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DEPARTMENT OF THE ENVIRONMENT, HERITAGE
AND LOCAL GOVERNMENT

Characterization of Microbial Communities Removing Nitrogen within an Integrated Constructed Wetland Treating Agricultural Runoff

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INTRODUCTION

- Agricultural activities are a potential source of diffuse water pollution, and degrade urban and rural waters.

- In Ireland, nutrient inputs from agriculture are an important source of water pollution.

- The majority of the recorded instances of water pollution can be attributed to the impact of ammonia-nitrogen and ortho-phosphate-phosphorus inputs from agriculture sources such as farm yard runoff.

ENVIRONMENT AND AGRICULTURE

- The central aim of the European Unions Common Agricultural Policy is to avoid water pollution through agricultural activity.
- Water quality protection is a key issue of the Common Agricultural Policy.
- The Common Agricultural Policy has identified three priority areas for action to protect and enhance the European Union’s rural heritage.

ENVIRONMENT AND AGRICULTURE

- Priority areas for action are as follows:

  1.Biodiversity and the preservation and development of 'natural' farming and forestry systems, and traditional agricultural landscapes;

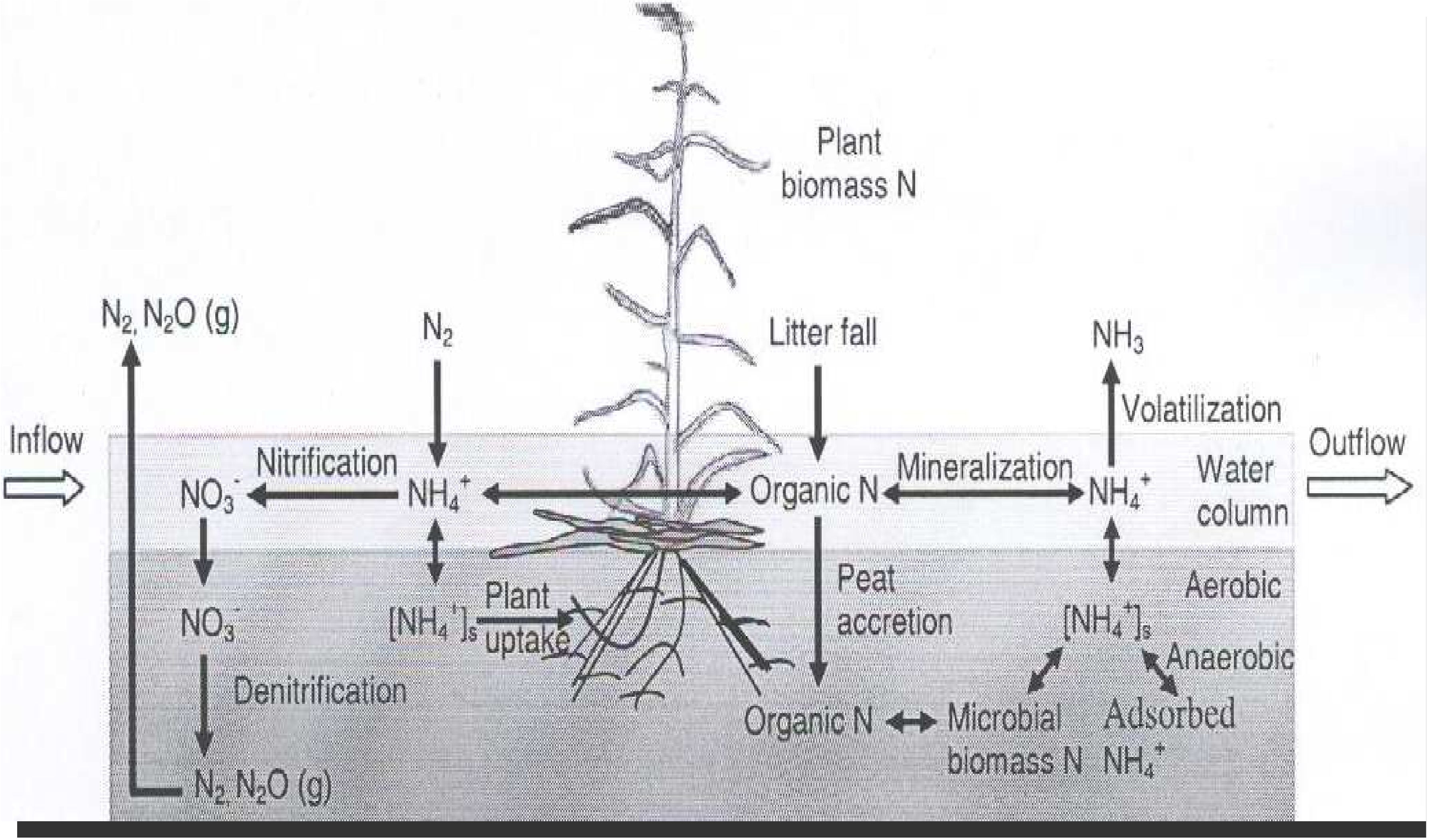
  2. Water management and use; and

  3. Tackling climate change.

- Legal driver is the *Water Framework Directive.*

- The primary challenge that all European Union member states including Ireland face over the next decade is to achieve “good water status” for all waters by 2015.

NITROGEN CYCLING IN WETLANDS



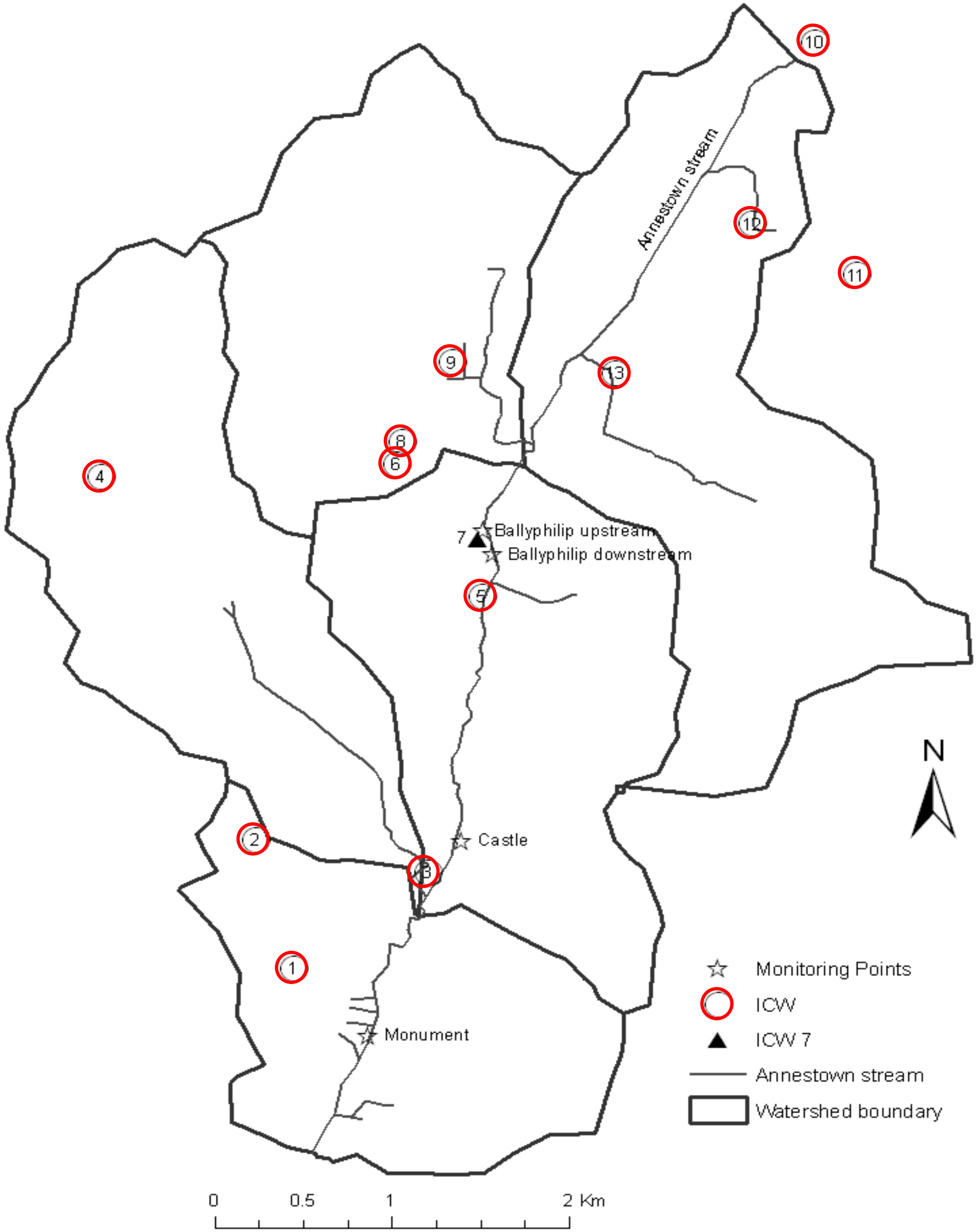
OBJECTIVES

- To characterise the microbial diversity responsible for nitrogen removal in different parts and components of an ICW.

- To compare the microbial diversity responsible for nitrogen removal in different parts and components of an ICW.

- To identify relationships between water quality variables and the microbial diversity.

ICW SITES AT WATERFORD, IRELAND



STUDY SITE

- Area 7660 m^2
- Number of cells: 4
- Dairy farm (77 cows)
- Commissioned in 2001
- Natural liner
- Emergent plant species

METHODOLGY

Water treatment

- Grab samples for each wetland cell inlet and outlet were taken at an approximately fortnightly basis.

- Samples were analysed for pH, temperature, dissolved oxygen, biochemical oxygen demand, chemical oxygen demand, ammonia-nitrogen, nitrate-nitrogen, molybdate reactive phosphorus (soluble reactive phosphorus) and *Escherichia coli*.

METHODOLGY

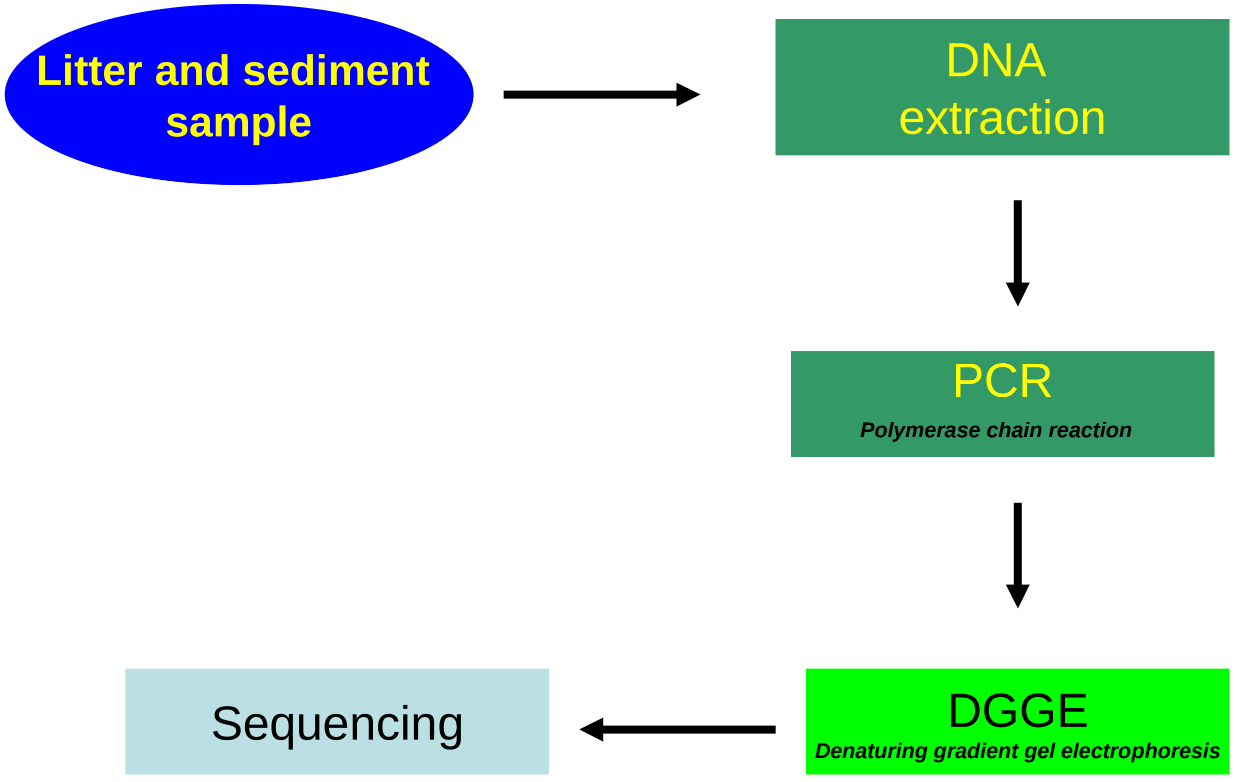
Molecular toolbox

- Molecular methods were employed to study ammonia-oxidisers and denitrifiers in the wetland environment.

- PCR based methods were used for the nitrogen removing bacteria community analysis.

METHODOLGY

Molecular toolbox



Litter and sediment sample

DNA extraction

PCR

Polymerase chain reaction

DGGE

Denaturing gradient gel electrophoresis

Sequencing

METHODOLGY

Sample collection

- Duplicate field litter and sediment samples were collected from each wetland cell of the ICW system.

- For each sampling location, all buried litter in an area of 0.2 m^2 was collected.

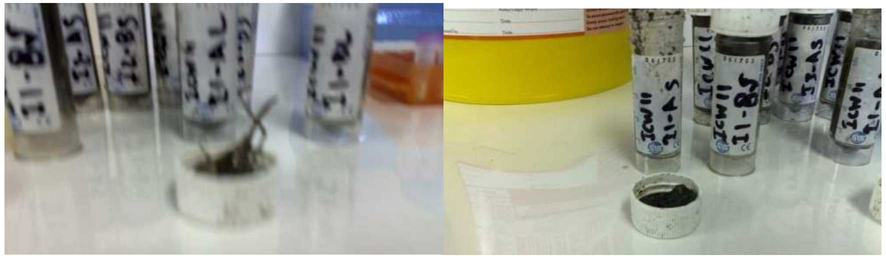
- Sediment samples were collected from the same area with a sediment sampler (diameter of 4 cm). The upper 3 cm of sediment located below the sediment-water interface were used for analysis.

METHODOLGY

Sample collection

- The samples were collected near the influent point of each cell with an additional sample at the outlet of the last cell.

- All samples were frozen immediately after collection and transported to the University of Newcastle for subsequent molecular microbiological analysis.



METHODOLGY

DNA extraction

- The duplicate sediment and litter samples were subjected to deoxyribonucleic acid (DNA) extraction using the FastDNA® SPIN kit for Soil (MP Biomedical Inc., USA) according to the manufacturer's protocol.

METHODOLGY

PCR and agarose gel electrophoresis

- Polymerase chain reaction is a method to multiply DNA segments by repeating cycles of high and low temperature to separate DNA strands and to synthesize new strands.

- Agarose gel electrophoresis is a method to separate DNA molecules by size.

METHODOLGY

PCR and agarose gel electrophoresis

- The ammonia-oxidising bacterial community was assessed using primers (Kowalchuk et al.1997).

- The denitrifying bacterial community was assessed using functional gene primers (Throback et al., 2004).

METHODOLGY

Denaturing gradient gel electrophoresis (DGGE)

- DGGE is a molecular fingerprinting method that separates polymerase chain reaction-generated DNA products.
- DGGE analyses were employed for the separation of double-stranded DNA fragments that are identical in length, but differ in sequence.
- Polyacrylamide gels (120×120×1 mm) were prepared with a denaturing gradient.

METHODOLGY

Denaturing gradient gel electrophoresis (DGGE)

- The composition of 100% denaturant was defined as 7M urea and 40% (vol/vol) formamide (Muyzer et al., 1993).
- The gels were polymerised with 15 µL of TEMED and 150 µL of ammonium persulphate.

METHODOLGY

Sequencing

- The DGGE bands were excised using a sterile tip.

- The excised DGGE bands plus TE buffer were melted in a heating block at 95°C for 10 min.

- 5 µL of post-PCR reaction product was mixed with 2 µL of Exonuclease I/Shrimp Alkaline Phosphate (ExoSAP-IT) and initially incubated at 37°C for 15 min, and later incubated at 80°C for 15 min to inactivate ExoSAP-IT.

METHODOLGY

Sequencing

- The cleaned PCR products were then sequenced.

- The sequences were then BLAST analysed; NCBI BLAST (http://www.ncbi.nih.gov) was used to find the closely related sequences available in the public databases.

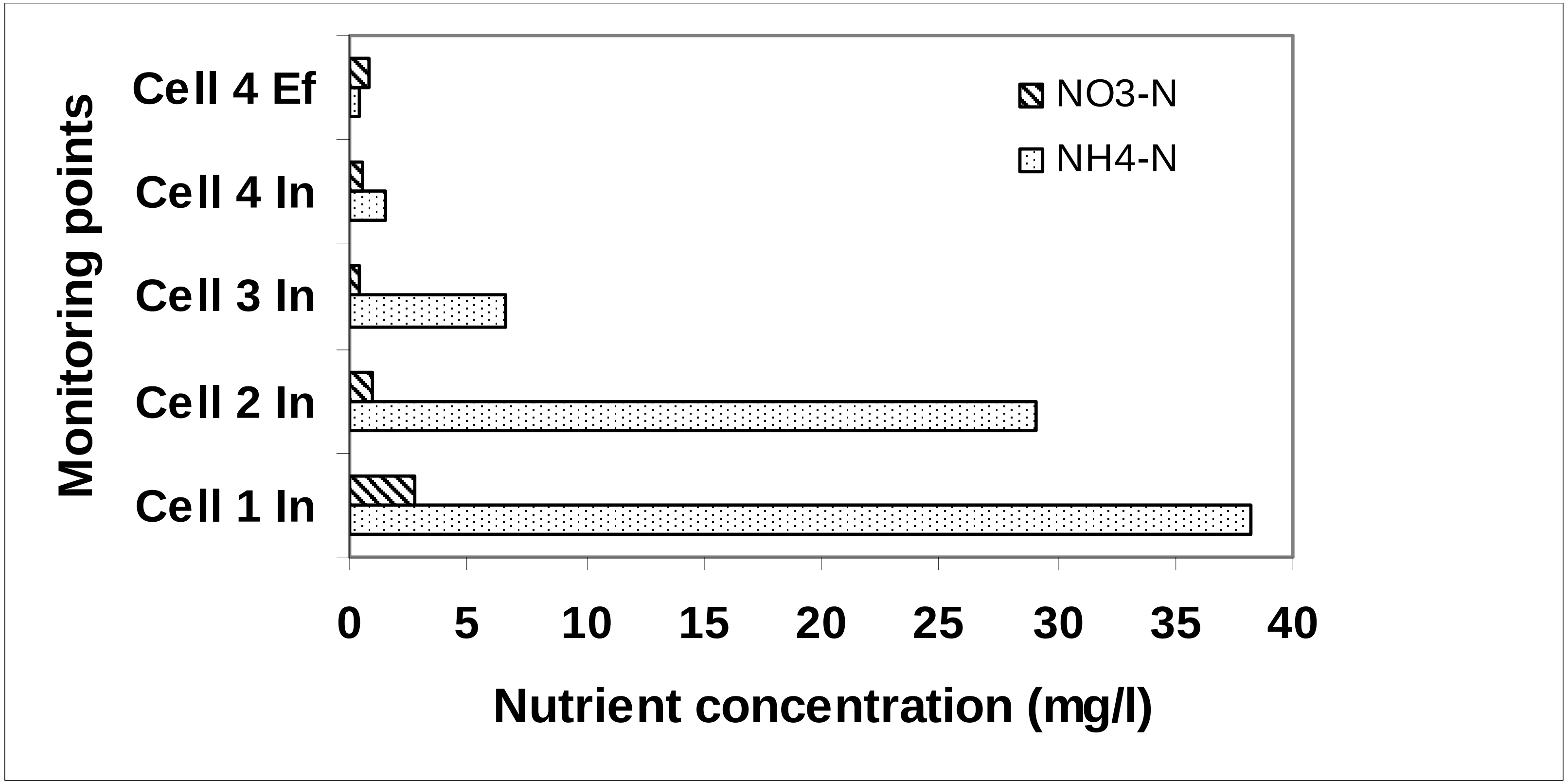
RESULTS AND DISCUSSION

Water treatment potential

Parameter	ICW 11		
	Inlet	Outlet	RR %
Temperature (ºC)	13.8	14.9	-
pH	8.12	7.37	-
Electrical Cond. (µS)	1469	373	-
SS (mg/l)	78.4	15.3	80.5
BOD_5 (mg/l)	593.1	5.8	99.0
COD (mg/l)	1341.5	50.4	96.2
NH_4 –N (mg/l)	28.60	0.39	98.6
NO_3 –N (mg/l)	2.60	0.83	68.0
MRP (mg/l)	8.13	0.83	89.8

RESULTS AND DISCUSSION

Nitrogen removal potential

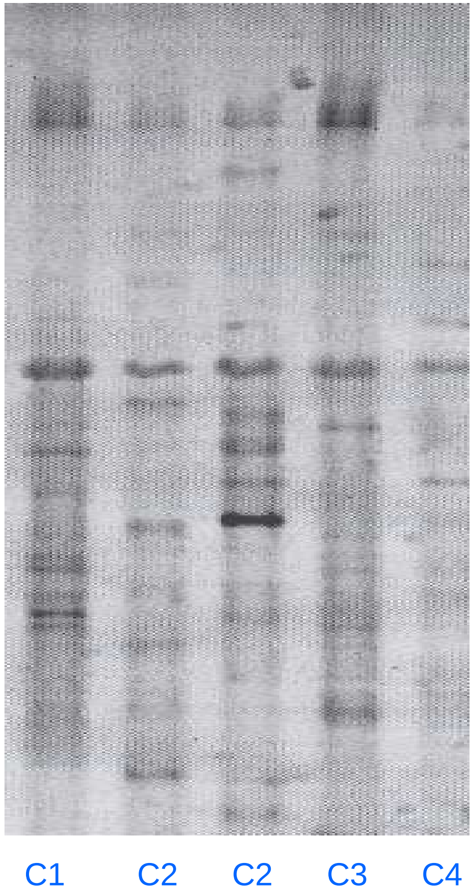


Nutrient reductions in selected ICW cells (In, influent; Ef, effluent).

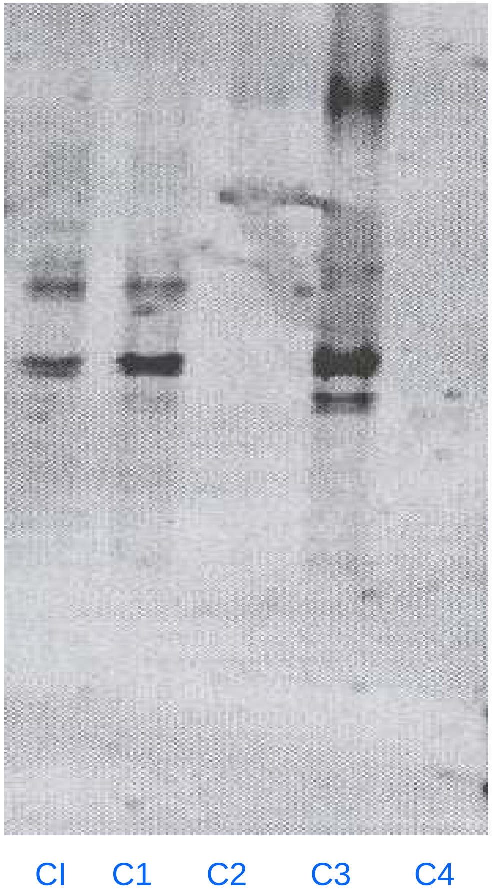
RESULTS AND DISCUSSION

DGGE profiles of PCR products

Denitrifying bacteria



Ammonia oxidising bacteria



RESULTS AND DISCUSSION

Sequencing

Sequence	ICW 11										Accession number	% Similarity	Strain
	C1 In		C2 In		C3 In		C4 In		C4 Ef				
	L	S	L	S	L	S	L	S	L	S			
C1			+		+						AY123811	97	Nitrosomonas sp. Nm59
C2	+	+			+			+			AY123801	99	Nitrosospira sp. Nsp12
C3	+				+						AY727031	100	Nitrosospira sp. En271
C4	+				+						AY792265	98	Uncultured beta proteobacterium clone

L= Litter S= Sediment

RESULTS AND DISCUSSION

DNA Sequencing Similarity

Sequence	ICW 11										Accession number	% Similarity	Strain
	C1 In		C2 In		C3 In		C4 In		C4 Ef				
	L	S	L	S	L	S	L	S	L	S			
nirK													
C8	+		+								EU448024	81	Uncultured denitrifying bacterium clone T23_D5 nitrite reductase (nirK) gene
C9	+		+								EU448024	81	Uncultured denitrifying bacterium clone T23_D5 nitrite reductase (nirK) gene
C10	+		+								FM209186	86	Pseudomonas aeruginosa LESB58 complete genome sequence
C11			+								AY345247	78	Pseudomonas aeruginosa strain DN24 copper-dependent nitrite reductase
C12										+	EF623501	100	Uncultured bacterium clone LK22mK-28 nitrite reductase (nirK) gene
C13		+						+			AM230857	77	Paracoccus sp. R-26824 nirK gene for nitrite reductase
C14								+			DQ783326	96	Uncultured bacterium clone T1R2_0-7cm_038 NirK (nirK) gene
C16		+				+					AM419485	89	Uncultured organism partial nirK gene for putative copper containing dissimilatory nitrite reductase, clone Fin28
C18	+										EF615316	86	Uncultured bacterium clone P1m_nirK-33 nitrite reductase (nirK) gene
C19	+										DQ337794	87	Uncultured bacterium clone S12m_nirK-33 NirK (nirK) gene
C21	+		+		+				+		AM230832	82	Rhizobium sp. R-24663 nirK gene for nitrite reductase
C22			+								DQ337762	89	Uncultured bacterium clone P7m_nirK-25 NirK-lke (nirK) gene
C23	+		+		+						DQ304404	88	Uncultured bacterium clone Ag100-6 putative nitrite reductase (nirK) gene
nirS													
C24											AY078267	85	Thauera terpenica strain 21Mol putative dissimilatory nitrite reductase (nirS) gene,
C25	+		+		+		+				AM230919	90	Dechloromonas sp. R-28451 nirS gene for nitrite reductase
C26	+		+								AM230913	84	Dechloromonas sp. R-28400 nirS gene for nitrite reductase

RESULTS AND DISCUSSION

DNA Sequencing Similarity

Sequence	ICW 11										Accession number	% Similarity	Strain
	C1 In		C2 In		C3 In		C4 In		C4 Ef				
	L	S	L	S	L	S	L	S	L	S			
nirK													
C8	+		+								EU448024	81	Uncultured denitrifying bacterium clone T23_D5 nitrite reductase (nirK) gene
C9	+		+								EU448024	81	Uncultured denitrifying bacterium clone T23_D5 nitrite reductase (nirK) gene
C10	+		+								FM209186	86	Pseudomonas aeruginosa LESB58 complete genome sequence
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C13		+						+			AM230857	77	Paracoccus sp. R-26824 nirK gene for nitrite reductase
C14								+			DQ783326	96	Uncultured bacterium clone T1R2_0-7cm_038 NirK (nirK) gene
C16		+				+					AM419485	89	Uncultured organism partial nirK gene for putative copper containing dissimilatory nitrite reductase, clone Fin28

RESULTS AND DISCUSSION

Diversity

Diversity indices for the ammonia-oxidising and denitrifying bacterial communities in sediment and litter of the ICW system (mean ± SD)

Primer/ Genes	Component	Shannon's Index (H)
CTO (Ammonia-oxidisers)	Litter	0.68 ± 0.80
	Sediment	n.d.
*nir*K (Denitrifiers)	Litter	2.04 ± 0.29
	Sediment	0.89 ± 0.80
*nir*S (Denitrifiers)	Litter	2.31 ± 0.18
	Sediment	1.60 ± 0.68

n.d. no data

CONCLUSIONS

- For AOB, both *Nitrosospira* and *Nitrosomonas* were detected in the studied wetland system.

- Concerning DNB, *Paracoccus, Pseudomonas, Rhizobium* and *Dechloromonas* were identified.

- The litter component of the studied wetland system supported more diverse nitrogen removing bacteria (ammonia-oxidising and denitrifying) than the sediments.

CONCLUSIONS

- The overall nitrogen transforming and removing bacterial diversity near the inlet (where ammonia-nitrogen and nitrate-nitrogen concentrations were high) was higher than near the outlet of the ICW system.

- This supports the water quality data derived from earlier and concurrent assessments of ICW performance, indicating that they are effective in the removal of water-vectored mineral nitrogen.

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