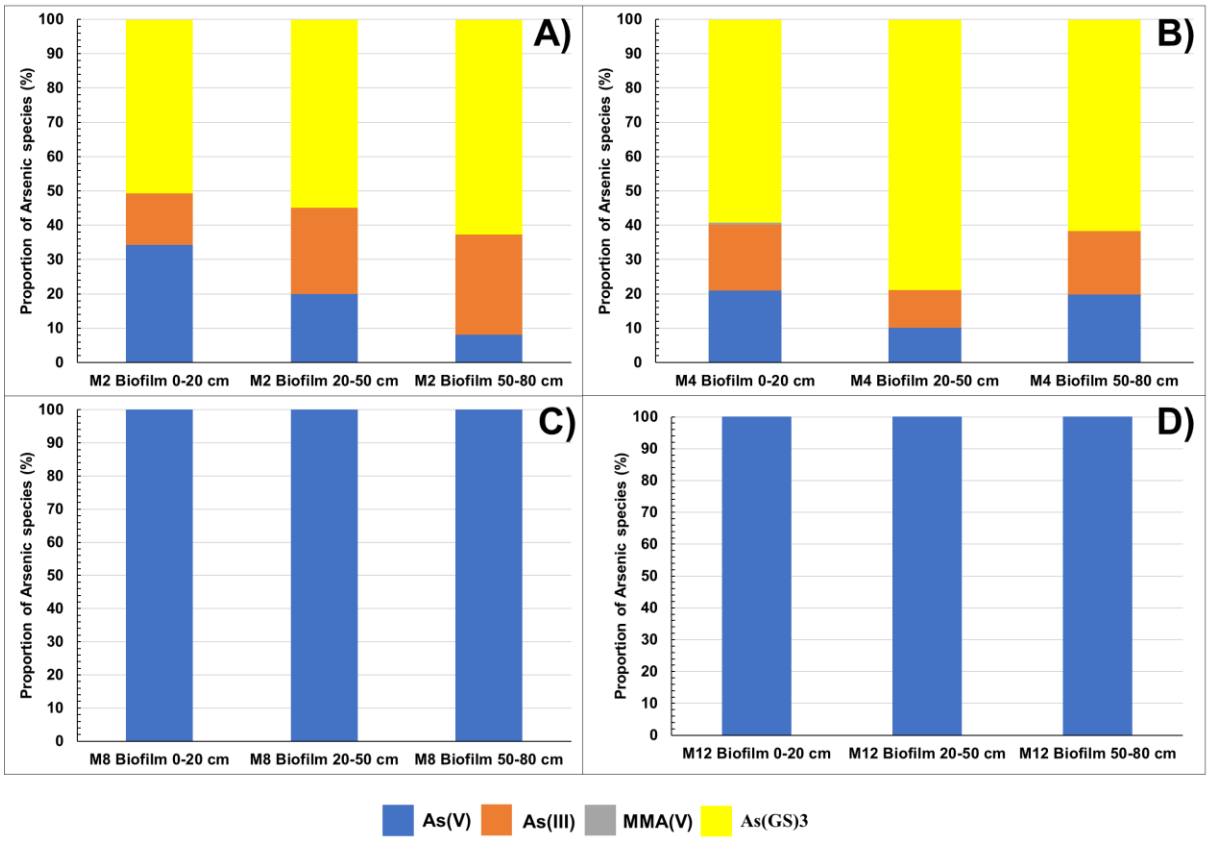

Figure 5-2: The proportion of arsenic species determined by linear combination fitting of XANES data found in detached biofilms from all three gravel layers (0-20 cm, 20-50 cm and 50 – 80 cm) for A) a non-aerated planted (M2); B) a non-aerated unplanted (M4); C) an aerated planted (M8); and D) an aerated unplanted (M12) mesocosm. As(GS)3 = arsenic (III) glutathione, used for fitting; its presence is assumed to indicate As(III) complexed to unknown thiol compounds.

5.3.3 Arsenic speciation in plants

In Figure 5-3 A–C, linear combination fitting of XANES data shows that whole *M. polymorpha* plants along with *E. hyemale* tissues contained 100% As(V) for non-aerated mesocosms M1–M3 except for the root tissues found in *E. hyemale* in M3 that contained 36% As(V), 11% As(III) and 53% As-S species. Root tissues of *E. hyemale* growing in the aerated planted mesocosms M7 and M8 shown in Figure 5-3 D and E contained 71 and 100% As(V) respectively, but *E. hyemale* roots in M8 also contained 29% As-S species. *E. hyemale* shoot tissues from M8 showed 5% As(V), 11% DMA(V), 38% As(III) and 46% As-S species but arsenic was not detectable in *E. hyemale* shoots from M7. No data were collected for *E. hyemale* shoots for M9 because arsenic concentration was below detection limits for XAS analysis. Linear combination fittings and arsenic species proportions along with a measure of goodness of fit for each individual sample that we analyzed is shown in the supplementary

Table S9.

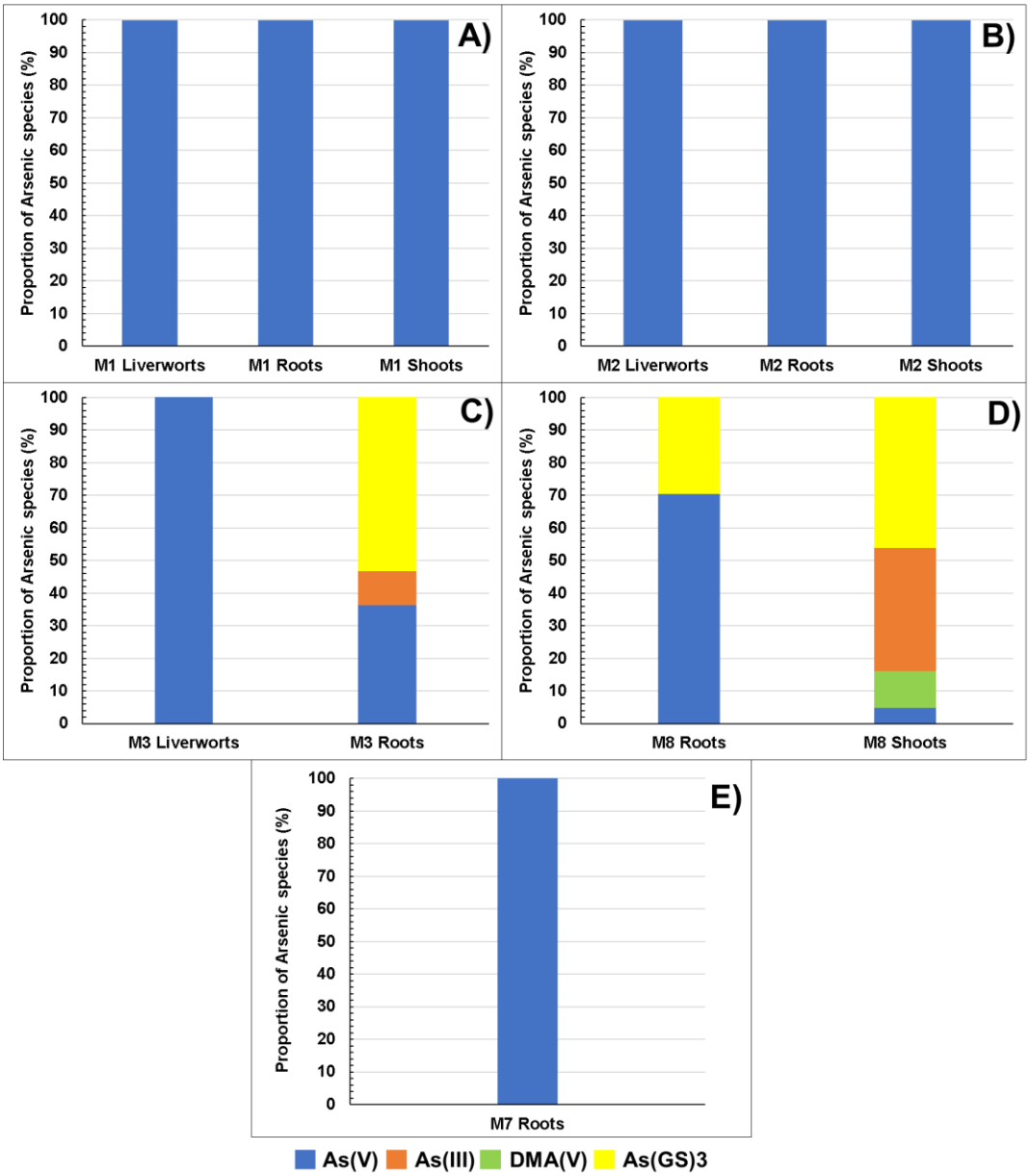

Figure 5-3: Proportion of arsenic compounds determined by linear combination fitting of XANES data for *M. polymorpha* plants and *E. hyemale* tissues from A) non-aerated planted mesocosm M1; B) non-aerated planted mesocosm M2; C) non-aerated planted mesocosm M3; and in E. *hyemale* tissues from D) aerated planted mesocosm M7; and E) aerated planted mesocosm M8. No M. polymorpha plants grew in aerated mesocosms. XANES spectra for E. *hyemale* shoots in M3 and M7 are missing because arsenic was not detectable in these samples.

5.4 Discussion

It has previously been shown (Chapter 4) that the porewater conditions in the non-aerated and aerated mesocosms in the present study were very different. Reducing conditions prevailed in the non-aerated mesocosms and were characterized by having low dissolved oxygen concentrations and redox potentials which allowed for the growth and activity of denitrifying bacteria that removed most of the nitrate from the porewater, and sulfate reducing bacteria that produced a strong smell of hydrogen sulfide in the water. All of the total organic carbon in fresh nutrients was likely used by these bacteria along with fermenters which produced high amounts of carbon dioxide that led to the high total inorganic carbon in the porewater and may have caused the pH to be lower in these mesocosms. As shown in Table 5-1, non-aerated mesocosms were able to remove the arsenic more rapidly than aerated mesocosms for weeks 1, 6 and 14 where almost no increase in removal was seen after day 1, even though the concentration of arsenic removed by day 6 apparently decreased with increasing week number.

The speciation of arsenic in the porewater may be important in trying to understand how arsenic is removed in non-aerated mesocosms. Although we expected that a high proportion of the added As(V) to be reduced to As(III) based on the pH and redox conditions of the porewater in the non-aerated mesocosms, there were interacting abiotic and biotic factors that likely interfered with this prediction. Abiotic factors could include reactions of arsenic with other dissolved organic and inorganic chemicals already present in the porewater or reactions with surface minerals present in the substrate. Biotic factors could include direct uptake and transformation of arsenic by bacteria or indirect reactions with inorganic and organic byproducts of bacterial metabolism which may include reactions with biogenic minerals. From Figure 5-1, we see that by day 6 in weeks 1 and 6, DMA(V) and MMA(V) formed in the porewater. Column recoveries were between 70 and 130%, for most samples with only a few anomalies whose data was excluded from Figure 5-1 (see speciation analysis QA/QC Procedures section and Table S10 of Appendix C). The bacteria in the gravel biofilms, shown in Figure 5-2 A and B, were likely the source of As(III) for bacteria in the porewater to rapidly transform As(III) into methylated arsenic species through the Challenger pathway (Challenger et al., 1933; Cullen, 2014; Cullen & Reimer, 1989). A higher proportion of As(III) with increasing gravel depth was seen in the non-aerated planted mesocosm in Figure 5-2 A than the non-aerated unplanted mesocosm in Figure 5-2 B. Initial transport of As(V) from the porewater into the gravel bacteria likely occurred through transport proteins that were meant for the transport of phosphate (William & Magpantay, 2023). As(V) was then reduced to As(III) by cytoplasmic enzymes such as glutaredoxin, thioredoxin and tyrosine phosphatases and then released through efflux transporters (Silver & Phung, 2005; William & Magpantay, 2023; Zegers et al., 2001). The other possibility is that As(III) was formed and released by dissimilatory arsenate respiring bacteria that use arsenate and a carbon source to derive energy via arsenate reductases located in their membranes or intermembrane spaces (William & Magpantay, 2023). As(III) is then taken up by porewater bacteria through aquaglyceroporins and transformed into highly toxic MMA(III) and DMA(III), which is again exported out of the bacteria by efflux transporters where the trivalent methylated species are either abiotically transformed into MMA(V) and DMA(V) due to their unstable nature or oxidized through bacterial oxidases (Chen et al., 2019, 2015b; Chen & Rosen, 2020; Garbinski et al., 2019). The absence of methylated arsenic species on day 6 of week 14 may have been due to inadvertent oxidation of the methylated arsenic species to As(V) during sample preparation or during analysis.

Based on our previous findings in Chapter 4, where XRD analysis of dried biofilms from non-aerated mesocosms revealed that large quantities of pyrite were formed, which may have helped remove As(V) from the wastewater, we could not rule out the possibility that As(III) that was formed by the gravel biofilm bacteria also reacted with hydrogen sulfide to form As-S precipitates, such as amorphous orpiment (As_2S_3), realgar (AsS), or arsenian pyrite (FeAsS) due to their non-crystalline nature and/or relatively low concentrations. Some evidence for the formation of As-S precipitates is shown in gravel biofilms from non-aerated mesocosms in Figure 5-2 A and B on the basis of arsenic (III) triglutathione ($As(Glu)_3$) as the standard for our linear combination fittings, as the XANES spectra for $As(Glu)_3$ cannot be distinguished from other As-S compounds as demonstrated in Figure S6. The high sulfide to iron ratio as well as the fact that most of the total organic carbon was consumed by the bacteria 1 day prior to As(V) addition to the

mesocosms would have likely favoured the formation of large quantities of pyrite prior to any formation of realgar (AsS), or arsenian pyrite (FeAsS) (Alam & McPhedran, 2019; Fischer et al., 2021; O'Day et al., 2004; Rodriguez-Freire et al., 2014, 2016; Saunders et al., 2018). It is possible that the small quantities of As-S precipitates that formed in the non-aerated biofilms were small quantities of abiotically produced orpiment (Alam & McPhedran, 2019; Newman et al., 1997; O'Day et al., 2004). Many constructed wetland studies only speculate at the presence of As-S precipitates in their wetlands as a potential arsenic removal mechanism but do not show any evidence of their presence (K. Z. Rahman et al., 2014, 2008aa; Singhakant et al., 2009a; Z. Zhang et al., 2017b, 2017a). The only constructed wetland study to show the formation of a small proportion of As-S precipitates in their substrates through XANES analysis was done by Park et al., 2022. They showed that very small quantities (<5%) of As-S precipitates appeared in substrates of planted but not their unplanted mesocosms which were both treating DMA(V) under mostly reducing conditions. The investigators did not believe that the As-S precipitates enhanced arsenic removal in the planted systems because both types of systems removed all of the DMA(V) through demethylation and formation of As(V) and As(III), which were then adsorbed to their substrates. Unlike the Park et al., 2022 study, our study showed a much higher proportion of As-S species (51 – 79%) in both of our non-aerated planted and unplanted mesocosms. Biofilms from our non-aerated unplanted mesocosms in Figure 5-2 B had a higher proportion of As-S species in all layers compared to non-aerated planted mesocosms in Figure 5-2 A, but the proportion of As-S in the biofilms from the unplanted mesocosm did not show any pattern with gravel depth, whereas the biofilms from the planted mesocosm showed an increase in the proportion of As-S with depth. It may be that root zone oxygenation in the non-aerated planted mesocosm reduced the amount of As-S formed by allowing for other aerobic bacteria to compete against anaerobic/anoxic sulfate reducing and fermentative bacteria (Stottmeister et al., 2003).

Our previous study (Chapter 4) is the first to maintain oxidizing conditions through active aeration in lab scale constructed wetlands treating arsenic contaminated wastewater. In contrast to the non-aerated mesocosms, the aerated mesocosms had high dissolved oxygen concentrations and redox potentials which created an oxidizing environment that likely supported the growth of aerobic bacteria that consumed most of the total organic carbon in fresh nutrients. The carbon dioxide produced by the aerobic bacteria was likely pushed out of the water due to active aeration which may have helped keep the pH relatively high.

Nitrification was occurring in these mesocosms but no removal of nitrate was occurring. As shown in Table 5-1, aerated mesocosms were able to remove less arsenic than non-aerated mesocosms. The most arsenic for aerated mesocosms was removed by day 6 of each week, but there was the same apparent degradation in arsenic removal by day 6 with increasing week number. Likely as a consequence of the oxidizing environment of the aerated mesocosms, most of the arsenic was found as As(V) in the porewaters for weeks 1, 6 and 14, as shown in Figure 5-1. It is likely that any As(III) released by bacteria in either the porewater or biofilms may have been immediately oxidized to As(V). Similarly, 100% of the arsenic in biofilms from all layers in both the aerated planted and unplanted mesocosms was found as As(V). This may indicate the presence of As(III) oxidizing bacteria in the aerated gravel biofilms (Liu et al., 2022) or there may have been inadvertent conversion of any As(III) in the biofilm samples during sample preparation and analysis. As stated in the study in Chapter 4, we propose that As(V), which is less mobile than As(III), was removed through adsorption on to carbonate substrates and through biosorption onto extracellular polymeric substances (EPS) produced by the aerated gravel biofilms.

Under most conditions, As(V) and As(III) are generally considered to be the most phytoavailable and hence most common species of arsenic found in plants (Li et al., 2016). As shown in Figure 5-3 A–E, most of the arsenic in *M. polymorpha* and *E. hyemale* remained as As(V), but some arsenic in some *E. hyemale* tissues from both non-aerated and aerated mesocosms was transformed into As(III), As-S species and even small quantities of DMA(V). In plant roots, As(V) is an analog of phosphate and may be transported through phosphate transporters while As(III) may be transported into cells via membrane proteins (aquaglyceroporins) that transport compounds such as glycerol, urea and silicic acid (Kumar et al., 2022). Once As(V) enters plant roots, it may be reduced to As(III) by enzymes such as CDC25-like tyrosine phosphatase and/or High Arsenic Content 1 proteins (Bleeker et al., 2006; Chao et al., 2014; Duan et al., 2007; Shi et al., 2016; Xu et al., 2017).The DMA(V) in the shoots of the aerated mesocosm M8 in Figure

5-3 D was proposed to have been produced by aerobic bacteria and then taken up by *E. hyemale* through aquaporin transporters in the roots that are also responsible for the transport of MMA(V) and As(III) into roots (Di et al., 2019; Farooq et al., 2016; N. Li et al., 2016). The presence of As-S species in non-hyperaccumulating plants may be due to the formation of thiol rich phytochelatins of various lengths, that are usually sequestered in root vacuoles to neutralize As(III) toxicity and limit the translocation of arsenic to shoots (Farooq et al., 2016; N. Li et al., 2016; Raab et al., 2005, 2007a). The As-S species in the shoots of *E. hyemale* from aerated mesocosm in Figure 5-3 D may reflect the fact that *Equisetum* sp. may have the ability to also store phytochelatins in their shoots as they are closely allied with the arsenic hyperaccumulating genus *Pteris* sp. (Indriolo et al., 2010; Meharg, 2003; Pickering et al., 2006). The lack of As(V) transformation in some of our samples may be due to a dose dependent response by the plants, where the arsenic concentration around the roots may not have been high enough to trigger a physiological response by the plants (Bleeker et al., 2006; Raab et al., 2005). This is especially true for *E. hyemale* tissues shown in Figure 5-3 A and B for non-aerated mesocosms where As(V) concentrations in Table 5-1 dropped, by 50% or more after 1 day of treatment. This dose dependence was also seen in our study in Chapter 3, where 10 mg/L As(V) was required for different arsenic species to appear in *E. hyemale* tissues while a 1 mg/L dose resulted mostly in little to no transformation of As(V) in the tissues. Based on our findings in Chapter 4, *M. polymorpha* was able to take up a higher mass of arsenic than *E. hyemale.* No previous arsenic speciation work has been done with *M. polymorpha* with respect to arsenic speciation but these plants have been shown to be extremely tolerant of exposure to high arsenic concentrations and can mount a robust physiological response to arsenic toxicity (Dutta et al., 2024; M. Li et al., 2024).

5.5 Conclusions

Arsenic in non-aerated mesocosms was removed more quickly and consistently than in aerated mesocosms between day 1 and 6 for weeks 1, 6 and 14. Both types of mesocosms showed a loss in arsenic removal efficiency by week 14. The speciation of arsenic in the different components of a constructed wetlands is largely mediated by the environmental conditions and type of microbial activity found in the wetlands. The presence and release of As(III) from all gravel biofilm layers was likely what led to the formation of methylated arsenic species in the porewater of non-aerated mesocosms on day 6 for weeks 1 and 6. The presence of As-S precipitates in all layers of non-aerated gravel biofilms suggested some removal of arsenic, proposed to be by the formation of amorphous orpiment. The orpiment likely formed abiotically because most of the carbon in the porewater was consumed by day 1 and arsenic was added after the carbon was depleted. This likely did not allow for the formation of arsenian pyrite. Realgar was also unlikely to form as it requires a carbon source along with high iron(II)-to-sulfide ratios. These findings further support our hypothesis in Chapter 4 that conditions in the mesocosms favored the formation of pyrite which removed most of the arsenic from the porewater. Conversely, there was no changes in arsenic speciation in aerated porewaters or in all gravel biofilm layers, and the arsenic remained as As(V). These findings further support our hypothesis in Chapter 4 that arsenic was likely removed through adsorption to carbonate substrates and the extracellular polymeric substances (EPS) produced by the aerated gravel biofilms.

There was no clear pattern of arsenic speciation in *E. hyemale* with respect to tissue type and mesocosm type. This may have been due to the confluence of inconsistent plant growth as shown in Chapter 4 and perhaps low arsenic exposure concentrations, which did not always induce arsenic transformations in the plants. Arsenic in *M. polymorpha* plants, which grew well at the surface of non-aerated mesocosms, remained as As(V) and likely did not have a large influence on how arsenic was removed in these mesocosms.

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Author Contributions

Antoine Hnain: Conceptualization, methodology, investigation, formal analysis, data curation, writing - original draft.; **Debora Meira:** Data acquisition, methodology, data curation; **Iris Koch:** Conceptualization, writing – review & editing, supervision, funding acquisition; **Kela Weber:** Resources, supervision, funding acquisition.

Chapter 6 – Activity, identification and ecophysiological analysis of prokaryotes found in porewaters, root and gravel biofilms from non-aerated and aerated, planted and unplanted mesocosms treating arsenic

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Abstract

For the first time in the literature, we examine the ecophysiology of prokaryotes found in non-aerated and aerated, planted and unplanted laboratory scale mesocosms treating arsenic from water. Specifically, we used 16s rRNA sequencing to identify (genus level) the prokaryotes found in the substrates and porewaters to then use this information to predict their potential carbon, nitrogen, sulfur and arsenic related metabolisms. This was ultimately done to try and explain how arsenic may be removed in the two types of wetlands. Our results show that non-aerated mesocosms removed more arsenic than aerated mesocosms and that planting had no influence on removal, in either type of mesocosm. The week-to-week Alpha diversity measure comparisons (Richness, Shannon's diversity and Pielou's Evenness) for porewater for both types of wetlands did not reveal any major changes indicating a relatively stable microbial community, although these measures were higher for detached gravel biofilms than for porewaters. Taxonomic classification to genera level of the prokaryotes found in detached gravel biofilms and porewaters from non-aerated mesocosms revealed that the reducing conditions in these mesocosms supported highly versatile anaerobic/facultatively anaerobic prokaryotes capable of chemoorganotrophic, chemolithoautrophic growth, or had the ability to switch between metabolic modes. This microbial community was capable of removing nitrogen and carbon through nitrate reduction/denitrification and fermentation as well as sulfate reduction within the first 24 hrs after nutrient addition. The dissolution of iron from the ankerite containing substrate through the formation of organic acids and carbon dioxide by fermentative prokaryotes along with the formation of sulfide by sulfate reducers may have led to the formation of pyrite. This pyrite formed and accumulated in the 3.9 yrs prior to and during arsenic exposure. The presence of pyrite likely enhanced the removal of arsenic in the non-aerated mesocosms through adsorption of As(V). Aerated mesocosms largely supported aerobic chemoorganotrophic prokaryotes that could remove most of the carbon but not the nitrogen 24 hrs after nutrient addition. The prokaryotes in the aerated mesocosms did not cause the precipitation of any mineral phases that would aid in As(V) removal and most of the removal in aerated mesocosms was attributed to arsenic adsorbing to substrates and biofilms. Both types of mesocosms supported a community of microbes that were highly resistant to arsenic exposure and have previously been found in arsenic contaminated environments.

6.1 Introduction

The speciation of arsenic in constructed wetlands (CWs) is determined by the interactions of abiotic and biotic factors which ultimately determines how well CWs can remove arsenic. Some of the same interacting abiotic and biotic factors that govern arsenic speciation may also govern the kind of microbial communities that exist in CWs. These abiotic factors include the dimensions of the CWs, pH, redox, temperature as well as the wastewater composition. The only biotic factors identified in CWs treating arsenic would include whether or not they are planted, the species of plant used in the CWs as well as the species of microbes present in the substrates and/or porewaters.

Only six studies have examined the microbial community that are found in constructed wetlands (CWs) that are treating arsenic (Arroyo et al., 2013; Liu et al., 2022; Nguyen et al., 2021; Park et al., 2022; Zhang et al., 2017b, 2017a). Park et al., (2022) found that both of their planted and unplanted CWs removed almost all of the added dimethylarsinic acid (DMA(V)) through demethylation to As(V) and As(III) and retention in their substrates, but they found no difference in the microbial composition of their planted and

unplanted CWs and they could not identify what bacteria were responsible for demethylation. They presumed that most of the arsenic was adsorbed to the substrate, despite not knowing the composition of their substrate. In a study by Liu et al., 2022, planted CWs with iron rich particles in the substrate treating As(V) removed more arsenic than planted CWs with no iron rich particles. In the CWs with iron, the genera *Myxococcus* was shown to have the ability to be able to anaerobically oxidize As(III) and reduce iron(III) with increasing depth, yet most of the arsenic was found adsorbed to iron(III) in the first 10 cm of the CWs. The removal of arsenic in CWs with iron containing substrates has also been shown to be influenced by the concentration of dissolved phosphate and sulfate in the water being treated. Zhang et al., (2017a) has shown that unplanted and low phosphate treatments removed more arsenic than planted and high phosphate treatments, irrespective of the concentration of iron added to the substrate, potentially because high phosphate enhances the activity of iron reducing and unidentified arsenate respiring bacteria which would enhance the mobility of arsenic. In a similar study, Zhang et al., 2017b showed that high sulfate concentrations in the wastewater enhanced arsenic removal in planted and unplanted CWs irrespective of the iron concentrations in the substrate. This was mainly due to high gene copy numbers for respiratory arsenate reductase and unidentified sulfate reducing bacteria, which together may have formed arsenic sulfide precipitates. The high sulfate concentrations also enhanced arsenic uptake in roots of planted CWs. The remaining CW studies by Nguyen et al., 2021 and Arroyo et al., 2013 showed very poor removal of arsenic relative to the other studies. Nevertheless, Nguyen et al., 2021 was able to show that there was a higher diversity of prokaryotes identified to the phyla level in porewater water from unplanted CWs than CWs planted with *Typha* sp. and the opposite was true for prokaryotes identified to the phyla level in their substrates. The study by Arroyo et al., 2013 showed that free water flow and vertical subsurface flow CWs planted with *P. australis* with either arlita clay or gravel as a substrate showed the highest arsenic removal and highest prokaryotic richness and diversity in comparison to equivalent CWs planted with *T. latifolia* and unplanted controls.

In this study, we identified the most common and dominant prokaryotes found in porewaters and attached to our substrates using next generation 16s rRNA sequencing that were found in planted and unplanted, non-aerated and aerated mesocosms treating arsenate from water for 33 weeks. Based on the identity of the most common and dominant prokaryotes in our mesocosms, we then inferred their potential ecophysiological roles with respect to carbon, nitrogen and sulfur cycling during weekly transitions from nutrient rich to nutrient poor conditions. We also compare the removal efficiencies of non-aerated and aerated mesocosms and suggest potential removal mechanisms for arsenic.

6.2 Materials and Methods

A thorough description of the design, construction and operation of our non-aerated and aerated planted and unplanted mesocosms exposed to 1 mg/L As(V) has previously been described in detail in Chapter 4. This chapter also describes the measurements that were made to evaluate wastewater conditions, nutrient dynamics and arsenic removal through measurements of temperature, conductivity, dissolved oxygen (DO), redox potential (ORP), pH, NO_3^--N. NH_4^+-N, total organic carbon (TOC), total inorganic carbon (TIC) and total arsenic concentrations. The procedures for measuring whole mesocosm microbial activity and performing 16s ribosomal RNA gene sequencing for the identification of the prokaryotes in our porewaters, gravel and root biofilms are described below.

6.2.1 Fluoresceine diacetate microbial activity test for whole mesocosms

Total microbial activity of all the mesocosms were assessed by monitoring *in-situ* fluorescein-diacetate (FDA) hydrolysis on weeks 2, 4, 6, 8, 12 and 16 on Day 4 (Fridays) well after most carbon sources have been utilized by the mesocosms. Briefly, following the methods developed in Weber et al., (2008), a 5 mM stock solution of FDA (Sigma-Aldrich Chemical, Milwaukee, WI) was prepared in acetone and 1 mL of this solution was added to each mesocosm. Aliquots of 2.6 mL samples were taken every minute for 30 min from the sampling port of the mesocosms and placed in plastic cuvettes to obtain fluorescence readings using a handheld fluorometer (Turner Designs, AquaFluor® 490 nm excitation, 520 nm emission).

The fluorometer was calibrated according to the manufacturers instructions where the data output is in µg/L of fluorescein. A 3-point calibration curve was created by using a 500 µM stock solution of fluorescein, prepared from fluorescein dye sodium salt (Sigma-Aldrich Chemical, Milwaukee, WI) and was used to make 0, 0.25 and 0.5 µM fluorescein calibration solutions (corresponding to 0, 94 and 188 µg/L of fluorescein). The 0.5 µM fluorescein solution was considered to be the approximate concentration we expected to see if all of the FDA is hydrolyzed to fluorescein in each mesocosm. After the fluorometer was calibrated, fluorometric interference produced by the mesocosm water from each mesocosm was accounted for by preparing a 0 and 0.25 µM fluorescein solution using the mesocosm water from each mesocosm and measuring the fluorescence. This data was then used to normalize and convert the data recorded from the assay to fit within the 0 to 188 µg/L range expected for the assay. The reaction rate for the conversion of FDA to fluorescein (ug/L*min) was measured by taking the slope of the microbial activity between 8 and 15 min for each mesocosm and for each week tested.

6.2.2 16S ribosomal RNA gene sequencing

To extract microbial DNA from mesocosm water, 150 mL of water was sampled on weeks 2, 4, 8, 12, 16 and 33 on day 4 of treatment for all mesocosms and the water was filtered through 0.22 µm filter paper (EZFlow® 47 mm nylon membrane, Foxx Life Sciences, NH, U.S.A) using a vacuum pump (Buchi V-500, BUCHI Corporation, DE, U.S.A) to capture biofilms. Fifty mililiters of detached gravel biofilms in phosphate buffered saline solution from layers 0-20, 20-50 and 50-80 cm from each mesocosm were also filtered in the same way. Filter papers with biofilm were stored at -20ºC.

To detach biofilms from gravel particles from each gravel layer, a procedure originally designed by Weber & Legge, 2013 was used. Briefly, 25 g gravel samples from each layer from each mesocosm were placed in 100 mL of sterilized phosphate buffered saline solution in Erlenmeyer flasks and shaken at 100 rpm for 3 hrs (Innova 2100 Platform shaker, New Brunswick Scientific Inc., CT, U.S.A.). Up to 50 mL of detached biofilm solution was then filtered on to 0.22 µm filter paper (EZFlow® 47 mm nylon membrane, Foxx Life Sciences, NH, U.S.A) stored in sterile vials and stored at -20ºC.

FastDNA® SPIN Kits for Soil (MP Biomedicals, OH, U.S.A.) were used to extract DNA from the biofilms on the filter papers following the manufacturers protocol. The extracted DNA samples were stored at -20ºC.

The samples were divided into three separate sequencing runs to ensure a large enough read depth. The variable V3 and V4 regions of the 16S rRNA were amplified following the Illumina 16S Metagenomic Sequencing Library Preparation guide (version B, Illumina Canada Ulc., Victoria, BC, Canada) including amplification and bead purification steps. The concentration of each sample library was measured on the Qubit fluorometer. Samples were each normalized to 4 nM, pooled together, denatured, and diluted to 4 pM before sequencing with the MiSeq Reagent v3 600 cycle kit on the MiSeq (Illumina Canada Ulc., Victoria, BC, Canada) generating 2 × 300 bp reads. A 10% PhiX control library was included as a positive control for sequencing quality.

De-multiplexed fastq files were imported and analyzed using QIIME2 version 2019.4 (Caporaso et al., 2010) on VirtualBox. For quality control, sequences were filtered, denoised, merged and chimeras were removed using DADA2 (Callahan et al., 2016). Sequences were classified using the Greengenes database 13_8 (99% OTUs full-length sequences) and features related to mitochondria or chloroplast were removed. The resulting median frequency was 25,667 (min 2 – max 145,903) reads. Therefore, sequences were rarefied to 6520 reads for further diversity analyses. Estimated alpha diversity metrics included OTU richness (S), Shannon's diversity index (H) and Pielou's Evenness (E). Beta diversity was assessed through comparing the functional profiles of the most common and dominant genera from porewaters and biofilms from each of the mesocosm treatments.

6.2.3 Selection of OTUs for functional and metabolic analysis

For each of the samples that were sequenced, the % relative abundance (% RA) for all identified OTUs was calculated by dividing the sequence counts of each OTU by the total sum of all sequence counts for all OTUs and multiplying by one hundred.

The average % relative abundance (Ave. % RA) for all OTUs from each individual porewater sample were calculated for each of the four treatment types, non-aerated planted (M1 – M3), non-aerated unplanted (M4 – M6), aerated planted (M7 – M9) and aerated unplanted (M10 – M12). The same treatment grouping was also used to calculate Ave. % RA for all OTUs from detached biofilm samples from layer 1 (0 – 20 cm), layer 2 (20 – 50), layer 3 (50 – 80 cm) and plant roots from the plants in M1 – M3 and M7 – M9. The top twenty genera with the highest Ave. % RA along with any genera level OTUs that shared a common Family were selected to display in the heat maps shown in the results (herein referred to as the selected prokaryotic genera).

For functional profiling of the selected prokaryotic genera, we decided to compare the non-aerated (M1-M6) to aerated (M7-M12) mesocosms and ignoring the effect of planting because many of the genera identified in the planted systems where also found in the unplanted systems, for each respective treatment type. The functional profiling was done by using the readily available scientific literature to classify each of the selected prokaryotic genera by metabolic mode, respiratory mode as well as their nitrogen and sulfur metabolisms. The functional profiling was done for each of the porewater samples (weeks 2, 4, 8, 12, 16 and 33) as well as for the detached biofilm samples from gravels (layer 1, 2 and 3) and roots, separately.

The metabolic mode of prokaryotes refers to the different ways in which prokaryotes can obtain energy as well as utilize carbon sources for growth. The selected prokaryotic genera were classified into five main metabolic modes: Chemoorganotrophs, chemolithoautotrophs, autotrophs, the ability to switch between modes (Multiple) as well as prokaryotes with very little information on their metabolic mode in the literature because they are uncultured and less well understood (Unknown).

The respiratory mode mainly refers to the type of oxygen environment the prokaryotes are able to live in. The selected prokaryotic genera were classified into five main categories: Obligate aerobes that require oxygen, obligate anaerobes that grow in the absence of oxygen, facultative anaerobes that can grow in both environments, pathogen/symbiotic prokaryotes that require a host organism to survive and finally, prokaryotes where their growth environment is not well characterized mainly because these prokaryotes have not been isolated and cultured on their own or the information is not clear because a particular genera is a host pathogen/symbiont (Unknown).

Nitrogen and sulfur metabolism refers to the potential ways in which prokaryotes transform nitrogen and sulfur in their environment which is largely driven by whether the prokaryotes are using a particular nitrogen and sulfur source for assimilatory and/or dissimilatory processes. The potential types of nitrogen metabolism used by the selected prokaryotic genera include nitrate reduction, denitrification, ammonia oxidation, anaerobic ammonia oxidation (Anammox), ammonification, nitrite oxidation, dissimilatory nitrate reduction to ammonium (DNRA), nitrogen fixation and ammonia assimilation. The potential types of sulfur metabolism used by the selected prokaryotic genera include sulfate reduction, sulfur oxidation, microbial sulfur disproportionation (MSD) and sulfate assimilation. In other cases, the metabolic pathways of prokaryotic genera were not clear because they are host pathogen/symbiont, information is not available on their N and S metabolisms or because they are uncultured and less well understood (Unknown).

Based on the above set of classifications for each of the modes and metabolisms, this information was quantified by determining what proportion of the total Ave. %RA of all the selected prokaryotic genera in each of the porewater, gravel and root biofilm samples contributed to each classification within each mode or metabolism.

6.3 Results

6.3.1 Microbial activity

From Figure 6-1 A, we can see that the average microbial activity of planted and unplanted non-aerated mesocosms were largely the same and the microbial activity decreased over time. Similar to the non-aerated mesocosms, the microbial activity of planted and unplanted aerated mesocosms in Figure 6-1 B were the same but the microbial activity remained stable over time. The weekly average microbial activity in non-aerated planted (6.99 ± 1.42 µg/L*min) and unplanted (7.53 ± 1.63 µg/L*min) mesocosms were not

significantly different from each other (ANOVA post hoc Tukey test $p>0.05$). The same relationship was seen when comparing the weekly average microbial activity of aerated planted (4.10 ± 0.61 µg/L*min) and unplanted (3.46 ± 0.58 µg/L*min) mesocosms (ANOVA post hoc Tukey test $p>0.05$). When comparing the weekly average microbial activity of non-aerated to aerated mesocosms, the non-aerated planted and unplanted mesocosms had a significantly higher microbial activity compared to aerated planted and unplanted mesocosms (ANOVA post hoc Tukey test $p<0.05$).

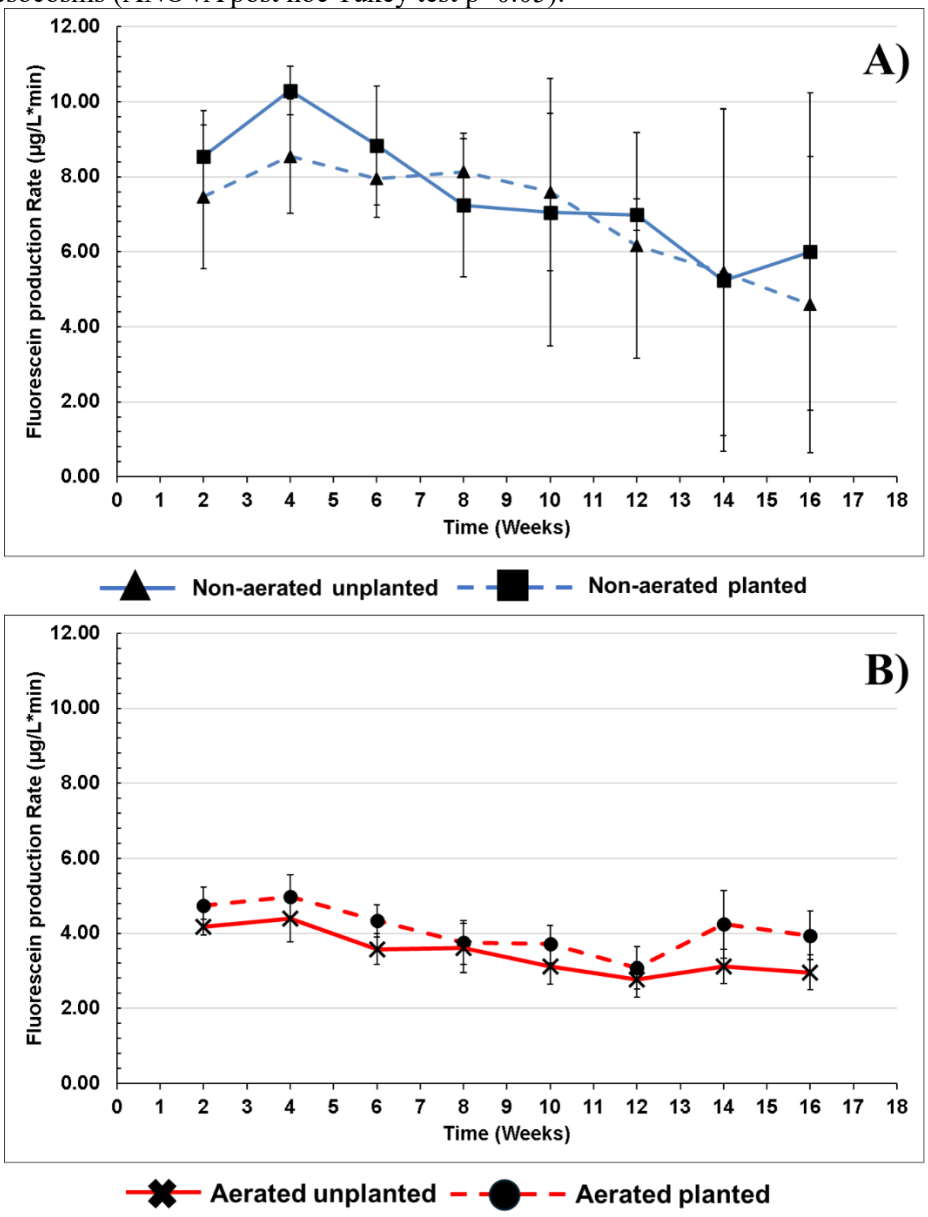

Figure 6-1: Whole mesocosm average microbial activity measured every two weeks over a sixteen-week period for a) non-aerated planted (M1-3) and unplanted (M4-6) mesocosms as well as B) aerated planted (M7-9) and unplanted mesocosms (M10-12).

6.3.2 Weekly carbon and nitrogen cycling kinetics

The data in Figure 6-2 A shows that all mesocosms significantly reduced the concentration of TOC that was initially present in the fresh nutrient solution at the start of each week and this occurred 1 day after addition ($p<0.05$ ANOVA post hoc Tukey test). TOC concentrations in the porewaters of all mesocosms

remained significantly low for the remaining treatment period, as shown by measurements for days 4 and 7 ($p<0.05$ ANOVA post hoc Tukey test).

With the decrease in TOC concentrations there was a concomitant and significant increase in TIC concentrations in the porewater of non-aerated mesocosms in relation to the TOC concentration present in fresh nutrients ($p<0.05$ ANOVA post hoc Tukey test), as shown in Figure 6-2 B. The direct opposite pattern was observed for porewaters from aerated mesocosms, where there was a concomitant and significant decrease in TIC concentration in relation to the TOC concentration present in fresh nutrients ($p<0.05$ ANOVA post hoc Tukey test). TIC concentrations remained significantly high in non-aerated mesocosms and significantly low in aerated mesocosms for the remaining treatment period as demonstrated by the measurements on days 4 and 7 ($p<0.05$ ANOVA post hoc Tukey test).

As shown in Figure 6-2 C, the non-aerated mesocosms significantly reduced the concentration of NO_3^--N that was initially present in fresh nutrients, 1 day after addition ($p<0.05$ ANOVA post hoc Tukey test) while there was no significant change in the NO_3^--N concentrations in relation to what was present in fresh nutrients for porewaters from aerated mesocosms ($p>0.05$ ANOVA post hoc Tukey test). The NO_3^--N concentrations remained significantly low in porewaters from non-aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test) while NO_3^--N concentrations in porewaters from aerated mesocosms remained unchanged ($p>0.05$ ANOVA post hoc Tukey test), as demonstrated by the measurements on days 4 and 7.

In Figure 6-2 D, the aerated mesocosms significantly reduced the concentration of NH_4^+-N that was initially present in fresh nutrients, 1 day after addition ($p<0.05$ ANOVA post hoc Tukey test) while there was no significant change in the NH_4^+-N concentrations in relation to what was present in fresh nutrients for porewaters from aerated mesocosms ($p>0.05$ ANOVA post hoc Tukey test). The NH_4^+-N concentrations remained significantly low in porewaters from aerated mesocosms ($p<0.05$ ANOVA post hoc Tukey test) while NH_4^+-N concentrations in porewaters from non-aerated mesocosms remained unchanged ($p>0.05$ ANOVA post hoc Tukey test), as demonstrated by the measurements on days 4 and 7.

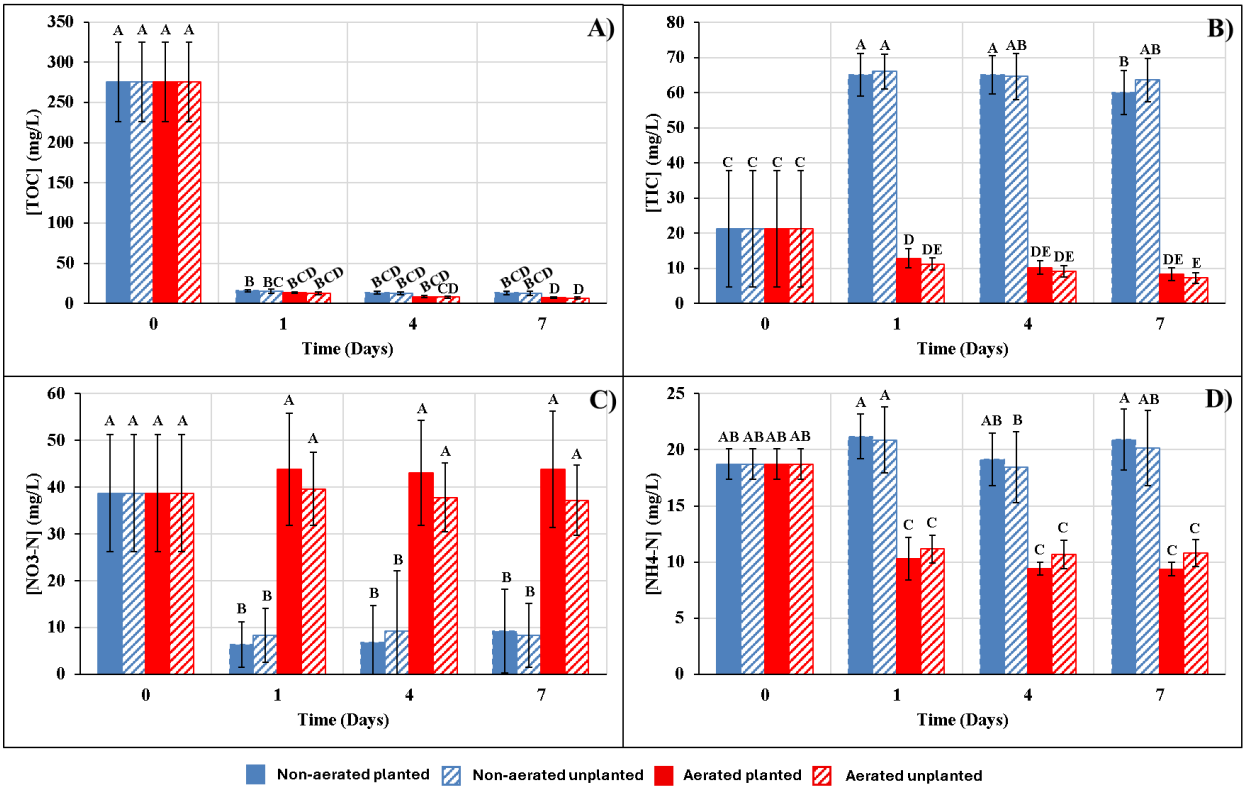

Figure 6-2: The average concentration of A) total organic carbon (TOC), B) Total inorganic carbon (TIC) and C) nitrate nitrogen (NO3--N) and D) ammonium nitrogen (NH4+-N) measured in porewater for days 0, 1, 4 and 7 days after addition for weeks 1, 3, 5, 7, 9, 11, 13 and 15. Day 0 measurements represent the average of measurements (n=8) taken from the nutrient tank prior to addition to each set of mesocosms. Day 1, 4 and 7 measurements represent the average measurements for n=24 measurements (8 time points x 3 mesocosms per treatment). Separate ANOVA post hoc Tukey tests were used for each measurement to determine any significant differences in averages between treatments. Averages denoted with different letters are significantly different from each other ($p<0.05$).

6.3.3 Alpha diversity measurements

In Table 6-1, we can see that all of the significant differences in average OTU richness for the different sample types appeared within the different treatments. Specifically, gravel biofilms had a significantly greater average OTU richness than porewaters in all treatments ($p<0.05$ ANOVA post hoc Tukey test). Root biofilms had a significantly greater average OTU richness and diversity than porewaters only in non-aerated planted mesocosms ($p<0.05$ ANOVA post hoc Tukey test).

Both aerated planted and unplanted porewaters had a significantly greater average OTU diversity (H) than both planted and unplanted non-aerated porewaters ($p<0.05$ ANOVA post hoc Tukey test). For all of the treatments, gravel biofilms had a significantly greater average OTU diversity than porewaters ($p<0.05$ ANOVA post hoc Tukey test). Root biofilms had a significantly greater average OTU diversity than porewaters only in non-aerated planted mesocosms ($p<0.05$ ANOVA post hoc Tukey test).

All of the significant differences in average OTU evenness for the different sample types appeared only within non-aerated planted mesocosms where gravel and root biofilms had a significantly greater average OTU evenness than porewaters ($p<0.05$ ANOVA post hoc Tukey test).

Table 6-1: Measures of the average Richness (S), Shannon's Diversity Index (H) and Pielou's Evenness (E) for prokaryotic OTUs identified in porewater, grave and root biofilms

Treatment	Sample	S	H	E
Non-aerated planted (M1-3)	Porewater	165 ± 53^{D}	3.4 ± 0.3^{F}	0.66 ± 0.05^{D}
	Gravel biofilms	328 ± 63^{AB}	4.2 ± 0.3^{ABC}	0.73 ± 0.03^{ABC}
	Root biofilms	387 ± 22^{A}	4.7 ± 0.2^{A}	0.79 ± 0.02^{A}
Non-aerated unplanted (M4-6)	Porewater	169 ± 65^{D}	3.5 ± 0.3^{EF}	0.69 ± 0.04^{CD}
	Gravel biofilms	303 ± 112^{ABC}	4.1 ± 0.5^{BCD}	0.72 ± 0.03^{BCD}
Aerated planted (M7-9)	Porewater	214 ± 61^{CD}	3.9 ± 0.2^{CD}	0.73 ± 0.04^{BC}
	Gravel biofilms	356 ± 40^{AB}	4.4 ± 0.3^{AB}	0.75 ± 0.04^{AB}
	Root biofilms	295 ± 34^{ABC}	4.3 ± 0.5^{ABC}	0.75 ± 0.07^{AB}
Aerated unplanted (M7-9)	Porewater	195 ± 73^{CD}	3.7 ± 0.2^{DE}	0.71 ± 0.03^{BCD}
	Gravel biofilms	282 ± 66^{BC}	4.2 ± 0.2^{ABC}	0.76 ± 0.04^{AB}

*Please note that the data for average richness, diversity and evenness were based on an n = 6 for porewaters (Weeks 2, 4, 8, 12, 16 and 33), n =9 for detached gravel biofilms (3 layers x 3 mesocosms per treatment except non-aerated planted mesocosm M3 layer 3, non-aerated unplanted mesocosm M5 layer 1 and aerated unplanted mesocosm M10 layer 2 where n=8 because the data produced by the sequencing run was bad) and n=3 for detached root biofilms. Separate ANOVA post hoc Tukey tests were used to determine if there were significant differences in average richness, diversity and evenness among the porewater samples as well as the gravel and root biofilms. Averages denoted with different superscripted letter(s) are significantly different from each other ($p<0.05$).

As shown in the Figure 6-3 heat map for porewaters from non-aerated mesocosms, the weekly Ave. %RA of *Candidatus* Omnitrophus, *Sulfuricurvum*, Nanoarchaeota archaeon SCGC AAA011-D5 and *Sulfurimonas*, in non-aerated unplanted mesocosms was significantly greater than the weekly Ave. %RA found in non-aerated planted mesocosms (Student t-test $p<0.05$). The opposite was true for *Magnetospirillum* and Blvii28 wastewater sludge group, where the weekly Ave. %RA in non-aerated planted mesocosms was significantly greater than the weekly Ave. %RA in non-aerated unplanted mesocosms (Student t-test $p<0.05$). The most common (genera found in >60% of the samples) and dominant genera (have a weekly Ave. %RA ≥ 1) found in porewaters from non-aerated planted and unplanted mesocosms were *Candidatus* Omnitrophus, *Thiovirga*, *Endomicrobium* and *Sulfuricurvum*. The only difference between non-aerated planted and unplanted mesocosms was that *Magnetospirillum* was more common and dominant in planted mesocosms while the same was true for Nanoarchaeota archaeon SCGC AAA011-D5 in unplanted mesocosms.

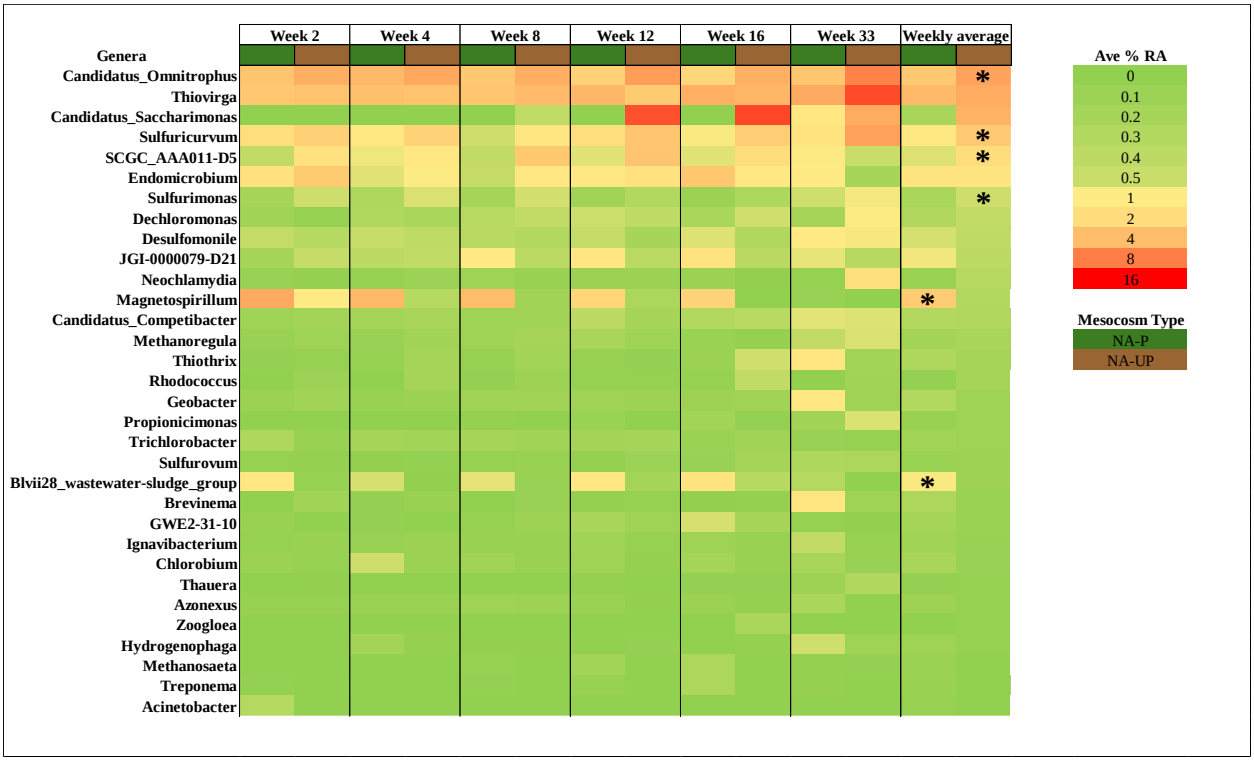

Figure 6-3: A heat map representing the Ave. %RA of 32 prokaryotic genera identified in porewater samples from non-aerated planted (M1– M3, dark green) and unplanted (M4 – M6, brown) mesocosms that were sampled on weeks 2, 4, 8, 12, 16 and 33. A weekly average %RA for each prokaryotic genera found in non-aerated planted and unplanted mesocosms is also represented in the last two columns of the map on the right. The list of genera shown in the heat map were sorted by the

weekly average %RA for non-aerated unplanted mesocosms in descending order. The * indicates what treatment had a significantly greater weekly average %RA for a particular genus (Student t-test p<0.05). Please note that Azonexus was formerly Dechloromonas but is represented as two separate genera in this figure.

Similarly, in the heat map shown in Figure 6-4 for porewaters from aerated mesocosms, the weekly Ave. %RA of *TM7a*, *Nakamurella*, SWB02, *Candidatus* Xiphinematobacter, *Candidatus* Protochlamydia, *Candidatus* Nitrosotenuis, *Neochlamydia* and Nanoarchaeota archaeon SCGC AAA011-D5 were significantly greater in aerated unplanted mesocosms than in aerated planted mesocosms (Student t-test p<0.05). The opposite pattern is true for *Micropruina*, *Nocardioides*, *Oligoflexus*, *Amphiplicatus*, *Pirellula*, *Cytophaga*, *Microlunatus* and *Ferruginibacter* where the weekly Ave. %RA for these genera was significantly greater in aerated planted mesocosms than in aerated unplanted mesocosms (Student t-test p<0.05). The most common (genera found in >60% of the samples) and dominant genera (have a weekly Ave. %RA ≥ 1) found in porewaters from aerated planted and unplanted mesocosms were Pir4 lineage, TM7a, *Nakamurella*, *Chryseobacterium* and *Mycobacterium*. The only difference between aerated planted and unplanted mesocosms was that *Micropruina* and *Nocardioides* were more common and dominant in the planted mesocosms while the same was true for SWB02 in unplanted mesocosms.

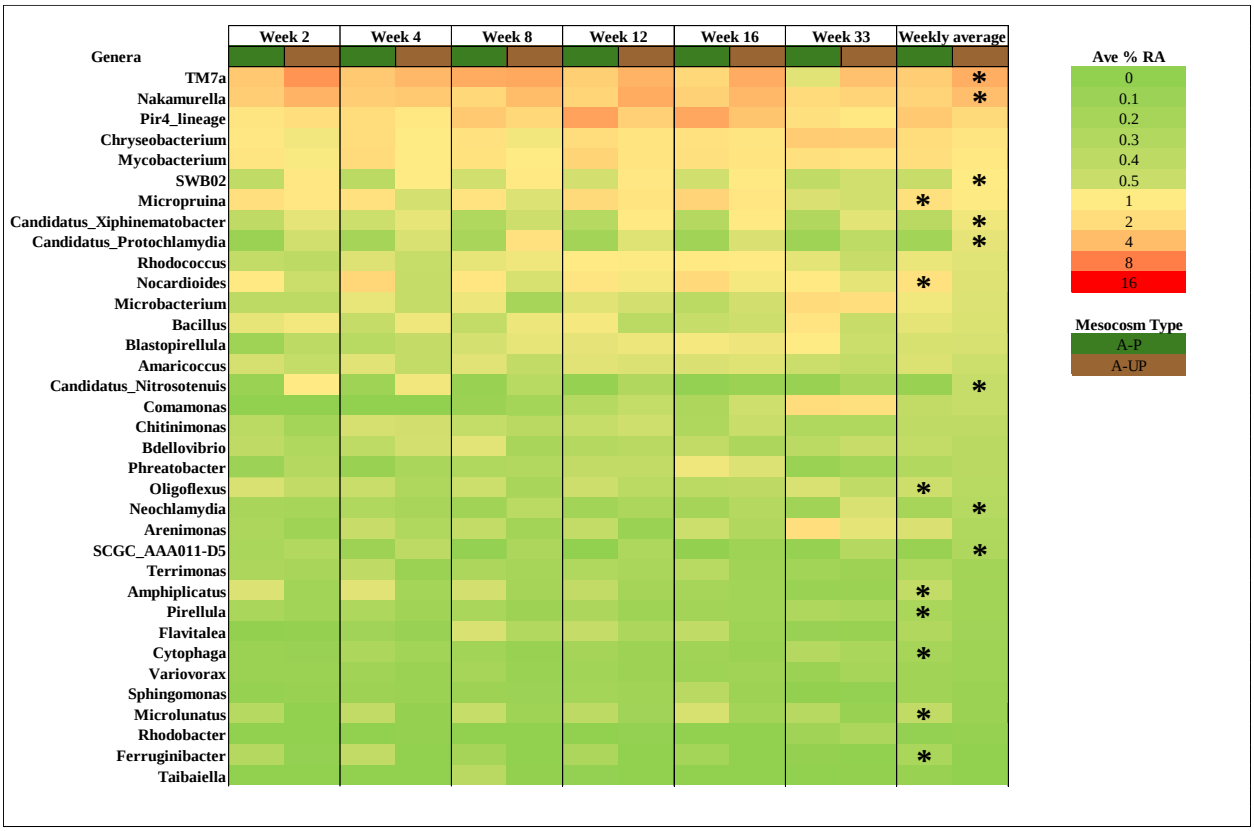

Figure 6-4: A heat map representing the Ave. %RA of 35 prokaryotic genera identified in porewater samples from aerated planted (M7 – 9, dark green) and unplanted (M10 – 12, brown) mesocosms that were sampled on weeks 2, 4, 8, 12, 16 and 33. A weekly average %RA for each prokaryotic genera

found in non-aerated planted and unplanted mesocosms is also represented in the last two columns of the map on the right. The list of genera shown in the heat map were sorted by the weekly average %RA for aerated unplanted mesocosms in descending order. The * indicates what treatment had a significantly greater weekly average %RA for a particular genus (Student t-test p<0.05).

In the heat map shown in Figure 6-5 of Ave. %RA for detached gravel biofilms from non-aerated mesocosms, we see that *Candidatus* Omnitrophus, *Methanoregula*, *Candidatus* Saccharimonas and *Desulfovibrio* on average had significantly greater %RAs in all gravel layers from non-aerated unplanted mesocosms than in gravels layers from non-aerated planted mesocosms ($p<0.05$ ANOVA post hoc Tukey test). The opposite was true for *Leptolinea*, which on average had a significantly greater %RA in gravel layers from non-aerated planted mesocosms than from non-aerated unplanted mesocosms ($p<0.05$ ANOVA post hoc Tukey test). The most common (genera found in >60% of the gravel layers) and dominant genera (have a layer Ave. %RA ≥ 1) found in gravel biofilms from both non-aerated planted and unplanted mesocosms were *Candidatus* Competibacter, *Thauera*, *Spirochaeta* and *Desulfomonile*. Genera 966-1 was common and dominant only to gravel biofilms from non-aerated planted mesocosms while *Neochlamydia*, *Candidatus* Omnitrophus, *Syntrophobacter*, *Candidatus* Saccharimonas and *Methanoregula* were common and dominant to gravel biofilms from non-aerated unplanted mesocosms.

It is clear that the prokaryotic genera detached from plant roots had a different Ave. %RA abundance pattern than biofilms detached from the gravel layers from either planted or unplanted non-aerated mesocosms. The genera found in plant roots clearly had significantly greater %RAs for *Amaricoccus*, *Nakamurella*, *Nocardioides*, *Nitrospira*, Pir4 lineage, *Mycobacterium*, *Thiobacillus*, *Candidatus* Xiphinematobacter, *Candidatus* Alysiosphaera, SWB02, Ellin6067, *Microlunatus*, MND1, SM1A02, *Pirellula*, *Luteolibacter*, *Nitrosomonas*, OLB12 and *Chryseolinea* than in the gravel layers for the non-aerated planted and unplanted mesocosms ($p<0.05$ ANOVA post hoc Tukey test). Many of the common and dominant genera found in the gravel biofilms of the non-aerated planted and unplanted mesocosms, were different from those found in root biofilms. They included *Candidatus* Competibacter, *Defluviicoccus*, *Amaricoccus*, *Nakamurella*, *Nocardioides*, *Sulfurovum*, *Nitrospira*, Pir4 lineage, *Mycobacterium*, *Candidatus* Xiphinematobacter, 966-1 and *Candidatus* Alysiosphaera.

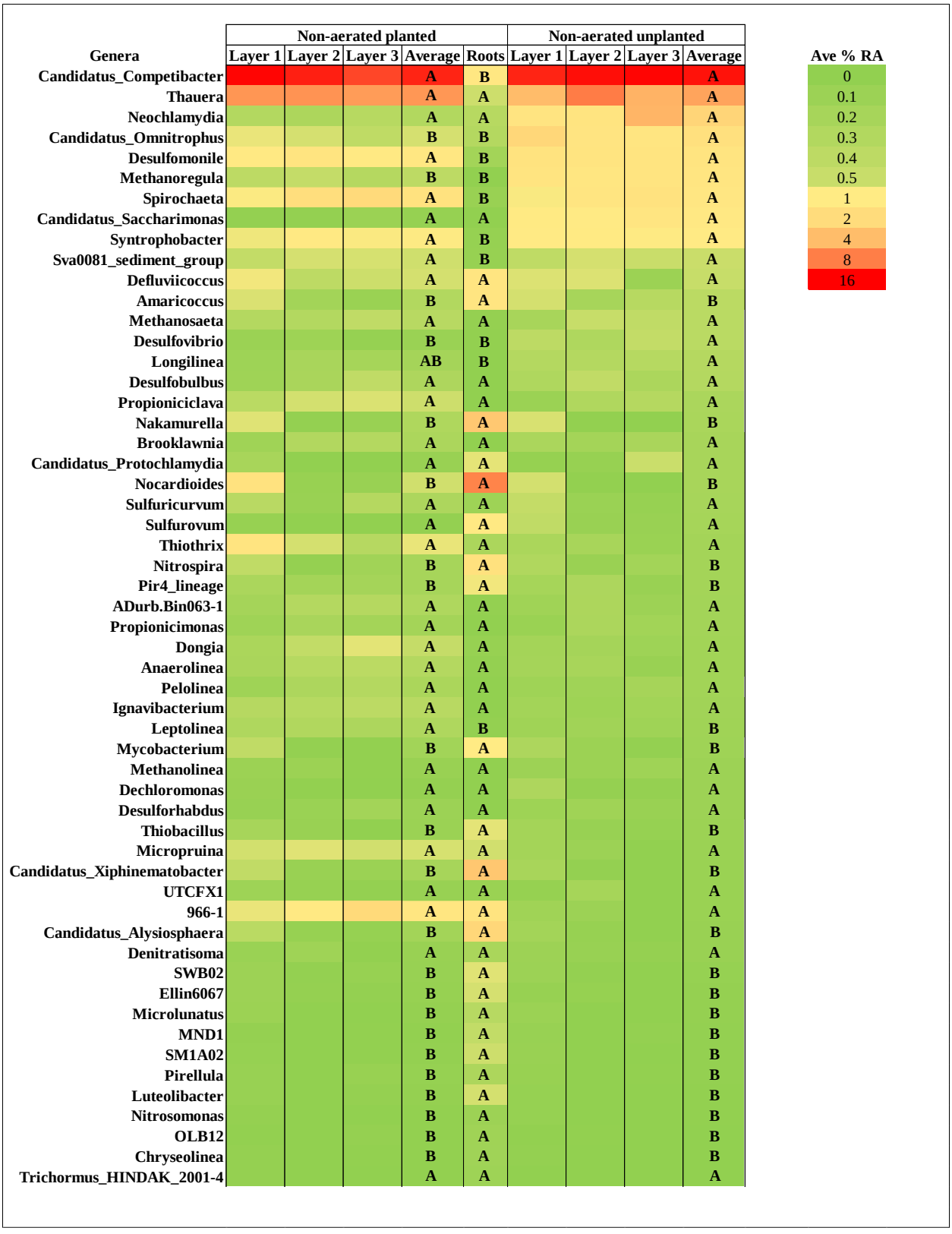

Figure 6-5: A heat map representing the Ave. %RA of 55 prokaryotic genera identified in detached gravel biofilms from three different depths (Layer 1, 0 – 20 cm, Layer 2, 20 – 50 cm and Layer 3, 50

– 80 cm) from non-aerated planted and unplanted mesocosms as well as plant roots (M1 – M3). The list of genera shown in the heat map were sorted by the %RA layer average for non-aerated unplanted mesocosms in descending order. Please note that the Ave. %RA were based on an n=2 for layer 3 NA-P and layer 1 NA-UP because the data from M3 and M5, respectively, were determined to be bad data. ANOVA post hoc Tukey tests for each genus was used to determine if there was a significant difference between Ave. %RAs for gravel and root biofilms from non-aerated planted and unplanted mesocosms. Cells in the heat map labelled with different letters represent significant differences in Ave. %RA between treatments for each genus.

In the heat map shown in Figure 6-6 of Ave. %RA for detached gravel biofilms from aerated mesocosms, we see that *Nocardioides* and *Microlunatus* were the only genera that on average had significantly greater %RAs in all gravel layers from aerated planted mesocosms than in aerated unplanted mesocosms ($p<0.05$ ANOVA post hoc Tukey test). The most common (genera found in >60% of the gravel layers) and dominant genera (have a layer Ave. %RA ≥ 1) found in gravel biofilms from both aerated planted and unplanted mesocosms were *Chryseobacterium*, *Nakamurella*, *Candidatus* Xiphinematobacter, *Nocardioides*, *Comamonas*, *Amaricoccus*, *Mycobacterium*, *Nitrospira*, *Pseudonocardia* and *Rhodococcus*. *Microbacterium*, *Micropruina*, *Stenotrophobacter*, *Microlunatus*, *Blastopirellula* and Pir4 lineage was more common and dominant in biofilms from aerated planted mesocosms.

Unlike the genera identified in root biofilms from non-aerated planted mesocosms, the Ave. %RA abundance pattern for genera identified in root biofilms from aerated planted mesocosms were not as different as the Ave. %RA abundance patterns for gravel layers from both aerated planted and unplanted mesocosms. Only *Comamonas* and *Microlunatus* from the root biofilms from aerated planted mesocosms showed a significantly greater Ave. %RA in comparison to the Ave. %RA for gravels in aerated unplanted mesocosms. Many of the common and dominant genera found in root biofilms from aerated planted mesocosms are mostly the same as those found in gravel biofilms from both aerated planted and unplanted mesocosms, as listed in the previous paragraph.

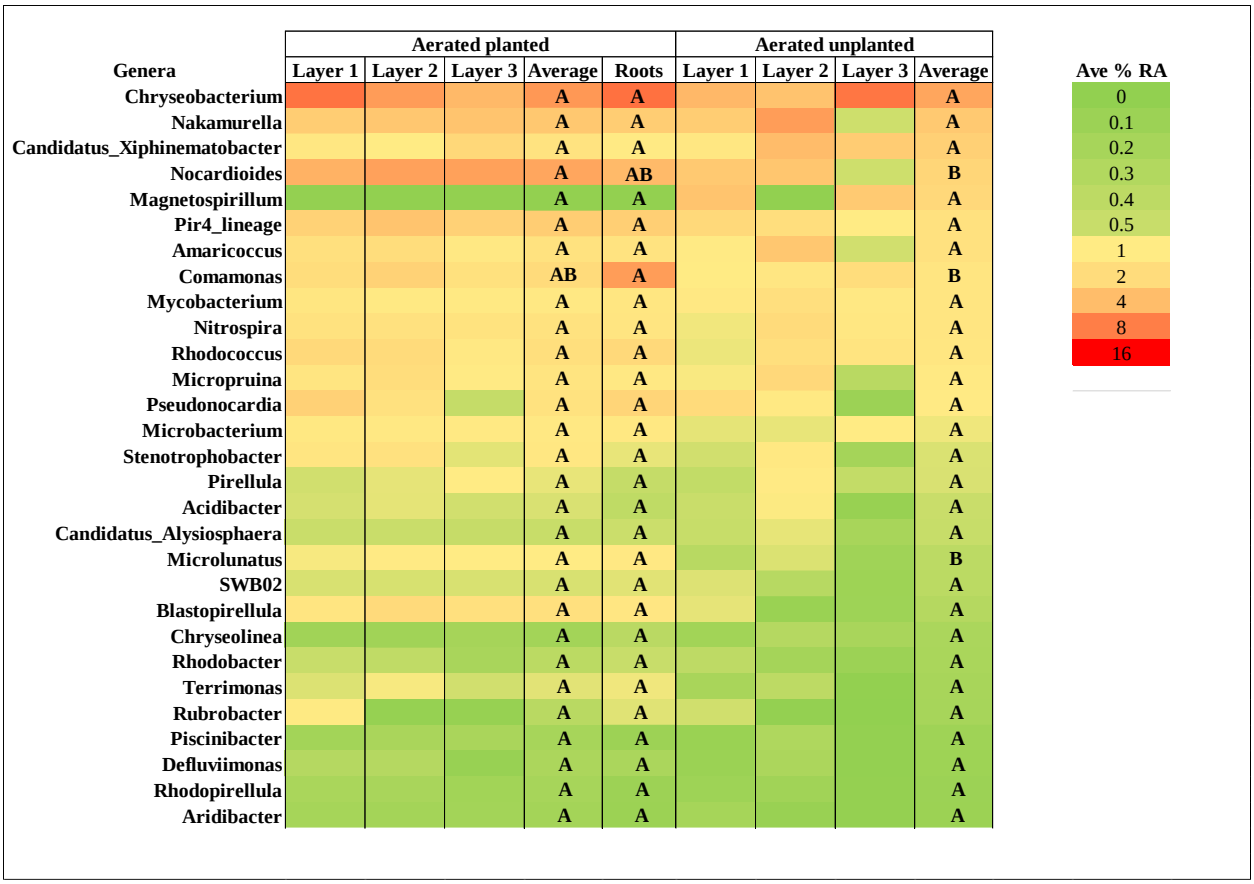

Figure 6-6: A heat map representing the Ave. %RA of 29 prokaryotic genera identified in detached gravel biofilms from three different depths (Layer 1, 0 – 20 cm, Layer 2, 20 – 50 cm and Layer 3, 50 – 80 cm) and plant roots from aerated planted and unplanted mesocosms. The list of genera shown in the heat map were sorted by the %RA layer average for aerated unplanted mesocosms in descending order. Please note that the Ave. %RA were based on an n=2 for layer 2 A-UP because the data from M10 was determined to be bad data due to sequencing errors. ANOVA post hoc Tukey tests for each genus was used to determine if there was a significant difference in Ave. %RAs between gravel and root biofilms from aerated planted and unplanted mesocosms. Cells in the heat map labelled with different letters represent significant differences in Ave. %RA between treatments for each genus.

6.3.4 Functional and metabolic characterization of prokaryotes in the mesocosms

In Figure 6-7 A, we can see that a large proportion of prokaryotes found in porewaters from non-aerated mesocosms for all weeks sampled are largely chemolithoautrophs or can switch between multiple metabolic modes, depending on mesocosm conditions. Prokaryotes found in porewaters from aerated mesocosms, on the other hand, are largely dominated by chemoorganotrophs. In contrast to the prokaryotes found in porewater samples from the two types of mesocosms, the prokaryotes found in detached biofilm samples from both non-aerated and aerated mesocosms were similar and largely dominated by chemoorganotrophs, as shown in Figure 6-7 B.

In Figure 6-7 C, we can see that a large proportion of prokaryotes found in porewaters from non-aerated mesocosms for all weeks sampled are facultative anaerobes as well as less well characterized uncultured genera for which no specific respiratory mode is known. The prokaryotes detected in aerated porewaters were largely dominated by obligate aerobes and facultative anaerobes. There was also a higher proportion of pathogenic or symbiotic prokaryotic genera detected in aerated porewaters in comparison to

non-aerated porewaters. For the detached genera detected in biofilms from the first gravel layer in non-aerated biofilms, shown in Figure 6-7 D, the first layer largely mirrors the pattern shown for non-aerated porewaters where there is a high proportion of genera that are facultative anaerobes and those with unknown respiratory modes as some of the genera identified are uncultured prokaryotes whose metabolisms are not well understood. For biofilms from layers two and three for non-aerated mesocosms, there is a high proportion of genera that are facultative anaerobes and obligate anaerobes while the roots showed a similar pattern to all the aerated detached biofilms from aerated mesocosms that had a very high proportion of obligate aerobes. Pathogenic or symbiotic genera existed in all detached biofilm samples from both non-aerated and aerated biofilms.

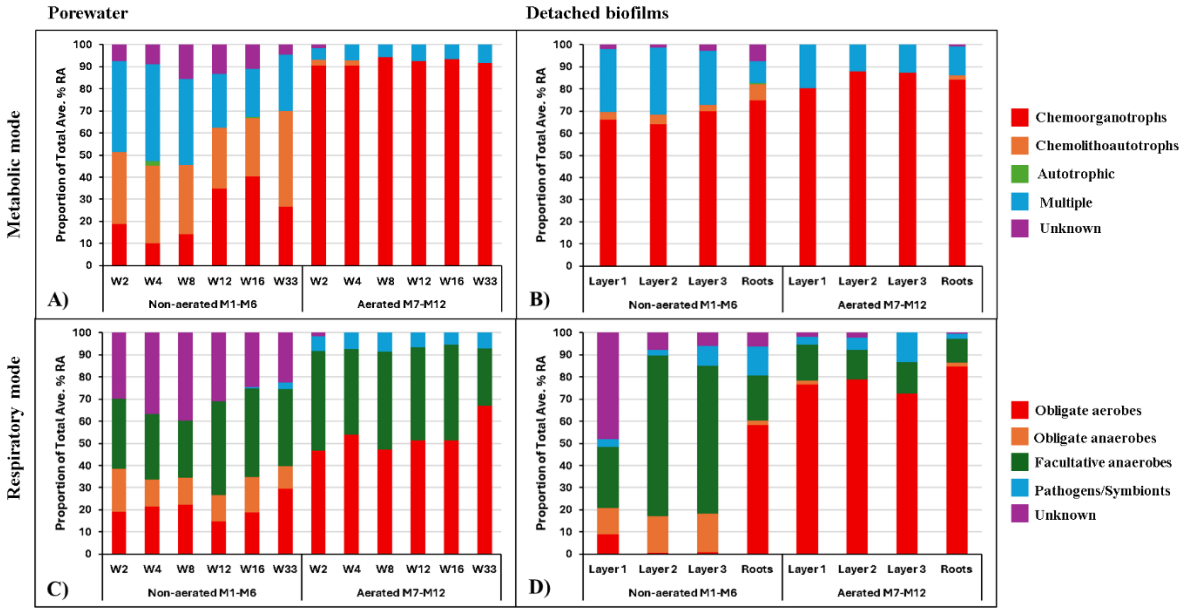

Figure 6-7: The metabolic mode and respiratory mode of common prokaryotes found in porewaters (A and C) for all weeks sampled and for detached biofilms (B and D) from all three layers and roots, shown as a proportion of the total Ave.% RA of all selected genera, for non-aerated and aerated mesocosms.

In Figure 6-8 A , we can see that the nitrogen metabolism and nitrogen cycling of prokaryotic genera from porewaters in non-aerated mesocosms was largely dominated by the reduction of nitrate and/or denitrification but there were also prokaryotic genera capable of switching to nitrogen fixation and/or DNRA under low nitrogen conditions (multiple) to allow for the generation of ammonium that may have allowed for sustained ammonium assimilation by the microbial community. In the aerated mesocosms, the genera in the porewaters were largely composed of ammonia assimilating bacteria and aerobic nitrifying bacteria.

In Figure 6-8 B, we can see that the prokaryotic genera identified in detached biofilms from all three gravel layers from the non-aerated mesocosms were able to transform nitrogen in multiple ways and make up the largest proportion of the total % RA. Most of this activity is oriented towards nitrate reduction and/or denitrification. The genera from biofilms detached from roots in non-aerated mesocosms was largely dominated by genera that assimilate ammonia or can switch to ammonification, ammonia oxidation, nitrite oxidation and nitrogen fixation (multiple). The nitrogen metabolism for genera found in all detached biofilms from gravel and roots from aerated mesocosms is largely the same as what was found for aerated porewaters, and is composed of aerobic nitrifying bacteria and ammonia assimilating bacteria.

In Figure 6-8 C, we can see that the sulfur metabolism and sulfur cycling of prokaryotic genera from porewater samples from non-aerated mesocosms is largely oriented towards sulfur oxidation activity while genera from aerated porewater samples were oriented towards sulfate assimilation. The genera found in non-aerated detached biofilms, shown in Figure 6-8 D, were able to oxidize sulfur, reduce sulfur or switch between sulfur reduction and sulfur disproportionation due to the highly reducing environment in these mesocosms (multiple). Prokaryotic genera found around roots in non-aerated mesocosms largely resembled the sulfur transformation pattern in biofilms detached from gravels and roots from aerated mesocosms which was dominated by sulfate assimilation. For genera identified in both non-aerated and aerated porewaters and biofilms that were identified as pathogenic or symbiotic genera, it was not possible to determine how they influenced sulfur cycling in the mesocosms and were classified as unknown.

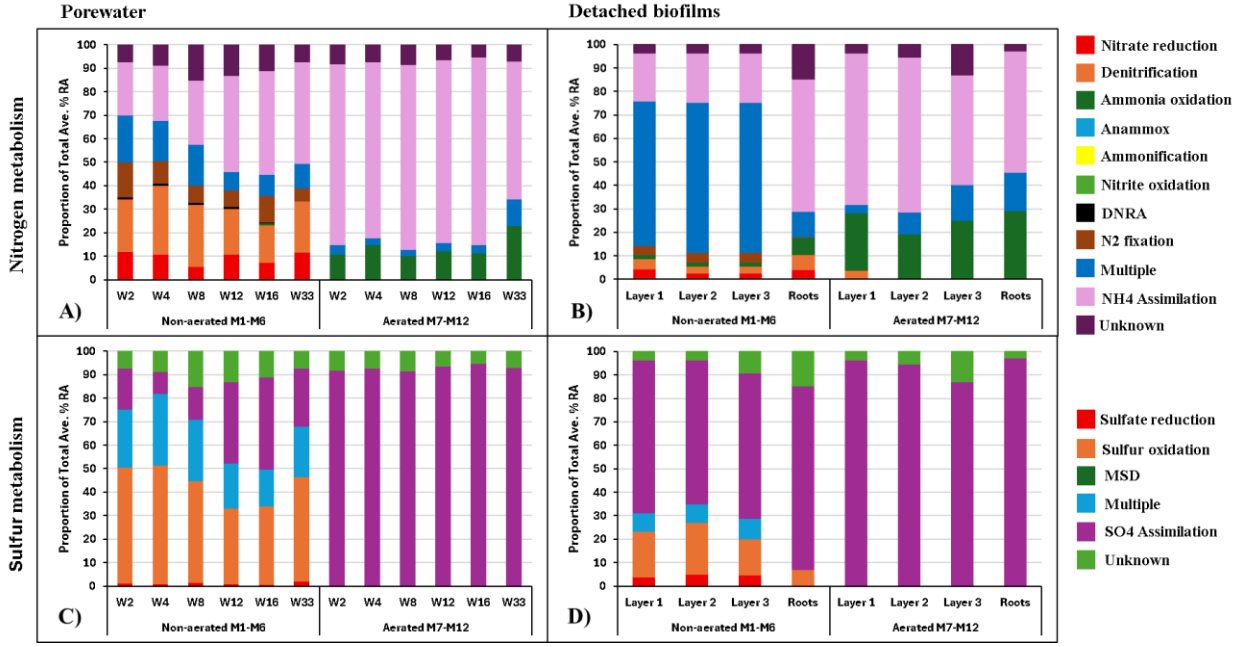

Figure 6-8: The nitrogen and sulfur metabolisms of common prokaryotic genera found in porewaters (A and C) and detached biofilms (B and D) shown as a proportion of the total Ave.% RA of all selected genera for non-aerated and aerated mesocosms.

6.4 Discussion

6.4.1 Microbial activity and alpha diversity

From Table 6-1, the reason we observed a significantly greater average OTU richness in gravel biofilms compared to porewater in all mesocosm types is likely because most of the prokaryotes in our mesocosms were able to form biofilms. Many prokaryotes form biofilms under adverse growth conditions, including exposure to heavy metals and metalloids (Damyanova & Paunova-Krasteva, 2025; Teitzel & Parsek, 2003). The higher concentration of prokaryotes in the biofilms compared to porewaters also likely made the recovery of more prokaryotic DNA from the biofilms easier than for porewaters that had fewer prokaryotes simply because they were more diluted.

Gravel and root biofilms in non-aerated planted mesocosms likely had a significantly greater average OTU richness and evenness than porewaters likely because the roots may have created a microenvironment that supported a wider variety of prokaryotes. It is possible that the roots of plants in non-aerated mesocosms were able to release both oxygen and carbon rich root exudates to support the growth of aerobic to aerotolerant prokaryotic species (Badri & Vivanco, 2009; Rehman et al., 2017). Indeed we see the same highly abundant genera such as *Amaricoccus*, *Nakamurella*, *Nocardioides*, *Nitrospira*, Pir4 lineage, *Mycobacterium*, *Candidatus* Xiphinematobacter and *Candidatus* Alysiosphaera in biofilms from the first gravel layer and roots from non-aerated planted mesocosms as we do in the gravel and root biofilms of aerated mesocosms, as shown in Figure 6-5 and Figure 6-6 respectively.

The fact that porewater from both aerated planted and unplanted mesocosms had a significantly greater average OTU diversity may be because the porewater environment within the aerated mesocosms was more stable than that found in non-aerated mesocosms. The formation of toxic compounds such as hydrogen sulfide by sulfate reducing bacteria as well as thiolated arsenic species may have acted as a selection force in non-aerated porewaters, killing any prokaryotes that were not resistant to these toxic compounds (D C Rubin et al., 2014; Herath et al., 2018). The weekly FDA microbial activity assays for the non-aerated and aerated mesocosms shown in Figure 6-1 A and B respectively provides some evidence to support these claims. Although the microbial activity of non-aerated mesocosms was higher than for aerated mesocosms, there was a consistently higher weekly variability in activity which gradually decreased over time. In contrast, the weekly microbial activity in aerated mesocosms had a much lower variability and remained relatively stable over time.

6.4.2 The ecophysiology of prokaryotes in non-aerated mesocosms

Based on the water quality data that was measured in Chapter 4, we successfully created highly reducing conditions in the non-aerated mesocosms which was characterised by low DO concentrations of less than 1 mg/L and low ORP measurements of less than -200 mV. In the porewater and biofilms from Figure 6-7 A and B respectively, this allowed for the growth of chemoorganotrophic, chemolithoautotrophic prokaryotes and/or prokaryotes that could switch between different metabolic modes depending on nutrient conditions. As shown in Figure 6-7 C and D respectively, there was a wider variety of prokaryotes capable of surviving under different respiratory conditions in the porewater compared to the attached biofilms perhaps because the porewater in the mesocosms was being constantly recirculated in the non-aerated mesocosms, and were exposed to more of an oxygen gradient than the prokaryotes found in biofilms that remained attached to gravel particles.

Under the reducing conditions in the non-aerated systems, where the initial concentration of $TOC:NO_3^-:SO_4^-$ was 1:0.2:0.05, it would have been energetically favourable for nitrate reducing processes (nitrate reduction/denitrification) to occur first, followed by fermentation and sulfate reduction (Dolfing & Hubert, 2017; Egami, 1974; Laverman et al., 2012). Most of the carbon and nitrogen in the nutrient solution was removed within the first 24 hrs of nutrient addition, as shown in Figure 6-2 A and C respectively. The molasses that was added to fresh nutrient solution at a concentration of 1 g/L at the start of each week likely contained high quantities of sucrose, glucose and fructose and were the main available carbon sources used for nitrate reducing/denitrifying microbes (Jamir et al., 2021; Palmonari et al., 2020; Pang & Wang, 2021; Teclu et al., 2009). Many studies have shown that addition of molasses can enhance the removal of nitrate

and carbon from various treatment systems through heterotrophic denitrification (Horová et al., 2020; B. S. Lee et al., 2014; Paul et al., 1989; Quan et al., 2005; Smyk & Ignatowicz, 2017; Tepuš et al., 2014; Ueda et al., 2006).

In genera found to be common and dominant in non-aerated porewater, shown in Figure 6-3, denitrification was likely driven by *Magnetospirillum* (Bazylinski & Blakemore, 1983; Escalante-Semerena et al., 1980; J. Hu et al., 2022; Yamazaki et al., 1995). Other less abundant (%RA <1) and/or less common genera in porewater that were involved in denitrifying activities included *Candidatus* Competibacter (Brand et al., 2019; Y. Kong et al., 2006; McIlroy et al., 2014, 2015, 2015; Rubio-Rincón et al., 2017), and *Azonexus* (formerly *Dechloromonas*) (Kondrotaite et al., 2025; Quan et al., 2006). Figure 6-5 shows genera that were identified in biofilms that had high abundance and commonly found in most biofilm samples from non-aerated mesocosms. In these biofilms, denitrifying activity was likely driven by *Candidatus* Competibacter (Brand et al., 2019; Y. Kong et al., 2006; McIlroy et al., 2014, 2015, 2015; Rubio-Rincón et al., 2017) and *Thaurea* (Liu et al., 2013a; Liu et al., 2013b). Other less abundant (%RA <1) genera that were found in biofilms and distributed in different layers that had the potential for nitrate reduction/denitrifying activity included *Defluviicoccus* (X. Wang et al., 2008), *Micropruina* (Shintani et al., 2000), *Azonexus* (Kondrotaite et al., 2025; Quan et al., 2006), *Pir4 lineage* (Dedysh et al., 2020), *Denitratisoma* (Fahrbach et al., 2006; Xia et al., 2019) and *Nitrosomonas* (Bock et al., 1995; Shrestha et al., 2002).

The denitrification activities of biofilms in the deeper gravel layers were likely supported by less abundant (%RA <1) but common genera associated with plant roots and/or the first gravel layer where oxygen was more available. These genera mainly consisted of AOBs that fix carbon dioxide and oxidize ammonia to nitrite. The AOBs found in these biofilm samples include *Ellin6067* (Z. Jiang et al., 2024), *MND1* (Tang et al., 2023) and *Nitrosomonas* (Bock et al., 1995; Shrestha et al., 2002). The highly versatile CO_2 fixing *Nitrospira* genera, found in the first gravel layer and associated with plant roots, are potentially capable of nitrite oxidation, complete ammonia oxidation to nitrate (Comammox) and even ammonification of urea (Bayer et al., 2021; Daims et al., 2015; H. Koch et al., 2015; Lawson et al., 2021; Lücker et al., 2010). The remaining genera found in the first gravel layer and/or roots may have contributed to carbon removal through aerobic respiration and included the genera *Candidatus* Alysiosphaera (Kragelund et al., 2006; McIlroy et al., 2011), *Luteolibacter* (F. Jiang et al., 2012; M. Kim et al., 2015), *Microlunatus* (Kämpfer et al., 2010), *Pirellula* (Clum et al., 2009), *Chryseolinea* (Kim et al., 2013), *OLB12* (Begmatov et al., 2022), *Nocardioides* (Govindasamy et al., 2014; Y. Ma et al., 2023), *Nakamurella* (Paudel et al., 2022; Tice et al., 2010), *Amaricoccus* (Maszenan et al., 1997) and *Mycobacterium* (Gomila et al., 2008).

Once the nitrate was depleted, microbial genera capable of fermentation or switching to fermentation may have increased their activities. Common and dominant genera capable of fermentation in the non-aerated porewater included *Candidatus* Omnitrophus (Perez-Molphe-Montoya et al., 2022; Seymour et al., 2023; Williams et al., 2021), *Endomicrobium* (Méheust et al., 2020; Zheng et al., 2016, 2017) along with less common and less dominant genera that included *Candidatus* Saccharimonas (Albertsen et al., 2013; Kindaichi et al., 2016), Blvii28 wastewater-sludge group (X.-L. Su et al., 2014), *Candidatus* Competibacter (McIlroy et al., 2014) and *Dechloromonas* (Horn et al., 2005). Fermenters that were common and dominant in the non-aerated biofilm gravel layers included *Candidatus* Competibacter (McIlroy et al., 2014), *Spirochaeta* (Breznak & Canale-Parola, 1972, 1975; Hespell & Canale-Parola, 1970) and *Candidatus* Omnitrophus (Perez-Molphe-Montoya et al., 2022; Seymour et al., 2023; Williams et al., 2021). Other less common and less common fermenting genera in the biofilms included *Micropruina* (McIlroy et al., 2018), *Anaerolinea* (McIlroy et al., 2017), *Brooklawnia* (Akita et al., 2024), *Leptolinea* (Ward et al., 2015), *Pelolinea* (Imachi et al., 2014) and *Propionicimonas* (Akasaka et al., 2003). The acidogenic fermentation of molasses likely produced volatile fatty acids (acetic, citric, butyric, succinic, lactic, and propionic acid), alcohols, carbon dioxide and hydrogen gas (Chaves et al., 2021; Palmonari et al., 2020; Ramos-Suarez et al., 2021; S. Zhang et al., 2021). Some of the genera identified in porewater and/or biofilms likely utilized the energy derived from fermentation to drive nitrogen fixation to provide assimilable ammonia to the microbial community. Some common and dominant nitrogen fixers that came from non-aerated porewaters included *Endomicrobium* (Méheust et al., 2020) and *Magnetospirillum* (Bazylinski et al., 2000) while

Candidatus Competibacter was the most common and domimant nitrogen fixer in non-aerated biofilms (McIlroy et al., 2014).

The production of carbon dioxide and VFAs likely contributed to a lower pH in porewater from non-aerated mesocosms. The high TIC concentrations shown in Figure 6-2 B provides evidence for high carbon dioxide concentrations in non-aerated porewaters. The production of VFAs by fermenters was likely essential for the growth and activity of gravel associated SRB genera that utilized VFAs as a carbon source to reduce sulfate to hydrogen sulfide, producing a smell of rotten eggs. These genera included common and dominant SRBs such as *Desulfomonile* (DeWeerd et al., 1990) and *Syntrophobacter* (Plugge et al., 2012) as well as lower abundance genera that included *Desulfovibrio* (Vita et al., 2015), *Desulfobulbus* (El Houari et al., 2017) and *Desulforhabdus* (Oude Elferink et al., 1995). All of these SRB genera are incomplete carbon oxidizers that likely produced acetate and carbon dioxide from VFAs while using sulfate as a final electron acceptor (Ahmed et al., 2021; Alam & McPhedran, 2019; Westerholm et al., 2022). The metabolic activity of these SRB likely allowed for syntrophic relationships with archaeal methanogenic genera, that included *Methanoregula*, *Methanolinea* and *Methanothrix* (formerly *Methanosaeta*).

After 24 hrs, the prokaryotes in non-aerated mesocosms had to be adapted to surviving in a sulfidic, low C and N environment for the remaining 6 days of treatment. The low carbon environment selected for prokaryotic genera that were able to generate electron donors from sources other than organic molecules. As shown in Figure 6-8 C and D, porewaters and biofilms from non-aerated mesocosms had a high number of sulfur oxidizing bacteria (SOB) or bacterial genera capable of switching to sulfur oxidation. SOB are chemolithoautotrophic and derive their energy from the oxidation of sulfide, sulfur and thiosulfate to sulfate while fixing carbon dioxide (Klatt & Polerecky, 2015). The common and dominant SOB genera found in porewaters included *Thiovirga* (Ito et al., 2005), *Candidatus* Omnitrophus (Perez-Molphe-Montoya et al., 2022; Williams et al., 2021), *Sulfuricurvum* (Kodama & Watanabe, 2004), *Magnetospirillum* (Geelhoed et al., 2010; Koziaeva et al., 2023) while in gravel biofilms they included *Thauera* (Alsanea et al., 2023; Y. Zhang et al., 2017), *Candidatus* Omnitrophus (Perez-Molphe-Montoya et al., 2022; Williams et al., 2021), and root associated *Sulfurovum* (Alamoudi et al., 2025). Oxygen is typically used as a final electron acceptor under aerobic conditions while nitrate, Fe(III) and even sulfur (S^0) are used under anoxic/anaerobic conditions (Can-Dogan et al., 2010; Malik & Hedrich, 2022; L. Zhang et al., 2021). The SOB found under the sulfidic low C and N environment may have provided energy for the generation of ammonia through DNRA activity, which would help maintain ammonia concentrations for ammonia assimilation by the prokaryotes in the non-aerated mesocosms (Murphy et al., 2020; C. B. Pandey et al., 2020; van den Berg et al., 2016). SOB capable of DNRA activity included *Thauera* (Tec-Campos et al., 2025; Zhou et al., 2022). Although there were prokaryotes that included *Endomicrobium*, *Magnetospirillum* and *Candidatus* Competibacter that were capable of N_2 fixation, the presence of sulfide likely inhibited this activity (Tat-Yee et al., 1982).

There are a number of other ways that bacteria adapt to low nutrient (oligotrophic) conditions which could not be represented by the data in this study. Many organisms, including bacteria, are able take up carbon sources and converting them into readily available storage products that act as a source of energy under adverse conditions. Many bacteria are able to store glycogen, polyphosphates and polyhydroxyalkanoate (PHAs) or a combination thereof under such conditions (Ruiz-Haddad et al., 2024; Strange, 1968; B. Zhao et al., 2023). The most common and dominant genera capable of storing carbon in these forms from non-aerated porewaters and/or biofilms included *Magnetospirillum* (Su et al., 2023), *Candidatus* Competibacter (McIlroy et al., 2014) and *Thauera* (Ren et al., 2021). Other potential physiological adaptations for survival under oligotrophic conditions used by prokaryotes include slower growth, dormancy, substrate switching as well as other stress tolerance mechanisms (Hirsch, 1986; Pechter et al., 2017; Yin et al., 2024; Zhu & Dai, 2024).

6.4.3 The ecophysiology of prokaryotes in aerated mesocosms

Based on the water quality data that was measured in Chapter 4, we successfully created highly oxidizing conditions in the aerated mesocosms which was characterised by high DO concentrations of greater than 7 mg/L and ORP measurements of greater than 50 mV. In the aerated mesocosms,

chemoorganotrophic prokaryotes dominated in the porewater and biofilms, as shown in Figure 6-7 A and B. Porewaters had a combination of aerobic and facultatively anaerobic prokaryotic genera, while biofilms mainly consisted of aerobic prokaryotes, as shown in Figure 6-7 C and D, respectively.

The overall metabolic activity of the prokaryotes found in aerated mesocosms, in both porewaters and biofilms, can be described as being anabolic and assimilative in nature for nitrogen and sulfur, as shown in Figure 6-8 A – D. The reduction in NH_4^+-N, as shown in Figure 6-8 C is likely attributed to the assimilation of ammonia by the prokaryotes in the aerated mesocosms which likely occurred concurrently with TOC removal within the first 24 hrs, as shown in Figure 6-2 A. Common and dominant bacterial genera found in aerated porewaters included *TM7a, Nakamurella, Pir4 lineage, Chryseobacterium, Mycobacterium, Micropruina and Nocardioides*. All the genera found in aerated porewaters, except for *TM7a*, were also found in detached gravel biofilms, along with genera that included *Comamonas*, *Candidatus* Xiphinematobacter, *Rhodococcus*, *Americoccus*, *Nitrospira*, *Pseudonocardia*, *Micropruina*, *Stenotrophobacter*, *Microlunatus*, *Blastopirellula* and *Microbacterium*. The low TOC to nitrogen ratio found in our nutrient solution also likely could not support the removal of nitrogen through heterotrophic nitrification and aerobic denitrification (HN-AD) by highly abundant and common genera that included *Comamonas, Pseudomonas*, *Rhodococcus*, *Chryseobacterium*, *Bacillus*, *Microbacterium* and *Zoogloea* (Bucci et al., 2021; Chen et al., 2024; Huo et al., 2025; Motamedi & Jafari, 2020; Ning et al., 2025; Qiao et al., 2020; D. Zhang et al., 2016). Sulfate was also likely assimilated by prokaryotes in the aerated mesocosms, as shown in Figure 6-8 C and D and no other transformations of sulfate was expected to occur in these mesocosms. Similar to genera found in non-aerated mesocosms, the genera found in aerated mesocosms may have utilized energy storage molecules as well as other physiological strategies to survive oligotrophic conditions after 24 hrs. The most common and dominant genera capable of energy molecule storage included *Micropruina* (McIlroy et al., 2018), *Comamonas* (Zakaria et al., 2013), *Rhodococcus* (Hernández et al., 2008) and *Amaricoccus* (Clagnan & Adani, 2023).

6.4.4 Arsenic resistance in prokaryotes from non-aerated and aerated mesocosms

Arsenic resistance in bacteria is as ubiquitous as the very existence of arsenic in the environment. Broadly speaking, the most common arsenic resistance mechanisms in prokaryotes involves the enzymatic reduction of As(V) to As(III) (Keren et al., 2022). As(III) is either excreted or methylated and then excreted to the environment (Keren et al., 2022). If prokaryotes are exposed to methylated arsenic species, enzymatic demethylation to As(III) or As(V) may occur (Keren et al., 2022). Some of the most arsenic resistant prokaryotes may also have other physiological and physical mechanisms that may confer arsenic resistance, such as a constitutively expressed and highly effective oxidative stress response or formation of a polysaccharide coat and/or formation of a biofilm which may help adsorb arsenic on the outside of their cells (Andres & Bertin, 2016).

Unsurprisingly, many of the prokaryotes found in samples from porewaters and biofilms in non-aerated and aerated mesocosms have also been found in arsenic contaminated environments. Common and dominant arsenic resistant prokaryotic genera found in porewater from non-aerated mesocosms include *Thiovirga* (Ke et al., 2022a; X. Sun et al., 2024) and *Sulfuricurvum* (Cavalca et al., 2019; Guan et al., 2017; P. Li et al., 2015). Common and dominant arsenic resistant prokaryotic genera found in biofilms from non-aerated mesocosms included *Thauera* (Cai et al., 2009; Zhang et al., 2017), *Spirochaeta* (Shivani et al., 2015), *Sulfurovum* (Ding et al., 2024; S. Kong et al., 2025; Luo, Zhong, et al., 2025) and *Nocardioides* (Bagade et al., 2016; Naseem et al., 2024). Common and dominant arsenic resistant prokaryotic genera found in porewater from aerated mesocosms include *Chryseobacterium* (Akoijam & Joshi, 2023; Bandopadhyay et al., 2017; Sultana et al., 2017), *Mycobacterium* (Lehr et al., 2003) and *Nocardioides* (Bagade et al., 2016). Common and dominant arsenic resistant prokaryotic genera found in biofilms from aerated mesocosms included *Chryseobacterium* (Akoijam & Joshi, 2023; Bandopadhyay et al., 2017; Sultana et al., 2017), *Mycobacterium* (Lehr et al., 2003), *Nocardioides* (Bagade et al., 2016), *Comamonas* (J.-S. Chang et al., 2010; Sultana et al., 2011), *Rhodococcus* (J.-S. Chang et al., 2010; Retamal-Morales et al., 2021; Sultana et al., 2011), *Nitrospira* (Ke et al., 2022b), *Blastopirellula* (Y. Yang et al., 2024), *Microbacterium* (Achour-Rokbani et al., 2010; Fekih et al., 2018; Mohd Bahari et al., 2017; Sher et al.,

2022), *Pseudomonas* (He et al., 2023; Miranda-Carrazco et al., 2018; Wevar Oller et al., 2025) and *Pseudonocardia* (Bose et al., 2022; L.-Z. Zhang et al., 2024).

6.4.5 Potential arsenic removal mechanisms from non-aerated and aerated mesocosms

Based on the results in Chapter 4, non-aerated mesocosms removed more arsenic than aerated mesocosms. There may be two main mechanisms for removal of arsenic in non-aerated mesocosms. The first is the potential formation of arsenic sulfide precipitate mixtures composed of orpiment (As_2S_3) and realgar (As_4S_4) (Alam & McPhedran, 2019; Jackson et al., 2013; Rodriguez-Freire et al., 2016). The other potential removal mechanism for arsenic in the non-aerated mesocosms is the formation of iron(II) sulfide (pyrite) and the subsequent adsorption of arsenate to that pyrite. We believe the former removal mechanism was the primary arsenic removal mechanism because high concentrations of pyrite were shown in detached gravel biofilms, as shown in Chapter 4. Both biogenic and laboratory synthesized pyrite have been shown to effectively remove As(V) and As(III) from water (Bostick & Fendorf, 2003; Bulut et al., 2014; Chowdhury, 2017; Fischer et al., 2021; E. J. Kim & Batchelor, 2009; M.-K. Lee et al., 2018; Saunders et al., 2018; X. Zhang et al., 2022).

The removal mechanisms in aerated mesocosms could not be attributed to any *de novo* mineral phase produced by the prokaryotes found in detached biofilms from these mesocosms and can largely be attributed to As(V) adsorption to the gravel substrates and some adsorption to organic components of biofilms, such as the extracellular polymeric substances (Flemming, 1995; Tournay et al., 2023).

6.5 Conclusions

Although the microbial communities in non-aerated and aerated mesocosms remained largely active, stable and equally diverse in composition, the functional roles of the most abundant and common genera in each type of mesocosm influenced the carbon, nitrogen and sulfur cycling which may have also indirectly influenced arsenic removal in the mesocosms. In the non-aerated mesocosms, the microbial community was incredibly versatile and was capable of performing denitrification, fermentation and sulfate reduction within the first 24 hrs of fresh nutrients being added, removing most of the carbon and nitrogen from the solution. The low nutrient and sulfidic environment that resulted for the remaining six days of treatment allowed for sulfur oxidizing bacteria to dominate. The sulfide produced by sulfate reducing bacteria in combination with the release of iron from ankerite as the result of the byproducts of fermentation may have resulted in the formation of pyrite which may have removed As(V) from the water through adsorption. On the other hand, the aerated mesocosms removed less arsenic than the non-aerated mesocosms and removal was likely attributed to adsorption of As(V) onto the gravel substrates and onto extracellular polymeric substances in the biofilms. By understanding the ecophysiology of the microbial communities found in CWs removing arsenic under different redox conditions, we may be better able to predict the fate of the arsenic as well as how effective the wetlands will be at removing arsenic.

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Author Contribution

Antoine Hnain: Conceptualization, methodology, investigation, formal analysis, writing - original draft; **Sarah Wallace:** Methodology, investigation, formal analysis, data curation, writing – review & editing; **Iris Koch:** Supervision, funding acquisition; **Kela Weber:** Resources, conceptualization, writing – review & editing, supervision, funding acquisition.

Chapter 7: Principal Outcomes and Recommendations

7.1 Objective 1: To test the ability of three horsetail plant species (*E. fluviatile*, *E. hyemale* and *E. scirpoides*) to grow in and remove arsenate from a hydroponic solution and select the species with the highest removal capabilities for use in laboratory scale CWs treating arsenic.

Based on the hydroponic uptake of arsenic in three horsetail species it was discovered that *E. hyemale* was able to remove the most arsenic in their tissues at both the 1 and 10 mg/L As(V) dose. All *Equisetum* sp. removed more arsenic in their roots than in their shoots but it was clear that when looking at the speciation of arsenic in *E. fluviatile* and *E. hyemale* in the first hydroponic experiment, that *E. fluviatile* restricted the movement of arsenic to the shoots by reducing As(V) to As(III) which formed As(III)-S complexes that were likely stored in root vacuoles. Meanwhile, *E. hyemale* produced little to no As(III) or As(III)-S complexes, which allowed for more translocation of arsenic to the shoots. *E. hyemale* was therefore selected for planting in non-aerated and aerated mesocosms and compared concurrently to a set of unplanted mesocosms treating arsenic from water.

7.2 Objective 2: Directly compare the ability of planted and unplanted, non-aerated (reducing) and actively aerated (oxidizing) mesocosms to remove arsenic from water under controlled laboratory conditions and attempt to elucidate the mechanisms of arsenic removal by incorporating the amount of arsenic found in biofilms into mass balance calculations and examining the mineral composition of biofilms.

Reducing and oxidizing environments in the non-aerated and aerated mesocosms were created respectively. The porewater in non-aerated mesocosms was characterized by having low dissolved oxygen concentrations and highly negative redox potentials with high denitrifying and sulfate reducing activity while the aerated mesocosms were characterized by having high dissolved oxygen concentrations, a positive redox potential and very little nitrogen removal. The porewater in non-aerated mesocosms was slightly more acidic than in aerated mesocosms likely due to the production of carbon dioxide and reduced organic acids. Over the 33-week As(V) exposure period, non-aerated mesocosms consistently removed more arsenic than aerated mesocosms by the end of each week (day 6) based on sampling of mesocosm water for weeks 1, 6, 14, 16, 25 and 33. Through mass balance calculations we revealed that detached gravel biofilms from the non-aerated mesocosms contained a higher amount of arsenic than aerated gravel biofilms. The presence of plants in both types of mesocosms did not have any measurable influence on the amount of arsenic removed when compared to unplanted mesocosms. Despite plants in non-aerated mesocosms removing more arsenic than plants in aerated mesocosms, the mass removal of arsenic in both types of mesocosms was negligible ($\leq 0.4\%$). The mineral composition of the gravel biofilms was examined and revealed that non-aerated biofilms contained a high amount of pyrite which may directly adsorb arsenic or As(III) may have co-precipitated with Fe(III) on the surface of the pyrite through oxidizing reactions. The pyrite in non-aerated mesocosms was likely formed through the dissolution of iron (II) from ankerite in the gravel substrate due to the more acidic conditions in these mesocosms and reaction with sulfide produced by sulfate reducing bacteria. Most of the arsenic found in aerated biofilms was likely due to adsorption onto extracellular polymeric substances (EPS) found on the surface of bacteria in the biofilms because no arsenic adsorbing minerals or arsenic precipitates were found in biofilms from aerated mesocosms.

7.3 Objective 3: Examine the speciation of arsenic in the porewater as well as the speciation of arsenic in flash frozen plants and biofilms

The concentration of arsenic was measured in porewater on weeks 1, 6 and 14 at three different time points that included 20 min, 1 day and 6 days after treatment and we discovered that treatment efficiency degraded over time, despite non-aerated mesocosms removing more arsenic than aerated mesocosms by day 6. The speciation of arsenic in porewater by day 6 for weeks 1, 6 and 14 revealed that the porewater

for non-aerated mesocosms showed large proportions of As(V) conversion to DMA(V) and smaller quantities of MMA(V) for weeks 1 and 6 and no conversion of As(V) or demethylation of methylated arsenic back to As(V) by microbes for week 14. Almost all of the arsenic remained as As(V) in the aerated mesocosms for all weeks sampled.

The speciation of arsenic in biofilms showed that As(V) was reduced to As(III) and As(III)-S complexes in both non-aerated planted and unplanted mesocosms but no transformation of As(V) to other arsenic species in aerated planted and unplanted mesocosms. The As(III)-S species formed in the non-aerated biofilms is proposed to be small quantities of orpiment that were formed through reaction of sulfide and As(III) produced by bacteria. The reason we believe that orpiment contributed very little to arsenic removal is primarily due to the fact that orpiment formation is favoured under carbon rich, acidic conditions and the fact that orpiment was not detected in high quantities using XRD analysis of the biofilms.

With respect to arsenic speciation in the plants from our mesocosms, most of the arsenic in *E. hyemale* and *M. polymorpha* was found as As(V) and only a few tissue samples from *E. hyemale* showed the presence of the two reduced arsenic species As(III) and $As(GS)_3$ and, less commonly, DMA(V). There was no discernable pattern of speciation based on treatment type (aerated vs. non-aerated) or tissue type, despite the fact that *E. hyemale* plants removed more arsenic in non-aerated mesocosms than in aerated mesocosms. This may have been due to the confluence of inconsistent plant growth and low arsenic concentrations that did not induce arsenic transformations in the plants.

7.4 Objective 4: Determine the microbial activity of and identity of the most common and dominant prokaryotes found in porewaters and gravel attached biofilms found in our mesocosms and attempt to infer their functional and ecophysiological roles with respect to carbon, nitrogen and sulfur cycling and how that may influence arsenic removal.

The microbial activity of non-aerated mesocosms was greater than for aerated mesocosms when using the fluoresceine diacetate test to measure whole mesocosms microbial activity. The presence of plants did not appear to influence the microbial activity in either type of mesocosm. Using 16s rRNA sequencing of bacteria found in porewaters and detached gravel/root biofilms revealed relatively stable richness, diversity and evenness measures over time and with gravel depth. The presence of plants did not have an influence on these measures.

The bacteria from non-aerated porewaters were largely composed of bacteria that were obligate anaerobes or facultative anaerobes that were capable of chemoorganotrophic/ chemolithoautrophic growth or were able to switch between metabolic modes depending on porewater nutrient conditions. The porewater bacteria such as the highly abundant and common genera *Magnetospirillum* largely contributed to nitrogen and carbon removal through nitrate reduction or denitrification as well as fix nitrogen or were able two switch between these modes depending on porewater nutrient conditions. The highly abundant and common bacterial genera *Candidatus* Omnitrophus, *Endomicrobium* sp. and *Candidatus* Saccharimonas were likely largely responsible for fermentation. With respect to sulfur metabolism, common and abundant bacterial genera that included *Thiovirga*, *Candidatus* Omnitrophus, *Sulfuricurvum*, *Magnetospirillum* found in non-aerated porewaters were largely involved in sulfide oxidation. Biofilms detached from gravel/roots from the non-aerated mesocosms were largely composed of bacteria that were obligate and facultative anaerobes with some aerobes appearing only in the first layer and in biofilms around plant roots. Common and abundant bacterial genera from gravel and roots in the non-aerated mesocosms allowed for nitrate reduction/denitrification (*Candidatus* Competibacter, *Thaurea*), fermentation (*Candidatus* Competibacter, *Spirochaeta*, *Candidatus* Omnitrophus), sulfate reduction (*Desulfomonile*, *Syntrophobacter*) and sulfur oxidation (*Thauera*, *Candidatus* Omnitrophus and root associated *Sulfurovum*).

The bacteria from aerated porewaters were largely composed of bacteria that were obligate chemoorganotrophic aerobes and facultative anaerobes largely consuming carbon using aerobic metabolic pathways and assimilating small amounts of ammonia and sulfate. This resulted in almost no nitrogen removal from porewater in aerated mesocosms. Common and abundant bacterial genera found in aerated

porewaters included TM7a, *Nakamurella*, Pir4 lineage, *Chryseobacterium*, *Mycobacterium*, *Micropruina* and *Nocardioides*. All the genera found in aerated porewaters, except for TM7a, were also found in detached gravel biofilms, along with genera that included *Comamonas*, *Candidatus* Xiphinematobacter, *Rhodococcus*, *Americoccus*, *Nitrospira*, *Micropruina*, *Blastopirellula* and *Microbacterium*.

7.5 Scientific Contribution

The thesis advances our knowledge and understanding of arsenic removal in constructed wetlands by first directly comparing arsenic removal in non-aerated and aerated mesocosms as well as examining the potential influence of plants. We discovered that planting had little influence on arsenic removal and that the conditions in the mesocosms and the types of bacteria that grew under these conditions had more of an influence on the removal of arsenic. For the first time in the literature, we considered the mass removal of arsenic by gravel attached biofilms and discovered that non-aerated biofilms contained more arsenic than aerated biofilms. We believe that the biotic byproducts of fermentation which likely led to the release of iron from ankerite in the substrate reacted with sulfide due to the activity of sulfate reducing bacteria, which mostly formed pyrite which may have removed arsenic through adsorption or co-precipitation with iron oxides formed on the surface of the pyrite particles. We believe that the feast to famine cycles of the mesocosms likely favoured the formation of pyrite within the first 24hrs of nutrient addition over the formation of orpiment, which required lower pHs and a more consistent supply of carbon. Both types of mesocosms selected for microbes that are arsenic resistant and can withstand long periods of starvation.

This thesis may help determine what some of the most important design and optimization parameters are for designing field scale constructed wetlands for the removal of arsenic from water. Some important design and optimization parameters may include ensuring that the wastewater containing arsenic is supplemented with nutrients to support microbes, particularly under anaerobic conditions. Anaerobic systems offer the potential for high removal rates of arsenic but particular attention needs to be paid to the ratio of iron, sulfur and carbon added to the systems, whether it is found in the wastewater or is released by or part of the substrates used in the wetlands. It is also important to pay attention to when the arsenic is loaded into the wetland, as the presence or absence of nutrients may influence how and if arsenic is removed.

The aerated systems may be best suited for removal of acid mine drainage waters, particularly those that contain Fe(II) and/or Mn(II) as the carbonate minerals in my wetlands would be able to neutralize the acidity of the wastewater and cause the precipitation of iron and manganese oxyhydroxides that may remove arsenic through adsorption/coprecipitation. For other neutral wastewaters such as ground waters highly contaminated with arsenic, non-aerated mesocosms could effectively be used to remove the arsenic if the mesocosms are amended with a source of carbon, iron and sulfate, either in the wastewater and/or in the substrate. Arsenic removal may then proceed through removal by formation of pyrite/arsenian pyrite if all three amendments are present or if carbon and sulfate are added only, removal may proceed through removal through the precipitation of arsenic sulfides.

7.6 Future Research Recommendations

Future research directions for optimizing removal of arsenic under non-aerated and aerated conditions using recirculating constructed wetlands should attempt to:

1) Compare whether other iron containing carbonate mineral substrates such as siderite, ferroan calcite and aragonite can enhance the formation of pyrite
2) Determine what kind of carbon feeding regime, continuous vs batch, would remove the most arsenic and examine what biogenic minerals are formed as a result.
3) Future studies that use CWs to treat arsenic from water should attempt to filter the outflow water to capture any particulates that contain arsenic or recover particulates that settle at the bottom of the CWs, which may help address the issue of missing/or unaccounted arsenic and may help close the mass balance.

4) Compare the effect of connecting non-aerated and aerated wetlands in series separately and/or combining both systems in series to examine the effects on arsenic removal.
5) Grow and test different *Equisetum* sp. at higher initial plant densities to determine if there are any direct or indirect effects on arsenic removal in CWs.
6) Repeat the hydroponic experiments with as many *Equisetum* sp. as possible and test the effects of adding phosphate and other nutrients to see whether it is possible to optimize arsenic removal and biomass growth.

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Appendix A.

Supplementary Information for Chapter 3
Uptake and speciation of arsenic in hydroponically exposed horsetail plants (*Equisetum* sp.)

Hnain, A.K., Nearing, M., Koch, I., Weber, K. P.

Calculations

Whole plant dry weights:

To calculate the dry weight whole plant arsenic concentrations in our *Equisetum* sp., we used the following weighted average equation (the same was done with wet weight arsenic concentrations for Table S1):

Whole plant dry weight [As] in mg/kg = {(Shoot dry weight/Total dry weight) x Dry weight shoot [As]} + {(Root dry weight/Total dry weight) x Dry weight root [As]} **Eq. S1**

Bioconcentration and translocation factors:

Tissue BCF = Dry weight [As] in tissue in mg/kg / initial [As] in the hydroponic solution in mg/L **Eq. S2**

Plant BCF = Whole plant dry weight average [As] in mg/kg / starting [As] in the hydroponic solution in mg/L **Eq. S3**

TF = Dry weight [As] in shoot tissue in mg/kg / wet weight [As] in root tissue in mg/kg **Eq. S4**

Similar calculations were done with wet weight arsenic concentrations for Table S2.

Quality Assurance and Quality Control (QA/QC)

Tissue Digests: There was a total of 36 tissue samples subjected to digestion and analysis in each arsenic uptake experiment. In the first experiment, quality control samples consisted of 4 blanks (empty tubes with no tissue material added), 4 replicates of standard reference material (Bush Twigs and Leaves GBW 07603, with 1.25 mg/kg certified As) and 4 duplicate digestions of samples which accompanied the tissue samples; this equated to 1 blank, 1 SRM, and 1 duplicate for every 9 tissue samples. A similar set of quality control samples were prepared for the second experiment except that there were no duplicate digestions because there was not enough tissue biomass to prepare them. In both experiments, matrix spikes were prepared immediately before ICP-MS analysis by adding a known concentration of As(V) to tissue digest samples not treated with arsenic (i.e. controls) to determine if the tissue digest matrix interfered with the detection and quantification of arsenic in our samples.

All digestion blanks remained well below the LOD of the instrument indicating that digestion tubes and materials (acid, filters, syringes etc.) did not introduce arsenic to the analysis. The average arsenic recovery from the standard reference material was 80% (n = 10, range 51–112%), which was considered acceptable. All digestion duplicates had relative percent differences (RPD) <20%, indicating acceptable precision. All matrix spikes had an average arsenic recovery of 100% (n = 11 range 78 – 119%) indicating little interference by other chemicals in the digest matrix.

Water Samples: In each experiment, water samples were taken on Day 0 and the last day (i.e. Day 16 for Part 1 and Day 45 for Part 2). Quality control samples consisted of 1 duplicate and 1 matrix spike for every 9 samples. The matrix spikes were prepared before ICP-MS analysis by adding a known amount of As(V) to control treatment water samples. All duplicates had relative percentage differences (RPD) <20%, indicating acceptable precision. All matrix spikes had acceptable average arsenic recoveries between 70 – 130% indicating minimal interferences by the sample matrixes.

The ICP-MS instrument analysis conditions were as follows. Calibration standards of 0.1, 0.5, 1, 5, 10, 50, 100 and 500 µg/kg of As(V) were prepared from 1000 µg/kg stocks (Inorganic Ventures) for total

arsenic analysis. The nebulizer was concentric-type, the flow of argon was 0.87 L/min, the RF power was 1300W, and the lens voltage was 6V. The analysis mode used was peak hopping, the dwell time was 100 ms, and the instrument read 10 sweeps and three replicates per sample. The internal standard was 10 ppb rhodium made from a 1000 µg/ml stock solution (SCP Science). The masses monitored were ^{75}As with ^{103}Rh. For quality control, two standard solutions of 5 and 50 µg/L arsenic were analyzed every 10 samples, and results accepted only if recovery was in an acceptable range of 70 – 130%. The limit of detection (LOD) of the instrument was estimated to be 0.5 µg/L.

Table S1: Average wet weight (mg/kg ww) tissue arsenic concentrations in *Equisetum* sp. at the end of each experiment

		[As] (mg/kg ww)		
Species	**Treatment (mg/L)**	**Shoot**	**Root**	**Plant[a]**
E. fluviatile	**0**	0.09 ± 0.02	0.3 ± 0.1	0.3 ± 0.1
	1	0.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
	10	12 ± 9	13 ± 2†§	13 ± 2†§
E. hyemale	**0**	0.11 ± 0.03	1.9 ± 1.8	1.6 ± 1.5
	1	2.5 ± 0.2$^{\dagger\#\square}$	4.5 ± 0.8$^{\Delta\#\square}$	4.0 ± 0.6$^{\infty}$
	10	11± 2†§	43 ± 22$^{\dagger\S\Delta}$	37 ± 17†§
E. hyemale	**0**	0.11 ± 0.08	0.06 ± 0.09	0.06 ± 0.02
	1	2.5 ± 1.1	2.3 ± 0.7	2.5 ± 0.7
	10	27 ± 6†§	37 ± 26†	30 ± 12†§
E. scirpoides	**0**	0.11 ± 0.09	0.25 ± 0.34	0.16 ± 0.18
	1	3.6 ± 1.0	2.0 ± 1.0	2.9 ± 1.2
	10	19 ± 9†§	48 ± 13†§	30 ± 11†§

All values are average ± SD for n=3 plants per treatment.
a= Calculated using weighted averages of the shoot and root tissue.
†= Indicates the treatment dose for which there was a significantly higher tissue/plant arsenic concentration compared to the control treatment for each *Equisetum sp.* in each experiment ($p<0.05$ ANOVA post hoc Dunnett test).
§= Indicates significantly higher tissue/plant arsenic concentration at the 10 mg/L As(V) dose compared to the 1 mg/L As(V) dose for each *Equisetum sp.* in each experiment ($p<0.05$ ANOVA post hoc Tukey test).
Δ= Indicates root arsenic concentrations significantly higher than shoot arsenic concentrations for the same *Equisetum* sp. within the same experiment ($p<0.05$ ANOVA post hoc Tukey test).
□= Indicates what *Equisetum* sp. had a significantly greater arsenic concentration for the same tissue type and treatment dose in each experiment ($p<0.05$ ANOVA post hoc Tukey test).
∞= Indicates in which experiment *E. hyemale* plants had a significantly higher arsenic concentration when exposed to the same arsenic dose.

Table S2: Calculated average wet weight tissue bioconcentration factors (BCFs) and translocation factors (TFs) in *E. fluviatile*, *E. hyemale* and *E. scirpoides*

Species	Treatment (mg/L)	Shoot BCF	Root BCF	Plant BCF	TF
E. fluviatile	**1**	1 ± 0.1	2 ± 0.2$^{\#}$	2 ± 0.2	0.5 ± 0.1
	10	1.6 ± 1.3	2 ± 0.3	2 ± 0.4	0.9 ± 0.6
E. hyemale	**1**	3 ± 0.2†§	6 ± 1$^{\S\Delta\#}$	6 ± 1$^{\S\Delta}$	0.6 ± 0.2
	10	2 ± 0.1	6 ± 4	5 ± 3	0.3 ± 0.2
E. hyemale	**1**	2 ± 1	2.0 ± 1	2 ± 1	1.1 ± 0.5
	10	3 ± 1Δ	3 ± 2	3 ± 1	0.9 ± 0.4
E. scirpoides	**1**	3 ± 1	2 ± 1	3 ± 1	2.1 ± 0.9
	10	2 ± 1	4 ± 1†$^{\#}$	3 ± 1	0.4 ± 0.1

All values are average ± SD for n=3 plants per treatment
† = Indicates which treatment concentration yielded a statistically greater BCFs or TFs for each *Equisetum* sp. ($p<0.05$ Welch's t-test).
§ = Indicates which *Equisetum* sp. yielded a statistically greater BCFs or TFs for the same treatment concentration within each experiment ($p<0.05$ Welch's t-test).
Δ = Indicates in which experiment *E. hyemale* yielded a statistically greater BCFs or TFs for the same treatment concentration ($p<0.05$ Welch's t-test).
= Indicates that root BCFs were significantly greater than shoot BCFs for the same treatment for each *Equisetum* sp. ($p<0.05$ Welch's t-test).

Table S3: The proportion of each arsenic species found in *E. fluviatile* and *E. hyemale* using XANES spectra and linear combination fitting of the data. Reduced chi-square ($\chi 2$) is an indication of fit (less than 0.02 is considered acceptable).

Species	Tissue	Treatment (mg/L)	% of Arsenic species			Total	$\chi 2$
			As(V)	As(III)	$As(SG)_3$		
E. fluviatile	**Shoot**	1	100	0	0	100	N.A.
		10	46	54	0	100	0.0180
	Root	1	45	41	15	100	0.0093
		10	15	24	62	100	0.0060
E. hyemale	**Shoot**	1	N.D.	N.D.	N.D.	N.D.	N.A.
		10	54	46	0	100	0.0416
	Root	1	100	0	0	100	N.A.
		10	91	0	9	100	0.0091

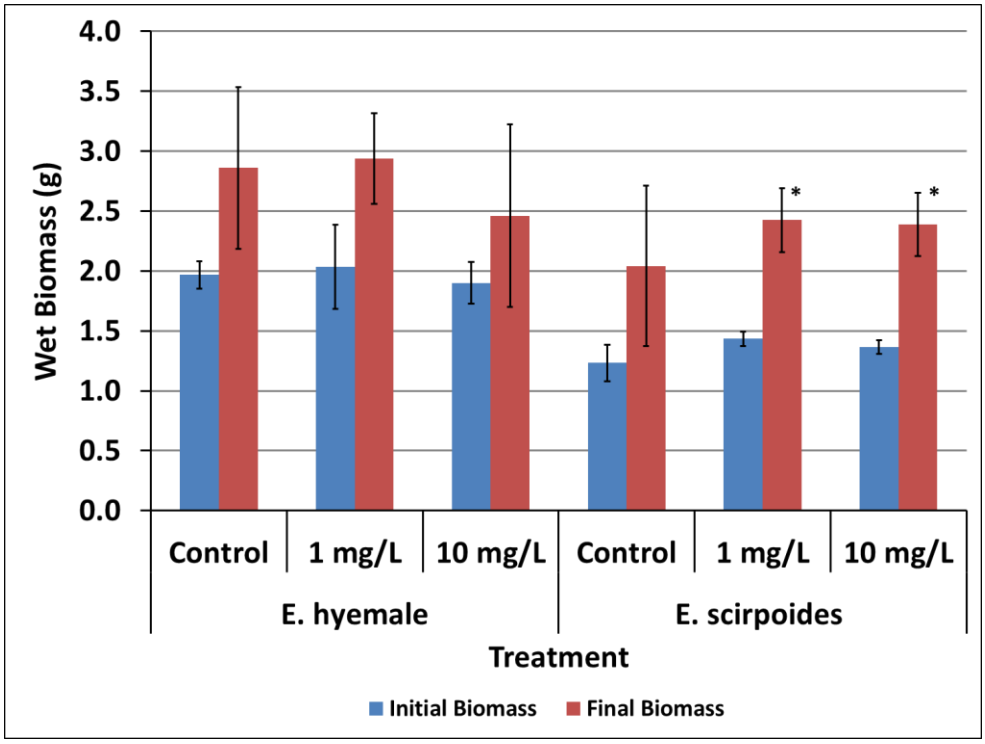

Figure S1: A comparison of the initial and final average wet biomass for *E. hyemale* and *E. scirpoides* in the second experiment treated at 0, 1 and 10 mg/L As(V) for 45 days under hydroponic conditions (All values are average ± SD for n=3 replicates). *= The final wet biomass was significantly greater than the initial biomass based on an ANOVA post hock Tukey test ($p<0.05$).

Appendix B.

Supplementary Information for Chapter 4
Removal and fate of arsenic in non-aerated and aerated treatment wetland mesocosms
Hnain, A.K., Koch, I., Weber, K.P.

Table S4: Simulated domestic wastewater recipe from Weber & Legge, (2011)

Macronutrient Stock Solutions	Concentration per mesocosm (mg/L)
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	47.3
Potassium nitrate (KNO_3)	151.5
Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$)	236.0
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	123.3
Urea ($CO(NH_2)_2$)	49.0
Ethylenediaminetetraacetic acid iron (III) sodium salt (FeNaEDTA)	9.2
Molasses	1000
Micronutrients	
Boric acid (H_3BO_3)	0.72
Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	0.45
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	0.06
Copper (II) sulphate ($CuSO_4$)	0.01
Ammonium molybdate hydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$)	0.005

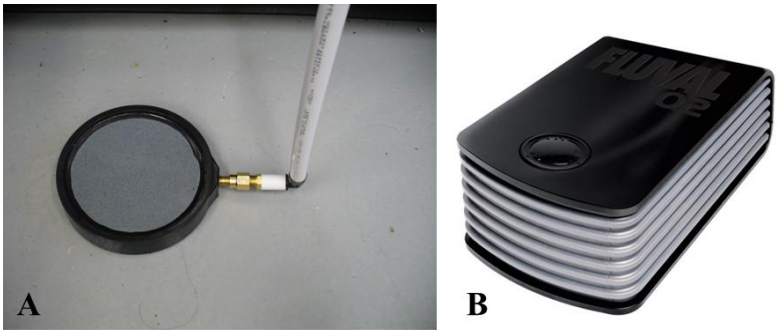

Figure S2: Picture of the a) six-inch aeration stones placed at the bottom of each aerated mesocosm and connected to b) the air pump that distributed air to each mesocosm via a 6-way manifold.

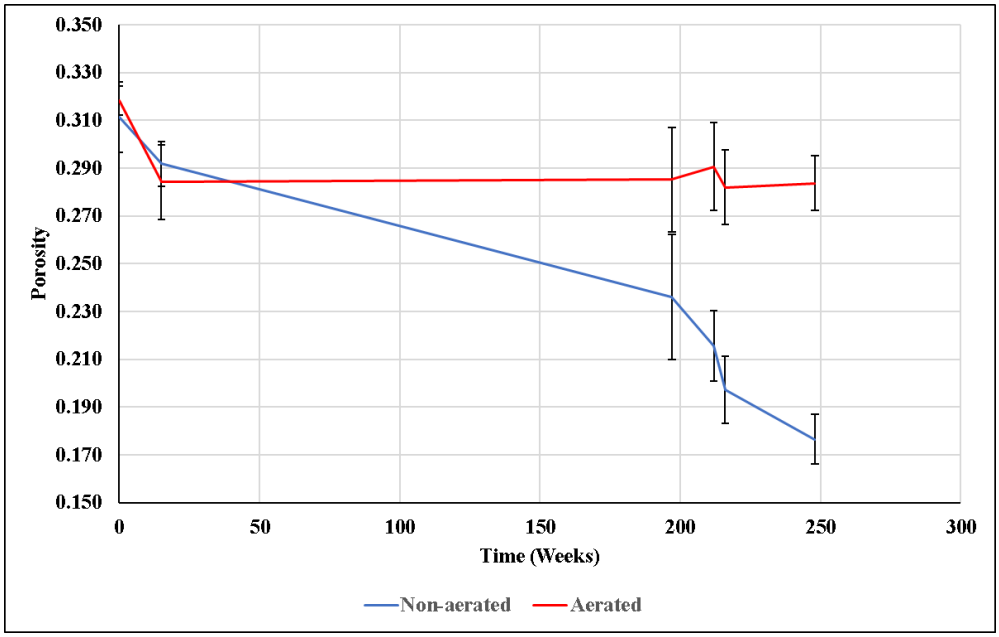

Figure S3: The porosity of non-aerated (M1-6) and aerated mesocosms (M7-12) for weeks 0, 15, 197, 212, 216, 248. The arsenic exposure experiment occurred from week 216 to week 248. Each data point is the average porosity ± S.D. for n=6 mesocosms from each system type.

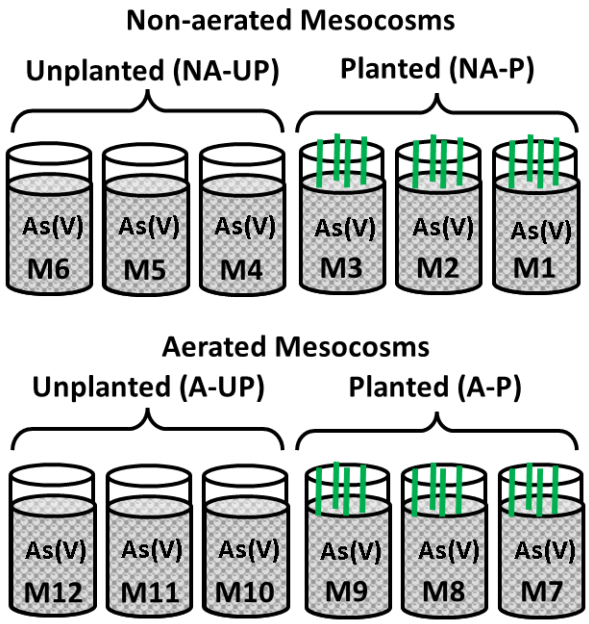

Figure S4: Arsenic exposure experimental design

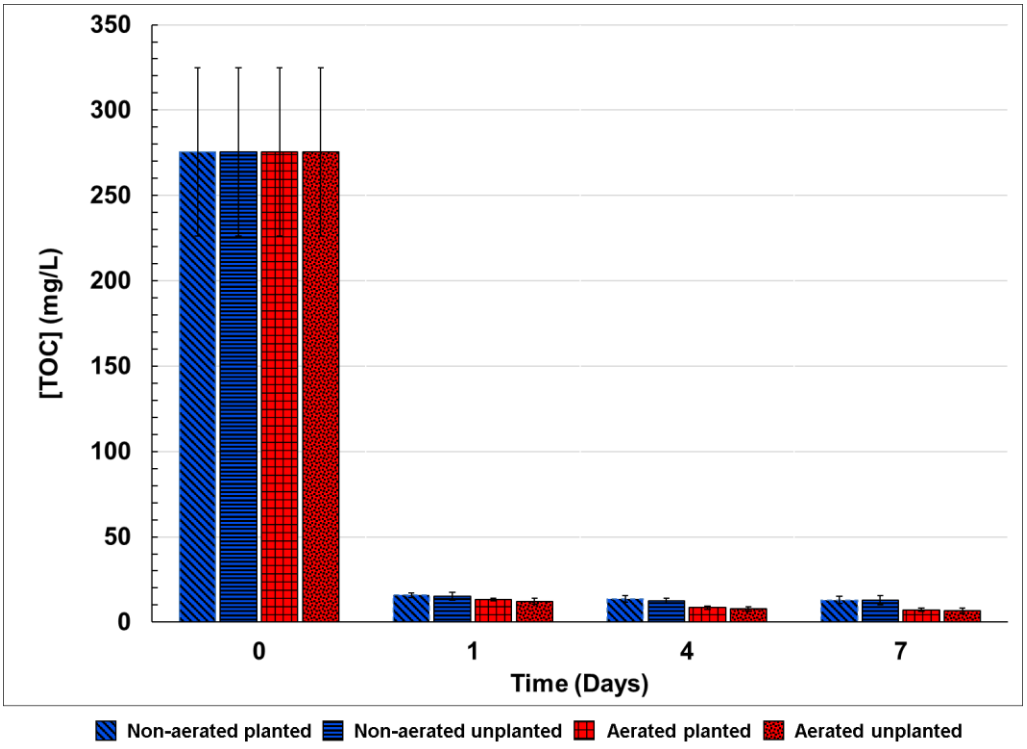

Figure S5: The average concentration of total organic carbon (TOC) measured in porewater for days 0, 1, 4 and 7 days after addition for weeks 1, 3, 5, 7, 9, 11, 13 and 15.

Table S5: The average total mass of arsenic treated (in mg) for each type of mesocosm, calculated from the average total mass of wastewater added and an As(V) concentration of 1 mg/L (Average ± S.D., n=33)

Wetland	Total volume treated (L)	[As(V)] (mg/L)	Mass of As treated (mg)
Non-aerated planted (M1–3)	250 ± 10	1.013 ± 0.0014	250 ± 10
Non-aerated unplanted (M4–6)	240 ± 20	1.010 ± 0.004	240 ± 20
Aerated planted (M7–9)	380 ± 6	1.03 ± 0.01	390 ± 10
Aerated unplanted (M10–12)	356 ± 7	1.024 ± 0.003	364 ± 6

Table S6: ICP-MS operating conditions for total arsenic analysis

	ICP-MS Instrument	
	PerkinElmer Elan DRC II	**PerkinElmer NexION 310D**
ICP Operating Parameters		
RF power	1300 W	1600 W
Lens voltage	6 V	No ion lens, ion deflector (1000 V)
Nebulizer	Concentric	Concentric
Nebulizer gas flow	Ar, 0.87 L/min	Ar, 1.5 L/min
Auxiliary gas flow	2 L/min	2 L/min
Plasma gas flow	20 L/min	20 L/min
Mass Spectroscopy Parameters		
Internal standard	^{103}Rh	^{103}Rh
Masses monitored	^{75}As, ^{103}Rh	^{75}As, ^{103}Rh
Dwell time	100 ms	50 ms
Scan mode	Peak hopping	Peak hopping
Sweeps/reading	10	10
Replicates per sample	3	3
Limit of Detection	0.5 ug/L	0.1 ug/L

Table S7: Characteristics of influent wastewater and pore water in the mesocosms prior to addition of arsenate (Average ± S.D.)

Parameter	**Initial Wastewater Characteristics**	**Overall Porewater Characteristics**			
		NA-P	**NA-UP**	**A-P**	**A-UP**
Temperature (ºC)	22 ± 3	24 ± 2	24 ± 2	24 ± 2	24 ± 2
Conductivity (µS/cm)	743 ± 33	802 ± 47	763 ± 53	731 ± 37	737 ± 38
DO (mg/L)	4.9 ± 1.6	0.05 ± 0.06	0.10 ± 0.15	5.92 ± 0.89	5.99 ± 0.90
ORP (mV)	100 ± 55	-228 ± 18.5	-228 ± 36	45 ± 12	58 ± 12
pH	7.12 ± 0.10	7.00 ± 0.13	6.94 ± 0.11	7.54 ± 0.07	7.54 ± 0.07
NH_4^+–N (mg/L)	20 ± 3	24 ± 5	23 ± 6	9 ± 1	10 ± 2
NO_3^-–N (mg/L)	38 ± 10	7 ± 5	6 ± 3	48 ± 15	46 ± 14
TOC (mg/L)	261 ± 26	24 ± 11	19 ± 6	17 ± 22	11 ± 5
IC (mg/L)	15 ± 3	62 ± 10	58 ± 13	7 ± 2	6 ± 2

Note: All fresh wastewater measurements were taken on a weekly basis (n= 33) from the nutrient tank before loading into the mesocosms while pore water measurements were taken on a daily basis from the sampling port from Mon-to Fri of each week of the experiment (n = 165). TOC and IC measurements are the average of day 7 measurements that were taken on odd weeks 1, 3, 5, 7, 9, 11 and 15 (n=8) for all mesocosms. The total arsenic concentrations for each of the mesocosm types are the average of n = 18 samples where day 6 samples were taken on weeks 1, 6, 14, 16, 25 and 33 (n = 3 mesocosms per treatment x 6 sampling points).

QA/QC Procedures

TOC Analysis: The instrument measured total carbon (TC) and inorganic carbon (IC) where TOC was derived from subtracting TC from IC. The instrument was calibrated every 3 to 4 weeks with a mixture composed of potassium hydrogen phthalate at concentrations of 0, 9, 18, 45, 90, 180, 450 and 900 ppm for TC, carbonate buffer composed of Na_2CO_3 and $NaHCO_3$ (in a 1:1.2 ratio) to obtain concentrations of 0, 4.5, 9, 22.5, 45, 90, 225 and 450 ppm for IC. For every 49 water samples we analyzed one 100 ppm TOC and

one ddH_2O blank were used as quality control checks. All standards had acceptable recoveries of between 80–120% and all blanks were below detection limits.

ICP-MS Analysis: Diluted mesocosm water samples, PB and CBE filtrates, as well as biofilm digests were run on a NexION 310D ICP-MS (PerkinElmer Shelton, CT, USA) while the plant digest were run on an Elan DRC II ICP-MS (PerkinElmer Shelton, CT, USA). Calibration curves with 0, 2.5, 25, 50 and 100 µg/L of As(V) ($Na_2HAsO_4 \cdot 7H_2O$, 98% Alfa Aesar) were used to calculate arsenic totals in all samples. Calibration curves with 0, 2.5, 25, 50 and 100 ppb of As(V) ($Na_2HAsO_4 \cdot 7H_2O$, 98% Alfa Aesar) were used to calculate arsenic totals in all samples. Quality control samples consisted of 1 duplicate and 1 matrix spike for every 9 samples. The matrix spikes were prepared before ICP-MS analysis by adding a known amount of As(V) to randomly chosen samples. All duplicates had relative percentage differences (RPD) <20%, indicating acceptable precision (except for one sample, week 6 M4 20 min, that had a %RPD of 102%). All matrix spikes had acceptable recoveries between 70–130% indicating minimal interferences by the sample matrices. The % recoveries for SRMs digested at the same time as plant tissues and detached biofilms were within an acceptable range of 70–130%.

XRF Analysis: Prior to analysis of dried biofilm samples and every 10 samples, the XRF instrument was standardized using an XRF standardization alloy (Stainless steel grade 316 alloy), a silica oxide blank and a standard reference material (NIST 2711A Montana II soil) in this order. The percent recovery for As, Fe and Mn from the standard reference material was within the acceptable limits of 80–120%.

Appendix C.

Supplementary Information for Chapter 5
Arsenic speciation in wastewater, plants and biofilms from non-aerated and aerated laboratory scale constructed wetlands

Hnain, A.K., Koch, I., Meira, D.M., Weber, K.P.

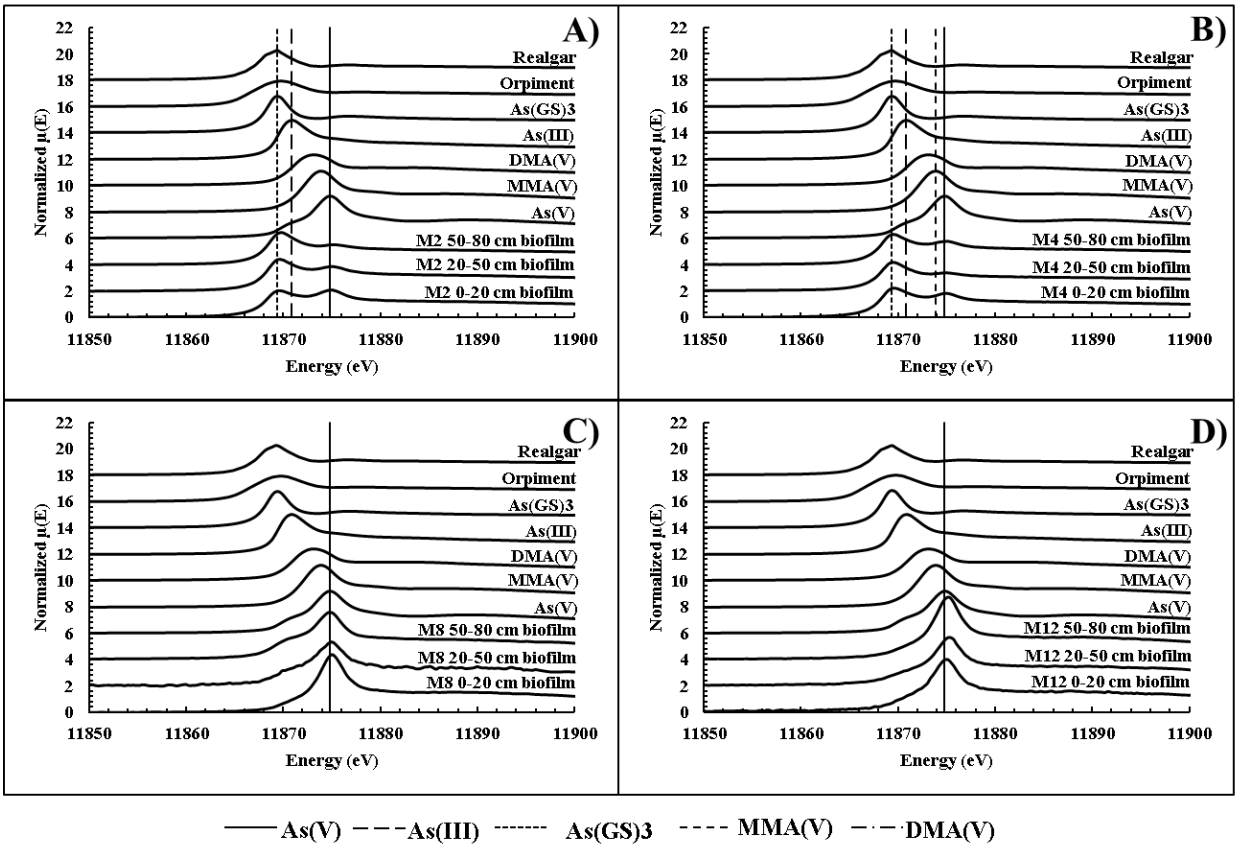

Figure S6: XANES spectra of arsenic in detached gravel biofilms from A) non-aerated planted, B) non-aerated unplanted, C) aerated planted and D) aerated unplanted mesocosms from three different gravel depths along with the corresponding XANES spectra for arsenic standards.

Table S8: X-ray absorption near-edge structure fitting results for detached gravel biofilms coming from mesocosms M2, M4, M8 and M12 at increasing gravel layer depths (0-20, 20-50 and 50-80 cm). Reduced chi-square ($\chi 2$) is an indication of fit (less than 0.02 is considered acceptable).

Mesocosm Type (ID)	Gravel depth (cm)	Proportion determined by XAS (%)					Total	$\chi 2$
		As(V)	As(III)	DMA(V)	MMA(V)	As(SG)3		
NA-P (M2)	0-20	34	15	0	0	51	100	0.004
	20-50	20	25	0	0	55	100	0.007
	50-80	8	29	0	0	63	100	0.0004
NA-UP (M4)	0-20	21	19	0	1	59	100	0.002
	20-50	10	11	0	0	79	100	0.008
	50-80	20	19	0	0	62	100	0.003
A-P (M8)	0-20	100	0	0	0	0	100	0.05
	20-50	100	0	0	0	0	100	0.006
	50-80	100	0	0	0	0	100	0.04
A-UP (M12)	0-20	100	0	0	0	0	100	0.02
	20-50	100	0	0	0	0	100	0.03
	50-80	100	0	0	0	0	100	0.07

Table S9: X-ray absorption near-edge structure fitting results for plants from planted non-aerated (M1-3) and aerated (M7-8) mesocosms. Reduced chi-square ($\chi 2$) is an indication of fit (less than 0.02 is considered acceptable).

Mesocosm Type (ID)	Sample	Proportion determined by XAS (%)					Total	χ^2
		As(V)	As(III)	DMA(V)	MMA(V)	As(SG)3		
NA-P (M1)	*M. polymorpha* (W)	100	0	0	0	0	100	0.03
	E. hyemale (R)	100	0	0	0	0	100	0.02
	E. hyemale (S)	100	0	0	0	0	100	0.04
NA-P (M2)	*M. polymorpha* (W)	100	0	0	0	0	100	0.007
	E. hyemale (R)	100	0	0	0	0	100	0.05
	E. hyemale (S)	100	0	0	0	0	100	0.01
NA-P (M3)	*M. polymorpha* (W)	100	0	0	0	0	100	0.01
	E. hyemale (R)	36	11	0	0	53	100	0.008
	E. hyemale (S)	N.D.	N.D.	N.D.	N.D.	N.D.	N.A.	N.A.
A-P (M7)	*E. hyemale* (R)	100	0	0	0	0	100	0.05
	E. hyemale (S)	N.D.	N.D.	N.D.	N.D.	N.D.	N.A.	N.A.
A-P (M8)	*E. hyemale* (R)	71	0	0	0	30	100	0.04
	E. hyemale (S)	5	38	11	0	46	100	0.004

W = whole plant, R = roots, S = shoot

QA/QC Procedures

ICP-MS Analysis: Diluted mesocosm water samples taken on day 6 for weeks 1, 6 and 14 were run on a NexION 310D ICP-MS (PerkinElmer, MA, USA.). Calibration curves with 0, 2.5, 25, 50 and 100 ppb of As(V) ($Na_2HAsO_4 \cdot 7H_2O$, 98% Alfa Aesar) were used to calculate arsenic totals in all samples. Quality control samples consisted of 1 duplicate and 1 matrix spike for every 9 samples. The matrix spikes were prepared before ICP-MS analysis by adding a known amount of As(V) to randomly chosen samples. All duplicates had relative percentage differences (RPD) <20%, indicating acceptable precision. All matrix spikes had acceptable recoveries between 70–130% indicating minimal interferences by the sample matrices (except for week 1 M3 day 6 that had a negative spike % recovery).

Speciation analysis: To run the week 1 day 6 speciation samples on our in-house HPLC-ICP-MS instrument, calibration curves with a mixture of As(V), As(III), MMA(V) and DMA(V) were prepared at concentrations of 1, 10, 25, 50 and 100 ppb for each species (standards were prepared from salts of $Na_2HAsO_4 \cdot 7H_2O$ 98% Alfa Aesar, $NaAsO_2$ Baker Analyzed Reagent, $CH_3AsNa_2O_3$ 95% Alfa Division, $C_2H_6AsNaO_2$ >99% Fluka).

Week 6 and 14 day 6 mesocosm water samples were shipped to ALS Environmental and the QA/QC report showed that the week 6 M10 day 6 duplicate %RPD was <20% for all arsenic species and week 6 M11 day 6 matrix spike showed no recovery of As(V). With respect to column recoveries shown in Table S10, samples for week 1 day 6 M1, M4 and M6 and week 14 day 6 M7 showed recoveries outside the 70–130% range and were excluded from Figure 5-1. We believe that the week 1 day 6 M1, M4 and M6 samples showed poor recoveries due to the low arsenic concentrations remaining in these samples which made it difficult to manually identify peaks and quantify the arsenic species using PeakFit v4.12 software. It is unclear why week 14 day 6 M7 column recoveries were so low.

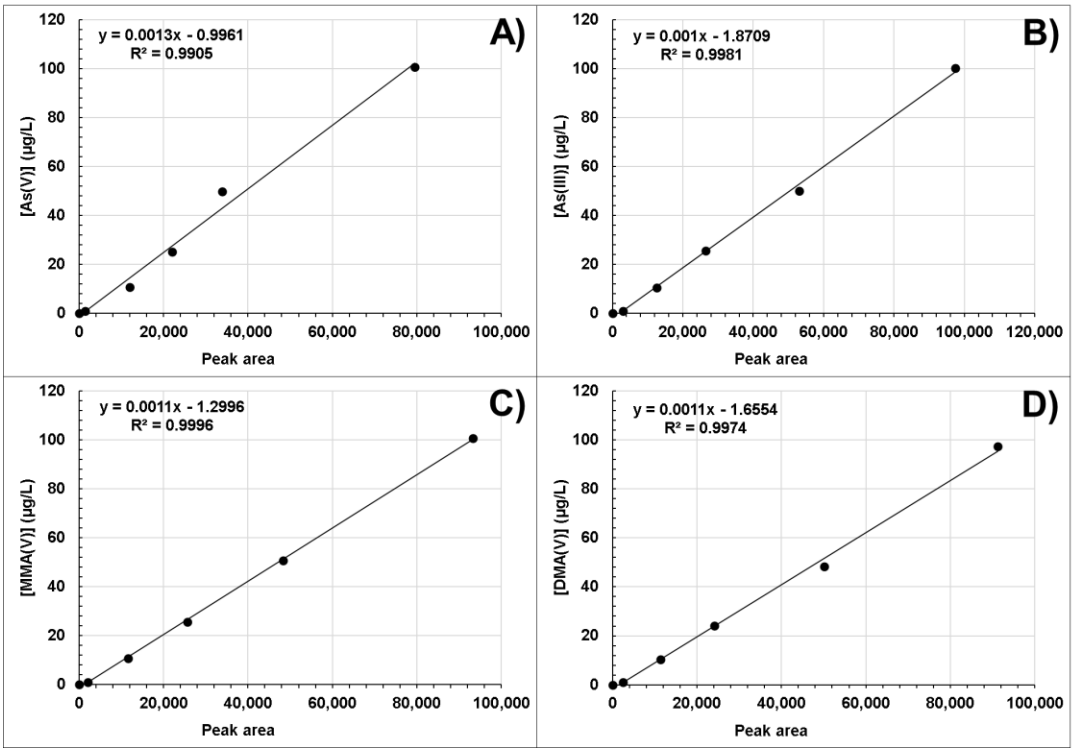

Figure S7: Calibration curves of peak area and the concentration of A) As(V), B) As(III), C) MMA(V) and D) DMA(V) used to estimate the concentration of these species in week 1 day 6 porewater in all mesocosms.

Table S10: % Recoveries from HPLC columns for mesocosm water samples collected on day 6 for weeks 1, 6 and 14

		Week 1	Week 6	Week 14
		Day 6	Day 6	Day 6
Mesocosm Type	**Mesocosm ID**	**% Recoveries**	**% Recoveries**	**% Recoveries**
NA-P	**M1**	9	92	95
	M2	96	95	92
	M3	112	100	95
NA-UP	**M4**	153	96	92
	M5	77	97	94
	M6	275	99	94
A-P	**M7**	117	99	47
	M8	121	100	91
	M9	125	98	94
A-UP	**M10**	84	97	92
	M11	98	102	91
	M12	82	96	94