CRANFIELD UNIVERSITY

Lloyd John James Thompson

Understanding the risk of iron and phosphorus release from constructed wetlands treating chemically-dosed wastewater

CRANFIELD WATER SCIENCE INSTITUTE
School of Water, Energy and Environment

PhD
Academic Year: 2014 - 2018

Supervisor: Gabriela Dotro
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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD
(NB. This section can be removed if the award of the degree is based solely on examination of the thesis)

ABSTRACT

Constructed wetlands (CW) consistently provide efficient wastewater treatment for the removal of organics and solids but have limitations when removing phosphorus. Recent changes in legislation have led to stricter consents for phosphorus discharge and the adaptation of CW based sites with the retrofitting of upstream dosing of chemical coagulant, such as iron salt. As part of this process, the sludge accumulating on the CW is enriched with phosphorus and iron and, although it was thought to be irreversible, evidence of release of both phosphorus and iron has been reported but the mechanisms remain unclear. This project then aimed to understand the risk, mechanisms, and appropriate management options of iron and phosphorus release from CWs treating chemically dosed wastewater. To meet this aim a variety of methodologies, including data mining for treatment performance, several biological assessment techniques to understand microbial dynamics, and controlled lab-scale trials to demonstrate the potential for release and evaluate remediation measures, were used. It was found that CW adaptation by iron dosing improved the robustness of the sites not only for phosphorus but also for BOD and suspended solids, at both current and potential future demand levels. The bacterial communities of CWs were unimpacted by the introduction of adaptations. The mechanism by which iron and phosphorus can be released from these sites was shown to require the establishment of appropriate environmental conditions that triggered the related microbial pathway, identified as the need for a source of iron, anaerobic conditions and an available carbon source. This triangle of needs gave rise to a mitigation strategy by dosing of nitrate which was demonstrated to be effective in supressing the release. This research showed that CW adaptation is required to robustly meet current and future demands however they must be operated correctly to ensure there is no re-release of pollutants.

Keywords:

Aeration; Dissimilatory Iron Reducing Bacteria; Ferric Coagulation; Iron Release; Preferential metabolism; q-PCR; Terminal Electron Adaptor; Terminal Electron Donor;

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I thank my Dad for the constant insults that were hiding his support, whilst I thank my Mam for getting us all through this

Finally, I want to thank Tina…

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LIST OF ABBREVIATIONS

Ag/AgCl	Silver / Silver Chloride
Al	Aluminium
As	Arsenic
BOD	Biochemical Oxygen Demand
C	Celsius
Ca	Calcium
cm	Centimetre
CO_2	Carbon Dioxide
CW	Constructed wetland
d	Day
DIR(B)	Dissimilatory Iron Reducing Bacteria
DO	Dissolved Oxygen
Dr	Doctor
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E_{50}	50th percentile
E_{95}	95th percentile
E_{goal}	Percentile at goal
EU	European Union
Fe	Iron
Fe^{2+}	Ferrous Iron
Fe_2O_3	Ferric Oxide
Fe^{3+}	Ferric Iron
$FePO_4$	Ferric Phosphate
FeRB	Iron (Fe) Reducing Bacteria
FeOB	Iron (Fe) Oxidising Bacteria
g	Gram
G	Goal
G%	Percentage time spend below goal
HCl	Hydrochloric Acid
HFCW	Horizontal Flow Constructed Wetland
HLR	Hydraulic Loading Rate
HSSF	Horizontal Sub Surface Flow

ICPMS	Inductively coupled plasma mass spectrometry
ID	Identification
kDa	Kilodalton
kg	Kilogram
KW (test)	Kruskal-Wallis
l	Litre
m	Metre
m2	Squared Metre
mg	Milligram
mm	Millimetre
mmol	Millimol
mV	Millivolt
µg	Micro gram
µm	Micro metre
µmol	Micro mol
n	Number
NO_3^-	Nitrate
N	No
N/A	Not Applicable
N_2	Nitrogen
NH_4^+	Ammonia
OTU	Operational Taxonomic Unit
P	Phosphorus
p.	Page
P.E.	Population Equivalent
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
pp.	Pages
q-PCR	quantitative Polymerase chain reaction
RBC	Rotating Biological Contactor
RPI	Robustness Performance Index
rpm	Revolutions per minute
S	Sulphur

s	Second
$NaNO_3$	Sodium Nitrate
SS	Suspended Solids
Stdev	Standard Deviation
TEA	Terminal Electron Acceptor
TED	Terminal Electron Donor
TP	Total Phosphorus
UK	United Kingdom
UWWTD	Urban Wastewater Treatment Directive
VF	Vertical flow
WFD	Water Framework Directive
Y	Yes

1 Introduction

Legislation has been introduced around the world to limit the uncontrolled discharge of phosphorus (P) onto water at risk of environmental damage (Jiang et al., 2008; United States Federal Law, 1962). Within the European Union, a significant step was the Urban Wastewater Treatment Directive (UWWTD; EEC Council, 1991), which was aimed at protecting the environment from the discharge of wastewater from urban environments into the natural environment. One of the requirements for this directive was performance monitoring of both wastewater treatment plants (WWTPs) and their receiving waterbodies (EC, 2000), with minimum treatment targets set for the discharge from WWTPs treating wastewater from over 2,000 population equivalents (P.E.). In the UK, the implementation of the UWWTD has resulted in most discharges from WWTP from agglomerations over 2,000 P.E. providing secondary treatment when discharging to freshwater and coastal waters, and tertiary treatment requirements when discharging to sensitive areas (DEFRA, 2012). Tertiary treatment was aimed at removing either nitrate or phosphorus to prevent eutrophication of receiving water bodies, primarily from WWTPs serving over 10,000 P.E. Subsequent legislation was introduced in the Water Framework Directive (WFD; EC, 2000), which established standards for river quality and a comprehensive river management basin system to help protect the ecological health of all water bodies (DEFRA, 2014). This directive was a major driver behind the UK Technical Advisory Groups' (UKTAG) initial recommendation on P standards for rivers in 2006, before revising these in 2013 (UKTAG, 2013).

Discharge standards for WWTPs in the UK are typically set on 5-day biochemical oxygen demand (BOD), suspended solids (TSS), and ammonia nitrogen (NH_4-N), with increasingly more WWTPs being required to remove (total) phosphorus. The UWWTD established TP standards of 1 mg/L and 2 mg/L for WWTPs serving over 100,000 P.E. and between 10,000 and 100,000 P.E., respectively. With the WFD and the identification of sensitive waters in the UK, a number of small (rural) WWTPs serving less than 2,000 P.E. have been required to meet TP consents on a case by case basis. Whereas BOD, TSS and NH_4-N are consented on 95th percentile effluent concentration in spot samples, TP consents are normally annual averages. The UK is unique in that when using iron or aluminium salts to remove TP, a discharge consent of total Fe or total Al will be required as well. This metal consent is typically 4 mg/L as 95% percentile (although it can be lower, depending on the receiving water body) and a maximum, “upper tier” consent of 8 mg/l which must never be exceeded. Therefore, the risk to compliance in the UK is higher for Fe than for TP in WWTPs required to provide tertiary treatment. For this reason, medium and large works, defined as WWTPs serving over 10,000 P.E., will typically have a sand filtration polishing step. For small works (<2,000 P.E.) with chemical dosing for TP removal, tertiary constructed wetlands that were originally built for polishing BOD and TSS from secondary effluents have been recently adapted to provide remove any residual particulate TP and Fe.

[redacted] Constructed wetlands and chemically-assisted phosphorus removal

Constructed wetlands (CWs) are a form of treatment technology engineered to promote the removal of specific pollutants from wastewater through a combination of biological, physical and chemical processes. Each of these processes can be enhanced by modifying the design and operating conditions of CWs, depending on the treatment application. Constructed wetlands are typically classified by the water level, divided into subsurface (horizontal and vertical direction of flow) and surface flow (open water) systems. In Europe, the most commonly used types are subsurface flow systems, with vertical flow (VF) wetlands commonly used for secondary treatment and, in some cases, primary treatment, and horizontal flow (HF) wetlands typically used for tertiary polishing of organics and solids (Dotro et al., 2017). In the UK, water utilities treating domestic wastewater typically favour tertiary HF wetlands in rural works serving up to 2,000 P.E. As the requirements for improved treated water quality have increased worldwide, CWs have been modified to help deliver those new targets. These are referred as adaptations in this thesis and denote a deviation from the original design features of a CW to help that asset deliver more treatment within the same footprint. As these changes are introduced, the predominant removal processes within the CW can shift too, resulting in unintentional impacts on the system microbiology and ability to meet treatment consents.

Current CW adaptations are aimed at enhanced removal of nutrients, in particular, NH_4-N and TP. For the former, artificial (forced) aeration of HF wetlands has been successfully employed and assessed in terms of its treatment robustness and impacts on wetland components (Butterworth et al., 2016). For TP, wetlands are either designed to contain reactive media or an additional pre-treatment stage of a metal salt added for chemical precipitation of P (Dotro et al 2017). The latter is the current solution for small works in the UK, where chemical addition can take place before primary settling or ahead of secondary (biological) treatment (Figure 1-11). The majority of the TP and Fe will be retained by the upstream processes, as the TP reacts with Fe species in the water, forms a floc and settles out in humus tanks. The sludge from these tanks is periodically removed either by direct tankering (e.g., from integral rotating biological contactors primary septic zones) or temporarily stored in a sludge holding tank before tankering away and treatment at larger works. The role of CWs is to provide removal of any residual particulate TP and Fe, as the water passes slowly through the wetland media. To date, there have only been very limited studies on the impact of adaptation of this CW flowsheet on the wetlands' internal pollutant transformation processes. Previous studies investigating sites with chemical dosing have shown CWs are generally effective but can experience sporadic events of P release (Dotro et al., 2015, Kim et al., 2016). As more small works are required to meet P and Fe removal targets, a better understanding is critical to develop management strategies to minimise risk to compliance whilst maximising the benefits of existing assets and to enable planning for future consents through the correct adaptation of technologies.

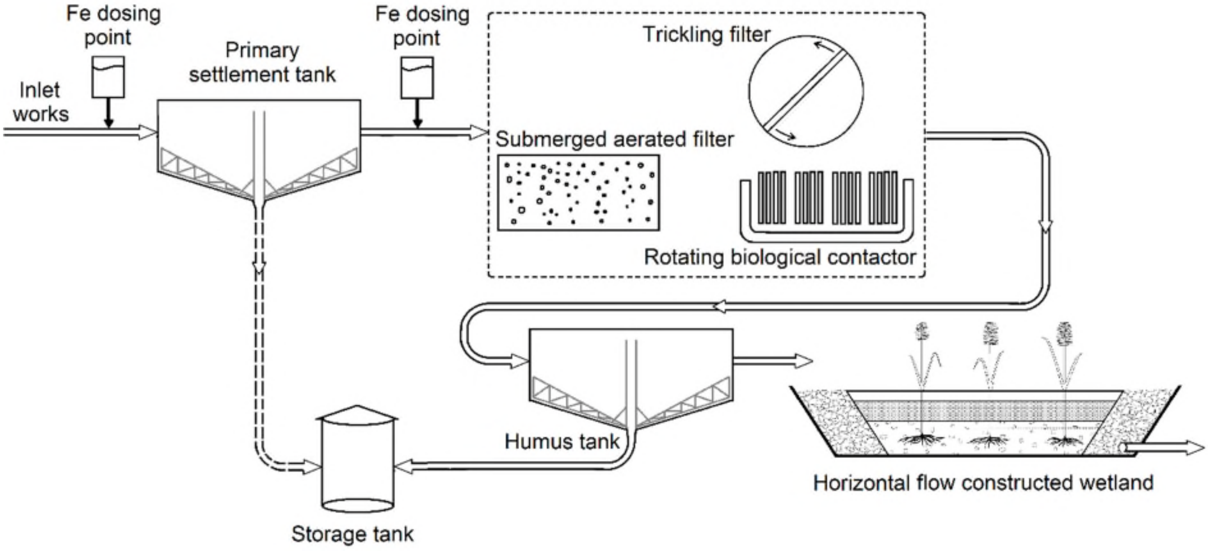


Figure 1-11 Typical UK rural works flowsheet where phosphorus removal is required

[redacted] Wetland treatment processes and implications for CW adaptations for tertiary P and Fe removal

Within CWs the main biological removal mechanisms are carried out by microorganisms either free flowing in the incoming wastewater or on the biofilm that grows attached to the media and roots of the plants. Whilst the media is responsible for the filtering of particulates such as suspended solids, it is the biological treatment steps that are responsible for the removal of a wider range of pollutants such as organic matter, nitrate, and ammonia. Whilst organic matter can be removed either aerobically (in the presence of oxygen) or anoxically (in the absence of oxygen and in the presence of nitrate through denitrification), nitrification requires an oxic environment. In conventional HF wetlands, there are zones that can be aerobic, anoxic, and anaerobic (Kadlec and Wallace, 2008). In particular, the inlet zone will receive the greater loads of pollutants and these would typically be anoxic, with strong anaerobic conditions expected with depth. Anaerobic processes such as iron and manganese reduction, sulphide and methane formation can occur depending on the abundance of each pollutant within these anaerobic zones. As the flow passes through the wetland and organic pollutants and nitrate (if sufficient carbon is available) are metabolised by the biofilm, the conditions can be anoxic with depth and close to aerobic at the media-water layer. Proximity to roots can also result in aerobic microzones (Reddy and Delaune, 2008).

Tertiary HF wetlands in the UK are planted with the common reed (*Phragmites australis*), which can partially contribute to the removal of nutrients during its growing season, although the proportion of nutrient uptake is deemed minimal compared to the typical loads (Vymazal and Kröpfelová, 2008). These plants are very rarely, if at all, harvested allowing plant detritus to form a sludge layer on the surface of the media. This sludge can be subjected to periods of oxygen availability (if not saturated with water) and anoxia (during flooded periods due to incorrect operation or storm events). The decaying plant matter plus any accumulated incoming solids on the sludge surface can thus act as both sink and source of pollutants, depending on the environmental conditions within that layer (Mesquita et al., 2017). The interactions between the surface sludge layer and the incoming and outgoing wastewater becomes of critical importance when adapting HF wetlands for tertiary removal of iron-bound P.

In conventional HF CW, Fe and P levels are typically in the order of 10-20 g P/kg of dried sludge and 9-17 g Fe/kg of dried sludge. This raises to around 30 g P/kg of dried sludge and as high as 178 Fe/kg of dried sludge in chemically-dosed sites (Dotro et al., 2015, 2017; Kim et al., 2016). Whilst the enriched levels following the introduction of CW adaptation are relatively new, significant efforts to understand P and Fe cycling has been done in anthropologically impacted natural environments (Supplementary information) and can be used as a basis for establishing critical knowledge gaps.

The three main factors affecting P and Fe cycling under waterlogged conditions are pH, dissolved oxygen (DO) levels and the redox potential (Figure 1-22). Whereas a change in pH from neutral to acid can result in P and Fe release from sediments, this would be unlikely to occur in chemically-dosed tertiary HF wetlands as the doses are too low to translate to a significant change in pH. A known relationship exists between DO levels, redox potential and microbial activity within wastewater treatment (Heduit et al., 2014), whereby microorganisms can create a redox environment that favours them by driving down the redox potential of sediments (Hunting and Kampfraath, 2013). This means isolating the individual factors is difficult. It was shown in studies of natural wetlands that the cycling or onset of anoxic conditions can induce release rates of P as high as 334 P mg/m^2/day (Olila, et al, 1997) Whilst these high levels are reported from natural wetlands impacted by P pollution, a greater pool of both Fe and P is present in chemically-dosed tertiary HF wetland sludge. The impact of higher

TP and Fe concentrations in sludge on the transformation processes that occur here is poorly understood.

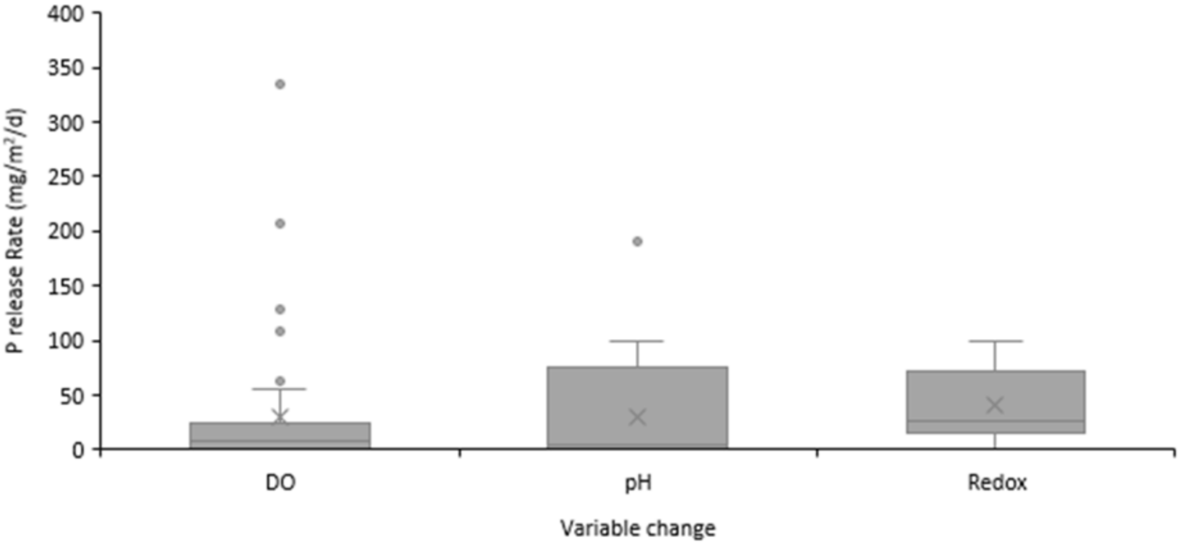


Figure 1-22 Phosphorus release rates attributed in the literature to various natural factors within the natural environment. The boxes show the interquartile range, whilst the median value is the mean release rate and cross is the mean release rate. The circles show anomalies and the whiskers are the outliers. The data points for this are taken from the table in supplementary information. The sample size was 70, 19 and 6 respectively

Within natural environments there is a well-accepted and defined link between the iron and phosphorus cycle (Jahnke, 1992). Due to the high affinity of Fe for complexing to P, Fe-P complexes are commonly found in nature. When the solubility of Fe increases due to changes in environmental conditions, P release coincides with it (Huang et al., 2005; Rothe, et al ., 2016). The cycling of Fe is primarily linked to its switch from insoluble (Fe^{+3}) to soluble (Fe^{2+}) species and vice versa (O'Connor, et al., 1971). This can be either microbially-mediated, with a large group of microorganisms known as iron reducing bacteria (FeRB) involved in the former and iron oxidising (FeOB) bacteria being involved in the latter, or chemically induced. Species from both of these bacterial groups have been reported in CW for wastewater treatment (Adrados et al., 2014) however, there have been no studies into their presence or abundance in CWs following adaptation for nutrient removal. Understanding the relationship between bacterial group presence and Fe and P cycling would enable the identification of risk of release and allow improved management of sites implementing Fe dosing for the removal of P. This would facilitate the continuation of robust and reliable treatment of domestic wastewater.

Based on knowledge from natural systems, a theoretical model was built for the surface sludge layer of tertiary HF wetlands adapted for chemically assisted removal of P (Figure 1-3). Significant knowledge gaps identified were the presence and activity of iron reducing bacteria found in the sludge and its relevance to the risk and management of P and Fe release from these systems. Understanding these is the basis for this research work.

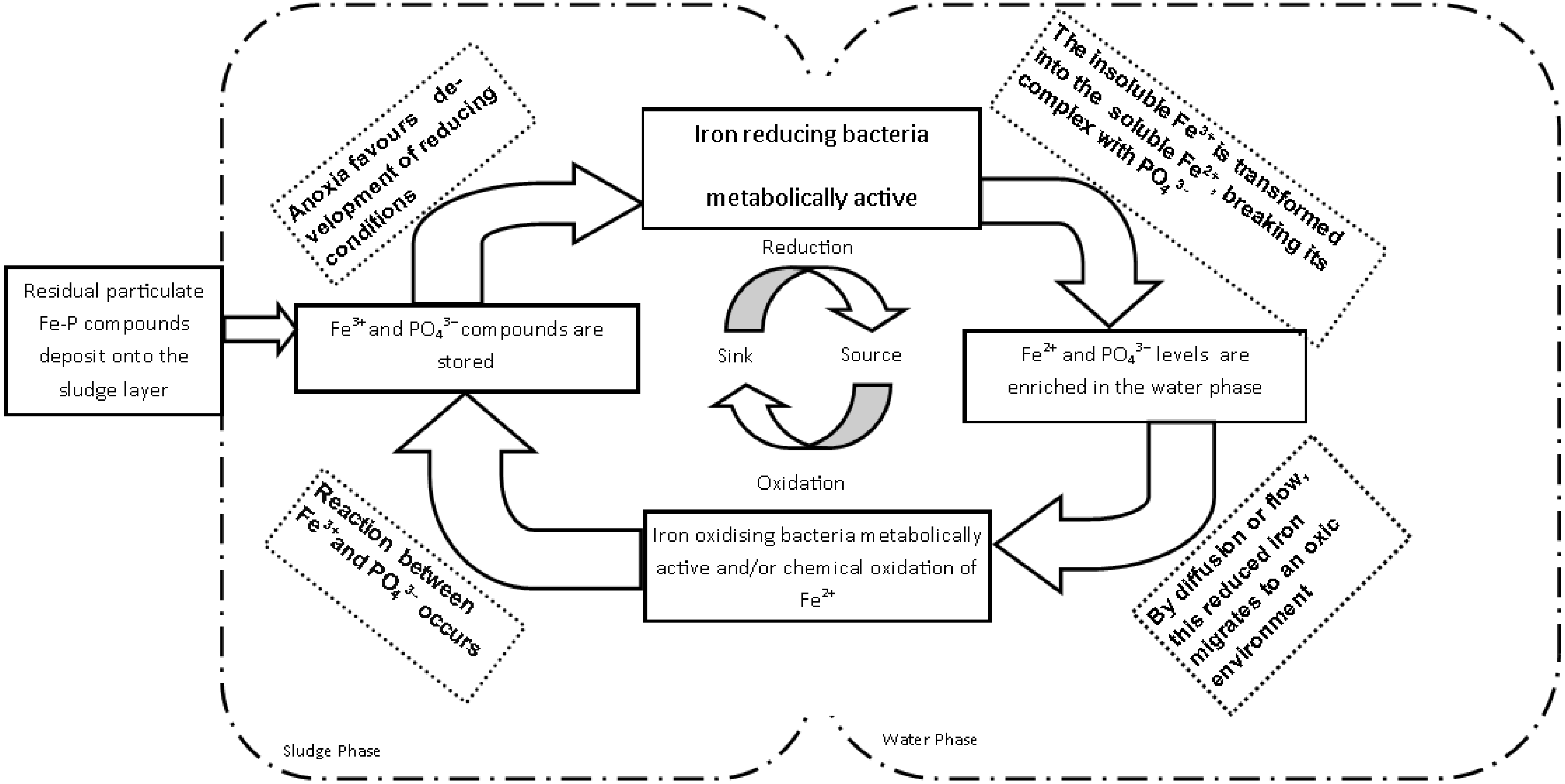


Figure 1-33 Theoretical model of P and Fe transformations in the surface sludge layer of a HF CW with chemical dosing for P removal

[redacted] Aims and objectives

This overall thesis aim is to understand the risk, mechanisms, and appropriate management options of iron and phosphorus release from constructed wetlands treating wastewater that has undergone chemical phosphorus removal.

To fulfil this aim, four objectives were defined:

1) Assess the robustness of tertiary treatment CWs adapted for nutrient removal to meet current and potential future standards;
2) Determine the qualitative and quantitative changes induced in bacterial communities in the surface sludge layer of tertiary HF CW adapted for nutrient removal;
3) Investigate the conditions and associated potential pathways for iron and phosphorus release from tertiary HF CW adapted for P removal;
4) Establish the iron release rates from solid phase ferric iron compounds metabolised by a pure culture of iron reducing bacteria found in adapted HF CWs and determine the impact of nitrate addition on observed iron transformations.

[redacted] Thesis structure

This thesis was structured so that each chapter is written as a scientific paper, following formatting guidelines of the journal "Water Research", with an extended introduction to cover the theoretical background and context of the work. The discussion chapter considers the implications of the findings for the operation of HF CWs adapted for tertiary removal of particulate P and Fe.

All sections of this thesis were written by the first author, Lloyd Thompson, with supervisory comments by Dr. Gabriela Dotro, Dr. Marc Pidou, and Professor Bruce Jefferson. Industrial supervision from Severn Trent Water Plc. was provided by Mr. Pete Vale and Dr. Yadira Bajon Fernandez. Additional specialist guidance was provided by Dr. Lars Duester for Chapter 5 and Dr. Boyd McKew and Dr. Fred Coulon for Chapter 3. Whilst all laboratory work was undertaken by

Lloyd Thompson, the bacterial analysis in Chapter 3 was conducted by Dr. Boyd McKew's laboratory.

Chapter 2, entitled "Constructed wetland adaptation: Impact on robustness to future demands", uses long term final effluent records from 37 sites owned and operated by Severn Trent to evaluate the robustness to tightening consents on a variety of pollutants (BOD, TSS, NH_4-N, TP).

Chapter 3, entitled "Impact of tertiary constructed wetland adaptation on microbial communities", is a survey of 17 full scale operational sites, where chemical characterisation and high throughput sequencing of the bacteria from the surface sludge of HF CWs adapted for nutrient removal was conducted to investigate the impacts that CW adaptation has on the bacterial communities of these enriched pools of Fe and P.

Chapter 4, entitled "Release of iron from solid phase species when metabolised by Geobacter metallireducens", is a pure culture study to investigate whether bacteria identified during the full-scale survey could be responsible for the observed Fe and P cycling in adapted tertiary HF wetlands.

Chapter 4, entitled "Profiling iron and associated phosphorus dynamics in treatment wetland sludge under different biogeochemical conditions", investigates the impact of alternative terminal electron acceptor addition on Fe and P cycling from an adapted tertiary HF CWs.

Chapter 6 is a discussion of the implications of the scientific research, in particular, the management of biogeochemical conditions within the sludge of adapted sites.

Chapter 7 summarises the key conclusions, linked to the main aim and objectives of this work.

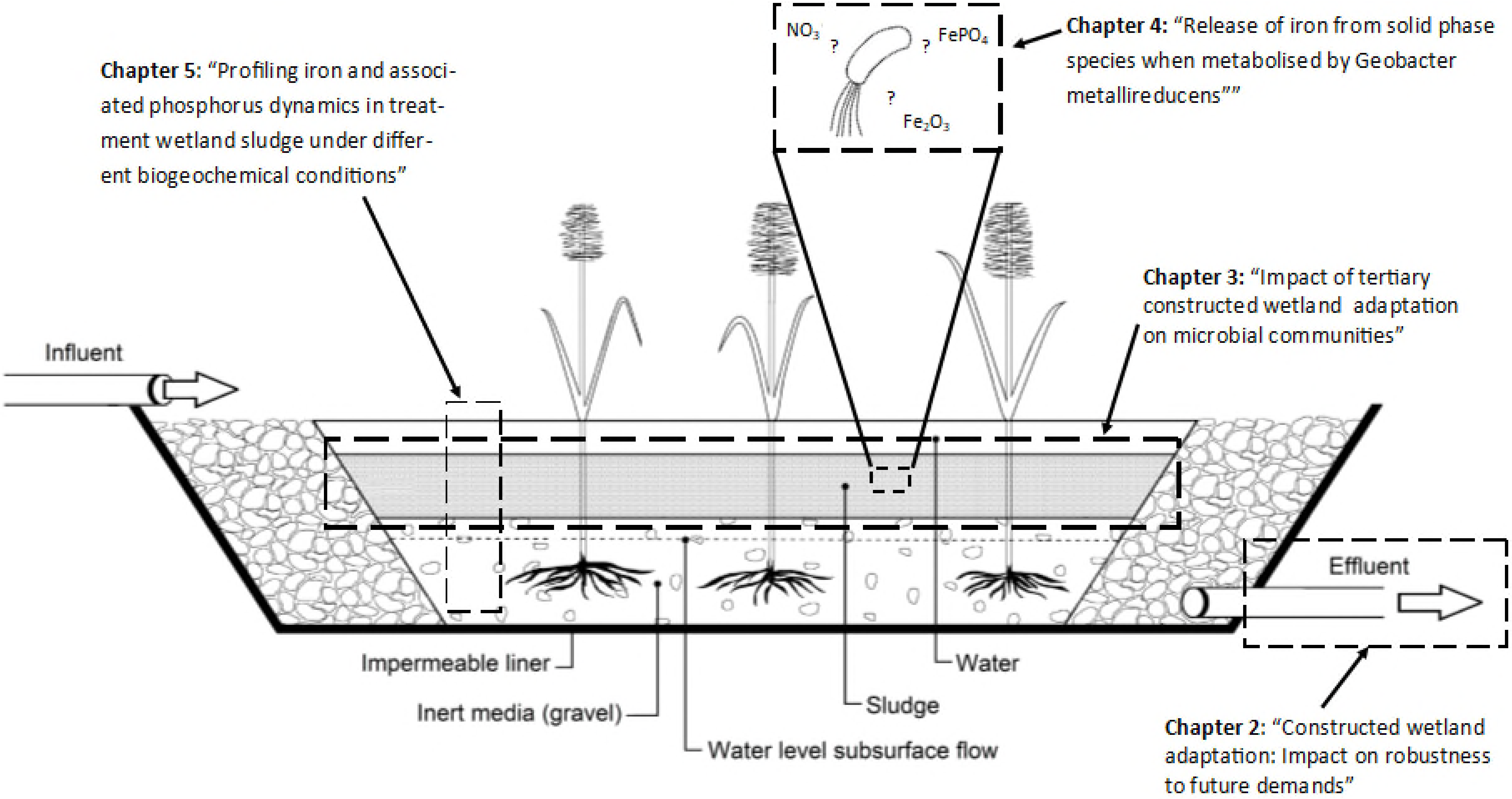
The contents and how each chapter contributes to fulfilling the proposed objectives are summarised in Table 1-11 and illustrated in Figure 1-44.

Table 1-11: Thesis plan

Chapter	Title	Chapter aim	Objectives	Hypothesis	Chapter interaction
1	Introduction	Provide a clear background of the thesis aim and objectives	Provide regulatory drivers for the research work Identify gaps in scientific understanding regarding the use of adapted CWs	n/a	Evaluates the current knowledge of iron and phosphorus release and the potential for this to occur within adapted constructed wetlands. This is used to inform the underlying hypothesis and experimental plans of every chapter
2	Constructed wetland adaptation: Impact on robustness to future demands	To show the impact on performance of CW adaptation for nutrient removal and investigate the resilience of these systems to changes in future consents.	Quantify the robustness of CWs across a range of consented pollutants Investigate the impact of adaptation on CW's resilience to tightening consent levels	Adaptation improves the performance of CWs against current and future consents	Allows the identification of sites potentially suffering P and Fe release, influencing the selection of site types in Chapter 3

Chapter	Title	Chapter aim	Objectives	Hypothesis	Chapter interaction
3	Impact of tertiary constructed wetland adaptation on microbial communities	To assess the impact of adaptation on the abundance, diversity and structure of microbial communities found in the sludge layer of horizontal flow CWs	Quantify bacterial levels found in wetlands, identifying any impacts caused by CW adaptation Quantify and understand any impacts of CW adaptation on microbially communities Identify the risk of phosphorus or iron release from CW based on iron reducing bacteria, especially those that have been adapted	The adaptation of CWs introduces a new biogeochemical environment to the sludge layer, significantly altering the microbial community structure	1) Reports on potential for CW adaptation to open the pathways for release identified within Chapter 1. 2) Explores variation in robustness between two sites operating in the same way
4	Release of iron from solid phase species when metabolised by Geobacter metallireducens	To provide a mechanism of iron and or phosphorus release from sediments	Show that iron can be released from a variety of solid Fe forms by a bacterium ubiquitous in constructed wetlands Investigate the potential for nitrate addition to supress iron and/or phosphorus release from the sludge found at constructed wetlands	Fe metabolism is less preferred when compared to nitrate utilisation, with Fe oxide species favoured over iron phosphate	1) Demonstrate the bacteria found in Chapter 3 can cause a release of Fe and P from CWs. 2) Tests a potential inhibition of Fe metabolism with an alternative electron acceptor to be tested with CW sludge in Chapter 5

Chapter	Title	Chapter aim	Objectives	Hypothesis	Chapter interaction
5	Profiling iron and associated phosphorus dynamics in treatment wetland sludge under different biogeochemical conditions	To investigate the impact of anaerobic incubation of surface sludge from adapted CW on Fe and P release, and test nitrate as a mitigation strategy	Characterise Fe and P release profiles from adapted CWs Demonstrate the ability of nitrate to supress Fe and P release	The microbial preference for nitrate that supresses the iron reduction pathway observed in Geobacter metallireducens pure cultures translates to the microbially diverse surface sludge layer environments of adapted CWs	Demonstrates the pathways identified in the pure culture study of Chapter 4 are transferable to biologically active surface sludge from a full-scale CWs
6	Implications of the work	To translate the scientific contribution to adapted CW management implications	Summarise the contribution to scientific knowledge arising from this thesis Translate findings into impact	N/A	Integrates the findings from each chapter into a coherent story and translates it into practical outputs
7	Conclusion	To provide an overall summary of the findings	Summarise the outputs from each objective addressed in this work Provide recommendations for further study	N/A	N/A



Chapter 5: “Profiling iron and associated phosphorus dynamics in treatment wetland sludge under different biogeochemical conditions”

Chapter 4: “Release of iron from solid phase species when metabolised by Geobacter metallireducens””

Chapter 3: “Impact of tertiary constructed wetland adaptation on microbial communities”

Chapter 2: “Constructed wetland adaptation: Impact on robustness to future demands”

Figure 1-44 A graphical representation of the research areas covered in each chapter of this thesis

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[redacted]Supplementary Information

Table S 1-11 A detailed list of previously recorded phosphorus release from the natural environment

Factor	Environment	Rate (mg/m2/d)	Reference
bacterial activity	Lake	0.0032	(Bates and Neafus, 1980)
biological (organic matter loading)	Lake	2	(Wang et al., 2008)
biological (organic matter loading)	Lake	1.5	(Wang *et al*., 2008)
biological (organic matter loading)	Lake	0.3	(Wang *et al*., 2008)
DO	Lake	10.3	(Appan and Ting, 1996)
DO	Lake	17	(Gomez et al., 1999)
DO	Lake	1.77	(Sen et al., 2007)
DO	Lake	0.89	(Sen et al., 2007)

Factor	Environment	Rate (mg/m2/d)	Reference
DO	lake	0.93	Wu *et al*., 2014)
DO	Lake (anoxic)	7.19	(Andersen and Ring, 1999)
DO	Lake (anoxic)	9.78	(Andersen and Ring, 1999)
DO	Lake (anoxic)	1.25	(Hoverson, 2008)
DO	Lake (anoxic)	12.3	(Moore *et al*., 1998)
DO	Lake (Eutrophic)	7	(Nürnberg, 1994)
DO	Lake (high DO)	0.0032	(Bates and Neafus, 1980)
DO	Lake (hypertrophic)	22	(Nürnberg, 1994)
DO	Lake (Low DO)	0.016	(Bates and Neafus, 1980)
DO	Lake (mesotrophic)	3	(Nürnberg, 1994)
DO	Lake (oligotrophic)	0.1	(Nürnberg, 1994)
DO	Lake (oxic)	1.27	(Andersen and Ring, 1999)
DO	Lake (oxic)	2.48	(Andersen and Ring, 1999)
DO	Lake (oxic)	0.25	(Hoverson, 2008)
DO	Lake (oxic)	5.79	(Moore, Reddy and Fisher, 1998)
DO	Marsh	38	(Lai and Lam, 2008)
DO	Peat soils	7.6	(Kjaergaard et al., 2012)
DO	Peat soils	11	(Kjaergaard et al., 2012)
DO	River	29.3	(Garban et al., 1995)
DO	River (anoxic)	15	(Kim, Choi and Stenstrom, 2003)
DO	River (anoxic)	31.7	(Lai and Lam, 2008)
DO	River (anoxic)	4.77	(Malecki, White and Reddy, 2004)
DO	River (oxic)	5.7	(Kim, Choi and Stenstrom, 2003)
DO	River (oxic)	9.4	(Lai and Lam, 2008)
DO	River (oxic)	0.13	(Malecki, White and Reddy, 2004)

Factor	Environment	Rate (mg/m2/d)	Reference
DO (Flooding)	agricultural soils	128	(Tanner, Clayton and Upsdell, 1995)
DO (Flooding)	fen sediments	52.3	(Zak et al., 2010)
DO (Flooding)	flood plains	9.9	(Banach et al., 2009)
DO (Flooding)	Marsh (flood)	109	(Corstanje and Reddy, 2004)
DO (Flooding)	Marsh (flood)	334	(Olila, Reddy and Stiles, 1997)
DO (Flooding)	Marsh (no flood)	0.7	(Corstanje and Reddy, 2004)
DO (Flooding)	Marsh (no flood)	7.6	(Olila, Reddy and Stiles, 1997)
DO (Flooding)	Organic solids	61.9	(Reddy, 1983)
DO (Flooding)	Peat soils	1.4	(Forsmann and Kjaergaard, 2014)
DO (Flooding)	Peat soils	2.9	(Forsmann and Kjaergaard, 2014)
DO (Flooding)	Peat soils	0.1	(Forsmann and Kjaergaard, 2014)
DO (seasonal)	Lake	15	(Selig and Schlungbaum, 2003)
DO (seasonal)	Lake	207	(Selig and Schlungbaum, 2003)
DO (seasonal)	Lake	22	(Selig and Schlungbaum, 2003)
DO (seasonal)	Lake	55	(Selig and Schlungbaum, 2003)
dredging	lake	0.21	(Yu et al., 2017)
flooding (DO)	restored wetland	0.025	(Aldous et al., 2007)
flooding (DO)	restored wetland	0.46	(Aldous et al., 2007)
flooding (DO)	restored wetland	0.13	(Aldous et al., 2007)
flooding (DO)	restored wetland	0.15	(Aldous et al., 2007)
Flow rate	Sands	5.8	(Kjaergaard et al., 2012)
Flow rate	Sands	24	(Kjaergaard et al., 2012)
harvesting	blanket peat	0.64	(Rodgers et al., 2010)
p equilibrium	lake	17	(Zhang et al., 2016)

Factor	Environment	Rate (mg/m2/d)	Reference
p equilibrium	lake	11	(Zhang et al., 2016)
pH	Lake	20	(Andersen, 1975)
pH	Lake	80	(Andersen, 1975)
pH	Lake	7.6	(Appan and Ting, 1996)
pH	Lake	4	(Boers, 1991)
pH	Lake	0.15	(Boström and Pettersson, 1982)
pH	Lake	75	(Drake, J.C., Heaney, S.I., 1987)
pH	Lake	2	(Drake, J.C., Heaney, S.I., 1987)
pH	Lake	75	(Drake, J.C., Heaney, S.I., 1987)
pH	Lake	204	(Gaoa, 2012)
pH	Lake	159	(Gaoa, 2012)
pH	Lake	190	(Gaoa, 2012)
pH	Lake	0.011	(Huang et al., 2005)
pH	Lake	4	(James and Barko, 1991)
pH	Lake	3.6	(James and Barko, 1991)
pH	lake	100	(Wu et al., 2014)
pH	lake	83.8	(Zhang et al., 2016)
pH and DO	Lake	14.8	(Appan and Ting, 1996)
pH/DO	Lake	1.5	(Christophoridis and Fytianos, 2006)
pH/DO	Lake	1	(Christophoridis and Fytianos, 2006)
pH/DO	Lake	0.7	(Christophoridis and Fytianos, 2006)
pH/DO	Lake	2	(Christophoridis and Fytianos, 2006)

Factor	Environment	Rate (mg/m2/d)	Reference
redox (dissolving Fe)	lake (high redox)	0.04	(Parker and Beck, 2003)
redox (dissolving Fe)	lake (low redox)	0.67	(Parker and Beck, 2003)
Redox (flooding)	freshwater ecosystem	16.5	(Kinsman-Costello, O'Brien and Hamilton, 2014)
Redox (seasonal change)	Lake	0.56	(Selig and Schlungbaum, 2003)
Resuspension	Lake	70	(Søndergaard, Jensen and Jeppesen, 2003)
sediment disturbance	aquatic sediments	72	(Dunn et al., 2017)
Temperature	Lake	5	(Boers and van Hese, 1988)
Temperature	Lake	0.7	(Wan, Jonasson and Bi, 2011)
Temperature	Lake	80	(Zhang et al., 2016)
Temperature	Lake	0.33	(Wu *et al*., 2014)
Temperature	Lake (10 degrees)	2.6	(Kelderman, 1984)
Temperature	Lake (15 degrees)	7	(Kelderman, 1984)
Temperature	Lake (20 degrees)	11	(Kelderman, 1984)
Temperature	Lake (5 degrees)	0.4	(Kelderman, 1984)
Temperature	River	14.3	(Kim, Choi and Stenstrom, 2003)
Redox (seasonal change)	Lake	15	(Jensen and Andersen, 1992)
Redox (seasonal change)	Lake	21	(Jensen and Andersen, 1992)
Redox (seasonal change)	Lake	33	(Jensen and Andersen, 1992)
Redox (seasonal change)	Lake	100	(Jensen and Andersen, 1992)

2 Constructed wetland adaptation: Impact on robustness to future demands

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[redacted] Abstract

Horizontal flow constructed wetlands (CWs) are utilised in the UK for tertiary treatment. Recent requirements to improve nutrient removal on sites with CWs has seen the adaptation of this technology. For the removal of ammonia this has been the inclusion of aeration systems, whereas for phosphorus, the sites have introduced chemical dosing upstream of the CW. The current paper explores the impact of the adaptations on the robustness of effluent quality through the analysis of long term discharge data on 37 treatment works. The sites have included those with no adaptation (16), iron dosed (15), aerated (4) and both adaptations (iron dosed and aerated) (2). Overall, the analysis revealed that the adaptation enhanced robustness both in terms of the specific target compound and for the other discharge parameters. In the case of iron dosed sites, enhanced robustness was also observed with regards to biochemical oxygen demand and suspended solids as well as phosphorus.

Keywords: 1) Treatment Performance 2) iron dosing 3) aeration 4) phosphorus control 5) ammonia control

[redacted] Introduction

Constructed Wetlands (CWs) are a near passive treatment system that utilizes natural processes within an engineered environment (Dotro et al., 2015). The technology can be configured in a variety of forms with regards to flow regime and size with most systems used for wastewater treatment in the UK configured as subsurface horizontal flow CWs. The predominant removal mechanisms in these systems are biological filtration under anoxic or anaerobic conditions and granular filtration. Accordingly, subsurface horizontal flow CW exhibit strong efficacy towards suspended solids (SS), organic (BOD) and pathogen reduction (Dotro *et al*, 2017). However, increasingly, additional requirements are being placed on the technology with regards to nutrient removal, mainly in regard to ammonia and total phosphorus (TP). Delivery of consistent nutrient removal thus requires adaptation of the original technology to provide the required removal pathways for nutrient removal.

Phosphorus removal in traditionally configured CWs is restricted as, whilst most particulate phosphorus can be removed by physical separation, the majority of phosphorus entering a CW is dissolved (Barak, 2014). A proportion is adsorbed onto the media (sand/gravel) but the uptake capacity of such materials is limited such that traditional CWs are not able to meet long term demands of phosphorus removal (Vymazal, 2004). In such cases, CWs can be adapted by replacing the standard media with reactive alternatives such as steel slag or apatite (Letshwenyo, 2014). However, operational concerns related to an associated pH increase and the long-term sustainability of removal means they are not yet considered a viable option (Jefferson, 2018). The alternative is to pre-precipitate the dissolved phosphorous with a metal precipitating agent (coagulant) and then utilise the physical separation capability of the CW. Suitable metals include calcium and aluminium, whilst within the UK iron is predominately used due to its improved selectivity toward phosphorus in complex matrices compared to the other metals (Dotro et al., 2015; Thistleton et al., 2002). Coagulant dosing is the adopted standard and has been shown to provide a robust and reliable method of phosphorus removal (Dotro et al., 2015). Recent work has however indicated a risk of release of iron and phosphorus from the accumulating sludge (Chapter 5).

The removal of ammonia occurs through biological utilisation under aerobic conditions (nitrification) and hence requires availability of dissolved oxygen to drive sufficient biological activity. Traditional horizontal subsurface flow CWs rarely have sufficient DO and hence the nitrification rate is inhibited (Butterworth *et al*., 2013). Consequently, reliable nitrification in CWs requires adaptation to include aeration, typically in the form of a coarse bubble aeration pipe at the bottom of the media bed fed by a blower. This has been shown to provide a reliable and robust treatment of ammonia enabling delivery of ammonia discharges equivalent to well operating activated sludge systems (Butterworth et al., 2016b).

The ability to adapt CWs to enable nutrient removal offers an opportunity to future proof wastewater treatment with minimal additional asset investment whilst delivering against tightening consents. However, the basic function of the CWs has been changed and raises questions as to whether the consistency and reliability of the effluent discharge is maintained for all water quality determinants. Such consideration connects to the general concepts of robustness and resilience which are more commonly applied to ecological systems (Janssen et al., 2007). There is a paucity of translation to treatment technology assessment, especially in relation to predicting future capability. However examples exist for depth filters in drinking water treatment (Hartshorn et al., 2015; Huck and Coffey, 2004; Li and Huck, 2007), clarifiers (Hurst et al., 2004), rotating biologcial contactors (RBCs) (Freeman, 2016) and aerated CWs (Butterworth *et al.*, 2013; 2016b). Whilst a number of definitions are used within the literature, the most commonly associated to treatment technology are that robustness relates to the consistency of the output against a variable input and resilience relates to the ability of a technology to recover from a perturbation that has caused a deterioration in the output (Sara and Mohammad, 2009; Upton et al., 2017; Zakarian et al., 2007). Consideration of robustness has led to a number of performance indices that are easy to calculate and utilise from regularly measured output data (Hartshorn et al., 2015). These include the robustness index, risk area profile and golden measures (equation 2-1; Butterworth et al., 2016b; Freeman, 2016; Upton et al., 2017). The use of the indices have been reported to enable simple management of large data sets generating a good decision tool. However, analysis of the indices has identified some weaknesses such as the robustness index being heavily dependent on the goal target and the percentile levels chosen (Huck and Coffey, 2004). This is, in part, resolved by relatively weighting the different components of the robustness index but does not indicate what happens either side of the goal target. Improved insight in this regard has been reported through calculating the area of the curve beyond the goal target which provides a combination of risk and robustness and so has been suggested as a better assessment when thinking about delivery of a set water quality goal (Hartshorn et al., 2015). The current study builds on this base and provides the first assessment of the robustness of tertiary treatment CWs with particular emphasis on the impacts of adaption to nutrient removal to meet current and predicted future standards.

[redacted] Materials and Methodology

2.3.1 Data gathering

Routine compliance sampling data was analysed from 37 wastewater treatment works within the midlands of England, that contained tertiary subsurface horizontal flow CWs over a period between 1993 and 2018. All CWs were based on a standard design sizing of 0.7 m^2/P.E. and a hydraulic loading rate of 200 mm/d. The beds were 0.6 m deep and filled with 10-15 mm gravel and planted with *Phragmites Australis* at a density of 4 plants/m^2. The residual reed material is not harvested and so accumulates on the bed. Water typically entered the CWs through a v notched trough and the water level is controlled through a swivel pipe. Detailed description is available in supporting information A, including the number of reed beds, overall sizes, daily flow rates, a partial refurbishment history, population equivalent serviced, consents applied to each site and from when, and long-term effluent data. A number of the beds had previously under gone adaptation for nutrient removal. Phosphorus adaption (n = 17 sites) involved the retro-fitting of a prior ferric sulphate coagulation stage, this coagulant is dosed either at the crude inlet stage or prior to the biological treatment upstream of the CW. Adaptation for ammonia removal (n = 6 sites) was accomplished by the site being refurbished (the total removal of the wetland, discarding everything except for the media which is washed and then put back), during which there is the addition of air diffusers along the base of the media. These diffusers are then operated either constantly or when anoxic environments are expected to be prevalent, thereby maintaining a constant oxic environment. The sites were grouped according to their adaptation into four groups: iron dosed, iron dosed and aerated, no adaptation and aerated (Table 2-11). The sites included a range of P.E. served and consents for SS, BOD, NH_4^+ and TP.

Table 2-11 Characteristics of the sites analysed in this study

Site characteristic	Iron Dosed	Iron Dosed and aerated	No adaptations	Aerated
Number of sites	15	2	16	4
P.E. served				
0<750	24%	100%	44%	75%
750<1500	35%	0%	38%	0%
1500<5000	41%	0%	19%	25%
TP Consent [P-mg/l]				
1	24%	0%	0%	0%
2	76%	100%	0%	0%
none	0%	0%	100%	100%
Fe consent [Fe-mg/l]				
4	25%	50%	0%	0%
8	75%	50%	0%	0%
none	0%	0%	100%	100%
Suspended solids consent [mg/l]				
20-30	22%	100%	24%	40%
31-40	28%	0%	24%	0%
40-55	50%	0%	52%	60%
Biochemical Oxygen Demand consent [BOD-mg/L]				
10-20	50%	0%	29%	60%
21-30	33%	100%	60%	20%
31-40	17%	0%	11%	20%
Ammonia consent [N-mg/L]				
0-5	55%	50%	29%	80%
6-10	20%	0%	24%	20%
11-15	5%	50%	12%	0%
16-20	20%	0%	0%	0%
None	0%	0%	35%	0%
Mean wetland sizing (plan area) [m^2/p.e.]	0.67	0.35	0.514	1.13

2.3.2 Robustness Performance Index (RPI) calculations

The distribution of the different concentration values was collated into cumulative frequency distributions from which the robustness index and the risk area percentage were calculated. The robustness index was calculated against a specified treatment goal (G) in relation to the percentile values at the 50th percentile (E_{50}), the 95th percentile (E_{95}), the percentile at the goal concentration (E_{goal}) and the percentage time spent below the goal (G%) in accordance to the work of Butterworth *et al.*, (2016a) and Freeman, (2016). Equation (2-11) represents the formula for this calculation.

$$\mathrm{RPI} = \left[\left(1 - \frac{\mathrm{G\%}}{100}\right) * \frac{\mathrm{E}_{95}}{\mathrm{E}_{50}}\right] + [\frac{\mathrm{E}_{50}}{\mathrm{E}_{\mathrm{goal}}} * \frac{\mathrm{G\%}}{100}] \qquad \textbf{(2-11)}$$

The above approach is acknowledged as an appropriate measure of the overall robustness of a technology but is sensitive to the goal target set. To reduce the impact of this, the current study follows the practice of Hartshorn *et al* (2015) by also determining the risk zone measurement which provides a more sensitive measure of the risk of exceeding the target goal.

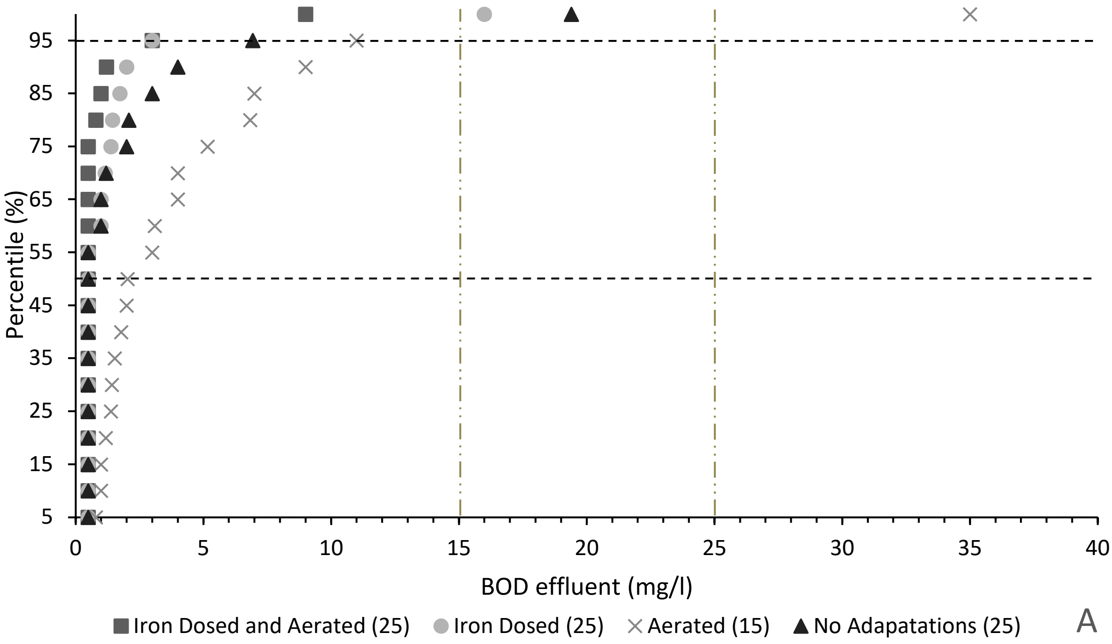
2.3.2.1 Statistical testing

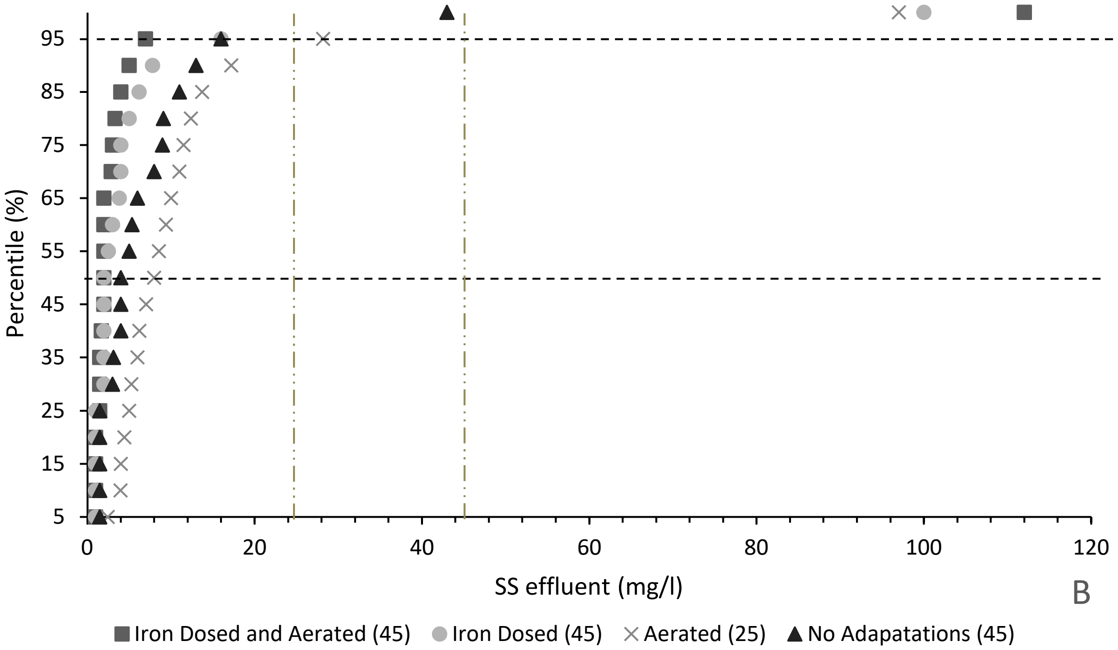
All statistical analysis was carried out using the software Minitab 17 (Minitab Inc, PA, USA). By using an Anderson-darling normality test, it was possible to show that none of the raw data was normally distributed, meaning that standard tests such as ANOVAs or T tests would not be valid. For this study, when analysing the differences in data between sites or site types, Kruskal-Wallis (KW) tests were used.

[redacted]Results and Discussion

2.4.1 Current robustness

All cumulative distribution curves (robustness curves) followed a similar pattern of an initial near vertical line followed by a tailing off at higher percentile values (Figure 2-11). The initial gradient and the inflexion point varied across the sites and the parameters measured with the greatest variation observed with regards to TP and the greatest similarity seen with SS. All sites met their compliance targets with generally 80% of the data at less than 50% of the consent value. The effluent profiles represent the overall impact of the whole works and so the observed robustness is generated through the design and operation of all the stages with BOD and ammonia removal being associated to both the main biological stage and the wetland and the SS and TP linked to both the clarifiers and the wetlands.





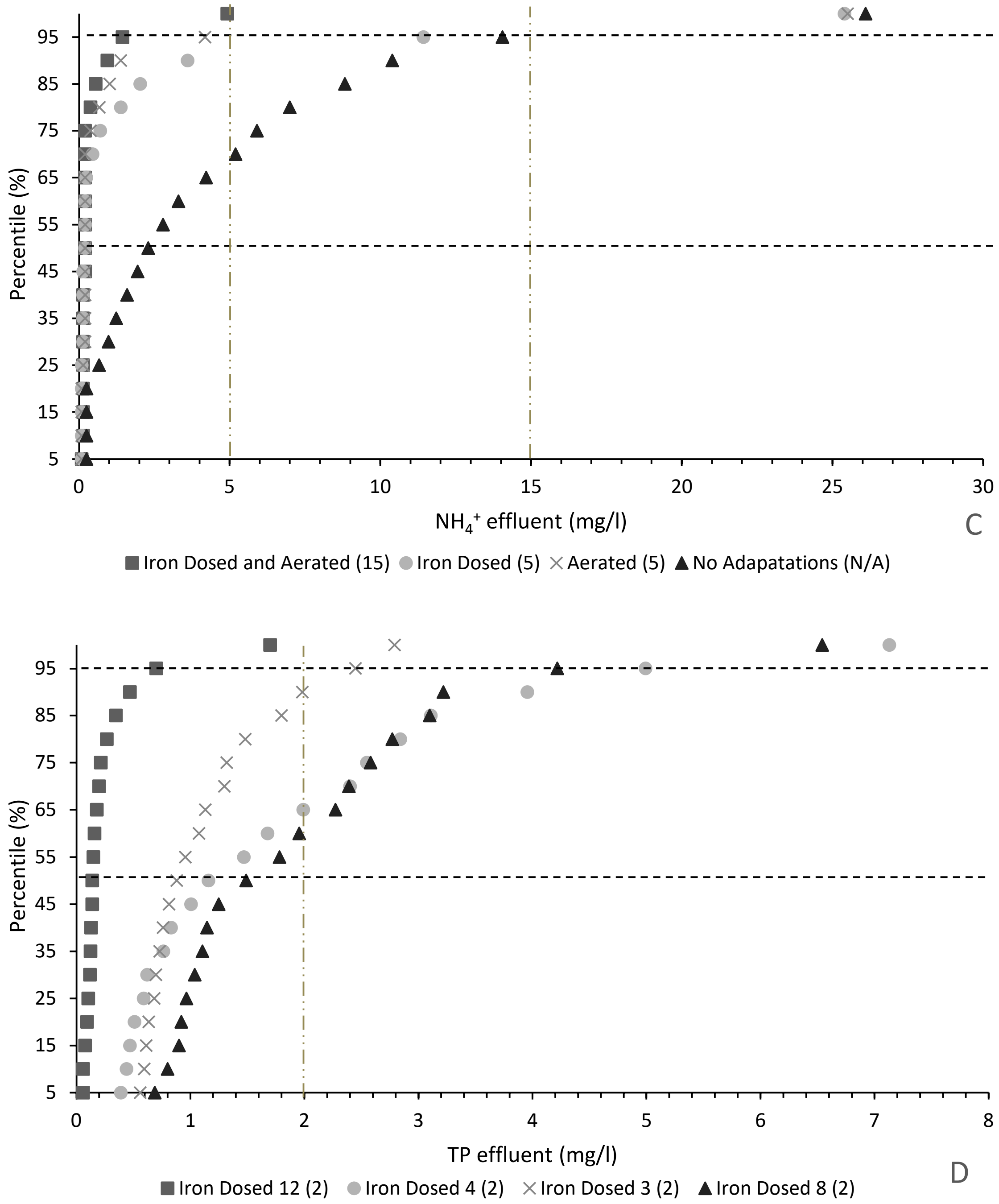


Figure 2-11 Effluent curves for representative sites for A) BOD, B) SS, C) NH_4^+, D) TP (only for a variety of Iron Dosed sites, as this group was the only group with reliable TP sampling). The effluent consents for each site is shown in the brackets, and demonstrated by the dashed lines.

In the case of BOD, the effluent data ranged with maximum values of 9, 16, 19 and 35 mg/L for the iron dosed and aerated, iron dosed, aerated and no adaption sites respectively (Figure 2-11A). The least robust system was identified as the aerated as it exhibited a much shallower gradient than the other systems. In contrast, the iron dosed systems exhibited profiles that are traditionally viewed as very robust with near vertical gradients for the majority of the distribution. Similar observations can be made with regards to SS but not nutrients removal. In the case of ammonia, the least robust system was the no adaption site with good performance for all the different adaptations. The robustness of the iron dosed system can be directly attributed to the upstream process due to the limited capacity for nitrification reported for unaerated CWs (Butterworth *et al.*, 2013). The requirement to remove phosphorus from these sites, is a recent change through the introduction of the Water Framework Directive (Dotro et al., 2015; UKTAG, 2013). Accordingly, TP is not routinely measured apart from sites with a TP consent. The effluent curves for TP were more distributed indicating a lower level of robustness compared to the other parameters. However, the TP consent target is set as an annual average and hence greater variability is possible whilst still meeting consent.

Comparison across all the sample sites is provided through analysis of the 95th percentile value obtained for the respective cumulative curves (Figure 2-22). For instance, the effectiveness of aeration in reducing residual ammonia can be seen by the reduced mean and range for the two aerated categories. To illustrate, the median values of the 95th percentile level across all sites within each category was 1.6 mg/L and 2.9 mg/L for the iron dosed and aerated and the aerated respectively. This compared to equivalent values of 14 mg/L for the no adaption sites and 5.1 mg/L for the iron dosed sites. In terms of BOD and SS, the no adaption and the iron dosed sites were observed to be statistically different from the other sites respectively as defined by a KW test ($p = <0.05$). In the case of BOD, the no adaption sites delivered a significantly higher 95th percentile values compared to the other sites with a median level of 13 mg/L compared to 5.1±0.5 mg/L. In contrast, comparison across all the iron dosed sites indicated a median value of 11 mg/L compared to 18 mg/L or above for all other category types. In part this is to be expected as the addition of coagulant will positively affect solids removal across the site in general and so have reduced solids loading rates to the CWs. Interestingly, this is not reflected in the iron dosed and aerated sites which in congruent with previously reported risks associated with operational water levels above the gravel when using aeration (Butterworth et al, 2016).

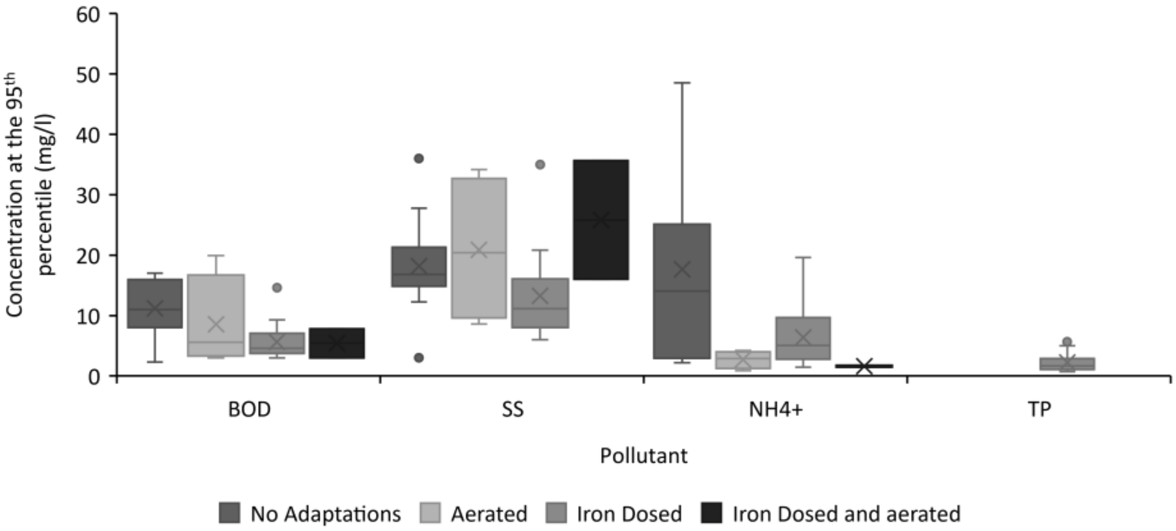


Figure 2-22 Box plot of the values of pollutant concentration at the 95th percentile for each site type. The cross represents the site types mean concentration, whilst the line is the median and the box is the interquartile range. The whiskers represent outliers whilst the circles are anomalies.

The overall impact is then related to the effluent consent through the robustness index (Figure 2-33). The distribution of RPI values for each category indicated that greatest overall robustness was observed in terms of SS for all types and iron dosed for all parameters. To illustrate, the mean RPIs for SS were 0.8, 0.2 and 0.6 for the no adaptions, iron dosed and aerated sites. In comparison, the equivalent mean RPI values for ammonia were 0.3, 0.6 and 1.1 and BOD were 3.2, 0.1 and 2.8. Typically, RPI values below 1-2 are indicative of highly robust systems where there is redundant capacity to meet tighter discharge consent without further adaption. Consequently, the CWs appeared more future proofed with regards to SS and ammonia than BOD and that the iron dosed sites offered the greatest potential to withstand tightening discharge standards without the need for asset investment.

The greater RPI scores identified for the aerated systems reflects the fact that these sites had the tightest compliance limits (Table 2-11) whereas those sites without adaptations had either no consent demands (and so were not considered for Figure 2-22), or were high enough that compliance failure was unlikely and/or had upstream nitrifying assets with sufficient capacity to ensure stable effluent performance. Further, the RPI value is known to be sensitive to the goal target and inherently increases when the target levels are very low (Hartshorn et al., 2015; Huck and Coffey, 2004).

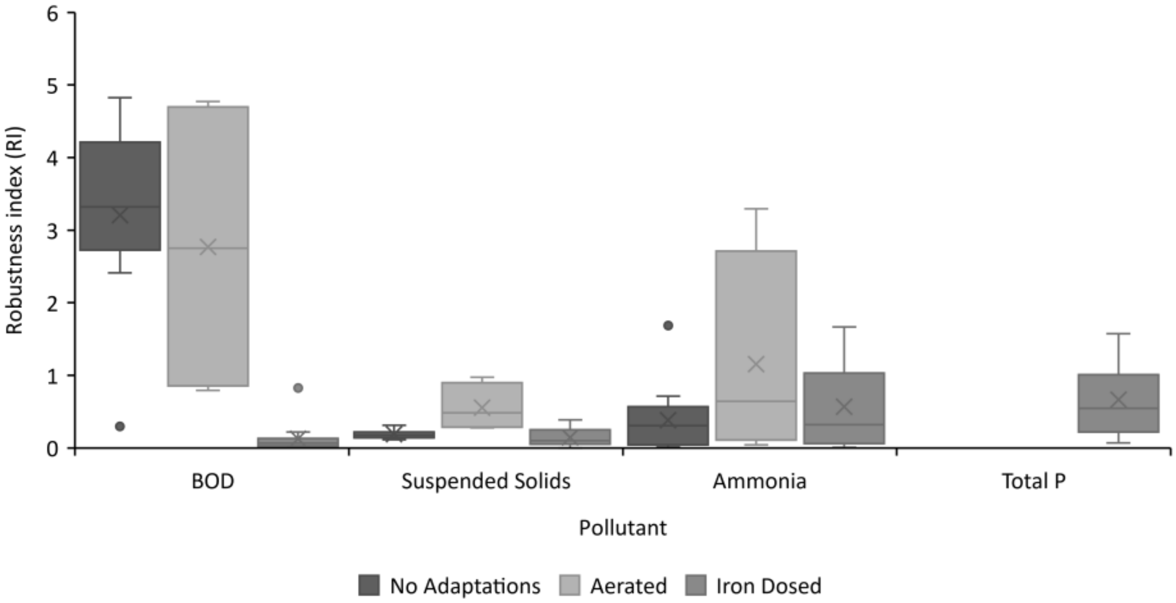
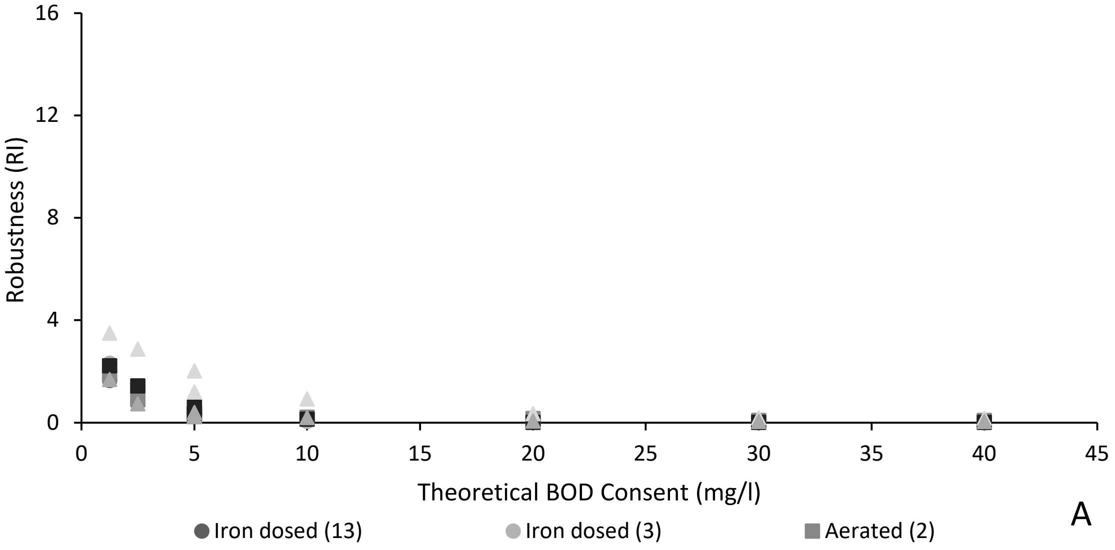


Figure 2-33 Robustness index for each site type at each site's independent consent level. The cross represents the site types mean robustness index, whilst the line is the median and the box is the interquartile range. The whiskers represent outliers whilst the circles are anomalies.

2.4.2 Impact of change of consent

To further consider the ability of the CWs to meet future compliance standards without further amendment the goal target was progressively reduced and the RPI recalculated (Figure 2-44). Across all sites, the greatest impact was seen when reducing the ammonia consent once the ammonia consent was reduced below 10 mg/L, except for the aerated sites were the systems remained robust to a consent level of 1 mg/l. This compares to the current consent levels of between 5 and 10 mg/l for all aerated sites, but as high as 16-20 mg/l for 20% of iron dosed sites, or not consented at all for 35% of sites with no adaptations (Table 2-11). Across all sites, greater robustness was observed with decreasing consent level with regards to SS and BOD were the systems remained robust down to effluent consent levels of 10 mg/l for SS and 5 mg/l for BOD as defined by all sites being below an RPI of 2, the upper RPI threshold to define a robust system. No significant difference could be observed as a function of the category type especially in terms of aerated and iron dosed showing both to be effective adaptions as a response to tightening consents.


16
12
8
4
0
Robustness (RI)
0
5
10
15
20
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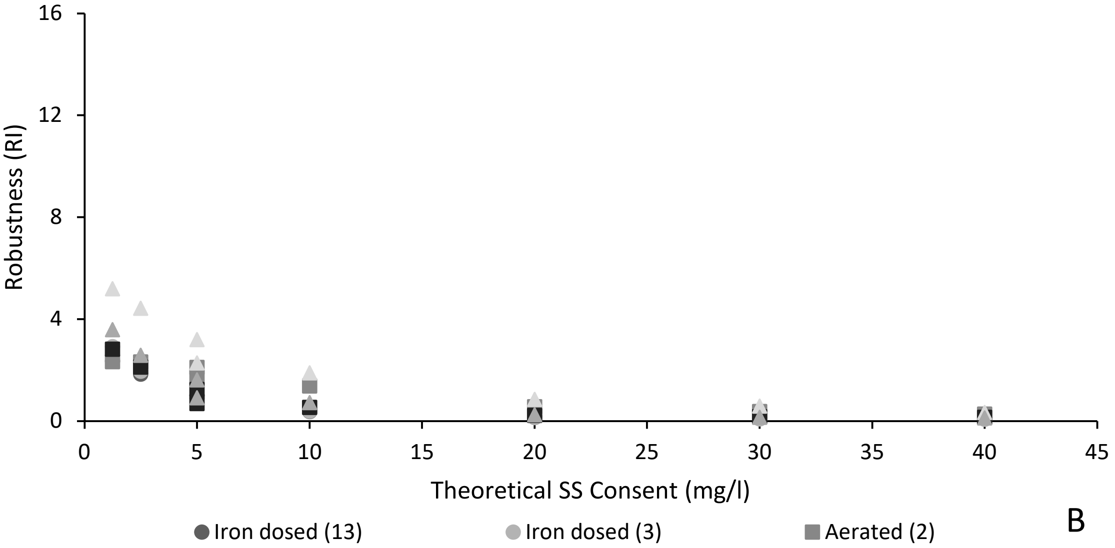
Theoretical BOD Consent (mg/l)
● Iron dosed (13)
● Iron dosed (3)
■ Aerated (2)
A


16
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Robustness (RI)
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Theoretical SS Consent (mg/l)
● Iron dosed (13)
● Iron dosed (3)
■ Aerated (2)
B

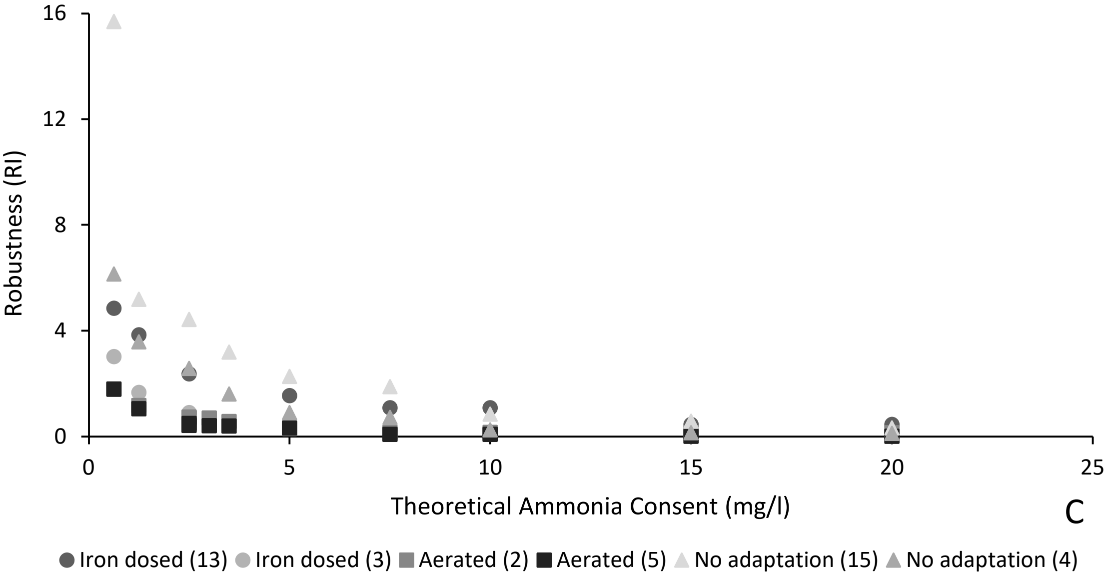
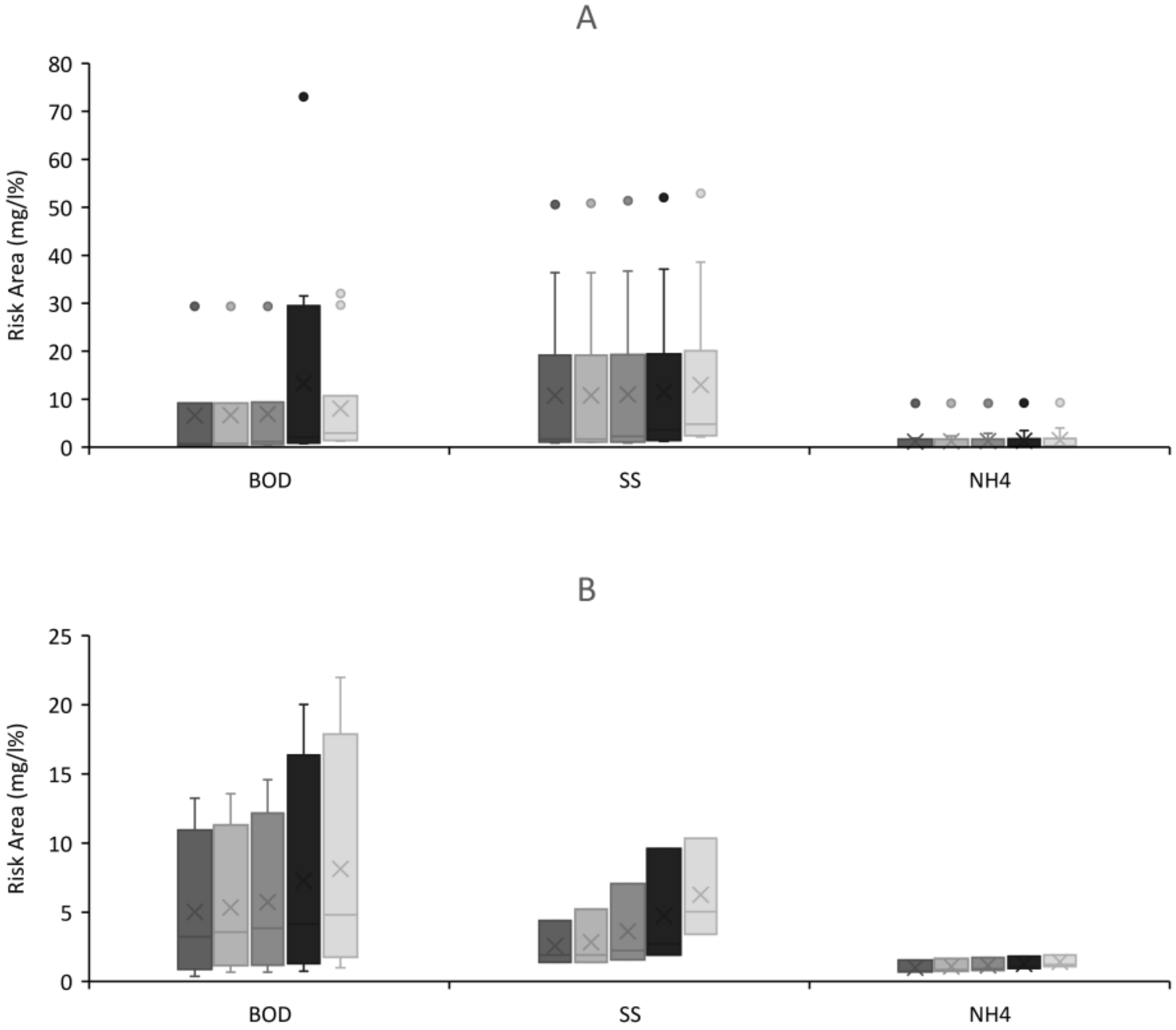


Figure 2-44 Robustness curves for theoretical consent levels for A) BOD, B) SS and C) ammonia. The site number within each group is presented in the brackets.

The RPI analysis indicated that the impact of a reduced consent appeared insensitive to the initial consent contrary to expectations. It was anticipated that the different sites would have a RPI that deteriorated in a proportional manner relative to their initial consent. To further examine this, the risk area for each site as a function of the percentage reduction of the current consent was calculated. The percentage reduction considered for the risk area calculations was 20% (4/5th of current consent), 40% (3/5th of current consent), 60% (2/5th of current consent), and 80% (1/5th of current consent). Analysis of the risk area indicated much clearer differences between the different categories of CWs and the parameters. The greatest impact on the risk area was observed with regards to the aerated sites in regards to BOD and SS but not ammonia. This is illustrated by comparing the mean risk area reported by the different categories for the reduction to a consent value that is 1/5th of the original value. In the case of the aerated sites the risk area was 4.8, 5.0 and 1.2 for BOD, SS and ammonia. This compares to equivalent values for 2.9, 4.8 and 0.3 for the un adapted sites and 0.4, 1. And 1.1 in the case of the iron dosed sites. The other area of impact is in regards to the SS for the iron dosed sites where the mean risk area increased from 0 to 1.2 as the consent value reduced from current to 1/5th of the value. Comparison with regards to TP for the dosed sites revealed consistently less risk as illustrated by the change in mean risk area from 0.14 to 0.68. A similar response is seen with regards to ammonia for the aerated sites and demonstrates the positive impacts of the adaptations on towards their selected targets.



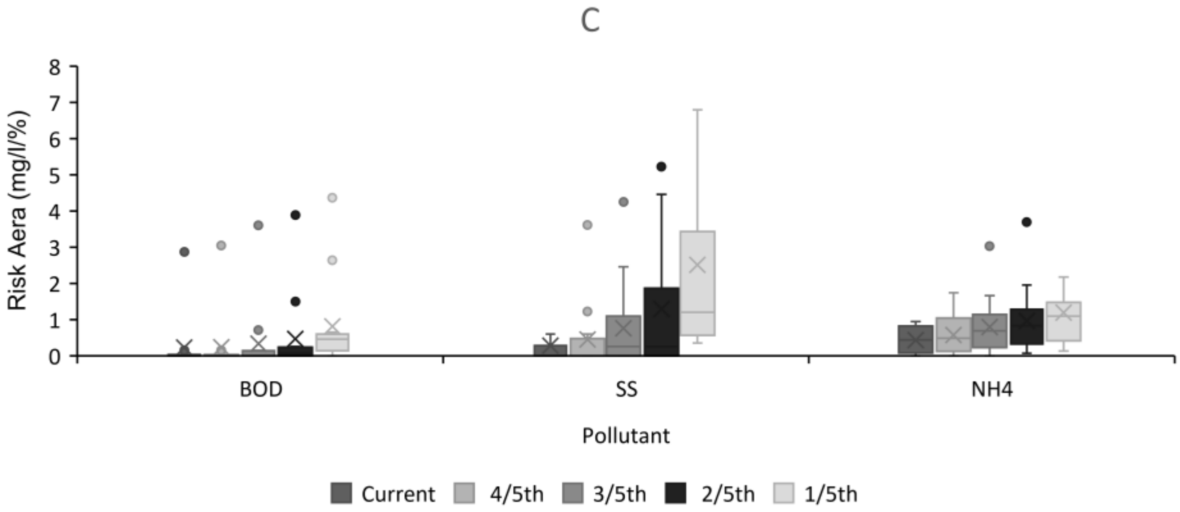


Figure 2-55 The risk area changes seen by decreasing consent levels by to 4/5th, 3/5th, 2/5th and 1/5th of their current values for A) sites with no adaptations, B) Sites that were aerated, C) and sites that had an iron dosing stage. The cross represents the site types mean robustness index, whilst the line is the median and the box is the interquartile range. The whiskers represent outliers whilst the circles are anomalies.

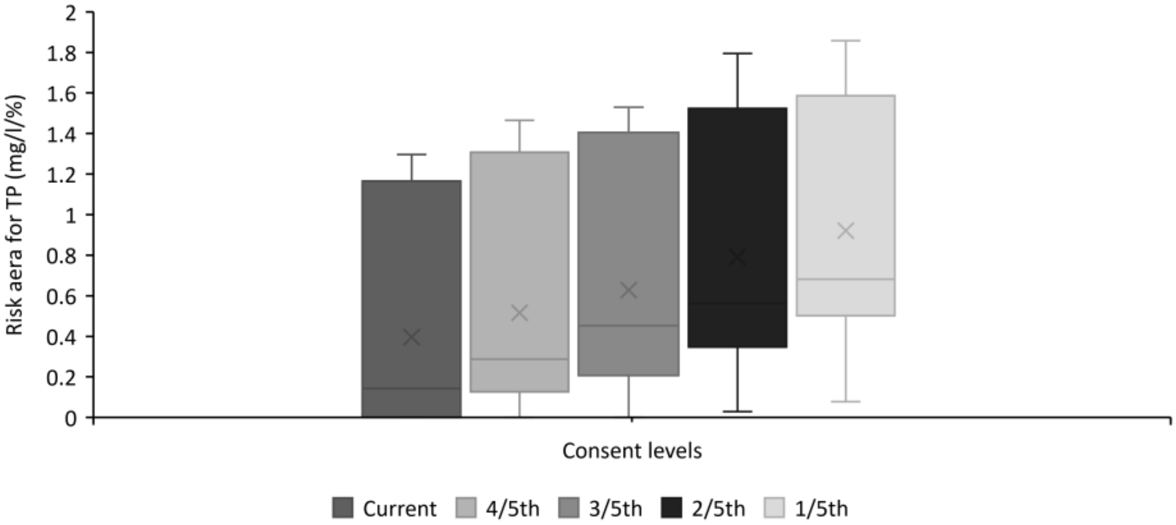


Figure 2-66 The values of risk area for iron dosed sites with regards to phosphorus as consents become tighter. The cross represents the site types mean risk area, whilst the line is the median and the box is the interquartile range. The few whiskers represent outliers whilst the few circles are anomalies.

2.4.3 Impact of adaptation

The previous analysis suggested a variability in the effluent profiles of CW site that had undergone the same adaptations (Figure 2-44). To further examine this the risk area profile based on the current consent was calculated pre and post the adaptation

(Figure 2-77). As expected aeration significantly improved the site performance with regards to ammonia, where post aerating risk area was roughly 10% of the pre-aerated levels, which held true throughout the tightening of consents for these sites ammonia effluent (The risk area following aeration was 11%, 10%, 11%, 11% and 15% of pre-aerated levels at the same consents). Whilst aeration is targeted at ammonia, there was also a positive effect on BOD effluent quality and to a lesser extent the SS. For instance, the aeration adaptation reduces the mean risk area of a site for SS by over 10% (mean pre= 2.56 mg/l% to 2.27 mg/l%), this improvement of the site is even better for tighter consents were the improvement is over 25%.

More significant impact was seen with the iron dosed sites with regards to SS, BOD and TP and to a lesser extent ammonia (Figure 2-88). This was observed in terms of the mean, median, inter-quartile range which all decreased significantly for each pollutant considered. To illustrate, the mean risk area decreased from 1.3 to 0.03 for BOD, 1.7 to 0.1 for SS, 0.8 to 0.4 for ammonia and 2.43 to 0.4 for TP.

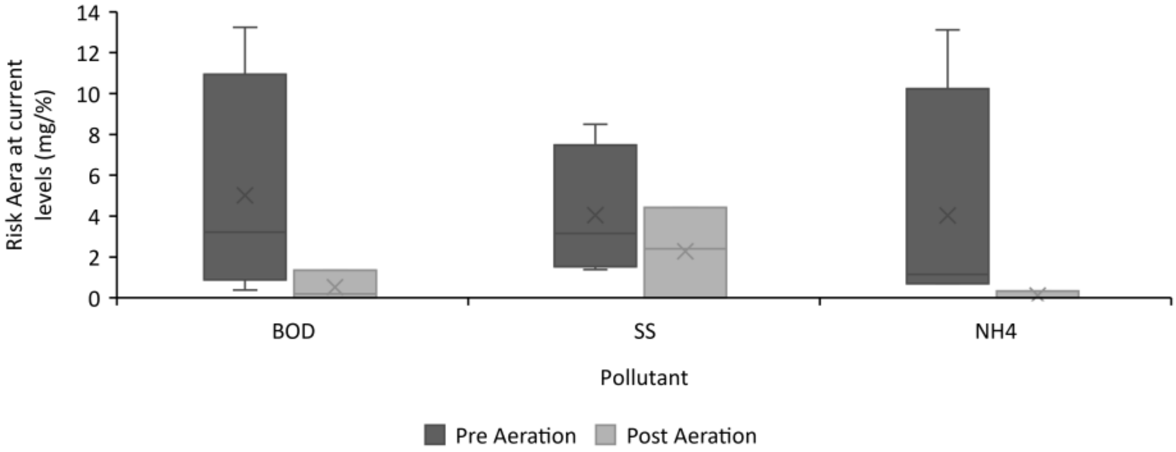


Figure 2-77 The risk area of sites pre and post the additiion of an addition adapatation

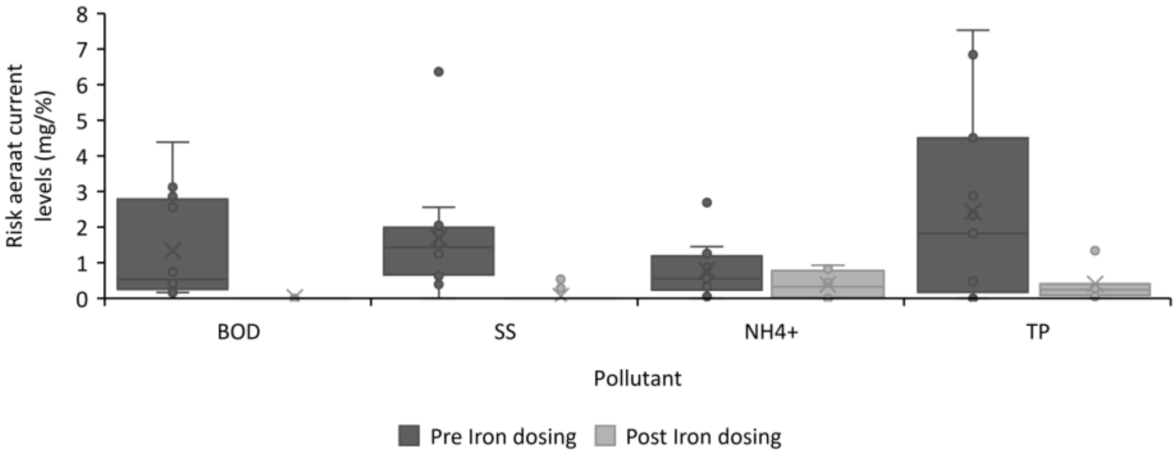


Figure 2-88 The risk area of sites pre and post the introduction of an iron dosing adapatation

2.4.4 Impact of size of the system

The risk area of the different wetlands were analysed to see if age, HLR, season or physical size had an impact on the robustness of the performance of the system. No discernible impact could be seen relative to age, season or HLR indicating that the original design was appropriate for the range of HLR actually experienced and that they were maintained appropriately with regards to refurbishment cycles. However, it should be noted that the analysis covers a timespan less than the initial life span estimates of 20 years (Green and Upton, 1995).

In contrast, impact was observed in relation to the size of the wetlands (Figure 9). This is expressed as the population equivalent (P.E.) served as this is the basis for the sizing used. Whilst low area risks were observed across all scales, an increasing number of sites exhibited higher risk areas as the P.E. served decreased. This was observed in regards to BOD and SS specifically where the risk area of some sites exceeded a value of 2 once the P.E. was reduced. Given that CWs are biological reactors, the decreased robustness could align to changes in the community diversity associated with smaller areas in line with the theories of island biology where the diversity of an ecological space follows a power law expression with regard to the size of the catchment. This has previously been demonstrated for membrane bioreactors (Van Der Gast et al., 2006) and the current results suggest that the same may apply for CWs. However, factors such as improved/increased maintenance and less input variability are also likely to be important factors.

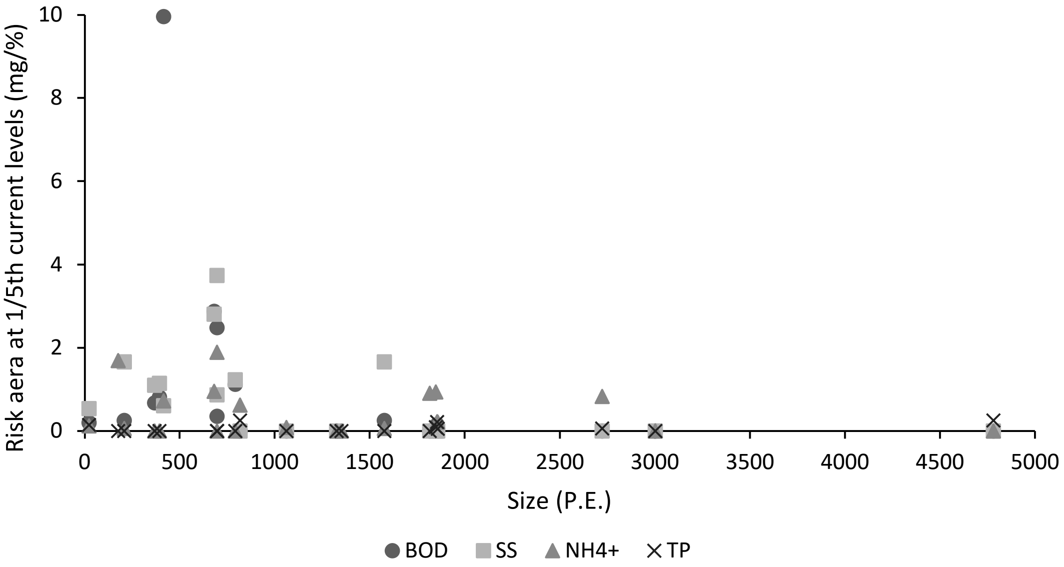


Figure 2-99 the relationship between site footprint and risk area at consents 1/5th current levels in all CWs.

[redacted] Conclusions

The aim of this study was to assess the robustness of CW based wastewater treatment processes and more specifically to look at how treatment adaptation impacted the robustness of the site towards the selected target and the other compliance variables. Overall the analysis has indicated that CW based sites deliver a robust effluent quality for small wastewater treatment works. Moreover, simple adaptations for nutrient removal are shown to be very robust in terms of their selected target and provide general improvements in robustness. Further analysis revealed that the current design approaches offer a reasonable degree of redundant capacity so that the sites should be able to cope with further effluent consent reductions without the need for further asset improvements.

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[redacted] Supplementary information

2.7.1 Site Details

Table 2-22 Full details of the sites included in this study

Site Name	P.E	Prior treatment	Distribution System	Number of Beds	Area (m²)	Volume (m^3)	flow rate (m3/d)	Consent Start Date	BOD consent (mg/l)	SS consent (mg/l)	Ammonia-N consent (mg/l)	TP consent (mg/l)
DU6	230	RBC	Port	2	800	480	3.7	11/03/2014	15	25	5	2
DU9	1848	Filter	Troughs	2	1400	840	8.5	26/02/2014	15	25	5	1
DU10	1857	Filter	Troughs	5	1250	750	6.9	25/02/2014	15	30	5	1
UA5	559	RBC	Troughs	3	622	373.2	5.4	01/01/2010	15	25	5	x
DU2	1855	RBC	Troughs	4	4273	2563.8	7.4	24/02/2014	15	30	5	2
DU3	1061	Filter	Troughs	3	1584	950.4	3.5	26/02/2014	25	45	5	2
DU11	4781	RBC	Troughs	2	140	84	22.9	11/03/2014	15	30	5	1
DU18	952	Filter	Troughs	1	945	567	4.2	01/01/2010	35	55	x	x
UU6	1576	Filter	Troughs	4	1800	1080	6.8	01/01/2010	20	30	5	
DU16	536	RBC	Risers	1	392	235.2	1.8	14/01/2014	40	60	x	2
DU14	894	RBC	Troughs	4	2400	1440	6.1	01/01/2010	15	30	5	x
DU15	3003	Filter	Troughs	3	1935	1161	13.7	11/03/2014	10	20	5	1
DU12	1816	Filter	Troughs	4	3006	1803.6	7.4	13/11/2013	15	25	5	2
DU7	817	RBC	Risers	2	495	297	2.3	02/06/2014	30	50	20	2
DA1	375	RBC	Risers	3	400	240	1.2	31/03/2015	21	45	4	x
UA1	350	RBC	Troughs	1	420	252	1.3	31/05/2013	14	45	3	x
UU3	991	RBC	Troughs	2	1100	660	2.0	01/01/2010	25	45	x	x
UU5	696	RBC	Troughs	2	676	405.6	2.7	x	x	x	x	x
UA4	215	RBC	Troughs	5	2100	1260	6.9	x	x	x	x	x

Site Name	P.E	Prior treatment	Distribution System	Number of Beds	Area (m^2)	Volume (m^3)	flow rate (m3/d)	Consent Start Date	BOD consent (mg/l)	SS consent (mg/l)	Ammonia-N consent (mg/l)	TP consent (mg/l)
DU8	375	RBC	Troughs	5	2228	1336.8	x	11/03/2014	25	45	15	2
UU14	249	RBC	Troughs	1	63	37.8	4.4	01/01/2010	25	45	x	x
UU10	2636	RBC	Troughs	3	1236	741.6	3.1	01/01/2010	15	30	10	
UU11	1351	RBC	No Data	1	675	405	5.0	01/01/2010	25	45	x	x
DU13	2723	RBC	Troughs	4	2400	1440	9.4	10/04/2014	15	35	10	2
DU4	1326	RBC	Troughs	2	2139	1283.4	3.6	21/02/2014	25	40	10	2
UU4	2019	Filter	Troughs	4	1656	993.6	9.4	31/12/2005	25	x	x	x
UU1	931	RBC	Risers	2	1108	664.8	3.8	01/01/2010	15	25	10	x
UU7	226	RBC	Port	1	800	480	6.8	31/03/2015	10	25	3	x
N	965	SAF	Troughs	1	210	126	1.1	29/04/2014	25	45	x	2
UU13	877	RBC	Risers	3	1339	803.4	x	01/01/2010	20	35	10	x
UU12	394	RBC	Troughs	1	170	102	x	01/01/2010	25	45	x	x
DA1	375	RBC	Port	4	724	434.4	1.3	01/04/2014	20	40	10	2
UU17	368	RBC	Risers	1	479	287.4	1.3	01/01/2010	25	45	x	x
UU2	792	RBC	Risers	2	340	204	2.1	31/03/2010	25	45	15	
UU18	1249	RBC	Port	1	320	192	1.5	01/01/2010	35	50	x	x
DU17	682	RBC	Troughs	4	1445	867	4.8	01/01/2010	20	45	5	2
UU15	379	RBC	Port	1	506	303.6	1.2	01/01/2010	25	45	x	x

3 Impact of Tertiary Constructed Wetland Adaptation on Microbial Communities

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[redacted]Abstract

Constructed Wetlands (CW) are a decentralised and sustainable treatment technology that are effective for BOD and SS when configured as horizontal flow subsurface wetlands for tertiary treatment. Recent adaptations have included chemical dosing upstream for phosphorus removal and aeration of the wetland for ammonia removal. The current study samples sludge layers from sixteen constructed wetlands with a range of adaptations to ascertain if this impacted the abundance and diversity of the microbial community. No significant impact on overall bacterial levels or diversity (between $6.91x10^9$ and $1.54x10^{11}$ cells per dried gram of sludge and Shannon diversity of over 6.5), or community structure was found across all adaptation types. Specifically, no difference was seen in regards to the availability of iron reducing bacteria in chemical dosed sized compared to traditional wetlands and even aerated systems.

Keywords: 1) q-PCR 2) dissimilatory iron reducing bacteria 3) ferric coagulation; 4) aeration

[redacted]Introduction

Constructed Wetlands (CW) are a biological filter technology that provide sustainable decentralised wastewater treatment solution as they require minimal maintenance (Dotro et al., 2017). Several CW configurations have been developed to treat waste water within a wide range of locations, environments, volumes, and to a variety of consent levels (Dotro et al., 2017; Kadlec et al., 2000). Vertical Flow (VF) treatment systems are common in, Austria, Germany, and Denmark, following similar guidelines (Brix and Arias, 2005a; Dotro et al., 2017; Jokic et al., 2003; Mitterer-Reichmann, 2012). Within Denmark they are extensively used following septic tanks to provide treatment of wastewater from single houses or small communities (Brix and Arias, 2005b). Within France VF wetlands, are designed distinctly different (Molle et al., 2005) and are often used in series for the complete treatment of coarsely pre-screened wastewater. Within the UK, the most commonly used configuration of CW is the horizontal sub-surface flow constructed wetland (HSSF), as a tertiary or polishing stage before emission into the aquatic environment typically for organics and suspended solids (Dotro et al., 2017; Green and Upton, 1995; Kadlec et al., 2000). However, recent legislative development around pollutant levels, discharge consents and water quality standards (EC, 2000; UKTAG, 2013) mean that CW need to meet tighter discharge consents especially for phosphorus (P) and ammonia.

Phosphorus removal poses some challenge as the media used has a low specific surface area and a limited number of adsorption sites (Kadlec et al., 2000). Over the last decade, studies have investigated chemical coagulation as a method to increase P removal from CW systems (Dotro et al., 2015). The typical coagulant used in the UK is ferric sulphate (Dotro et al., 2015), however globally there others used such as ferric chloride (Kim et al., 2016), aluminium oxide (Tanada et al., 2003) and lime (Mohammed and Abbas Shanshool, 2009). Iron (Fe), particularly Fe^{3+} has a strong affinity for phosphorus, especially phosphate and as such will form complexes with soluble phosphorus allowing it to be filtered out of the wastewater stream (Thistleton et al., 2002). The resultant sludge that accumulates within the bed is thus artificially enriched with Fe and P, with levels as high as 48 P g/kg and 178 Fe g/kg, compared to 2 P g/kg and 30 Fe g/kg found in natural wetlands (Dotro et al., 2015; Stockman, 2014). Whilst at lower levels of enrichment, the phosphorous within the natural sediments (rivers, lakes, and reservoirs) is known to re-release back into the water body under certain conditions (Malecki et al., 2004a; Parker and Beck, 2003; Withers and Jarvie, 2008; Zaragüeta and Acebes, 2017). The pathway is thought to be microbially mediated by dissimilatory iron reducing bacteria (DIRB) (Dotro et al., 2015; Jensen and Andersen, 1992; Kim et al., 2016; Malecki, White and Reddy, 2004b;

Moore, Reddy and Fisher, 1998; Lovley, 1993). In contrast, the precipitated phosphate solids formed during coagulation were thought to be effectively irreversible within waste water treatment systems (Dotro and Jefferson, 2013). This assumption has been proven not to be true in wetlands, with the phosphorus release observed occurring within the sludge accumulated within the systems (Dotro et al., 2015; Kim et al., 2016; Chapter 5). However, the presence of DIRB in the sludge layer of adapted constructed wetlands is currently unreported and hence the potential for re-release unclear.

Along with phosphorus, efforts are being undertaken to enhance ammonia removal from wastewater discharges. To remove ammonia sufficient oxygen must be provided in order to convert ammonia to nitrate/nitrite (Khuntia et al., 2013). Whilst the media of all VF system oscillates between oxic and anoxic conditions depending on their stage of treatment cycle, large variations exist constantly across a HFCW (Butterworth et al., 2016). To achieve a constantly oxic environment within HFCW artificial aeration is required. By dispersing air along the base of the wetland bed, an oxic environment can be maintained, and sufficient ammonia removed robustly attributed to the build-up of active ammonia oxidising biological communities (Butterworth et al., 2016; Ouellet-Plamondon et al., 2006; Zhang et al., 2010b).

Croatisation of the microbial community found within CW has only relatively recently been reported (Ibekwe et al., 2003b; Nurk et al., 2005; Truu et al., 2009). Focus has tended to be around characterising the community in close proximity to the media where it is believed the majority of treatment occurs (Adrados et al., 2014; Chang et al., 2015; Ramond et al., 2012; Zhang et al., 2010a). As such, the previous studies have not investigated the microbial community found within the accumulating sludge layers. Further, none of the previous studies have considered the impact of recent adaptations to enhance nutrient removal such as iron dosing or aeration. Accordingly, the objective of this study was to determine the qualitative and quantitative changes induced in bacterial communities following the introduction of iron dosing and/or aeration across 16 HFCW in order to gain a better understanding of the impacts of recent adaptations.

[redacted] Material and methods

3.3.1 Site description

Sixteen rural HFCW sites treating tertiary wastewater within the Seven Trent region (UK) were investigated. The selection of HSSFs included CWs which received iron dosing for P removal (8 sites), aeration (2 sites), a site that underwent both adaptations and six sites that have remained as conventional HFCW. This selection allowed sites to be classified as to their adaptation, with the four main groups: Both adaptations, Dosed, Aerated and No Adaptations (Table 3-11) Further to this, two sites, one with a French style VF treating crude wastewater and the second consisting of an iron dosed site receiving an additional dose of nitrate, designated N, were also investigated to identify any differences due to the differing regimes operated on these sites. Using long term effluent grab sampling data collected by site operators, there was the ability to identify sites that had previously struggled to meet compliance consents. This was done by calculating the robustness in the same way as Chapter 2. This led to the further segregation of Dosed into two more sub groups of Dosed sites with a Low Robustness Performance Index (RPI), consisting of sites that have consistently performed well, and Dosed sites with a High RPI, consisting of sites that have occasionally struggled to meet compliance consents. The details of these sites are included in Table 3-11.

Table 3-11 Showing which the basic physical details of each site chosen for this study

Site ID	DA1	DA2	DU1	DU2	DU3	DU4	DU5	DU6	DUP 1	DUP 2	N	VF	UA1	UU1	UU2	UU3	UU4	UU5
Iron dosed	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N
Aerated	Y	Y	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N
Population	375.0	275.0	817.0	1855.0	1061.0	1326.0	599.0	536.0	368.0	1271.0	965.0	794.0	350.0	931.0	792.0	991.0	2019.0	394.0
Number of Beds	3.0	1.0	2.0	4.0	3.0	2.0	2.0	1.0	1.0	2.0	3.0	1.0	1.0	2.0	1.0	2.0	4.0	3.0
Approx. Size (m^2)*	400.0	375*	495.0	4273.0	1584.0	2139.0	800.0	392.0	724.0	960*	210.0	350.0	420.0	1108.0	320.0	1100.0	1656.0	676.0
Size per person (m^2/p. e)	1.0	1.4	0.6	2.3	1.5	1.6	1.3	0.7	2.0	0.8	1.4	0.4	1.2	1.2	0.4	1.1	0.8	1.7
Mean Flow rate (m^3/day)	1.2	N.A.	2.2	7.4	3.5	3.6	3.7	1.8	1.3	4.4	1.2	N.A.	1.3	3.8	1.5	3.0	9.4	2.7
Loading rate (mm/d)	252.7	N.A.	384.0	150.0	188.7	145.0	398.5	390.1	155.1	396.9	481.4	N.A.	257.1	298.7	413.1	234.9	489.9	343.8
P g/kg dw	43.6	41.3	29.6	27.0	20.9	32.6	18.0	75.0	30.5	64.0	37.1	6.3	11.2	19.9	27.4	37.1	4.8	16.0
Stdev (n≥3) g/kg dw	22.7	2.6	9.4	12.1	3.5	5.3	7.7	34.3	12.9	27.8	16.5	1.1	1.6	2.8	11.9	36.1	3.6	0.7
S g/kg dw	11.8	11.9	11.0	9.2	8.8	9.0	8.9	343.5	8.4	11.8	9.5	9.8	8.4	8.8	9.8	18.8	8.2	11.2
StDev (n≥3) g/kg dw	2.8	1.6	1.5	0.9	1.9	1.6	0.7	393.3	1.2	5.6	1.4	2.5	2.6	1.2	1.6	6.3	0.9	2.3
Ca g/kg dw	37.9	26.0	32.0	21.0	27.9	32.0	17.0	23.1	23.7	51.0	29.7	15.8	24.8	21.5	27.6	28.7	15.1	31.0
StDev (n≥3) g/kg dw	15.6	1.0	9.0	3.7	5.0	4.8	0.0	20.2	6.0	22.1	9.3	5.2	4.9	3.1	4.9	40.7	13.2	4.4
Fe g/kg dw	127.6	112.1	82.1	82.1	61.7	94.3	42.7	122.4	77.5	176.1	91.6	4.1	15.9	36.4	23.9	25.8	3.5	12.5
StDev (n≥3) g/kg dw	62.3	15.7	27.9	37.8	22.6	25.9	37.3	46.2	35.5	76.3	51.9	1.7	10.4	12.3	17.5	19.7	5.2	4.1
Site Robustness (RI) at current TP consents**	X	x	0.7	0.6	0.2	0.3	0.5	0.7	1.5	1.4	x	x	x	x	x	x	x	x
P Influent (mg/l)	0.0	2.0	0.6	2.1	0.5	0.9	1.2	1.5	7.3	5.5		1.5	1.8	1.7	6.0	2.9	4.7	2.0
P Influent (<0.45 um, mg/l)	0.0	1.3	0.5	1.6	0.1	0.0	0.8	1.3	6.7	5.0	0.5		2.0	1.1	5.2	2.0	3.9	1.6
Ca Influent (mg/l)	56.0	18.9	48.6	60.0	76.1	38.2	73.5	33.9	40.2	50.9		13.9	48.5	13.6	30.5	21.4	17.7	30.8
Ca Influent (<0.45 um, mg/l)	54.6	19.4	45.9	54.1	81.8	35.3	77.0	32.9	40.5	49.7	43.2		45.6	12.4	29.4	20.1	16.6	73.6
Fe Influent (mg/l)	0.2	1.6	0.2	1.2	2.0	5.5	2.0	0.1	0.4	0.3		0.3	0.0	0.3	0.0	0.1	0.2	2.0
Fe Influent (<0.45 um, mg/l)	0.1	0.1	0.0	0.1	0.1	0.2	0.0	0.0	0.1	0.0	0.0		0.0	0.1	0.1	0.0	0.0	0.1

Site ID	DA1	DA2	DU1	DU2	DU3	DU4	DU5	DU6	DU P 1	DUP 2	N	VF	UA1	UU1	UU2	UU3	UU4	UU5
S Influent (mg/l)	113.	63.9	77.7	109.0	101.0	44.6	88.8	86.4	86.0	98.8		61.7	120.	40.6	98.6	113.0	72.9	72.5
S Influent (<0.45 um, mg/l)	111.	64.5	73.6	100.0	108.0	42.7	97.1	83.1	85.8	96.7	62.9		111.	36.6	93.9	104.0	69.8	88.0
P Effluent (mg/l)	0.2	1.0	0.7	1.4	0.3	5.1	0.1	2.0	5.4	4.3	1.6	1.6	1.6	1.1	3.8	2.3	4.1	1.1
P Effluent (<0.45 um, mg/l)	0.1		0.7	1.3	0.3	5.1	0.1	1.7	5.4	4.3	2.4	1.4	1.6	1.3	0.1	2.2	4.0	0.2
Ca Effluent (mg/l)	56.9	21.8	45.0	52.5	71.4	45.1	78.3	43.5	39.5	48.6	38.2	14.3	47.7	15.6	13.8	21.5	18.0	42.7
Ca Effluent (<0.45 um, mg/l)	60.5		45.0	44.9	70.2	45.3	80.4	39.6	39.2	47.2	42.1	15.6	46.2	16.2	37.1	20.7	18.0	88.3
Fe Effluent (mg/l)	0.4	0.6	0.0	0.6	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.3	0.0	0.0	0.1	0.0	0.1	1.2
Fe Effluent (<0.45 um, mg/l)	0.0		0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.8	0.2	0.0	0.0	0.1	0.0	0.0	0.1
S Effluent (mg/l)	111.	64.5	83.0	91.6	113.0	104.0	97.6	105	78.3	96.5	63.4	63.4	123	53.0	45.2	115.0	69.6	57.6
S Effluent (<0.45 um, mg/l)	119.		82.4	80.4	111.0	104.0	95.2	94.0	78.6	94.9	67.8	68.6	118	50.4	49.6	115.0	68.4	88.4
DO Influent (mg/l)	8.3	8.6	8.8	11.8	8.4	8.9	0.9	8.5	9.7	7.6	8.8	7.8	7.5	9.1	7.1	8.1	6.1	9.1
DO Effluent (mg/l)	11.4	7.4	9.5	2.1	5.8	11.5	7.7	10.9	8.9	8.8	7.8	8.2	6.3	4.9	7.3	6.7	7.6	8.9
pH Influent	7.9		7.4	7.1	7.1	7.5	8.2	7.2	8.5	7.2	7.3	x	7.3	6.9	7.1	7.1	6.5	7.2
pH Effluent	7.4	7.5	7.3	7.2	7.0	7.6	7.6	7.1	8.1	7.3	7.5	x	7.2	7.1	7.2	7.1	7.2	7.1

*Indicates that site size was an estimate from google maps; N.A. not available

** Chapter 2

3.3.2 Sample collection and analysis

Sludge samples from all sites were collected at the front, middle and end of the CW plant except for two sites with No Adaptations sites 1 and 2 and two Dosed sites (Table 3-11). For these four sites, samples were collected at the inlet, inlet/middle, middle/end, and end. Sludge samples (500 g) were collected from the surface of the wetland media using a grab sampling method with a trowel, before placing in airtight, translucent plastic containers. Sludge samples were then transported to the laboratory on the same day. Upon arrival, 50 g of the sludge was placed in a smaller airtight container and frozen at -80^{o}C for molecular analysis; the remaining sample was stored at 4^{o}C until further physicochemical analysis. The remaining 450 g of sludge was digested in an aqua regia solution and then measured for levels of phosphorus, calcium, sulphur and iron using an ICPMS (Perkin Elemer, UK).

3.3.3 Wastewater Characteristics

Prior to sampling, the pH value and DO of the influent and effluent were taken using an HQ40d portable Meter with a DO IntelliCAL LDO105 probe and pH IntelliCAL PHC10101 standard gel (Hach, Salford UK). At each site, a sample of the influent and effluent water stream of the CW was collected. Both filtered (0.45 µm Millipore filter) and unfiltered samples of the influent and effluent were collected. These were stored in airtight 50 ml centrifugal containers (VWR, UK), with a minimal amount of headspace. When returned to the Laboratory and awaiting measuring, the samples were stored at 4^{o}C. At the time of measuring the samples were acidified by adding 5% by volume of nitric acid (68%, sigma Aldrich, UK). The samples were then measured for levels of phosphorus, calcium, sulphur and iron using an ICPMS (Perkin Elemer, UK).

3.3.4 Molecular analysis

DNA was extracted from 0.25 g wet weight of sediment using a Powersoil DNA extraction kit (Qiagen) according to the manufacturers instructions. 16S rRNA gene abundance was quantified using the 16S rRNA primers Bakt_341F

(CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011) by SYBR Green assay on a CFX384 Real-Time PCR Detection System (BioRad) using a SensiFAST SYBR No-ROX Kit (Bioline). An absolute quantification method was used with an internal standard calibration curve of DNA standards of the target gene in 10 µl reactions containing 400 nM of primers and 1 µl of DNA template. Cycle conditions were 95 °C for 2 min followed by 40 cycles at 95 °C for 10s then 60 °C for 30 seconds. Amplification of a single product was confirmed by melting curve analysis.

Amplicon libraries were prepared by a 28-cycle PCR using the same primer set flanked with Illumina Nextera overhang sequences. The amplicons were purified using AMPure XP (Agencourt) SPRI bead protocols. Illumina flowcell adapter sequences and unique combinations of Nextera paired-end Indexes were added to the amplicons using an 8-cycle PCR, before purifying again with AMPure XP beads. Each sample was quantified using a Quant-iT Picogreen dsDNA assay kit (Life Technologies) on a Nanodrop 3300 fluorospectrometer (Thermo Scientific) and then pooled in equimolar concentrations.

Metagenetic amplicon sequencing was conducted on an Illumina Miseq platform using a 600 cycle v3 Reagent Kit v3. Sequencing reads were quality trimmed using Sickle (Joshi and Fass, 2011), before being error corrected within SPAdes (Nurk et al., 2013) by implementing the BayesHammer algorithm (Nikolenko and Alekseyev, 2011). The sequences were then pair-end aligned with PEAR (Zhang et al. 2014) within PANDASeq (Masella et al., 2012). These quality filtered, error corrected and pair-end aligned sequences were then dereplicated, sorted by their abundance, and operational taxonomic unit (OTU) centroids were picked using VSEARCH (Rognes et al., 2016) clustering at the 97% level. All chimeric sequences were removed using both denovo and reference based chimera checking with UCHIME (Edgar et al., 2011). Taxonomy assignment was performed with the RDP Classifier (Wang et al., 2007), using the principles laid out by (Yarza et al., 2014). The percentage of total reads assigned at each level is shown in Figure A.1 in the appendix. Mothur v.1.21 was used to produce rarefaction curves with all samples normalised to 30246 sequences, this was done to calculate the diversity indexes of each sample and ultimately each site, which was confirmed by using Microsoft Excel.

3.3.5 Data analysis

With the sites assigned according to Table 3-11, it was possible to then study any impacts of adaptation on the bacterial community of CW. 1-way ANOVA's using a Welch T test (Welch, 1947) was conducted within the Minitab 17 software (Minitab, 2000). The remaining graphs and standard deviations were calculated or produced using Microsoft Excel. Bacterial community analysis including PCA comparison of bacterial community's similarities was conducted using the STAMP program.

[redacted]Results and discussion

3.4.1 Effect of constructed wetland adaptation on overall bacteria levels

The bacterial concentration within the sludge layers of all sites used in this study was found to vary between 6.91x10^{9} and 1.54x10^{11} cells per dried gram of sludge, with most sites having an average concentration of between 1.66x10^{10} cells per dried gram of sludge and 5.66x10^{10} cells per dried gram of sludge. When each site was assigned to its respective site type, there was found to be no statistical difference (ANOVA analysis) between those with and without adaptations, suggesting that the was no impact on overall bacterial content of the sludge when sites are adapted (Figure 3-11).

This finding was unexpected. It was expected that bacterial levels would be affected either because; CW's have an abundance of potential terminal electron donors (such as the organic matter found in the sludges), and adaptation introduces a potential extra TEA (either oxygen or iron), this adaptation would the provide a boost to bacterial levels.

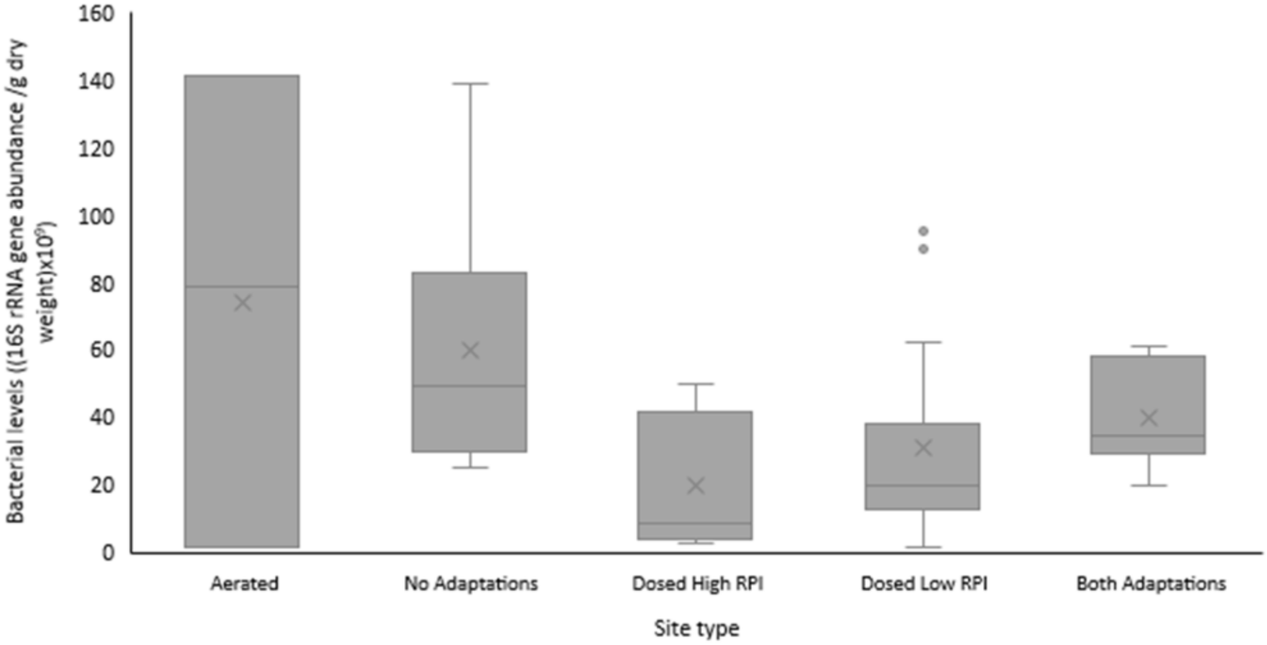

Figure 3-11 Category box and whisker plot of cells counted per dried gram of sludge. Boxes representative of the interquartile range, with whiskers showing the standard deviation

[redacted] Community structure

The community structure of the sludge from all sites was shown to be richly diverse with all site types recording an average Shannon diversity indexes of over 6.5, except from the two unique sites (VF= 5.1, and N= 5.3). No statistical difference with respect to diversity levels was observed between the conventional and adapted sites related to the high levels of overall diversity observed. Previous reports concerning the biofilm around the media indicate much lower diversity levels with Shannon diversity indexes as low as 1.5 (Calheiros et al., 2009). Given the general view that greater diversity means greater resilience and stability (Ibekwe, Grieve and Lyon, 2003) then the current findings suggest the role of the sludge layer may have been previously understated and requires further consideration. Further analysis was conducted to identify the major phylum, defined as contributing greater than 2% of a site's total bacteria (Figure 3-22). The total sum of all bacteria with a greater than 2% relative abundance, only made up 15-20% of the total community. Proteobacteria, Acidobacteria, and, Verrucomicrobia dominated all site types making up over 75% of the bacteria that was greater than 2% of total bacteria (Figure 3-22).

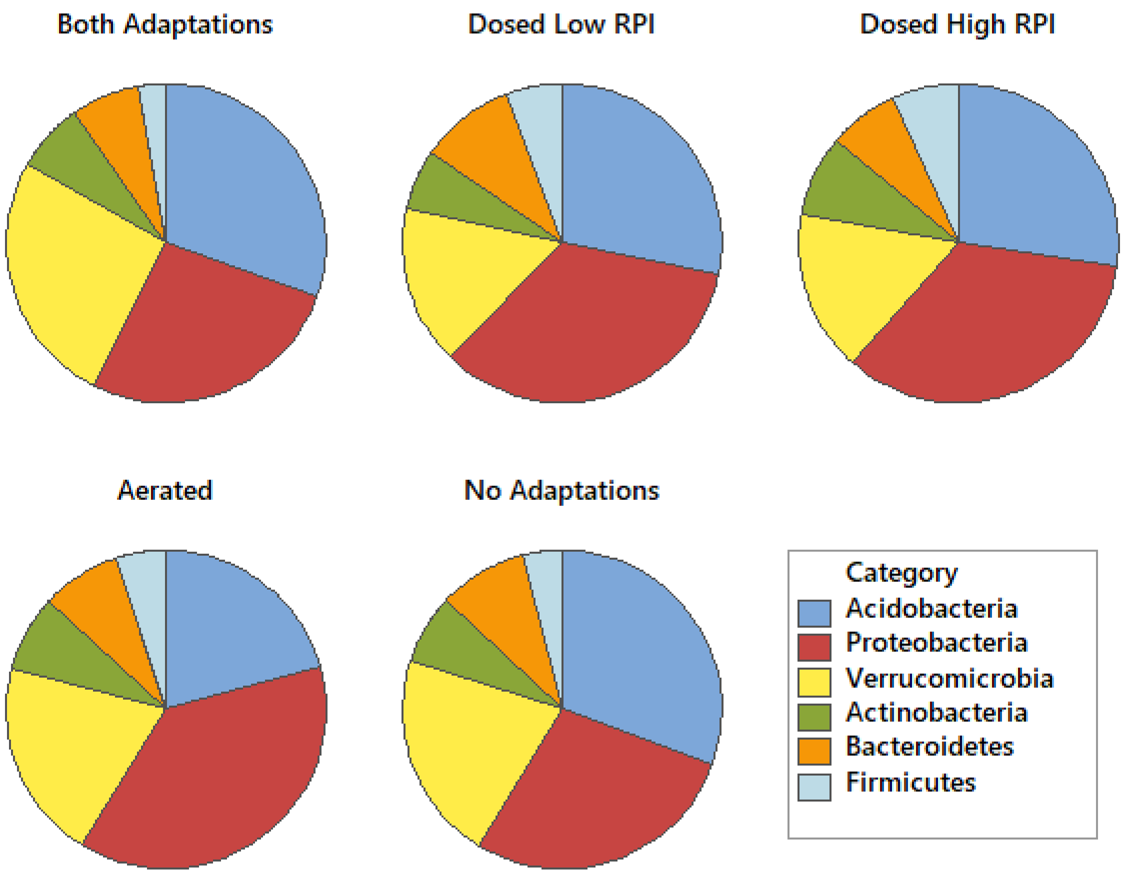

Figure 3-22 Pie charts of phylum level make up of each site type, due to the large diversity only those making up greater than 2% of any site type were selected.

Mirroring the findings of the abundance and overall diversity level, all sites showed significant similarity ($p<0.05$, ANOVA) to each other, which was further supported through PCA analysis that showed no significant trend associated with respect to community similarities caused by specific adaptations (Figure 3-33). No site type ordinated out, indicating that there was no trend between site type and overall community structure. This tied into the overall picture that there was no significant observable impact on the microbial community due to the adaptations required for enhanced nutrient removal.

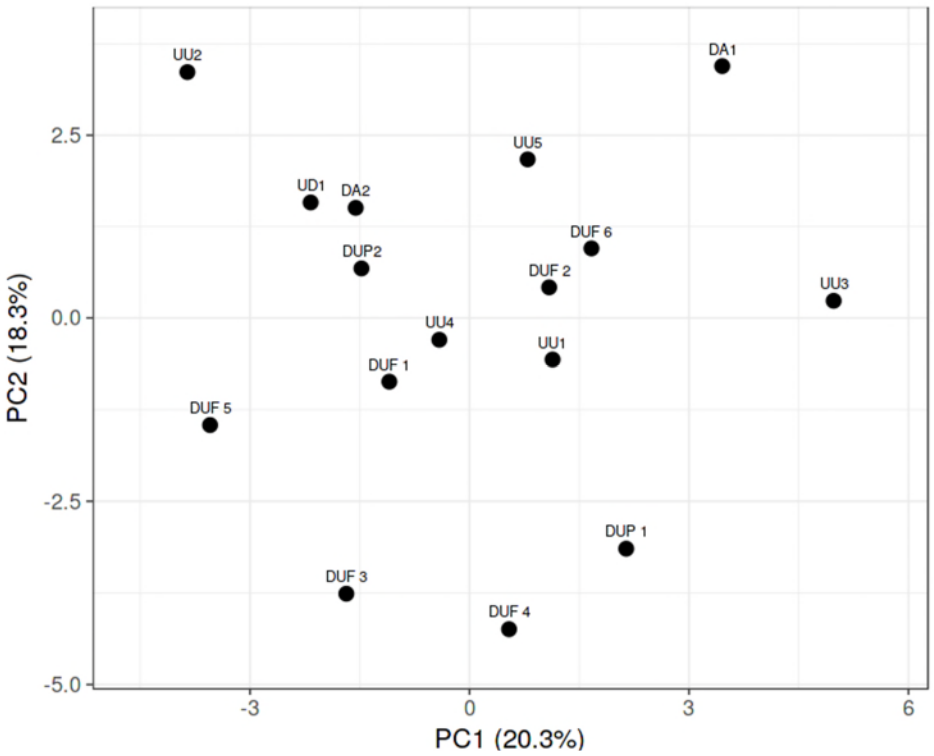

Figure 3-33 PCA plot based upon a sites community similarity, the code for each site is shown in Table 3-11

Acidobacteria are found in many diverse areas such as oceans and soils, even metal-contaminated soils (Thrash and Coates, 2015). An important member of this phylum with regards to iron release is the genus of Geothrix. This genus contains species such as Geothrix ferrnentans, which was found to be an anaerobic iron reducing bacterium, able to induce iron release from a hydrocarbon contaminated aquifer (Coates et al., 1999). Proteobacteria is perhaps a better-known phylum of bacteria as it includes many more common pathogens such as *Escherichia, Legionella, and Salmonella* (Brenner et al., 2005). Importantly, Geobacter, a common iron reducing bacterium that can dominate in wetlands (Adrados et al., 2014), is a genus of Proteobacteria.

In contrast with the similarity between all the HFCW, irrespective of adaptation, the VF site had a distinctly different profile (Figure 3-33). In this case the community consisted of 17% Proteobacteria compared to 26% in the nitrate dosed site. To further explore the community structure a heat map showing presence and absence of these abundantly smaller phylum was created (Figure

3-44). Particular attention was placed on the sites experiencing problems (sites 9 and 10) where it was suggested that the sites undergoing iron release caused by iron reducing bacteria (Dotro et al., 2015). However, no evidence was apparent indicating an enrichment of such bacteria. Instead elevated levels of Cyanobacteria, Chloroflexi were observed in these sites, neither related to iron reduction. Consequently, it indicates that all sludge sources potentially contain adequate availability of iron reducing bacteria to activate an iron reducing pathway.

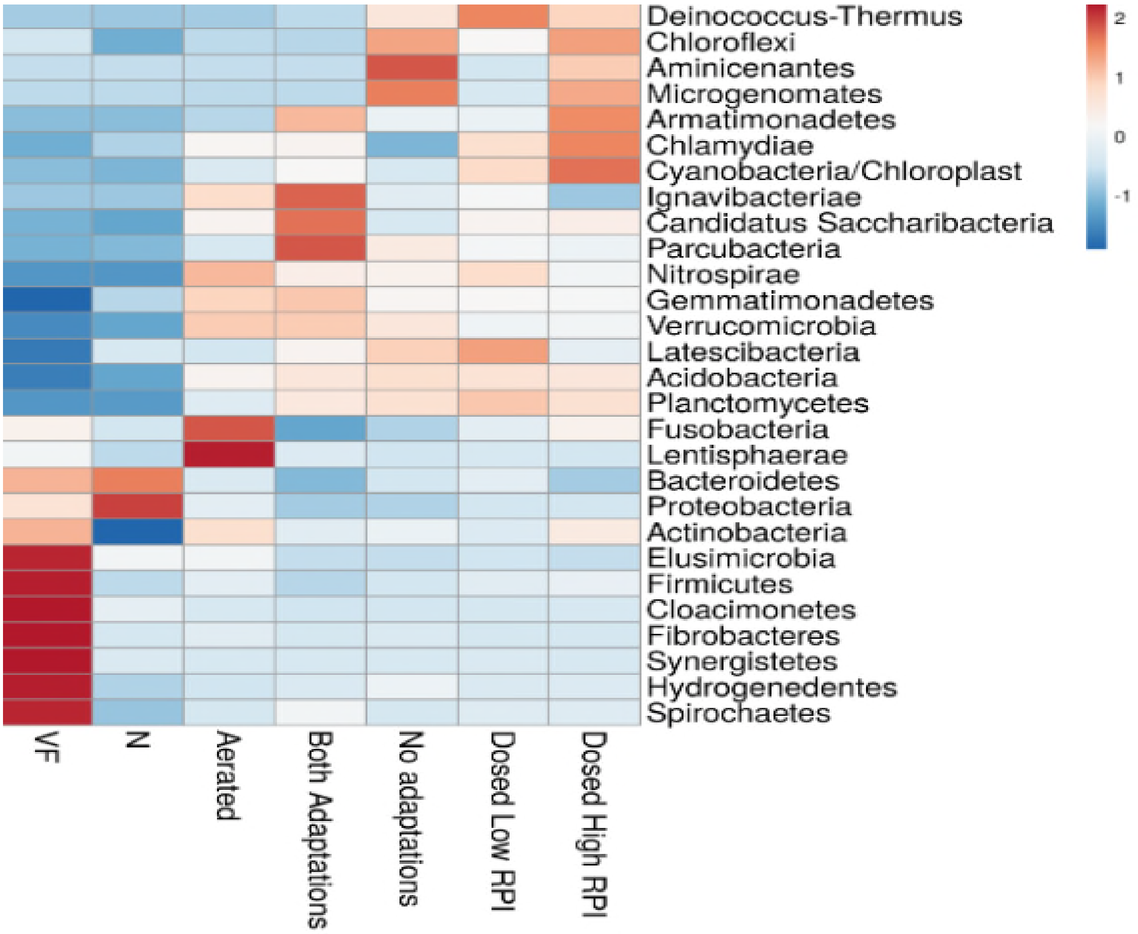

Figure 3-44 A heat map showing change between site types of each phylum per site type

The similarity between site types, irrespective of adaptation continued when considering the most dominant genus (Table 3-22). In all cases, Gemmatimonas was identified as the most dominant genus with mean relative abundances between 4 and 7%. The Gemmatimonadetes phylum is commonly found in sedimentary environments such as soils and is reported to grow better with constant moisture conditions (Fawaz, 2013).

However, significantly different profiles were observed for VF and N sites. In the case of the VF, the Paludibacter genus was identified as the dominant genus and is comprised of strictly anaerobic species such as *Paludibacter jiangxiensis* and *Paludibacter propionicigenes*, which were both isolated from rice paddy soils (Qiu et al., 2014; Ueki et al., 2006). This reflects that the VF bed is still in its early stages of acclimatisation and experienced periods of extended flooded operation in the initial year, restricting oxygen to the accumulating sludge. Whereas, in the case of the nitrate dosed sites, Thermomonas was identified as the most dominant genus at mean relative abundance of 16%.

Table 3-22 The most dominant genus, and relative abundance at each type of site

Site type	**Dominant genus**	**Mean relative abundance (%)**	**Phylum**	**Class**	**Order**	**Family**
Both adaptations	Gemmatimonas	7	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae
Dosed Low RPI	Gemmatimonas	5	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae
Dosed High RPI	Gemmatimonas	5	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae
Aerated	Gemmatimonas	4	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae
No Adaptations	Gemmatimonas	6	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae
VF	Paludibacter	17	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae
N	Thermomonas	16	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae

3.5.1 Iron reducing bacteria in detail

Specific emphasis was placed on iron reducing bacteria to understand if the abundance of such organisms was likely to be a factor when considering the risk of iron re-release. The relative abundance of DIRB, as identified by Ehrlich and Newmann (2009) varied between 1.2 and 2.3% of the population across the HFCWs (Figure 3-55). Interestingly, the highest abundance levels were observed for the aerated sites which was unexpected. Further, 97% of the identified DIRB in these sites were classified as strict anaerobes even though they are ostensibly oxic. However, the oxic conditions are defined by the dissolved oxygen in the liquid phase, not the sludge and it is known that within the inner sections of sludge samples different redox chemistry can prevail. An illustration of this is the reported case of methane release in an activated sludge system once the DO set point had been reduced. Analysis revealed that the reduced aeration rates lead to sludge accumulation in spots on the floor where anaerobic conditions developed. In addition recent discoveries have indicated an increase tolerance to oxygen than first thought with organism such as *Geobacter sulfurreducens* (Lin et al., 2004). However, importantly, there is no evidence to support increased abundance on iron dosed sites indicating that the natural levels of DIRB present within the sludge are likely to be sufficient. This implies that all wetlands are potential sites where re-release could occur and that management of the risk needs to focus on control of the operating microbial pathway. Interestingly, the lowest observed abundance was observed for the nitrate dosed site although they are known to be able to utilise nitrate as a terminal electron acceptor (Chapter 4).

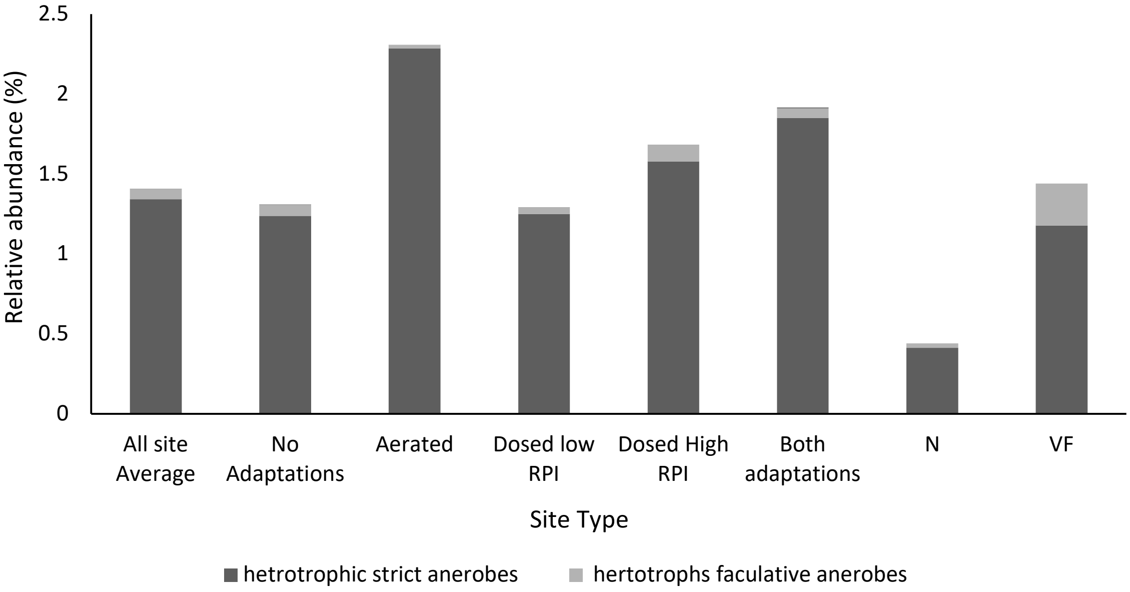

Figure 3-55 The relative abundances of genus of bacteria that have been reported as dissimilatory iron reducers (Ehrlich and Newmann, 2009)

[redacted]Conclusions

Adaptation of CWs for nutrient removal by addition of aeration of coagulant dosing upstream did not impact the overall levels of bacteria found or the community structure. Importantly this was observed with regards with NIRB where no evidence could be observed to indicate an enrichment of the bacterial species on sites that accumulate iron rich sludge. Accordingly, it appears all CWs treating municipal sewage are likely to contain sufficient NIRB to pose a risk of iron release if the prevailing environmental conditions are appropriate. Further, management of the potential risk needs to focus on inhibiting the microbial pathway for iron release.

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4 Release of iron from solid phase species when metabolised by Geobacter metallireducens

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[redacted] Abstract

Iron (Fe) cycling by iron reducing bacteria is recognised as a pathway for observed release of Fe and phosphorus (P) from sludge in waste water treatment systems. This study investigates the metabolic release of iron from two solids substrates: ferric oxide (Fe_2O_3) and ferric phosphate ($FePO_4$) as well as sodium nitrate ($NaNO_3$) on their utilization by *Geobacter metallireducens*. A pure culture of 1.4×10^9 cells of *G. metallireducens* was incubated with 10 ml of Fe-limited broth and eight varieties of reactors containing either a single terminal electron acceptor (TEA) (Fe_2O_3, $FePO_4$, or, $NaNO_3$), or an equimolar mix of two. After an initial period, both iron systems released Fe into the water although the Fe from ferric oxide was released at a significantly higher rate than from ferric phosphate. The presence of $NaNO_3$ supressed the reduction of solid phase iron solubilisation. Addition of $NaNO_3$ to an already releasing systems inhibited further release and started to reduce the soluble Fe concentration.

Keywords: 1) iron release 2) dissimilatory iron reduction 3) preferential metabolism 4) terminal electron donor 5) terminal electron acceptor

[redacted] Introduction

Iron (Fe) is one of the most abundant elements on the planet and accounts for roughly 5% of the crust (Rankama and Sahama, 1950). Its importance to life cannot be understated as almost every single or multicellular organism requires Fe nutritionally (Ehrlich and Newmann, 2009). Fe exists in several common oxidation states, however the Fe^{2+} and Fe^{3+} are most common. The Fe^{2+} species are chemically oxidised to Fe^{3+} in the presence of oxygen or an aerated solution at a pH level greater than 5 (Ehrlich and Newmann, 2009). Fe can also be microbially transformed, with several families of bacteria known to use it as a terminal electron acceptor (TEA). Model examples are the Geobacter and Shewanella genera (Esther et al., 2014) commonly found in aquatic sedimentary environments (Huang, Sun and Zhang, 2010; Lovley, 1997), and in recent studies also within constructed wetlands (CWs) used for waste water treatment (Adrados et al., 2014, Chapter 3). Their presence is particularly important in systems where Fe cycling can impact the water quality being discharged in relation to the required water quality, such as Fe-dosed systems for phosphorus (P) removal. Specific examples include *Geobacter metallireducens* which has been reported to couple the reduction of Fe^{3+} as TEA to the oxidation of organic matter such as acetate (Lovley and Phillips, 1988a). Further, *G. metallireducens* has been shown to grow pili in order to access insoluble Fe^{3+} (Childers, Ciufo and Lovley, 2002). The product of such reactions is Fe in the reduced form of Fe^{2+}, which is typically soluble and thus mobile.

Bacterially mediated release of Fe and P has been recorded from natural environments and the enriched sludge of iron dosed constructed wetlands (Boström et al., 1988; Dotro et al., 2015; Kim et al., 2016; Mortimer, 1940). Previous studies of iron dosed constructed wetlands have found iron in the sludge to exist at a molar ratio of roughly 3:1 with respect of phosphorus (Dotro et al, 2015). This exceeds the molar ratio found in iron phosphate (1:1) and indicates other iron complexes are formed, such as iron oxides or iron hydroxides. In experimental systems, the microbially-mediated release of Fe and P under anaerobic conditions was expected to follow a similar ratio; however, there has been an unexpectedly lower release of P from these relative to the iron release (Chapter 5). This suggested that other iron species are present contributing to the release of Fe. In initial studies of *G. metallireducens* (Lovley and Phillips, 1988b), a preferential order of TEA was established. Ferric oxides (Fe_2O_3) and other poorly crystalline structures were preferred to akageneite and crystalline Fe forms such as goethite and magnetite, however nitrate was metabolically preferred to all of these Fe forms. To the authors' knowledge, the ability for ferric phosphate ($FePO_4$) to be metabolised and the levels of Fe released when this is occurring is unknown. Establishing the relative metabolic release rates between Fe oxides and Fe phosphates by *G. metallireducens* would help to understand and potentially control Fe and P release in both natural and engineered environments.

The preferential hierarchy of TEAs used by *G. metallireducens* is not just limited to Fe, as manganese was demonstrated to be preferential to Fe (Lovley and Phillips, 1988a). Nitrate was also shown to be preferred over both of these for use as TEA (Lovley et al., 1992; Lovley and Phillips, 1988b, 1988a). In a recent study (Chapter 5), nitrate addition to a reactor releasing Fe from anaerobically incubated waste water sludge went further than just supressing release and led to a removal of Fe and P from the pore water. This removal was hypothesised to be bacterially-driven with nitrate utilization linked to Fe oxidation. The study aimed to establish release rates of Fe when metabolised by a pure culture of *G. metallireducens* and the impact which nitrate addition has on suppression or reversal of Fe release.

[redacted]Materials and Methods

4.3.1 Source of organism and growth conditions

G. metallireducens was acquired and grown in liquid broth in accordance with literature provided by the supplier (DMSV, Germany; Supplementary Information, Table S 4-11) using Milli-Q water (18.2 Ω). For the TEA, a soluble Fe source was added in the form of ferric citrate (13.7 g/l; Sigma-Aldrich, UK). The broth used sodium acetate (6.8 g/l; Sigma-Aldrich, UK) as the main carbon donor (TED). The concentration of chemicals used in the broth was developed from previous studies on *G. metallireducens* (Lovley et al., 1993; Lovley and Phillips, 1988b;).

To ensure that the levels of TEA, TED and other essential vitamin and minerals remained in excess, the growing culture was inoculated at a 10:1 rate into fresh liquid growth media every five days. Despite recent evidence that other species of the *Geobacter* genus can tolerate oxic environments, for this study, *G. metallireducens* was assumed to be strictly anaerobic (Coppi et al., 2001). All growth was conducted within a sterile anaerobic environment (95% N and 5% CO_2) kept at 30°C (MACS-cabinet, Don Whitley, England). To ensure that the growth media had no dissolved oxygen it was purged for 10 minutes with 100% nitrogen gas before introduction to the anaerobic chamber. The bacterium was grown in these conditions until there were 10 litres with a concentration of $1.4x10^8$ cells/ml, quantified with a flow cytometer (BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences, UK). The bacteria were centrifuged at 4500 rpm for 10 minutes, with the overlying Fe-rich growth media discarded and the bacterium pellet rinsed with Milli-Q water and resuspended in 10 litres of growth media without Fe. The latter was prepared in an identical manner to the Fe rich media, without Fe addition. pH was corrected to 7 prior to rinsing by addition of concentrated HCl (68%, Sigma-Aldrich, UK).

4.3.2 Experimental set up and sampling

To investigate any potential preferential metabolic pathways by *G. metallireducens* between iron oxide (Fe_2O_3), iron phosphate ($FePO_4$) and sodium nitrate ($NaNO_3$), the bacterium was exposed to six TEA environments and tested in triplicate with an additional six control experiments (Table 4-11). A 10-day sacrificial sampling campaign was used, thus for each TEA environment there were 30 reactors set up. The reactors consisted of 10 ml of the final growth media (detailed above) plus the corresponding TEA conditions (Table 4-11) in 15 ml centrifugal tubes (Sigma-Aldrich, U.K). Incubation was performed within the same sterile anaerobic environment (95% N and 5% CO_2) and kept at 30°C (MACS-cabinet, Don Whitley, England) as used during the initial growth phase. Once the set amount of TEA species and bacterium containing growth medium were added to the reactors, they were sealed with caps and shaken vigorously by hand. In the case of the delayed nitrate addition on day 10 (see *Delayed Experiment* in Table 4-11), a stock solution of 50 g/l sodium nitrate (reagent grade, Sigma-Aldrich, U.K) was prepared and 1 ml (containing 595 micromoles of nitrate) was pipetted into each reactor on day 10. Three replicates of each of the TEA were ran with Milli-Q water and used as controls.

For sampling, the reactors were shaken vigorously by hand and the entire contents passed through a 0.45 µm syringe filter (Millipore, England) within the anaerobic chamber. An aliquot of 2.5 ml of the filtrate was acidified with the addition of 50 µmol of concentrated nitric acid (68%, Sigma-Aldrich, U.K). The acidified sample was diluted with 5 ml of Milli-Q (18.2 Ω) water and stored at 4°C until the samples were analysed for Fe with an inductively coupled plasma mass spectrometer (NexION® 350D ICP-MS, Perkin Elmer, U.K). Standards were run every 12 sample readings and the method had a limit of detection of 3 µg/l. There was a problem during analyses on day 5 where all samples became contaminated and had to be discarded. The Fe release rates were calculated by applying a linear regression model for the variation in Fe concentration of the overlying broth between days 4 and 10 in the Fe_2O_3, day 6 and day 10 in the Fe_2PO_4, and between days 11 and day 18 in the delayed nitrate reactor. These days were selected as they had the strongest trends observed within each reactor with at least five data points. The gradient of each regression model gave the reactors' release rate per day quoted in this study. The consumption per cell was then calculated by dividing the release rate observed per cell of *G. metallireducens* measured at the start of the experiment.

4.3.3 Data analysis

To statistically analyse daily Fe concentrations of the overlying broth between different days within the same reactor and the same day between differing reactors, a one way ANOVA was used at the 0.05 significance level using Minitab 18 software (Minitab, 2000). This test implemented a Welch T test (Welch, 1947). A Tukey Test was then used to compare the release pattern from each reactor and group those that had seen a similar release pattern.

Table 4-11 Experimental Conditions

Terminal Electron Acceptor (TEA)	**Mass of Fe (mg)**	**Micro moles of Fe (µmol)**	**Purpose of reactor**	**Max concentration of Fe, if all released (mg/l)**	**Expected reactions**	**Reference**
Ferric phosphate ($FePO_4$)	40	720	Investigate if can be used as TEA	4000	Eq. (4-22)	Hypothesised by this study
Ferric oxide (Fe_2O_3)	40	720	For comparison of release rates to the ferric phosphate reactors and literature	4000	Eq. (4-11)	(Lovley and Phillips, 1988b)
Ferric phosphate ($FePO_4$)/ Ferric oxide (Fe_2O_3)	20/20	360 /360	Identify potential preferential reduction of these two TEAs	4000	Eq. (4-11) Eq. (4-22)	(Lovley and Phillips, 1988b)
Sodium nitrate ($NaNO_3$)/ Ferric Phosphate ($FePO_4$)	20	360 /360	To prevent Fe release into the liquid phase	2000	Eq. (4-44)	Hypothesised by this study, based on literature including (Lovley and Phillips, 1988b)
Sodium nitrate ($NaNO_3$)/ Ferric phosphate ($FePO_4$)/ Ferric oxide (Fe_2O_3)	13/13	120 /120	To prevent Fe release into the liquid phase	2600	Eq. (4-44)	(Lovley and Phillips, 1988b)

Terminal Electron Acceptor (TEA)	**Mass of Fe (mg)**	**Micro moles of Fe (µmol)**	**Purpose of reactor**	**Max con-centration of Fe, if all released (mg/l)**	**Expected reactions**	**Reference**
Delayed experiment						
Ferric phosphate ($FePO_4$) + Sodium nitrate ($NaNO_3$, day 10)	40	720	Stop Fe release into the liquid phase	4000	Eq. (4-22) Eq. (4-33) Eq. (4-44)	Hypothesised by this study, based on literature including (Lovley and Phillips, 1988b)
Experimental controls						
Ferric citrate ($FeC_6H_5O_7$)	30.3	600	Broth Control	4000	Eq. (4-55)	(Lovley and Phillips, 1988b)
Sodium nitrate ($NaNO_3$)	0	0	To measure iron release from cells, in the absence of soluble or solid phase iron, as a possible stress (osmotic) response	0	Eq. (4-44)	(Lovley and Phillips, 1988b)
Sodium nitrate ($NaNO_3$)[1]	0	0	Control of TEA solubility	0	N/A	Safety data sheet provided by supplier
Ferric phosphate ($FePO_4$)[1]	40	720	Control of TEA solubility	4000	N/A	Safety data sheet provided by supplier
Ferric oxide (Fe_2O_3)[1]	40	720	Control of TEA solubility	4000	N/A	Safety data sheet provided by supplier
Ferric Phosphate ($FePO_4$)/ Ferric oxide (Fe_2O_3)[1]	20/20	360 /360	Control of TEA solubility	4000	N/A	Safety data sheet provided by supplier

[1] These had no growth media, only ultrapure water, and acted as controls, for the solubility of these TEA in Milli-Q (18.2 Ω) water.

4.3.4 Expected reactions

As *G. metallireducens* has been extensively studied previously, it is possible to predict the reactions that would occur as the bacterium metabolises the TEA's found in each reactor.

4.3.4.1 Ferric oxide

Ferric oxide and in particular the amorphic source has been shown to be readily metabolizable by *G. metallireducens* as seen in reaction (4-11) (Lovley, 1993; Lovley and Phillips, 1988a, 1988b).

$$CH_3COO^- + 3Fe_2O_3 + 3H_2 0 \rightarrow\ 2Fe_3O_4 +\ HCO_3^- + CO_2 + H_2 0 + 6H^+ + 6e^-. \tag{4-11}$$

4.3.4.2 Ferric phosphate

The demonstration of the ability for *G. metallireducens* to metabolise ferric phosphate is expected to be a contribution of this study to the scientific literature. Whilst other iron forms, such as akageneite, goethite, and ferric citrate, have been shown to be able to be metabolised, no evidence exists in the case of ferric phosphate (Childers, Ciufo and Lovley, 2002; Finneran, Housewright and Lovley, 2002). The proposed reaction scheme, shown in reaction (4-22), indicates its importance through the potential to release both iron and phosphate.

$$CH_3COO^- + 8FePO_4 + 3H_2 0 \rightarrow\ 8Fe^{2+} + HCO_3^- + CO_2 + H_2 0 + 8PO_4^{3-} + 8H^+ \tag{4-22}$$

This has been hypothesised to be the system of iron release within nature by iron reducing bacteria (Dotro et al., 2015), although the metabolism has never been outlined in detail. The release of both ferrous and phosphate has the potential to result in the biologically mediated formation of vivianite, reaction (4-33) (Roussel and Carliell-marquet, 2016).

$$CH_3COO^- + 8FePO_4 + 3H_2 0 \rightarrow 2Fe_3(PO_4)_2 + HCO_3^- + 6PO_4^{3-} + 8H^+ + CO_2 + 2Fe^{2+} \tag{4-33}$$

The impact of this is a reduction in the resultant dissolved iron to one quarter of the level depicted in reaction (4-22). It was not possible to measure vivianite during these experiments but its generation is considered unlikely due to the lack of optimum conditions for its formation and its slow overall precipitation kinetic (Walpersdorf et al., 2013).

4.3.4.3 Nitrate

Nitrate is the main competing ion related to the metabolism of *G. metallireducens* showing two possible reaction pathways, shown in reaction (4-44) and (4-55) (Lovley and Phillips, 1988a, 1988b; Coby et al., 2011).

$$CH_3COO^- + NO_3^- + 2H^+ \rightarrow NH_4^+ + HCO_3^- + CO_2 \qquad \textbf{(4-44)}$$

$$Fe^{2+} + 0.125NO_3^- + 1.625H_20 \rightarrow 0.125NH_4^+ + 1.75H^+ + FeOOH \qquad \textbf{(4-55)}$$

The relative significance of the two relates to the availability of organic TED, depicted as acetate, which drives an oxidation pathway. In the absence of organics, *G. metallireducens* has been shown to have the ability to oxides ferrous iron whilst converting nitrate to ammonia. It is worth noting that it is reported that a bacterium thought to have been functioning as iron reducing cannot then switch pathways and become iron oxidising (Coby et al., 2011; Finneran, Housewright and Lovley, 2002).

[redacted]Results and Discussion

4.4.1 Release of iron

Incubation of solid Fe species with *G. metallireducens* resulted in an increase in Fe concentration in the liquid phase within six days, ultimately reaching 2.4 Fe mg/l (Fe_2O_3), and 0.6 Fe mg/l ($FePO_4$) after 10 days (Figure 4-11). The control experiment reactors confirmed that the Fe sources used in these reactors were insoluble, and no abiotic release was measured (data not shown). The observed Fe concentration pattern across the ten days revealed a lag for the first four days where the concentration oscillated leading to a Fe concentration of 0.36, 0.43 and 0.49 Fe mg/L for the oxide-phosphate mix, phosphate and oxide systems respectively. Thereafter, all systems released iron with a significantly greater amount released in the case of iron oxide. Normalised to the initial cell number the respective release rates were 2.26 x 10^{-15} g Fe/day/cell for Fe_2O_3 and 3.07 x 10^{-16} g Fe/day/cell for $FePO_4$. Such rates align to previous data concerning the release rate generated when using amorphic iron (III) oxide with *G. metallireducens* where a rate of 1.7 x 10^{-15} g Fe/day/cell (Lovley and Phillips, 1988b). The measured rates also fit between those reported for Akageneite at 5.05 x10^{-16} g Fe/day/cell and Goethite at 1.16 x10^{-16} g Fe/day/cell indicating a possible order of preference by rate of release (Lovley and Phillips, 1988a). The impact of the difference of the rates is illustrated by the fact that the time to generate an equivalent release of Fe from ferric oxide and ferric phosphate would be 6 and 25 days respectively.

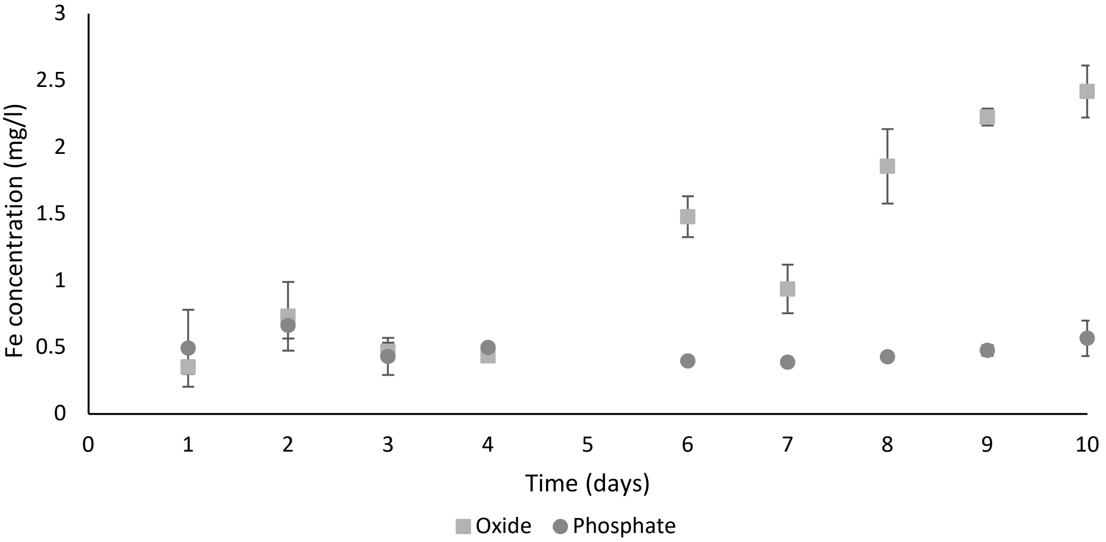

Figure 4-11 Iron concentration throughout the experimental period for the ferric phosphate and ferric oxide reactors. Error bars represent the standard deviation (n=3)

The release profile when using an equimolar mixture of ferric oxide and ferric phosphate was similar to that of the ferric phosphate alone. A slight difference was observed in relation to release rate which was 3.07x10^{-16} g Fe/day/cell with a corresponding final iron concentration of 0.59 mg/l on day 10 of the trial (Figure 4-22). Statistical analysis of the daily data using a one-way Anova revealed significantly difference between the ferric oxide and the other two systems and a statistically significant difference between the mixture and iron phosphate only on days 8 and 10. Further analysis using a Tukey post T test confirmed the similarity between the iron phosphate and the equimolar mixture of ferric phosphate/ferric oxide. Such similarity was unexpected and revealed that the mixed system operated over a more complex set of pathways than can be explained simply by the combination of the two individual ones. However, iron release was observed for all three systems, albeit at different rates per cell (Figure 4-22). Previous work has demonstrated that iron reducing bacteria, similar to *G. metallireducens,* are present in all wetlands (Chapter 2) and can be abundant in anoxic environments (Adrados et al., 2014). Accordingly, the likelihood of iron and potentially phosphate is high when conditions are favourable for the reported pathways. Further, management of the risk must focus on alteration of the local environment to disfavour/ inhibit the identified pathways.

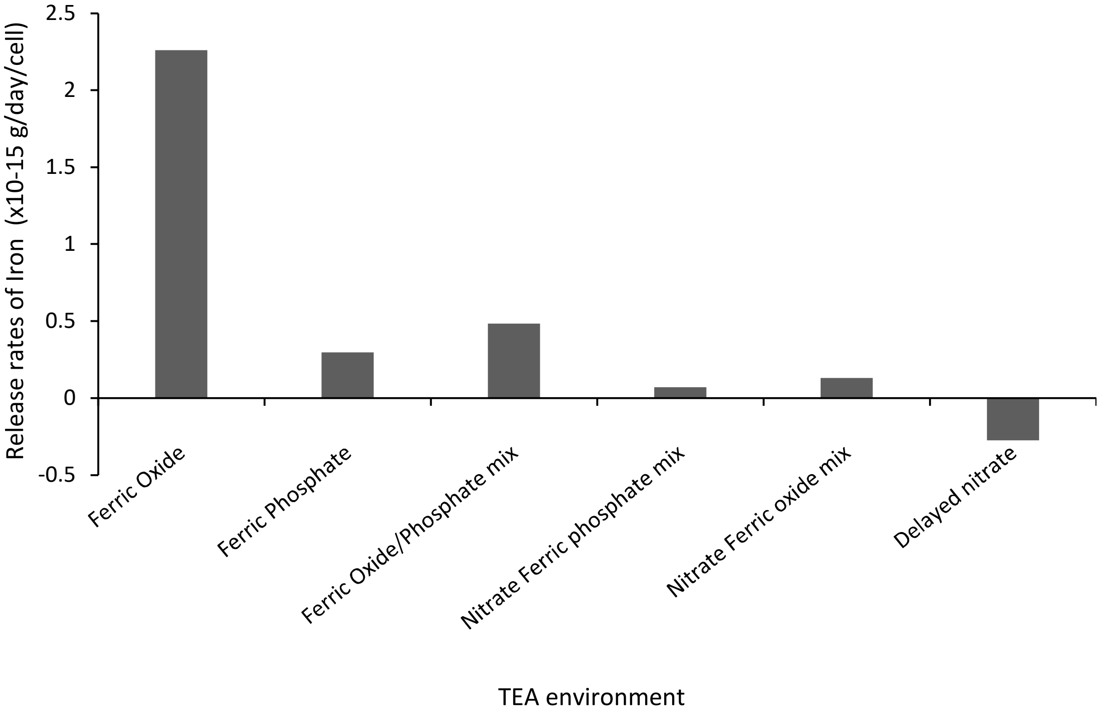

Figure 4-22 Rates of iron released from each TEA environment used in this study

The first four days for all reactors exhibited similar fluctuation patterns (Figure 4-11). This included the control reactors that contained no solid phase iron along with a broth that was absent in iron in all cases. Accordingly, it is suggested that the initial variation of iron reflects a release from the cells in order to establish an equilibrium between the solid and liquid phases. Previous work has shown a similar impact due to oxygen stress on the strictly anaerobic *G. metallireducens*

(Lovley and Phillips, 1988b). However, the broth was maintained oxygen free through the use of nitrogen bubbles prior to the experiments, negating this option. Further, the lag period is congruent with a previous study that demonstrated that the bacterium required three days to be able to metabolise the insoluble TEA (Childers, Ciufo and Lovley, 2002).

4.4.2 Impact of nitrate addition on iron utilisation

Inclusion of nitrate into the systems altered the profile of iron release (Figure 4-33). Whilst the initial four day period followed a similar pattern, the iron release remained relatively flat thereafter. The respective iron release rates per cell were 6.89×10^{-17} g Fe/day/cell and 1.31×10^{-16} g Fe/day/cell for nitrate-ferric phosphate and the nitrate-ferric oxide systems respectively. Importantly, the presence of nitrate inhibited the relatively fast rate of iron release previously seen with respect to the ferric oxide system (Figure 4-11). Restriction of iron utilisation in the presence of nitrate was further supported through a separate test using a ferric citrate rich broth containing *G. metallireducens* (Figure 4-44). Two tests were conducted, with and without a dose of 1 mmol of sodium nitrate. The non-dosed sample underwent a noticeable colour change from orange (fresh) to dark green/brown once the bacterium had been able to metabolise some of the ferric citrate. The nitrate dosed sample remained unchanged congruent with current understanding of TEA preference by *G. metallireducens* (Lovley and Phillips, 1988b).

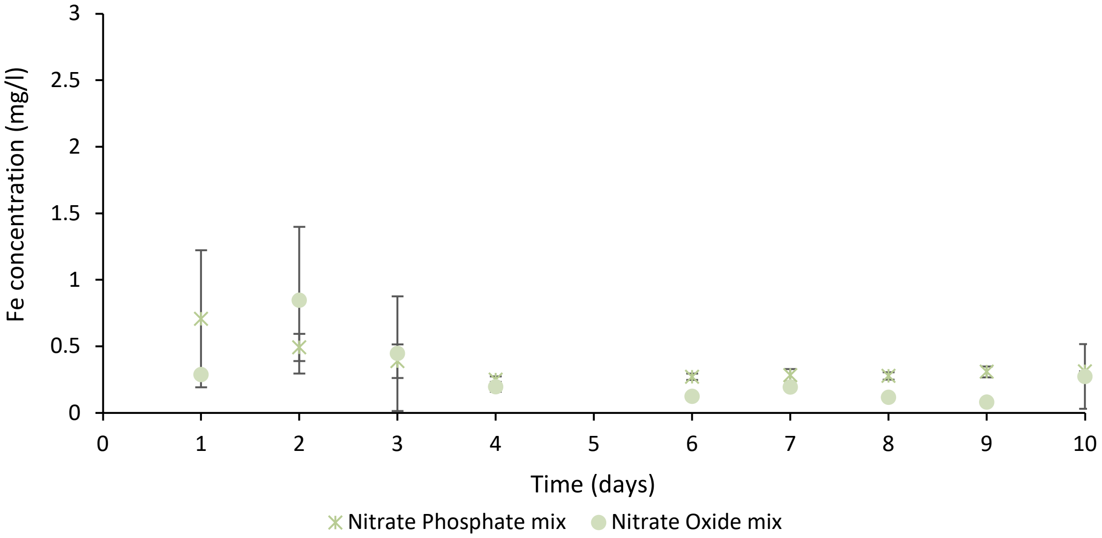

Figure 4-33 Impact of nitrate addition expressed as the absolute iron concentration profile during the experiment when nitrate is present from day zero. Error bars are the standard deviation (n=3)66

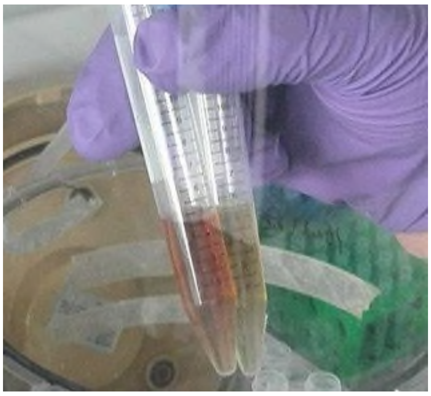

Figure 4-44 Impact of nitrate showing visually as colour change in reactors with ferric citrate

The impact of nitrate addition on an already releasing systems was then considered by dosing 1 mmol of nitrate into the ferric phosphate tests cells reported in Figure 4-11. The addition of nitrate on day 10 stopped further release of iron which then fluctuated but with a reducing tendency thereafter. This was confirmed through statistical testing which revealed that the iron concentration was significantly lower at the end of the experiment than prior to nitrate dosing with a concentration of 0.387 ±0.01 mg/l on day 19 and 0.56 ±0.1 mg/l on day 10. These observation match the natural environment where the addition of nitrate has been used as a methodology to limit P release (Foy, 1986). This was believed to be due to the ability of nitrate to supress additional release, allowing longer term natural process to remove the P from the water table. In the current case, the reduction is, in part, related to the measurement which is based on soluble Fe^{2+} by pre-filtration through a 0.45 μm filter. Accordingly, the reduction reflects a small increase in colloidal iron that is rejected by the filter paper. The generation of this precipitate of ferrous ions is congruent with previous research that has demonstrated that some nitrate reducing organisms have an innate ability to oxidise Fe by coupling it to the utilization of nitrate (Carlson et al., 2013) including *G. metallireducens* (Coby et al., 2011; Finneran, Housewright and Lovley, 2002). As such this implies a switch in both the TEA (Fe^{3+} to nitrate) and the TED (acetate to Fe^{2+}) used by *G. metallireducens*. This chemotrophic pathway has previously been shown to occur in organic depleted systems (low acetate, Coby et al., 2011) which does not occur in the current trial. Whilst interesting, further investigation is required to elucidate the potential for this pathway to occur and validate its viability in more organic rich systems.

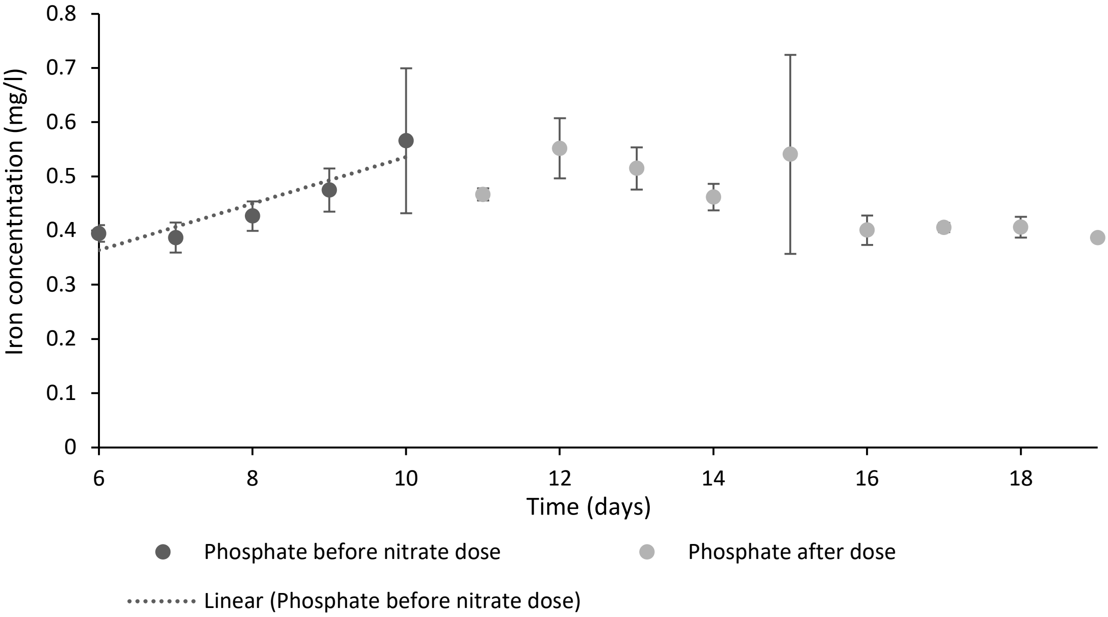

Figure 4-55 Concentration of iron in the overlying broth from the pure $FePO_4$ reactors both before and after the nitrate dose on day 10. Error bars are the standard deviation (n=3)

[redacted] Conclusions

The metabolism of *G. metallireducens* was shown to be capable of resolubilising iron from a pre-existing solid material. Differential rate of release were observed from comparative experiments with different ferric types. A greater rate of release was observed with ferric oxide compared to ferric phosphate. Comparison to a mix system showed similarity to the ferric phosphate indicating that the release in real systems is defined beyond just simple addition of the individual release rates of the different components. The generic presence of iron reducing bacteria means that management of the release risk must be accomplished through inhibition of the metabolic pathway associated with iron utilisation. This is achieved through simple nitrate dosing which was shown to both inhibit further release and then reduce the residual level. The latter offers the potential for a new mechanistic pathway whereby the organisms can switch to a chemotropic action.

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[redacted]Supplementary information

4.7.1 Broth

Heat about 400 ml of water on a hot stir plate to near boiling. Add ferric citrate, allow it to dissolve, then cool the solution to room temperature in a slurry of ice. Add 400 ml of water; this quickly cools the medium to room temperature. Adjust the pH to 6.0 using 10 N Sodium hydroxide; when the pH approaches 5.0, add Sodium hydroxide by drops until the pH level is established. Approximately 6 ml of 10 N Sodium hydroxide per liter will be needed per liter of medium. Add the remaining ingredients and bring the final volume up to 1.0 L with water. Bubble the medium with 80% N2 and 20% CO2. The final pH should be 6.8-7.0. Do not expose this medium to sunlight.

Geobacter growth broth components and quantities, as directed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Table S 4-11 Contents of bacterial growth broth as directed by the DSMZ

Component	**Quantity per Litre**
Ferric citrate	13.7 g
Wolfe's Vitamin Solution	10 ml
Wolfe's Mineral Solution	10 ml
Sodium bicarbonate	2.5 g
Ammonium chloride	0.25 g

Sodium Dihydrogen Phosphate Monohydrate	0.6 g
Potassium chloride	0.1 g
Sodium acetate	6.8 g
Distilled, deionized water	1 L

[redacted] Wolfe's Vitamin Solution

Table S 4-22: Contents of Wolfe's vitamin solution as directed by the DSMZ

Component	**Quantity per Litre**
Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Thiamine . HCl	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D-(+)-pantothenate	5 mg
Vitamin B12	0.1 mg
p-Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Distilled water	1 L

[redacted] Wolfe's Mineral Solution

Add nitrilotriacetic acid to approximately 500 ml of water and adjust to pH 6.5 with KOH to dissolve the compound. Bring volume to 1.0 L with remaining water and add remaining compounds one at a time.

Table S 4-33: Contents of Wolfe's mineral solution as directed by the DSMZ

Compound	**Quantity per Litre**
Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.1 g

CaCl2	0.1 g
ZnSO4 . 7H2O	0.1 g
CuSO4 . 5H2O	0.01 g
AlK(SO4)2 . 12H2O	0.01 g
H3BO3	0.01 g
Na2MoO4 . 2H2O	0.01 g
Distilled water	1 L

5 Profiling iron and associated phosphorus dynamics in treatment wetland sludge under different biogeochemical conditions

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[redacted] Abstract

The removal of phosphorus from sewage at small wastewater treatment works is typically achieved by the addition of iron or aluminium salts upstream of biological treatment processes, including constructed wetlands. Whilst this enables removal of phosphorus from the liquid, it creates a different biogeochemical environment within the constructed wetlands that is poorly understood. This research focused on the enriched sludges caused by iron dosing to study the potential for, and mitigation of, release due to iron and phosphorus transformations. To study this sand was stored in an open top reactor, before sludge and then water was placed above. This allowed the stratification of layers mimicking a constructed wetland. This reactor was then incubated at room temperature and filtered pore water samples were then taken daily. The pore-water sample was then analysed over time for phosphorus and iron contents as well as redox potential. Results showed that iron release followed a linear trend from day 4 at a rate of 82.8 mg/m^2/day (R^2 of 0.76), in comparison the phosphorus release was lower with 4.36 mg/m^2/day (R^2 of 0.72), from day 3. The addition of oxygen induced a positive redox change of over 60 mV (from -132 mV up to -68 mV at its maximum impact) within the overlying water column without significantly affecting the sludge layer redox. The addition of nitrate led to a steady increase in the redox potential of up to 482 mV within the sludge layer, whilst inducing variable changes above the sludge layer. These impacts were suggestive of a chemically driven process for oxygen and a biologically-mediated process for nitrate. Further, nitrate addition was found to remove both iron and phosphorus from the water column, despite not increasing the redox to a positive level. Results suggest nitrate addition could be considered a mitigation step for the release of iron and/or phosphorus and offer a remedial action. Finally, the lack of a consistent ratio between released iron and phosphorus suggests a preferential pathway for the release of iron speciation within the wetland sludge.

Keywords: Iron; Phosphorus; Release; Constructed Wetlands; Mitigation;
Nitrate

[redacted] Introduction

Constructed wetlands (CWs) are a treatment technology that can provide biological and physical treatment of a variety of wastewaters, including sewage. The removal of phosphorus (P) by CWs occurs primarily via adsorption, sedimentation and biomass uptake (García et al., 2010). In sewage applications, the P loading rates typically applied to CWs are 0.8 g/m^2/d (Tanner, Clayton and Upsdell, 1995; Wang et al., 2018), which far exceeds the capacity of the system to retain P in the long term (Vymazal, 2004). P removal from sewage is increasingly being demanded, driven by the introduction of stricter international legislation with for example The Urban Wastewater Treatment Directive, the Water Framework Directive in Europe and the 2012 Great Lakes Water Quality Agreement in North America (EC, 2000; EEC Council, 1991; Government of Canada and Government of the United States of America, 2013; UKTAG, 2013). As a result, the addition of a chemical precipitation step and settling prior to the wetland has been employed to enhance P removal (Brix and Arias, 2005; Dotro et al., 2015; Kim et al., 2015; Lauschmann et al., 2013). When using iron (Fe) salts, as commonly done in the UK, this additional step results in Fe and P rich sludge accumulating in the CW, with reported concentrations of 178 g Fe/kg of dried sludge and 48 g P/kg of dried sludge, compared to 30 g Fe/kg and 2 g P/kg in the sludges of non-dosed wetlands (Dotro et al., 2015; Stockman, 2007). As the chemical of choice for coagulation is ferric sulphate, this also increases the levels of sulphur found in these sludges with values as low as 0.057 g S/kg, in natural wetlands compared to consistently over 8 g S/kg in dosed CW (Barak, 2014; Jokic et al., 2003)

Fe is a redox sensitive element, typically existing in several valency forms, with the most important being ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) (Miller, 2013; Sano, 1990). Within the natural environment, Fe can change between these two valences multiple times depending on biogeochemical conditions (Ehrlich and Newmann, 2009). Whilst initially believed to be entirely driven by chemical transformation processes occurring at redox values below +200 mV (Boström et al., 1988), microbially-mediated Fe transformation is now believed to be the driver for Fe reduction seen in anoxic soils and sediments (Lovley et al., 1993; Nealson and Saffarini, 1994). The current understanding places Fe as a terminal electron acceptor (TEA) used by iron reducing bacteria (IRB), with this reduction occurring in the absence of higher energy yielding TEAs such as nitrate (Lovley and Phillips, 1988b), or manganese (Lovley and Phillips, 1988a). Whilst Fe cycling has been extensively studied in the natural environment, limited studies have attempted to characterise microbially-mediated Fe transformations in sewage treatment technologies. With chemically-dosed CW becoming large sinks of P and Fe, understanding the release risk is of paramount importance.

Although the majority of CW act as sinks, temporary or intermittent release of P and Fe has been reported in some systems (Dotro et al., 2015; Kim et al., 2015). The proposed mechanism was hypothesised to be microbially-mediated reduction of Fe leading to the breakdown of Fe based compounds such as ferric phosphate and consequently inducing the simultaneous release of Fe and P into the pore water. Although present in sludge and known to react with P to form precipitates such as calcium phosphate, calcium was unable to supress release of P at neutral pH, typical of chemically-dosed CW (Dunets, 2014). Indeed, higher pH, (greater than 10), have been shown to be necessary for calcium phosphate precipitation et al., 1971; Semerjian and Ayoub, 2003). In addition, P and Fe release associated with suspended solids in the effluent was discarded as the main contributor in conventional CWs as the systems exhibited good solids removal and the majority of P in the effluent was in the soluble phase (Dotro et al., 2015).

It is well-known that many IRB can utilise either nitrate (NO_3^-) or Fe^{3+} as TEA, with preference for nitrate utilisation under carbon-rich conditions. This is believed to be because of the higher energy yield of nitrate, at 95% of the aerobic yield compared to 20% of aerobic yield when using iron (Strohm et al., 2007). The use of oxygen for carbon oxidation is known to provide a higher yield TEA, however many IRB are strictly anaerobic so would be unable to utilize this (Ehrlich and Newmann, 2009). Also, Fe reducing organisms have been shown to be present within CW and, in some cases, to be the dominant group (Adrados et al., 2014). The addition of nitrate can then be proposed as a potential pathway to retain P and Fe in the sludge layer, as it can either chemically affect the redox of a waterlogged soil (Gardiner and James, 2012) or inhibit the metabolism of iron by IRB (Lovley and Phillips, 1988b). Ultimately, understanding the mechanisms associated with P and Fe release from sludges will have direct implications for sustainable management of chemically-dosed constructed wetlands for P removal from municipal wastewater and can also contribute to the current understanding of Fe and P cycling in impacted ecosystems in the natural environment.

The aim of this study was to investigate the potential for, and suppression of Fe and P release from the sludge accumulated at a Fe dosed CW. The first objective of this study was to show that Fe and P can be released from the sludge, whilst the second was to investigate if and then how this release could be supressed with the addition of an alternative TEA.

[redacted]Materials and Methods

5.3.1 Field sampling

Sludge was collected from two trough-fed horizontal subsurface wetlands (85 m x 12.5 m) at a sewage treatment works serving 1326 population equivalents in the Midlands region of the UK. The flowsheet at this site consisted of three integral rotating biological contactors (RBCs) dosed with ferric sulphate (40% by volume) at the inlet followed by a cross-flow settlement (humus) tank, which is desludged back into the primary zone on a timer, and then the subsurface horizontal flow (HF) wetlands. The HF wetlands are used as a tertiary filtration step to remove any remaining particulate organic matter and suspended solids, including any residual Fe and Fe-bound phosphate. In addition, the site has a storm overflow on the crude sewage to divert flows greater than 19.1 l/s. The discharge from this overflow bypasses the RBC, is screened by a cloth filter, and mixed with the secondary treated wastewater in the inlet of the wetland. The CW were refurbished in 2006, 10 years before this sampling took place, during which the gravel media (<10 mm) was removed, washed and returned to the site. The overlying sludge layer went to landfill. Common reed (*Phragmites Australis*) was planted at a density of 4 plants/m^2. The plants are not harvested so plant detritus can act as a source of P and organics, returning it to the sludge layer. The wetlands are designed for a hydraulic residence time of 24 hours, and for a feed flow rate of 3.6 l/s.

The sludge (8.5 kg) was collected from 17 completely randomised places at the surface of the wetland using a trowel, before placing in airtight plastic containers. Sludge samples were transported to the research laboratory (BfG, Koblenz, Germany) and stored at 4ºC in the dark. Prior to the experiments starting, the sludge was placed in a water tight barrel and homogenised by mixing, before being brought up to room temperature for two weeks. The control experiment followed the same procedure and was conducted within the laboratory at Cranfield University (UK).

5.3.2 Experimental setup

Three open top reactors (SOFIE®, Delatres, The Netherlands, as used by Duester et al., (2008) and Vink (2002)), were set up by filling them to a depth of 3 cm with sand, topped by an 8 cm layer of the homogenised wetland sludge and a 5 cm layer of tap water to force anoxic conditions to form (Figure 5-11). These were ran for 13 days for each test and control conditions, allowing the experiment to be repeated in triplicate. Time zero was defined as the day when the water and sludge were mixed in the reactor. A reversed aqua regia digestion (Duester et al., 2005) of the sludge found concentrations of 139 g/kg, 51 g/kg, and, 10 g/kg of Fe, P and sulphur, respectively, whilst the tap water used was shown to have a concentration of <0.03 mg/l Fe, <0.1 mg/l P, and, <0.24 mg/l sulphur.

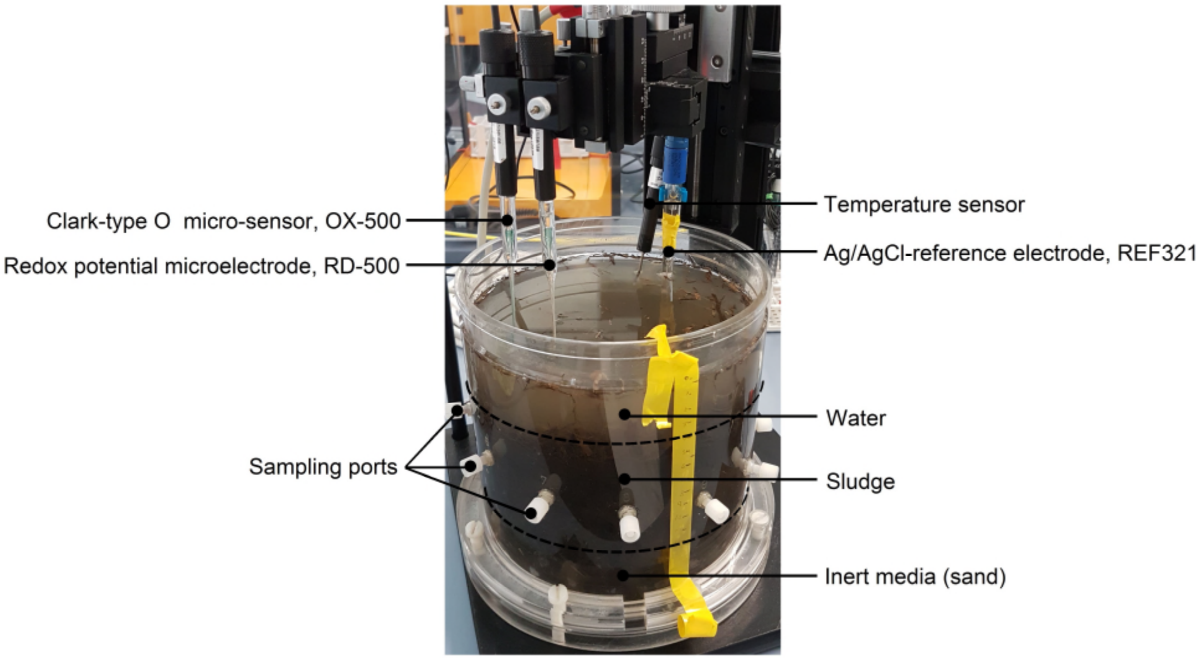

Figure 5-11 Reactors employed in this study, showing the location of the sand, sludge and water levels as well as sampling ports and probes.

The SOFIE® systems contains 13 ports in a spiral pattern that descend in 1 cm vertical increments with a 2 cm horizontal displacement between the ports. In six alternates ports, to allow for the sampling of pore water, 0.45 µm filter-probes (Pijpker, The Netherlands) were inserted. In relation to the sludge surface these filters were positioned at a depth of -2, 0, 2, 4, 6, 8, and 10 cms. A peristaltic pump (Ismatec, Germany) was attached to these ports for the removal of pore waters at a rate of 1 ml/hr, which was slow enough to avoid disturbing any potential sediment horizons.

For the test reactors, four concentrations of nitrate were sequentially added on days 4, 6, 8, and 11 to the system, namely: 500, 5000, 15000, and 30000 µmol corresponding to average nitrate concentration of 8.5, 93.5, 348, and 856 mg/l, respectively. To add nitrate to the system, aliquots from a stock solution of tap water fully saturated with sodium nitrate (912 g/l) were taken. On days when dosing occurred, the dose was applied 4 hours before that day's sample was starting to be taken. As there was three reactors set up, this allowed the effects of this nitrate addition to be studied in triplicate.

In a second experiment, that was ran sequentially, oxygen and an initial nitrate dose of 105 µmol each were added to the reactors, resulting in system concentrations of 1.7 mg/l and 9 mg/l, respectively. Oxygen dosing was achieved by adding 180 ml of fully saturated tap water (DO = 9.26 mg/l), after aeration for 10 minutes. This volume was chosen to be c. 5% of the reactor's total water volume. A further Nitrate dose was added, with the second dose after the redox change following the initial dose was depleted and consisted of a further addition of 105 mmol of nitrate. Both nitrate additions were achieved by dosing 10 µl, and 10 ml of the stock solution into the reactors overlying water column. These experiments were performed within an anoxic chamber purged with 100 % Argon, at room temperature (c. 20C).

5.3.3 Sample processing and analyses

For each pore water sample, a total volume of 5.5 ml was collected from each depth; of this, 2.5 ml was acidified with 50 µl of nitric acid (65% w/w, Sigma Aldrich, Germany), and analysed for phosphorus, calcium, sulphur, and iron content using an ICP OES (Perkin Elmer ICP OES Optima 8300, Germany) or ICP MS (Perkin Elmer, U.K), depending on the trial. Another 2.5 ml was further filtered to 0.1 µm (EMD Millipore™, Fisher Scientific, Germany) which involved being centrifuged at 450 G for 30 minutes, before being acidified as described above, before analysed by the same ICPMS as previously used. The remaining 0.5 ml was then discarded. Water sampling was conducted every 24 hours.

Measurements of the oxygen concentration and the redox potential were conducted by means of a micro-profiling system equipped with a computer controlled motorized micromanipulator. A Clark-type oxygen micro-sensor (OX-500, Unisense, Denmark) was used to measure the oxygen concentration with the redox profile conducted by the application of a redox potential microelectrode (standard hydrogen potential; RD-500, Unisense, Denmark) connected to an Ag/AgCl-reference electrode (REF321, Radiometer Analytical, Unisense, Denmark). These were inserted in the system from above (Figure 5-11) and took readings every 25 µm, on a cycle that was repeated at least every 12 hours.

The data was processed using Suite software provided by suppliers of the micro-sensors (Unisense, Denmark), Microsoft Excel and Minitab. Statistical analysis included a one-way ANOVA and Pearson correlations, both run at the 0.05 significance level.

[redacted]Results

5.4.1 Baseline iron and phosphorus dynamics

Results from the control system (not dosed with sodium nitrate) showed a release of soluble (<0.45 µm) Fe and P through time (Figure 5-22A). A greater increase in the concentration of soluble Fe occurred within the deeper layers of the sludge compared to the rest of the system, with the largest increase at a depth of 4 cm within the sludge layer. This corresponded to Fe concentrations of 47 mg/l to 119 mg/l at days 1 and 13, respectively (Figure 5-22B). Through time, the change in concentrations in depth becomes more distinct, showing an increase in absolute numbers and the area in which this elevated soluble Fe occurs. The smaller increase seen during the experiment at the shallower depth layers (such as 0 and 2 cm deep) compared to the increase seen deeper in the reactor (such as at 4 and 6 cm depth), indicates that a greater release of soluble Fe can be expected deep within the sludge layer. The greater release at depth also coincided with a decreasing redox potential through the sludge profile. A decreasing redox potential increases the likelihood of Fe being in its reduced and soluble Fe^{2+} phase, resulting in increased Fe in the water phase deeper in the sludge profile. Over 90% of all measured Fe was between 0.1 µm and 0.45 µm, suggesting a predominance of colloidal Fe forms.

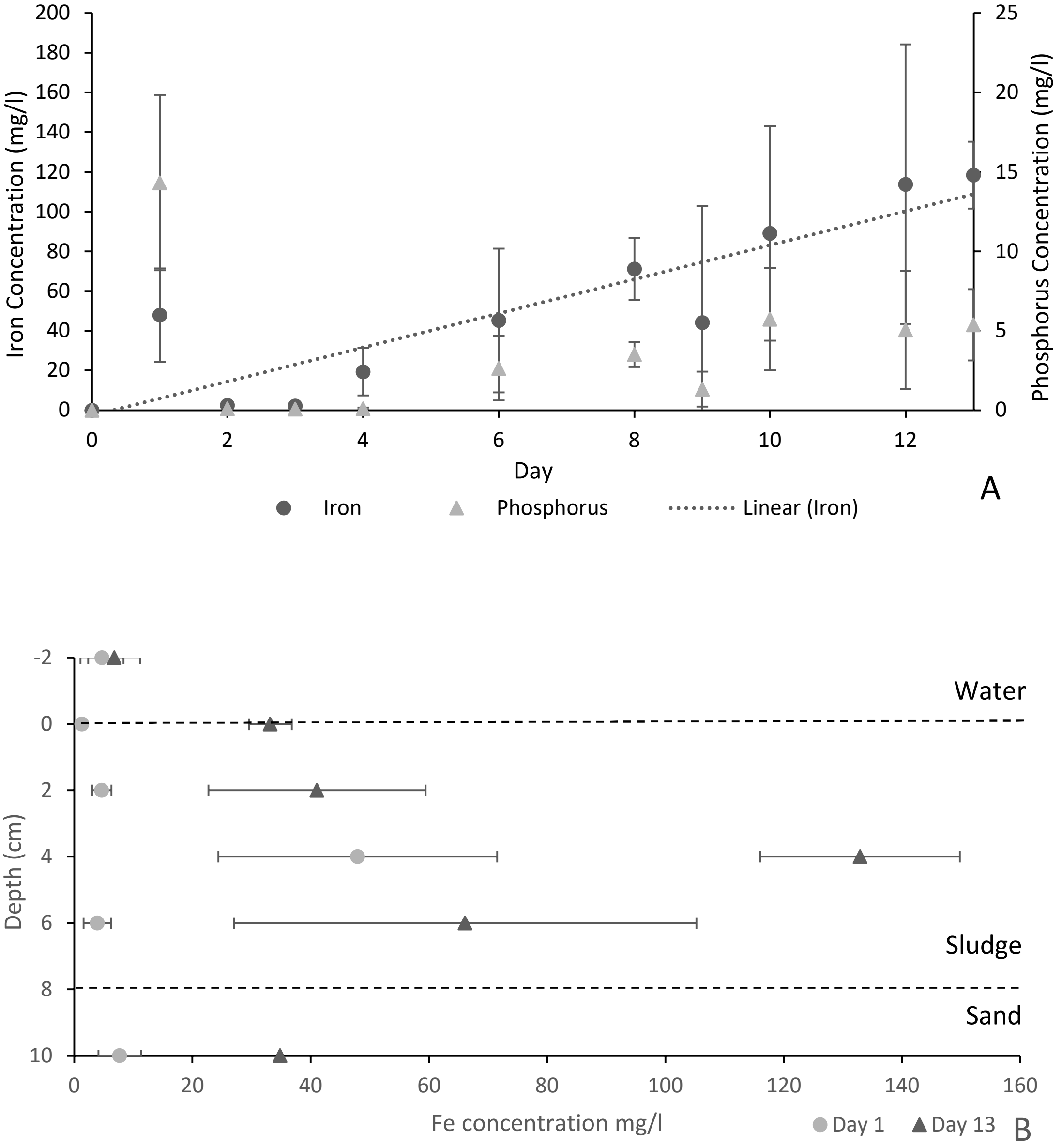

Figure 5-22 A) Iron and phosphorus concentrations evolution over time in the control experiment at a depth of 4 cm within the sludge. Error bars represent standard deviation, n=3. B) Iron concentrations in the pore water within the sand, sludge and water layers on days 1 and 13 of the control experiment. Error bars represent standard deviation, n=3.

When considering a depth of 4 cm within the sludge, there was an initial Fe release followed by a period of removal that lasted 24 hours, followed by a stable period of no net release for an additional 48 hours (Figure 5-22). On day four, Fe release was observed, which resulted in a linear increase of soluble Fe concentration over time in the pore water until the end of the experiment at day 14 ($R^2 = 0.76$). A slower release of P was observed which followed a linear trend from day 3 of the experiment ($R^2 = 0.72$). The release rate of Fe from the sludge within in the control experiment corresponded to area release rate of 82.8 mg/m^2/day, whilst P was 4.36 mg/m^2/day. These release rates contrasted with the significantly larger release recorded from a previous study on a Fe dosed CW (340 mg/m^2/day for Fe and between 12-52 mg/m^2/day for P (Dotro et al., 2015)). However the P release rate found was similar to the 4.77 mg/m^2/day found in river sediments (Malecki, White and Reddy, 2004). There was no direct relationship between Fe and P concentrations found, and a one-way ANOVA showed that the means of the Fe/P molar ratio for each day were statistically different ($P<0.001$), ranging between 1.8:1 and 111:1.

Sulphur showed a linear increase ($R^2 = 0.88$) from 111 mg/l to 1346 mg/l between days 2 and 13 (data not shown). The increasing amount of sulphur in the pore water was consistent with a continuously decreasing redox potential. Calcium showed no overall trend (data not shown), Calcium is known to be a redox inactive element unlike iron and sulphur (Lionetti and Agapie, 2014), so the lack of any consistent trend as redox decreased was expected. The pH peaked at 7.6, minimising any direct impacts of Calcium on P levels (pH greater than 10 needed for significant calcium and phosphorus interactions (Jenkins, et al, 1971). Whilst there has been reports of gypsum formation in both high saline natural and metal rich constructed wetlands (Karathanasis and Thompson, 1995; Steinwand and Richardson, 1989), indicating that there is the potential for sulphur and calcium interaction. It is hypothesised that this did not occur within the reactors as there was no significant trend between the two. Further investigation is required into the reason for the fluctuation of calcium levels as it is currently unknown.

5.4.2 Impact of nitrate and oxygen supplementation on biogeochemical conditions

Nitrate and oxygen additions to the bioreactors had significantly different impacts on the system's redox profile (Figure 5-33). The oxygen spike led to an instantaneous positive change in the redox potential above the sediment water interface, raising the redox in the overlying water layer by up to 60 mV, from -132 to -72 mV. Within the sludge layer, the effect was minimal and tailed off to an increase of 5 mV at a depth of 5.5 cm, three hours after the dose. An equivalent dose of nitrate resulted in an insignificant change in the redox profile of both the water and the sludge layer (data not shown). In contrast, the large dose of nitrate (9 g/l) induced a variable effect on the redox around the sediment water interface, and a steady and stable increase within the sludge layer by up to 462 mV (Figure 5-44), from -510 to -48 mV. The area of influence within the bioreactor was significantly different between the oxygen and the large nitrate dose, with nitrate affecting the redox profile from the lowest 1 cm of overlying water to 5.5 cm of sludge depth whereas the oxygen effect was greater between 1.5 and 2.5 cm above the sludge.

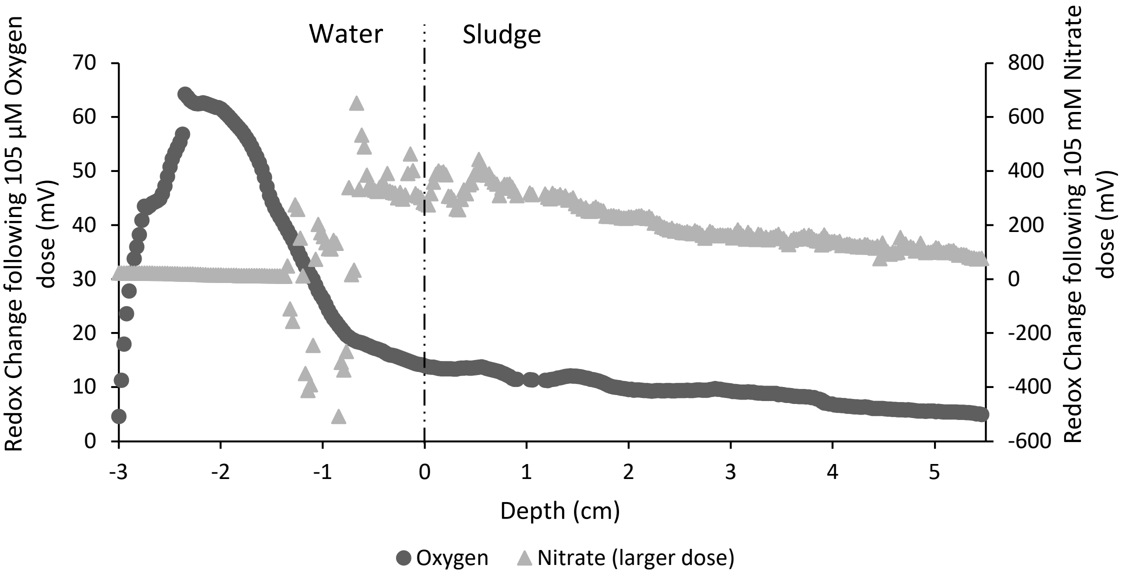

Figure 5-33 Comparison of redox change following addition of TEA as nitrate (9 g/L dose) and oxygen. These cycles of measurements took 6 hours and were taken immediately after the dosing occurred

The oxygen-induced redox change was short lived and disappeared within 8 hours. This was most likely chemically-induced with the oxygen added reacting with any reduced products encountered within the water levels. The nitrate-induced redox change was also temporary, however it lasted longer with a positive change to pre-dosage redox observed for 48 hours.

5.4.3 Impact of nitrate additions on P and Fe cycling

Sequential dosing of nitrate was found to lower the concentration of Fe and P measured within the pore water of the system (Figure 5-44A). The initial addition of nitrate (day 4, system concentration of 8.5 mg/l, 62 mg NO_3^-/kg sludge) caused a decrease of 16 mg/l in Fe concentration in the pore water between days 2 and 4. The following additions of nitrate (day 6, system concentration of 93.5 mg/l, or 682 mg NO_3^-/kg sludge) supressed any further release for 36 hours. The addition at day 8 (system concentration 348 mg/l, 2542 mg NO_3^-/kg sludge) was able to lower the pore water concentration of Fe down to 11 mg/l. The final addition at day 11 (system concentration of 856 mg/l, 6262 mg NO_3^-/kg sludge) virtually removed all Fe from the pore water. The cumulative nitrate doses had a negative linear relationship (R^2= 0.77) with pore water Fe concentration and P (R^2 = 0.92; Figure 5-44B). By providing a preferred TEA, nitrate addition was expected to limit the microbial reduction of Fe, supressing further release of both Fe and P. The observed removal of Fe and P from the pore water, however, was unexpected. To illustrate, on Days 9 and 13 Fe concentrations were 44.1 and 118.5 mg/l in the control, respectively, compared to 16.5 and 0.93 mg/l in the nitrate-dosed systems. Based on the Fe concentrations in the pore water at the time of first dosing, the nitrate additions represented doses in a molar ratio of 178:1, 1948:1, 7264:1 and 17895:1.

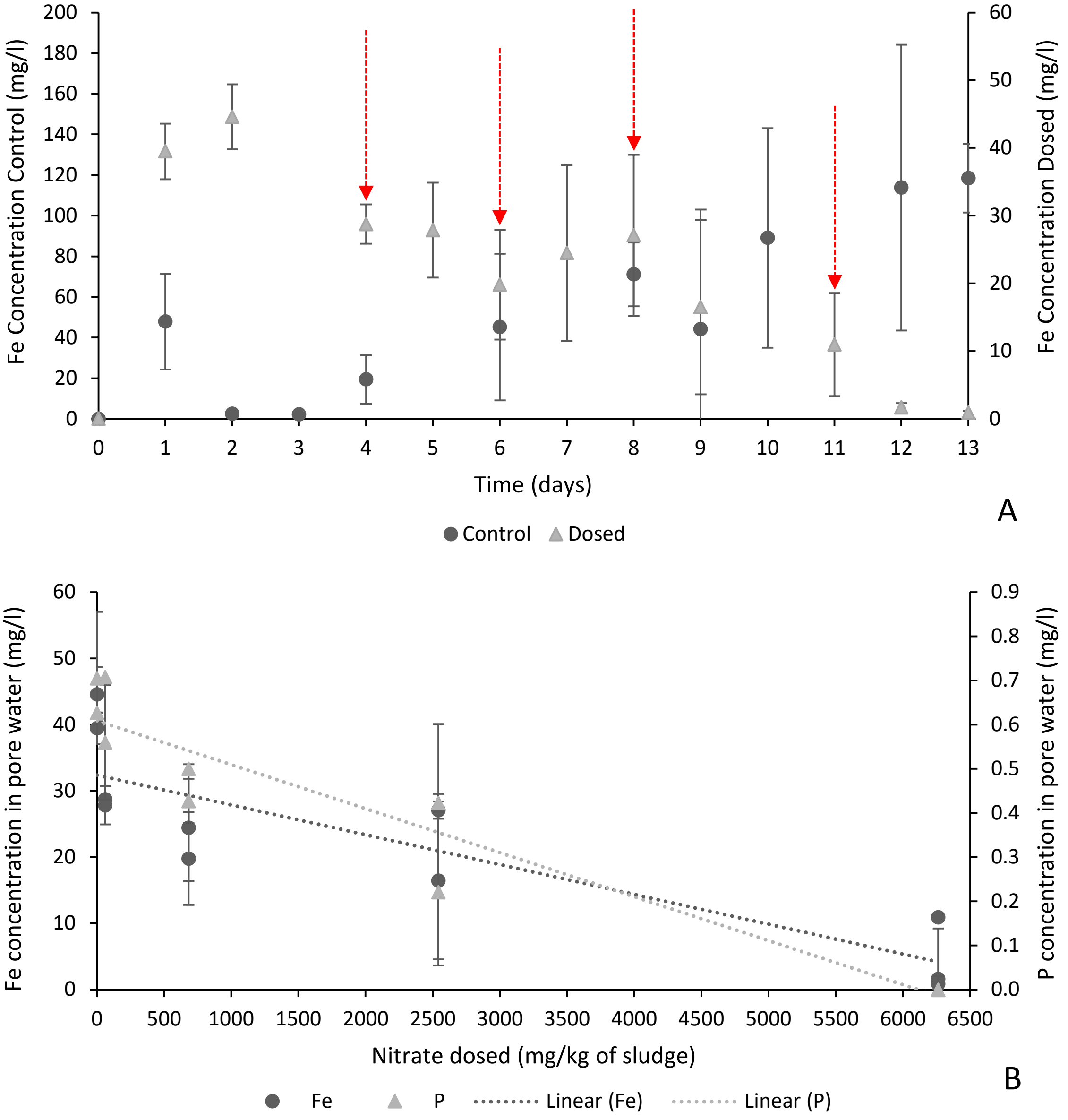

Figure 5-44 Impact of nitrate addition on Fe and P in the pore water at a depth of 4 cm (a) as compared to the control system (arrows indicate when dosing occurred), and (b) against cumulative nitrate dose. Error bars represent standard deviation, n=3

The impact of sequential nitrate addition was to generate significantly lower concentrations of both P and Fe in the pore water. In terms of biogeochemical conditions, oxygen was completely depleted before the experiment was started (DO < 0.1 mg/l). The highest redox at the start of the experiment was found within the water layer at 39 mV, with the lowest being -206 mV at 6 cm depth within the sludge. Overall the redox generally decreased throughout the experiment (except for short term increases that coincided with nitrate addition). The water phase showed a decrease from 71 mV to -32 mV, whilst the sludge layer showed a larger decrease from 154 mV to -400 mV. This is suggestive that the process driving the redox decrease within the sludge was likely to be, at least partially, microbially driven (ZoBell, 1946).

There was no clear correlation between redox and Fe concentration (Figure 5-55). Redox values were always below the 200 mV redox threshold, suggested for Fe reduction (Boström et al., 1988), and also below -100 mV suggested as the lowest redox for the instigation of ferrihydrite reduction (Thamdrup, et al, 2000). Fe release has been recorded from ferric Fe forms at redox potentials lower than -200 mV (Jacobs et al., 1989).

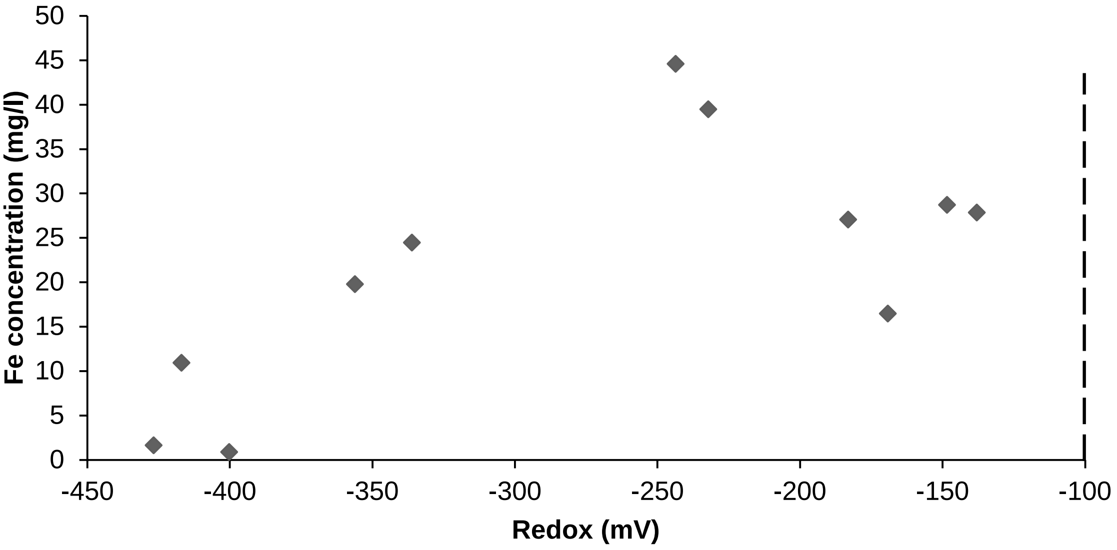

Figure 5-55 Pore water iron concentrations within the sludge layer (4 cm depth) against redox potential. The dashed line shows the minimum redox for initiation of ferrihydrite reduction

[redacted] Discussion

5.5.1 Iron transformations within wetland sludge are microbially-mediated

This study showed an increase in Fe and P pore water concentrations when incubating sludge of a Fe-dosed CW under anoxic conditions. This increase was greater at depth within the sludge profile, rather than at the sludge-water interface. The low redox values developed during sludge incubation favour a predominance of Fe^{2+} species in the pore water, although the change in redox alone is insufficient to explain the observed dynamic behaviour of Fe in and out of the pore water, especially following the dosage of nitrate. The findings on the environmental conditions during the nitrate dosing tests support the theory that scavenging of soluble Fe species was microbially-mediated.

There was a distinct pattern with an initial phase of Fe and P release and removal from the pore water as the system acclimated, followed by a period of no net release which lasted 48 and 72 hours for Fe and P, respectively, which was followed by sustained Fe and P release. This is a similar pattern observed in previous sludge incubation trials (Barak, 2014) and is consistent with a system moving towards chemical equilibrium, where mass transfer from the solid to the liquid phase is limited by diffusion (Withers and Jarvie, 2008). The diffusion rate of Fe within soils is reported as $1.5 \times 10^{-7}\ cm^2/s$ compared to $6.2 \times 10^{-6}\ cm^2/s$ within water (O’Connor, Lindsay and Olsen, 1971). Whilst previous studies that investigated iron and phosphorus release into the overlying water of incubated iron dosed constructed wetlands may have been chemical diffusion driven (Barak, 2014), the lack of rapid increase in concentrations observed in the overlying water in this study indicates that it was not. This could however be explained by the process of dissimilatory iron reducers inducing this release.

Further evidence of the predominance of microbially-mediated reactions in the reactors was found on nitrate dosing. If it was merely a chemical process, nitrate additions would leave the system unaffected, i.e., the release of both P and Fe would continue until the system reached equilibrium. If the release of Fe and P was at least partially microbially-mediated, then introducing nitrate would result in inhibition of the iron reducing microbial pathway, leaving only the chemical pathway in operation. The result would be a smaller change in the soluble Fe (and P) concentrations with incubation time. However, the addition of 68 mg NO_3/kg sludge in this study, equal to 178:1 NO_3:Fe in the liquid phase, resulted in net reduction of Fe and P from the pore water (Figure 5-44). This was unexpected as whilst chemically, the addition of nitrate provided a temporary increase in redox potential, it never reached the redox values where the oxidation of Fe^{2+} to Fe^{3+} would be chemically favourable (Figure 5-55). Therefore, the transformations observed are likely to be a combination of a microbially-mediated process that can oxidise Fe^{2+} species at the low redox values in these reactors at a faster rate than chemical reduction and diffusion of reduced Fe species from the sludge. There are many microbial groups that can transform Fe as part of their metabolism (Ehrlich and Newmann, 2009), some of which include species reported to be found within CW (Adrados et al., 2014). The pathways for Fe transformations are complex, with contradictory findings depending on the methods or conditions of the study, for example, *Geobacter metallireducens* is a dissimilatory metal reducer that can oxidise organic carbon with either NO_3^- or Fe^{+3} but can also link NO_3^- reduction to Fe oxidation (Coby et al., 2011; Finneran, et al, 2002; Lovley et al., 1993; Lovley and Lonergan, 1990). The activity of this bacteria could explain the apparent contradiction of Fe oxidation at redox levels that would favour reduction within the sludge (Coby et al., 2011). Sludge samples taken from the same CW system as used in this study was DNA sequenced and showed the presence of *Geobacter* (Chapter 3), which would support the proposed cycling pathway of Fe and P from Fe -rich sludge. However, in a previous study (Finneran, et al, 2002), *Geobacter metallireducens* was unable to oxidise Fe^{2+} if it had previously used Fe^{3+} as its TEA. The present study shows that even in an environment actively reducing Fe, the addition of NO_3^- could stop the release and, when applied in sufficient quantities, remove Fe from the pore water. To do this, the nitrate appears to be used as a TEA and the Fe^{2+} as the electron donor.

A greater understanding of the exact microbially-mediated pathway for removal of Fe by the rich diversity of microorganisms in wetland sludge (3, n.d.), cannot be established from this study, but would greatly aid the management of these types of CW.

The majority (>90%) of iron released within this experiment was found to be within the colloidal phase. This is in contrast to previous reports (Klar et al., 2017) that the concentration of Fe found in pore waters of a continental coastal shelf mainly (>84%) consisted of smaller colloids (<0.15 µm) or dissolved Fe. It also demonstrated that in warmer conditions (late spring), the dominate size fraction became smaller than 0.02 µm, which was attributed to the activity of dissimilatory iron reducing bacteria. The dominance of colloidal release found in the current study also contrasted with previous studies into the potential for iron release from sludges at CW where the majority of iron leaving the system was in the truly dissolved form (<1 kDa) and only a small amount in the colloidal form (Dotro et al., 2015). The difference could be due to the ability to sample without disturbance of sediment/layers, which was not possible in previous studies, however further investigation would be required to fully understand this.

The suggestion that the release of iron and phosphorus was bacterially mediated, matches with the redox measurements after TEA dosing. If the redox increase caused by nitrate addition was chemically-driven, a larger effect would be expected within the water phase, as the nitrate encountered any reduced chemical species. As there was a limited impact upon the water phase but a significant impact on the redox profile within the sludge, this is indicative of a microbially-driven process.

5.5.2 Iron can be released without an associated phosphorus release from iron-dosed wetland sludge

The release of P as a direct result of the reduction of Fe found in sediments is reported in a wide variety of environments such as lakes, rivers, reservoirs, and constructed wetlands (Kim, et al, 2003; Kinsman-Costello, et al., 2014; Zaragüeta and Acebes, 2017). In this study, after an initial lag period, P release was observed under anoxic conditions. This has been observed in other controlled studies with wetland sludge (Dotro et al., 2015). The lag period for P release was longer than for Fe release, suggesting other solid Fe species were targeted before P-associated Fe. Once P release was observed, the ratio between P and Fe in the pore water varied drastically from 3:1 to 201:1 (Figure 5-22B). A ratio of 2.7:1 between Fe and P was measured within the sludge of this study which falls within the range of ratios of 1.8:1 to 4.4:1 found for other Fe dosed CW (Dotro et al., 2015). If only the Fe species associated with P were transformed, the release of Fe would be expected to release P in similar proportion. Fe reducing bacteria such as *G. metallireducens* was shown to preferentially metabolise ferric oxide over ferric hydroxide or magnetite (Lovley and Phillips, 1988a). The addition of Fe salts in wastewater treatment plants is typically done in excess of the required Fe:P ratio based on stoichiometry. This is because the chemical dosing is not 100% efficient, with competition between phosphates and hydroxides for Fe combination. However, these hydroxides also have the potential to remove P through adsorption (Thistleton et al., 2002). Any reduction of these Fe oxides/hydroxides, in preference to ferric phosphate or P associated hydroxides, could result in limited P release, consistent with the findings from this study.

5.5.3 Iron-dosed constructed wetlands can act as sinks and sources of iron depending on operating conditions

Constructed wetlands with Fe dosing are typically considered to be a sink of Fe (Sexstone et al., 1993), and P (Lauschmann et al., 2013). This study hypothesised that when the redox values of this sludge were reducing (-100 mV; as reported by Thamdrup et al., 2000) then reduction of this Fe pool would occur. This would result in any reduced Fe and associated P being released into the pore water. If microbially-mediated, it was expected that the addition of nitrate would suppress this release. Whilst all these statements were proven true in these experiments, the lack of clear ratio between Fe release and P release, combined with the removal of Fe from pore water following nitrate addition, showed this system is complex.

The depth profile of Fe release found in this study highlighted a potential concern for the management of Fe dosed CW. In natural environments, such as lakes, the redox conditions of the sediment-water interface are of primary concern as the problem would be if the release of Fe/P was to pollute the water overlying the sediments. Without natural mixing, a concentration gradient would develop where most of the released P and Fe would be in proximity to the sediment layer. For CW, the effluents are taken from below this sediment (sludge) level. There are two scenarios that apply to CW: (a) when they are flooded or ponded, and (b) when run in true subsurface flow mode (Figure 5-66). For the latter, although the water enters the system from the top, it quickly infiltrates into the media. In subsurface HF systems, only the inlet portion should have an overlying water column during normal operation, whereas in vertical flow (VF) systems the overlying water column is only visible during batch feeding. When operated correctly, a HF CW should have no overlying water column, and a VF CW has rapid infiltration through the media during feeding, both ensuring that the sludge is kept in oxic conditions. However, if flooding or ponding occurs this will result in the possibility of the sludge becoming anoxic and reducing (Kim et al., 2015), as seen in this study (Figure 5-55). When this occurs, there is the potential for the release of P and Fe. This was demonstrated by the increasing levels of Fe within the sand layer in the experiment, in the absence of a Fe source that deep within the reactor (Figure 5-22B). Subsurface operation in chemically-dosed CW is then critical to minimise this risk. This agrees with previous recommendations of subsurface operation to minimise potential Fe and P release in particulate and colloidal forms through sludge lifting (Dotro et al 2015).

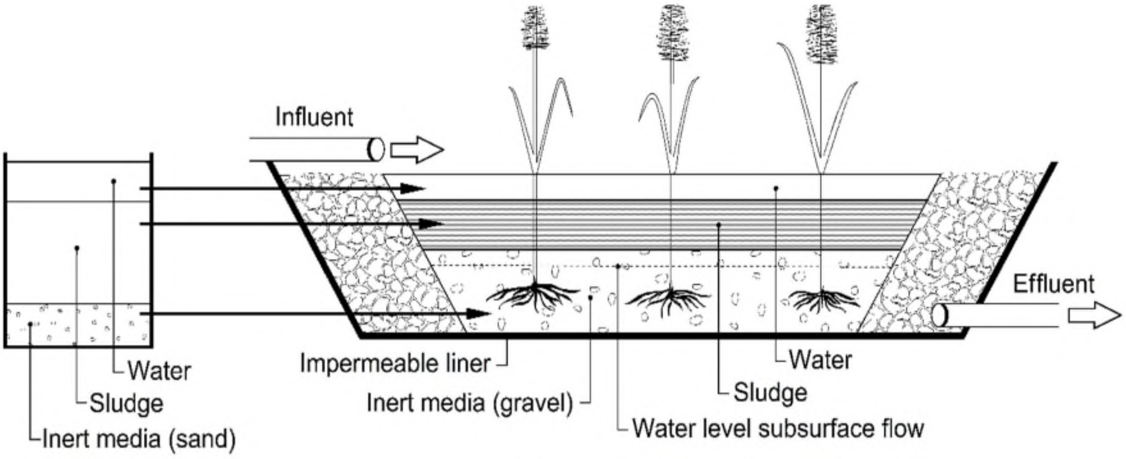

Figure 5-66 Schematic of the experimental set-up, on the left, and the corresponding layers in a HF CW on the right with water levels above and below the sludge layer, respectively

Under the controlled conditions of this experiment the increase (and subsequent decrease) of concentrations within the inert layer was probably a diffusion driven process. The water at this level was removed slowly and sparingly by the peristaltic pump, and so it is unlikely that this would have driven the migration of Fe and P to a significant level. In a full-scale HF CW this water passes through the (biologically-active) media typically within 24 hours (Green and Upton, 1995), with effluent exiting from below the sludge and media layer. When flooded, flowing water could draw the high Fe concentration from the sludge pore water towards the effluent, leading to elevated levels of Fe and P within the effluent. However, most CWs have effluents with lower concentrations of P and Fe than at the influent (Dotro et al 2015). This suggests other transformation processes can take place within the biologically active media (gravel in HF, sand in VF systems) and root environment. The potential and mechanisms of these transformations require further study.

The present study considered both the artificial addition of dissolved oxygen and nitrate to CW sludge. Artificial aeration of horizontal flow wetlands is increasingly being used for ammonia removal in the UK (Butterworth et al., 2016) and so offers an additional benefit. For lower energy systems with passive or no aeration, this study has shown that NO_3^- can also be used as a mitigation step and, more importantly, as a remedial step for suppressing release of Fe from wetland sludge. Whilst a dose of 62mg NO_3^-/kg sludge was able to suppress release in static conditions for three days (Figure 5-44), the optimal dose, duration and frequency would need to be determined based on the wetland's residence time, sludge and wastewater characteristics.

[redacted] Conclusions

This laboratory study into the release of iron and phosphorus from the sludge accumulated at an iron-dosed constructed wetland showed that the potential for release of iron and phosphorus from these enriched environments exists and was observed at a rate of 82.8 Fe mg/m2/day and 4.36 mg/m^2/day. This cycling from sediment into the water phase was shown to be microbially mediated. However, a lack of relationship between the quantities of iron and phosphorus released suggests a preference for different iron forms by the iron reducing bacteria.

The addition of oxygen was able to significantly increase the redox potential within the overlying water table, this would be sufficient to mitigate the problems of release. However, no impact on redox was observed at depth within the sludge profile, indicating that oxygen addition may not stop the underlying cause of release. Conversely, the addition of nitrate had limited impact in the overlying water phase but had an impact on the redox potential within the sludge layer. Although this impact could not make the prevailing redox condition positive, the presence of nitrate significantly lowered the levels of phosphorus and iron within the pore water. This means that nitrate addition can be used as a mitigation step to prevent release and, potentially, as a remedial step to re-entrap released iron and phosphorus in a malfunctioning wetland. Another potential opportunity to increase nitrate within the CW is to increase the efficacy of nitrification steps, such as a RBC, before the influent to these sites. This study highlights the importance to manage the subsurface conditions of CW to minimise the risk to effluent compliance posed by iron and phosphorus release.

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6 Implications of the work

The overall aim of this study was to *understand the risk, mechanisms and appropriate management options of iron and phosphorus release from constructed wetlands treating wastewater that has undergone chemical phosphorus removal*. In response to the aim a series of investigations were undertaken which led to the following main observations:

1) Adaptation of constructed wetlands for nutrient removal improved the treatment for both the target compound as well as the other standard parameters. The inclusion of iron dosing upstream for phosphorous removal improved the robustness of the overall flowsheet with specific emphasis towards phosphorous, BOD and suspended solids (Chapter 2).
2) The microbial community within the sludge layers found in constructed wetlands has a high overall bacterial count and is highly diverse. Neither component is significantly impacted by the different types of adaptation that can be applied and no difference in the abundance was observed in specific reference to iron reducing bacterial groups (Chapter 3).
3) The release of iron and phosphorus was linked to establishment of appropriate environmental conditions that triggered the related microbial pathway. This was identified as the need for a source of iron, anaerobic conditions and an available carbon source (Chapter 4).
4) The release of iron can occurr from both ferric oxide and ferric phosphate. The rate of release was faster with ferric oxide when tested individually compared to ferric phosphate but no difference could be seen when the two were mixed together (Chapter 4).
5) The release of iron was effectively inhibited in the presence of nitrate (Chapters 4 and 4).

These observations lead to key implications for both predicting the potential for P and Fe release as well as identification of appropriate management options for its control.

[redacted]Potential for release

As hypothesised in previous literature (Dotro et al., 2015; Kim et al., 2016) and subsequently identified in this this study, the potential for a constructed wetland to release iron and phosphorus is related to the biotic process of iron reducing bacteria utilising ferric phosphate precipitates as a terminal electron acceptor. Consequently, three components are critical if a release of iron and phosphorus is to occur: a sufficient community of iron reducing bacteria, the background environment that enables the metabolic pathway to be active and a source of precipitated ferric phosphate and organic material. The latter extends to other sources of precipitated ferric compounds in which case release will specifically relate to just iron (Chapter 4), and was previously identified in the literature (Coby et al., 2011; Jones, Gardener and Simon, 1984; Klar et al., 2017).

Whilst previous studies had identified that certain CW are dominated by iron reducing bacteria (Adrados et al., 2014), Chapter 3 of this study presented a survey of the bacterial community present in the sludge layers of a range of constructed wetlands and demonstrated the presence of sufficient iron reducing bacteria in all cases. This was unexpected as it included sites that do not receive ferric precipitates (site with no chemical P removal) and aerated sites. The overall abundance and diversity of the microbial communities within the CWs turned out to be sufficiently high that the required community of iron reducers is likely to always be present. Consequently, the risk of release then becomes only about the underlying chemical environment and the availability of source materials.

The requirement about source materials concerns both ferric precipitates and available organics. In terms of ferric, only sites that include chemical phosphorus removal with an iron based coagulant are at risk. Pure culture studies demonstrated that *Geobacter metallireducens* was able to reduce both ferric oxide and ferric phosphate, albeit at different rates (Chapter 4) congruent with literature data with other forms of solid phase iron (Akageneite, Goethite Finneran, Housewright and Lovley, 2002; Lovley et al., 1993). Phosphate release will only occur when there is ferric phosphate present but iron release will occur with a broader range of sources and as such iron release is more likely to occur. This is reflected in the measured pore water Fe:P ratios which did not match those associated to biological utilisation of ferric phosphate if it was the only source (Chapter 4).

In order to activate the metabolism of the bacterium (*Geobacter metallireducens)*, acetate (an acceptable terminal electron donor for this bacterium Childers, Ciufo and Lovley, 2002) was added in the pure culture experiments which was kept in excess to ensure organics did not become rate limiting (Chapter 4). Consequently, it remains unclear as to the exact limit below which a lack of utilisable organics would inhibit iron reduction. Whilst possible, it is suggested that in most cases (Brix, Schierup and Arias, 2007; Chan et al., 1982; Molle et al., 2005), sufficient organics are likely to be present as to not be rate limiting. The organics are available from the incoming feed water (both soluble and particulate) and from the decaying plant matter aligned to the fact that the reeds are not harvested. The former is reflected in the BOD consent with many small sewage works set reasonably relaxed consents of between 20-30 mg/l. The main risk associated with this is if an increase in organic matter become available in concert with the other required conditions such as when the upfront biological process becomes overloaded or has operational problems.

Consequently, it is suggested that the dominant factor in understanding the risk of release is related to the availability of alternative terminal electron acceptors to iron, namely oxygen and/or nitrate (Chapters 4, 4). Both have been shown to inhibit the microbial pathway required for iron release (Finneran, Housewright and Lovley, 2002; Lovley et al., 1992). In the course of the current experiments, limitations were observed in relation to oxygen penetration into the sludge layer (Chapter 4). In part, this reflects a combination of the fact that oxygen is preferentially used, over nitrate, and has a relatively low solubility limit in water. This means, maintaining oxic environments through the incoming DO in the water has limited scope and indeed unaerated CWs are known to operate in anoxic or anaerobic conditions. In contrast, nitrate has a much greater solubility limit and is less readily used and so offers a potentially more robust control. The smallest concentration seen to have an impact in the current work was 8.5 mg/l (Chapter 4) which is less than would be expected if the upstream works fully nitrifies to a reasonable tight ammonia consent (c5 mg/l) at 20-30 mg/L of nitrate. Accordingly, the level of nitrification can be considered a surrogate guide of risk. This can be manifested in two main ways, a relaxed ammonia consent and a deterioration in the upstream nitrification process. Chapter 2 demonstrates that there is a wide range of ammonia consents, with the highest at 20 mg/l and the lowest at 5 mg/l. Reduced nitrification can occur as a consequence of low temperatures (Hwang and Oleszkiewicz, 2007) or an organic overloading on the main biological process upstream of the CW (Caffrey et al., 1993), an occurrence commonly associated to population growth or seasonal migration issues (i.e. holiday resorts, Molinos-Senante et al., 2016).

Consideration of the above can be illustrated through the likely type of consent a works has and how this impacts the potential risk of release (Table 6-11). Total nitrogen consents are uncommon in the UK and very unlikely at small works that contain constructed wetlands and hence the predominant risk profile is associated to works with a relaxed ammonia consent or sites that experience nitrification problems.

Table 6-11 Risk matrix of iron and or phosphorus release

Situation	Carbon content in influent to wetland	Nitrate content in influent to wetland	Potential for release	Consent at works
1	High	High	Low	Standard small works
2	High	Low	High	Site with no or a very relaxed ammonia consent
3	Low	High	Low	Stringent ammonia consent
4	Low	Low	Medium	Total nitrogen consent

[redacted] Management of the risk

When release is occurring the imperative thing to do is alter the biogeochemical conditions so that release is no longer favoured by changing the redox environment. This needs to consider the full sludge audit associated to the CW and importantly the depth of sludge layer on top on the media. The two simplest ways are the introduction of O_2 or nitrate into the system.

The current work indicated a potential challenge with adding sufficient oxygen through the feed water, especially in relation to maintaining appropriate environmental conditions throughout the depth of the sludge layer (Chapter 4). However, aeration of a CW is currently employed to aid nitrification and this would generate an appropriately oxic environment to suppress the metabolic pathway(Butterworth, 2014; Butterworth et al., 2016). The aeration of CWs is done by placing a network of pipes along the base of the wetland media and providing gentle aeration with blowers (Zhang et al., 2010). The adaptation requires a retrofit associated with a refurbishment and so it not an appropriate short term/responsive solution. However, retrofitting CW to include aeration is considered a relatively small investment and so is recommended on sites where future chemical phosphorus removal is planned or envisaged in the future (Butterworth et al., 2016).

The alternative is to ensure there is sufficient nitrate present which can be effectively achieved through adding a nitrate dosing system to the feed chamber of the CW. This option requires no refurbishment of the wetland and can be implemented fairly routinely. The presence of nitrate was shown to supress the initial metabolism and release of iron (Chapter 4 and 4) but also to reverse this release when added to a system already undergoing P/Fe release (Chapter 4 and 4). The addition of nitrate had little to no chemical impact within the overlying water column but was able to impact the release profile within the sludge (Chapter 4). The impact was achieved with 8.5 mg/l, a small percentage of the solubility limit and so a small additions from a concentrated stock solution, with effective mixing, would be able to provide an effective strategy to supress the release a

CW. As outlined earlier, a fully functional nitrification plant ahead of the CW, should provide sufficient cover to protect against P/Fe release. Accordingly nitrate dosing can be used as a top up or seasonal component to ensure a sufficient and steady supply of nitrate to the CW.

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7 Conclusions

Adaptation of Constructed Wetlands (CWs) for nutrient removal by either chemical dosing in the upstream works (for phosphorus removal) or aeration (for ammonia removal (Butterworth et al., 2016; Dotro et al., 2015) resulted in an overall increase in the robustness of the sites delivery against its consent values (Chapter 2). In the case of chemical dosing, the improvements were seen in both the target component (phosphorus) and in other components (BOD, SS, Chapter 2). However, an undesired consequence is that iron and phosphorus can be re-released from the sludge layer (Chapter 5). The current thesis explored the mechanisms and management options to minimise the risk of this happening. The process was identified as a microbially mediated pathway driven by iron reducing bacteria in anaerobic environments (Chapters 4,5). The work explored aspects related to the availability of iron reducing bacteria, the source type of iron and the impact of different terminal electron acceptors (Chapter 4).

The microbial community found within the sludge layer of a range of HF CW has a high overall bacterial count and is highly diverse (Chapter 3), and was shown to be more diverse than that biofilms typically found at these types of sites (Adrados et al., 2014; Ibekwe, Grieve and Lyon, 2003). Adaptation of HF CW for nutrient removal through either upstream chemical dosing or aeration had no significant impact on the count or the diversity levels of the microbial community. Importantly, the presence of iron reducing bacteria was observed in all cases with no evidence of enrichment of iron reducer on sites that utilise chemical P removal. Accordingly, all wetlands have a risk of biologically mediated iron and phosphorus release if the environmental conditions are appropriate.

The release of iron was linked to the establishment of anaerobic conditions within the system, which matched previous studies (Hu et al., 2001; Hupfer and Lewandowski, 2008; Tammeorg et al., 2017). In pure culture experiments utilising *Geobacter metallireducens* in the presence of acetate the release rates were observed as $2.26\text{x}10^{-15}$ g Fe/day/cell and $3.07\text{x}10^{-16}$ g Fe/day/cell. In a mixed system of oxide and phosphate the overall release rate was similar to that of phosphate only indicating that the system is complex and cannot be defined in terms of the combination of the release for each individual source.

The presence of nitrate was shown to supress the release of iron including in a systems that was already actively releasing, similar to previous lake based studies (Foy, 1986). Further, the addition of nitrate was shown to lower the residual level of water phase iron over time, offering the potential for a remediation pathway. Comparison of terminal electron acceptors present in the liquid phase showed that nitrate was able to sustain a profile deeper into a sludge layer than oxygen.

Overall, it is suggested that the finding outlined in this thesis indicate that the management of the terminal electron acceptor is the key to understanding and managing the risk of iron and phosphorous release in constructed wetlands. The most likely situations where this may occur are chemical dosing sites which have a relaxed ammonia consent or are experiencing nitrification problems. It is recommended to adapt existing site for the inclusion of aeration or nitrate dosing to ensure effective management against the risk of release in the future.

[redacted]Further Work

The research presented here has highlighted the potential for the release of iron and phosphorus from the sludges contained within constructed wetlands treating wastewater that has undergone chemical phosphorus removal. It has also shown how the presence or addition of nitrate can be considered as a management tool for the mitigation of this release. There was however, still avenues of further work identified that are considered sensible next steps.

[redacted]Larger scale trials

CWs are a rural treatment system that are not enclosed and are subsequently exposed to the natural seasonal and daily variations of environment. Whilst Chapter 5 provided an accurate representation of a CW within the confines of a laboratory it was not able to recreate the exposure to the natural environment and hence the translation of the finding to real world systems requires validation.

Specifically, CWs typically operate continuously with a constant supply of fresh feed water entering the system (Dotro et al., 2017; Kadlec et al., 2000). Therefore, further investigation of both the potential for and mitigation of iron and phosphorus from the sludges within constructed wetlands treating wastewater that has undergone chemical phosphorus removal should be conducted by the implementation of a pilot scale trial. By providing these pilots with the influent that would be expected if a prior nitrification step was working ineffectively, the concepts shown in this thesis, especially regarding the mitigation of release could then be tested further.

[redacted] Profiling sludge

Chapter 5 started to provide interesting consideration of the impact of sludge depth on the changing environmental conditions that prevail. It is recommended that further work is conducted to fully characterise such profiles in a system that enables flow through so that both the spatial and temporal impacts can be properly characterised. Specifically it is posited that there may be a heterogeneity, in the chemical make up and bacterial communities, to the profile both in depth and in physical distance from the inlet. This reflects established understanding in plug flow fixed film biological processes but equivalent information in regards to the current topic is unavailable. As part of this, the role of sludge depth needs further investigation by trialling different sludge depth under otherwise identical conditions. This would establish if there was a critical depth beyond which the risk of release was significantly increased and may indicate a refurbishment frequency that is required as part of the overall management strategy.

[redacted] Terminal electron acceptor carbon link for criticality of metabolism

The thesis has identified the importance of the terminal electron acceptor on management of the release of iron. However, the specific limiting values require further investigation. In particular, controlled laboratory trials are recommended to establish limits below which organics become rate limiting. This needs to reflect both readily utilisable and more complex organics to establish an overall picture. Further, similar experiments should be conducted with nitrate and oxygen in relation to the available iron to establish if the limiting conditions are fixed at specific mass concentrations or more appropriately defined in relation to a molar ratio with iron.

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